Food Safety

CONTAMINANTS AND TOXINS

Edited by J.P.F. D'Mello

CAB Publishing

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J.P.F. D'Mello

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CABI *Publishing*

CABI *Publishing* is a division of CAB *International*

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Cambridge, MA 02138
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A catalogue record for this book is available from the British Library, London, UK.

Library of Congress Cataloging-in-Publication Data

Food Safety/edited by J.P.F. D'Mello.
p. cm.
Includes bibliographical references and index.
ISBN 0-85199-607-8
1. Food--Toxicology. 2. Food--Safety measures. I. D'Mello, J. P. Felix.
RA1258 F65 2002
615.9'54--dc21
2002004671

ISBN 0 85199 607 8

Typeset by AMA DataSet Ltd, UK
Printed and bound in the UK by Cromwell Press, Trowbridge

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Preface

Background

It is perhaps fitting that *Food Safety* should have its origins in the UK. The idea for this book was, indeed, conceived and developed at the height of the various food crises in the UK. However, the primary impetus for this book emerged with the stark realization that some 20 years after the initial food scares, college and university undergraduate curricula in agriculture, veterinary medicine and food science have remained quite impervious to food safety issues. There is still, unfortunately, the perception that food poisoning is rare and that denial and crisis management are effective strategies to restore consumer confidence. Yet we must appreciate that, in comparison with our predecessors, we live in a highly contaminated environment. There is a need to take stock and address the human health implications of food contaminants.

Although recent events may have given the impression of a nation enduring a malaise, the UK has also emerged as a hotbed of dissension regarding other issues such as the attributes and safety of genetically modified (GM) and organic foods. The current furore in the UK over these matters undoubtedly has helped in the globalization of food safety concerns in general, and *Food Safety* has been designed to crystallize the major themes now emerging in Europe, North America and Japan.

Policy

If educational policy in undergraduate training is in need of radical change, then current post-graduate research programmes in food safety can best be described as grossly inadequate. There is an urgent need to attract talented science graduates to undertake innovative work that will underpin future developments in food safety. Above all, it is critical that an integrated and coordinated policy is devised and implemented. Thus, there is a long-held philosophy among academic and research policy-makers that responsibilities in food production and quality assurance can be separated. It is often argued that the obligations of food producers cease at the farm gate. In this philosophy, matters relating to safety of farm produce are assumed to be the responsibility of a second sector, comprising food processors, manufacturers and retailers. Recent events around the world have served to demonstrate unequivocally the need for a holistic approach in food safety. It is not easy to discern how, for example, pesticide or fertilizer recommendations to arable farmers can be justified solely on agronomic efficacy.

Equally, the division of research priorities into 'strategic', 'public good' and 'near-market' categories patently has failed as a policy for ensuring that good science is undertaken and delivered in the interests of food safety. There are now compelling arguments and practical instances to show that this policy is discredited. At the very least, these issues are worth debating in governmental and academic circles.

Content

Food Safety is divided into sections that reflect the major toxins and contaminants in the plant and animal products that constitute our staple diets. The first part includes chapters on plant and microbial toxins that may contribute to common cases of food allergies, intolerance and poisoning. The second part deals with contaminants arising from anthropogenic activities and environmental pollution, while the third part comprises current topics of particular concern in food safety. Specific emphasis is placed on the nature of compounds, distribution of residues in common foods, uptake, toxicology and regulatory issues. Many food contaminants are now definitively associated with the induction of cancer and with neurotoxic, hepatotoxic and nephrotoxic effects. However, subtle effects of these contaminants on immunocompetence and endocrine disruption will be more difficult to establish. In the fourth part, a concluding chapter contains a synthesis of the worldwide and continuing concerns over food safety using information from all chapters in the book. Emerging issues and legislation are also addressed, and the chapter ends with a review of research priorities and action points.

Aims

The aims of this book are to provide a scientific documentation of recent advances with guidance on future directions in all matters relating to food safety, and to do this from a global perspective. As intimated above, this book has been designed to enhance the profile of food safety in college and university curricula. The book should be suitable for final year undergraduates in agriculture, food science, nutrition, dietetics and veterinary medicine. It is assumed that these readers will have a good working knowledge of organic chemistry and human biology. Although the book is structured in a particular way, each chapter is designed to be a self-contained unit to enable readers to make appropriate choices. Ideally, concerted efforts should now be directed at instituting a degree course in food safety, and it is my hope that this volume will provide the framework for such a course. An additional aim is to stimulate interest among our talented science graduates to become involved in research in all aspects of food safety including analytical methodologies, monitoring and development of diagnostics. I firmly believe that only sound scientific training and research will help to allay current apprehension about food safety and ensure consumer protection in the future.

Conclusions

I am delighted to have secured the services of expert authors from the major food safety agencies, research institutes and universities around the world. All of my authors are actively involved in and committed to innovative work, thus helping to underpin future advances in food safety. I commend their efforts to my readers. I am also pleased to express my gratitude to staff at CABI *Publishing* for the encouragement and support they have offered throughout the preparation of this book. To sum up, I believe that food safety teaching and research are still undertaken on an *ad hoc* basis. There is a clear need to formalize these activities into coherent

education and research programmes. In *Food Safety*, I have attempted to provide a text and framework to initiate such developments. Sound training and high-quality and sustained research are the best pre-emptive measures at our disposal to restore and perhaps even enhance consumer confidence in food.

J.P.F. D'Mello

Glossary

Introduction

As in most other scientific disciplines, understanding food safety involves an appreciation of the particular vocabulary and the technical language that are used to describe the diverse issues that constitute the subject of this book. Although many of the terms and acronyms used are now in common usage outside the scientific community, it was deemed important to provide as comprehensive a list as possible to assist those readers who are new to the field of food safety. Further information may be obtained from appropriate scientific dictionaries, in particular that by Hodgson *et al.* (1998). In addition, several reports by expert groups contain useful glossaries of terms associated with particular contaminants in food (e.g. Ministry of Agriculture, Fisheries and Food, 1992a,b,c, 1994; Pennington Group, 1997).

Definition of Terms and Acronyms

Table 1 lists the major terms and acronyms in alphabetical order. Cross-referencing to specific chapters in this volume is also provided to permit a fuller appreciation of the context of usage of selected terms.

Table 1. Explanation of major terms and acronyms used in this volume.

Term	Meaning
AChE	Acetylcholinesterase (Chapter 1)
Acute toxicity	Severe adverse effects occurring within a relatively short period of exposure to a harmful substance (Chapters 4 and 13)
Adduct	Covalent product of a toxicant or metabolite to large biomolecules such as proteins and DNA (Chapters 4 and 8)
ADI	Acceptable daily intake (Chapters 5, 11 and 12)
AFB_1, AFB_2, AFG_1, AFG_2	Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2: carcinogenic mycotoxins (Chapter 4)
a.i.	Active ingredient; as used in pesticide formulations (Chapter 5)
Anthropogenic	Arising from human activities, e.g. industry (Chapters 5–13, 17 and 18)
ASP	Amnesic shellfish poisoning (Chapter 3)
BA	Bisphenol A (Chapters 12 and 19)

continued

Table 1. *Continued.*

Term	Meaning
BADGE	Bisphenol A diglycidylether (Chapter 12)
BFDGE	Bisphenol F diglycidylether (Chapter 12)
BHA	Butylated hydroxyanisole: antioxidant (Chapter 11)
BHT	Butylated hydroxytoluene: antioxidant (Chapter 11)
Bq	Becquerel: unit of radioactivity; 1 Bq of a radioactive particle undergoes, on average, one radioactive decay per second (Chapter 17)
BSE	Bovine spongiform encephalopathy, also known as 'mad cow disease' (Chapters 14 and 19)
BW	Body weight
Carcinogenic	Causing cancer (Chapters 6, 7, 10 and 17–19)
CAST	Council of Agricultural Science and Technology (Chapter 2)
CEPA	Canadian Environmental Protection Act (Chapter 6)
Chronic toxicity	Adverse effects resulting from prolonged and repeated exposure to small quantities of a harmful substance (Chapters 4 and 13)
Cl_6DD	Hexachlorodibenzo-*p*-dioxin (Chapter 7)
Cl_7DD	Heptachlorodibenzo-*p*-dioxin (Chapter 7)
Cl_8DD	Octachlorodibenzo-*p*-dioxin (Chapter 7)
Cl_4DF	Tetrachlorodibenzofuran (Chapter 7)
Cl_5DF	Pentachlorodibenzofuran (Chapter 7)
Cl_6DF	Hexachlorodibenzofuran (Chapter 7)
Codex Alimentarius Commission	An international body formed by WHO and FAO responsible for establishing standards for food (Chapters 6, 7, 11 and 13)
Critical group	That part of the population which consumes a particular foodstuff at the highest rate (Chapter 17)
CRMs	Certified reference materials: used in quality assurance (Chapter 9)
Cutting plant	Premises used for cutting up fresh meat for human consumption (Chapter 19)
DA	Domoic acid (Chapter 3)
DDT	Dichlorodiphenyltrichloroethane (Chapter 5)
DEFRA	Department for Environment, Food and Rural Affairs (UK)
DEG	Diethylene glycol (Chapter 12)
DEHP	Di-(2-ethylhexyl)phthalate (Chapter 12)
DILs	Derived intervention levels (Chapter 18)
DM	Dry matter
DNA	Deoxyribonucleic acid (Chapter 15)
DON	Deoxynivalenol (Chapter 4)
DSP	Diarrhoetic shellfish poisoning (Chapter 3)
EC	European Commission
ECEH	European Centre for Environment and Health (of WHO; Chapter 7)
EEC	European Economic Community
ELISA	Enzyme-linked immunosorbent assay (Chapters 2 and 3)
EPA	Environmental Protection Agency (USA) (Chapter 6)
EU	European Union (Chapters 3 and 19)
External dose	The exposure of a person to radioactivity (or other contaminant) from outside the body, e.g. soil (Chapter 17)
FAO	Food and Agriculture Organization (of the United Nations)
FB_1, FB_2, FB_3, FB_4	Fumonisins B_1, B_2, B_3 and B_4: carcinogenic mycotoxins (Chapter 4)
FDA	Food and Drug Administration (USA) (Chapters 4, 6 and 19)
FD&C	Food Dye and Coloring (Act) (Chapter 11)
FSA	Food Standards Agency (UK) (Chapter 19)
FW	Fresh weight
GM	Genetically modified (Chapters 15 and 16)
GMP	Good manufacturing practice
GRAS	Generally recognized as safe

Term	Meaning
Gy	Grays; unit of absorbed radiation energy (Chapter 17)
h	Hour(s)
HACCP	Hazard analysis critical control point (Chapter 2)
Half-life	Time taken for the amount of radioactivity to decrease by one half due to physical decay (Chapter 17)
HAS	Hygiene assessment system: used in assessing hygiene standards in licensed slaughterhouses and cutting plants to yield HAS scores
HAV	Hepatitis A virus (Chapter 19)
HCN	Hydrogen cyanide (Chapter 1)
Heavy metals	Collective term for Pb, Hg, Cd and certain other inorganic elements (Chapter 9)
Hepatotoxic	Toxic to the liver (Chapter 4)
HPLC	High-performance liquid chromatography (Chapter 3)
IARC	International Agency for Research on Cancer (Chapters 4, 6, 7 and 10)
ID_{50}	The dose that infects or causes an infectious or toxic response in 50% of a population of test animals in a designated period of time (Chapter 2)
Internal dose	The exposure of a person to radioactivity ingested and incorporated in the body (Chapter 17)
IPCS	Intergovernmental Programme for Chemical Safety (Chapter 7)
JECFA	Joint (FAO/WHO) Expert Committee on Food Additives (Chapters 11 and 13)
LD_{50}	The dose that causes lethality in 50% of a population of test animals in a designated period of time (Chapters 2 and 5)
LOAEL	Lowest observed adverse effect level (Chapter 6)
LOC	Levels of concern (Chapters 18 and 19)
MAO	Monoamine oxidase (Chapter 1)
MBM	Meat and bone meal: now banned as a feedingstuff in EU Member States (Chapters 14 and 19)
MFO	Mixed-function oxidase (Chapter 8)
MHS	Meat Hygiene Service (UK; an agency of the FSA) (Chapter 19)
MJ	Megajoule
MPL	Maximum permitted level: reference level determined by calculating the mean activity concentration in a foodstuff which, assuming consumption over a 1-year period, would lead to an acceptably small dose (Chapter 17)
MRL(s)	Maximum residue limit(s) (Chapters 5, 13 and 19)
MSG	Monosodium glutamate (Chapter 11)
Mutagenic	Causing mutations (Chapters 17 and 18)
Nephrotoxic	Toxic to the kidney (Chapter 4)
NOAEL	No observed adverse effect level (Chapter 6)
NOCs	*N*-nitroso compounds: includes nitrosamines and nitrosamides (Chapter 10)
NOEL	No observed effect level (Chapter 5)
OA	Okadaic acid (Chapter 3)
OECD	Organization for Economic Cooperation and Development (Chapter 15)
OPs	Organophosphates (Chapter 5)
ORs	Odds ratios: used in epidemiological studies (Chapter 10)
OTA	Ochratoxin A (Chapter 4)
OTM rule	Over 30-month rule: in BSE legislation to prevent any OTM cattle (with limited exceptions) from entering the food chain (Chapter 14)
Outbreak	Two or more incidents of disease associated with a common cause (Chapter 19)
PAA	Primary aromatic amines (Chapter 12)
PAHs	Poly(cyclic) aromatic hydrocarbons (Chapters 8 and 19)
PCBs	Polychlorinated biphenyls (Chapters 6 and 19)
PCDDs	Polychlorinated dibenzo-*p*-dioxins (Chapters 6 and 7)
PCDFs	Polychlorinated dibenzofurans (Chapters 6 and 7)
PCR	Polymerase chain reaction (Chapters 2 and 15)

continued

Table 1. *Continued.*

Term	Meaning
Proteomes	Total complement of proteins within a cell (Chapter 15)
Proteomics	Involves use of two-dimensional gel analysis to separate individual proteins present in a particular tissue (Chapter 15)
PrP	Prion protein (Chapter 14)
PSP	Paralytic shellfish poisoning (Chapters 3 and 19)
PVC	Polyvinylchloride (Chapter 12)
RAST	Radioallergosorbent test (Chapter 16)
Reference level	Level of radioactivity (or other contaminant) in a foodstuff above which some action must be taken by regulatory authorities (Chapter 17)
Risk	Probability of ill effects (Chapters 14 and 19)
RLs	Reporting limits (Chapters 5 and 19)
SML	Specific migration limit (Chapter 12)
SRM	Specified risk material: relates to slaughter procedures and legislation controlling BSE contamination of meat (Chapters 14 and 19)
Sv	Sievert: unit of absorbed dose equivalent used to estimate radiation risk (Chapter 17)
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
TBHQ	Tertiary butylhydroquinone: antioxidant (Chapter 11)
TCDD	Tetrachlorodibenzo-*p*-dioxin (Chapters 6 and 7)
TDIs	Tolerable daily intakes (Chapters 4, 6, 7 and 12)
TEFs	Toxicity equivalency factors (Chapters 6 and 8)
TEQs	Toxic equivalents (Chapter 6)
Teratogenic	Causing birth defects (Chapters 4 and 6)
TSE	Transmissible spongiform encephalopathy (Chapter 14)
TWI	Tolerable weekly intake (Chapter 7)
UNEP	United Nations Environment Programme (Chapter 7)
vCJD	Variant Creutzfeldt–Jakob disease (Chapter 14)
WHO	World Health Organization (of United Nations)

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1 Plant Toxins and Human Health

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Introduction

Toxic plants and human illness

Natural substances in plants used for food impact the health of the human species. This chapter focuses on plant products associated with acute illness, chronic disease or developmental perturbation. The reader is referred elsewhere for accounts of the toxic effects of plants used as over-the-counter herbal medicines (Cupp, 2000). Fungal toxins are discussed elsewhere in this volume (Chapter 4).

Public comprehension of plant toxicology is simplistic and naive: a few plants are poisonous and should not be ingested, but all plants used for food are nutritious and lack toxic effects. It is understood that certain plant products when immature may contain poisonous principles, but these are assumed to disappear during maturation. Even pharmacologically active plants are considered health promoting because the chemicals are natural in origin. The public fails to consider the presence in plants of natural substances with toxic potential while clamouring for the exclusion of all traces of anthropogenic chemical contamination. Demand in affluent countries for the freshest fruit and vegetables minimizes postharvest chemical breakdown and therefore tends to magnify the dose of these natural toxins.

Those who study chemicals in plants are impressed with the ingenuity and variety of substances with toxic potential, including those that serve to defend against attack by predators. While chemical defence is a presumed function, the actual physiological roles of plant chemicals that adversely impact human health often are unknown. Individual plants may harbour more than one category of noxious agent; witness the presence of a convulsant (β-cyanoalanine) and cyanide-liberating glycosides (vicianin, prunasin) in the common vetch (*Vicia sativa*) (Roy *et al*., 1996; Ressler and Tatake, 2001). Reminiscent of the shape, size and coloration of the red lentil (*Lens culinaris*), this neurotoxic vetch has been marketed profitably to countries with pervasive poverty (Tate and Enneking, 1992; Tate *et al*., 1999). Similarly, the neurotoxic grass pea (*Lathyrus sativus*), the cause of a crippling motor system disease (lathyrism), has been used to adulterate non-toxic pulses (Dwivedi, 1989). These practices illustrate the importance of protein-rich legumes as a food source and the need for tighter controls on their distribution and use.

The vulnerability of disadvantaged populations in poor countries also arises from their tendency of necessity to rely on

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monotonous diets derived from cheap, environmentally tolerant and often potentially toxic plants. Drought and flood, but also civil disturbance or war, tend to increase dependency on such plants and foster florid disease traceable to natural plant toxins. The root crop cassava (*Manihot esculenta*) is of particular concern because the tuber and leaves of this hazardous plant feed an estimated 400 million people, half of whom reside in Africa (Rosling and Tylleskär, 2000). Outbreaks of irreversible crippling neurological disease among children and adults hallmark southern African communities that subsist on this plant. The drive to expand the production and consumption of the carbohydrate-rich but protein-poor cassava tuber must be accompanied by increased awareness of methods to remove its natural toxins; however, this is not likely to happen, in part because uneducated populations that survive on toxic plants tend to reject an association with illness. Even acutely poisonous species, such as cycads – which kill or paralyse animals after oral ingestion – may be cherished by communities that have used such plants to survive. Humans often cannot grasp the notion that disease may evolve and first appear long after ingestion of a plant product that is nourishing in the short term. The notion that disease may evolve years or decades after exposure to a plant product is a sophisticated concept that requires education to instil. Of course, overt illness is the tip of the iceberg, for populations with epidemic disease triggered by dietary reliance on toxic plants often display a gradation of clinical manifestations and may have symptoms that are subclinical on examination.

Susceptibility to plant chemical toxicity varies with factors such as the maturity of the plant component, soil characteristics and environmental conditions; the potency, dose and duration of exposure to the offending agent(s); differential (target) organ and cellular susceptibility; and factors intrinsic to the affected subject, notably sex and nutritional state. The interaction of these factors determines when disease appears, how severely individuals are affected and the potential for persistence of or recovery from illness. Whereas poorly nourished children and adults who subsist on incompletely detoxified cassava may suddenly develop crippling disease after a few months (Ministry of Health, Mozambique, Mantakassa, 1984), others who are exposed to smaller daily doses seem to experience a slowly developing gait disorder that appears in later years (Osuntokun, 1981).

Precursors or activators

While the bulk of this review is devoted to individual compounds with potential toxicity, plants also provide the precursors and activators of otherwise innocuous substances that, if modified, can act as target organ toxins. The toxic substance potentially could be formed during postharvest treatment or food processing, in the gastrointestinal tract, at stages in intermediary metabolism or in cells of the target organ itself.

Again, the nervous system is a convenient tissue with which to consider this unproven concept, particularly in relation to certain neurodegenerative disorders, notably Parkinson's disease (PD). β-Carbolines (BCs) and isoquinolines (IQs), which occur in a large number of angiosperms, are illustrative plant neurotoxin precursors. BCs, such as norharman and harman, are also formed during the cooking of foods, the elevated temperatures promoting a reaction of tryptophan with aldehyde compounds and subsequent oxidation leading to carboline formation (Collins and Neafsey, 2000). *N*-Methylation of BCs and IQs generates compounds structurally similar to the *N*-methyl-4-phenylpyridinium cation (MPP^+), a proven cause of a PD-like disorder in humans and animals (Fig. 1.1). While BCs and IQs are not taken up by the dopamine-containing nigrostriatal neurones that degenerate in PD, methylated ionic species (e.g. 2,9-dimethylnorharmanium cation) are, like MPP^+, substrates for the dopamine transporter of these nerve cells. *Phalaris tuberosa*, a grass that contains methylated BC-related indole alkaloids (gramine, methyltryptamine and 5-methoxydimethyltryptamine), is

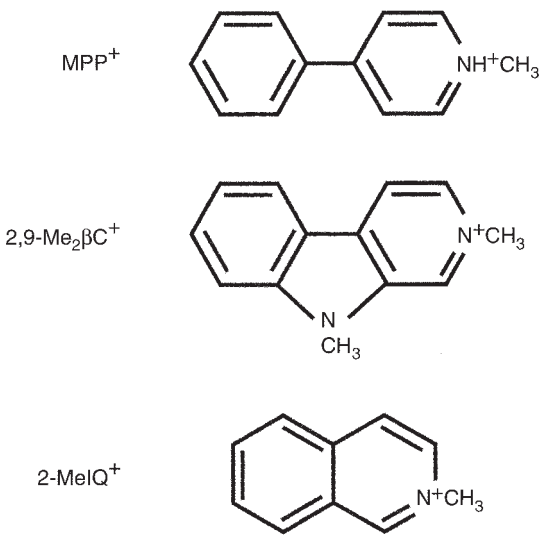

Fig. 1.1. Comparison of the structure of three agents that damage nigrostriatal nerve cells in humans and/or laboratory animals. *N*-Methyl-4-phenylpyridium cation (MPP^+) is the metabolite of a street drug contaminant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, that produced a parkinsonism-like disease in addicts. The neurotoxic, MPP^+-like 2,9-dimethyl norharmanium ion ($2,9\text{-}Me_2\beta C^+$) and 2-methyl-isoquinolinium ion ($2\text{-}MeIQ^+$) are generated by *N*-methylation of plant precursor molecules (Collins and Neafsey, 2000).

causally linked to neurological disease in cattle and sheep that use these plants for food.

Methylation of BCs and IQs conceivably might arise from postharvest seed treatment with a methylating agent (e.g. methyl bromide), through the action of an endogenous methyltransferase in animal tissue or, in a specific unique circumstance, co-exposure to a plant-derived methylating agent, such as methylazoxymethanol (MAM). MAM is the aglycone of cycasin, a toxic glucoside present in seed of the false sago palm (*Cycas* spp.), which through part of the 20th century was a significant source of food in parts of Oceania. On Guam, where the parkinsonism–dementia and amyotrophic lateral sclerosis complex has been rampant, there is a remarkably strong correlation between the historical incidence of this disease and the concentration of cycasin in flour samples used for food by these communities (Spencer, 2000a). Whether cycasin is the culpable agent or whether its aglycone MAM methylates a neurotoxic precursor is unknown.

Nitrogenous Compounds

Plant chemicals with toxic potential can be divided into those containing nitrogen and those lacking this element.

Non-protein amino acids

Plants synthesize hundreds of amino acids, but only about 20 are employed in proteins. The balance – amino acids, imino acids and amides – are secondary metabolites. Non-protein amino acids occur in many unrelated plant families, but they are particularly characteristic of legumes. Several disrupt the nervous system, and others damage the liver, kidney and other organs.

Some of the dicarboxylic plant amino acids mimic the action of glutamate, the principal excitatory neurotransmitter in the human central nervous system (CNS). In culture, micromolar concentrations of these 'excitotoxic' amino acids trigger the influx of

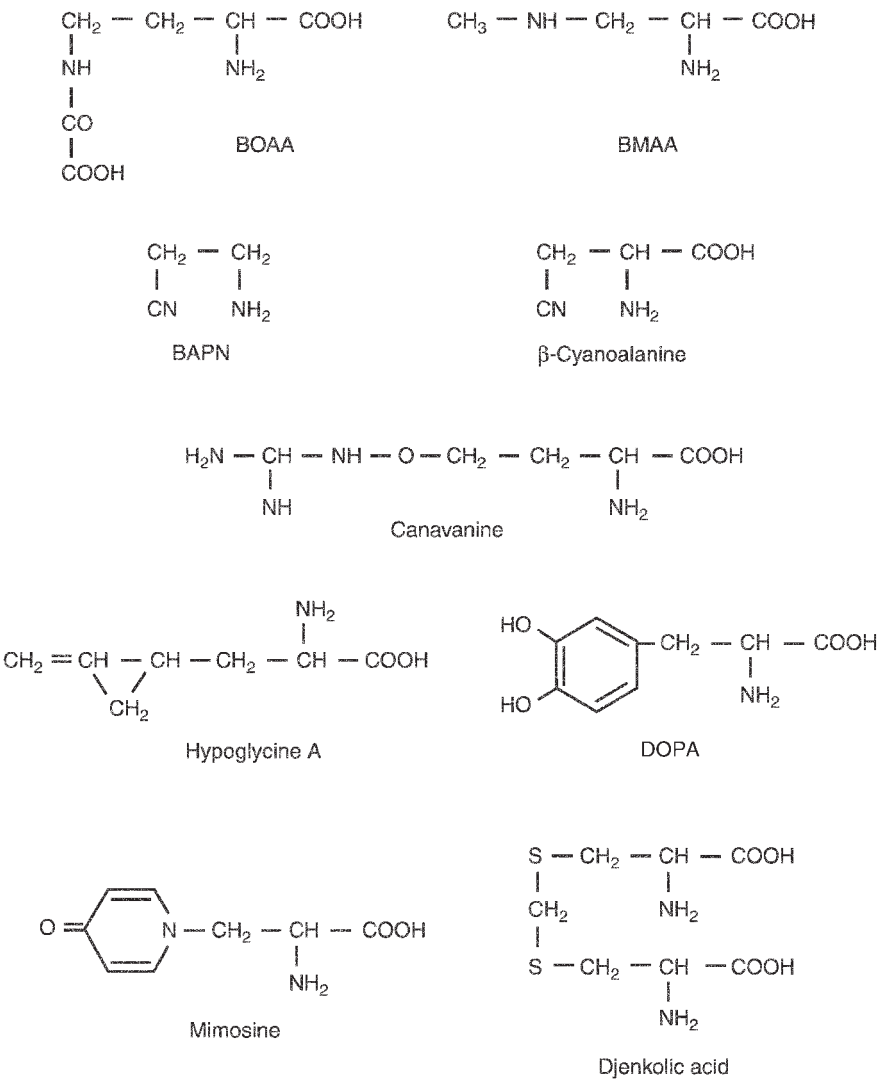

Fig. 1.2. Chemical structures of β-*N*-oxalylamino-L-alanine (BOAA) (*Lathyrus sativus*), β-*N*-methylamino-L-alanine (BMAA) (*Cycas circinalis*), β-aminopropionitrile (BAPN) (*Vicia sativa*), 2-amino-4-(guanidinooxy) butyric acid (canavanine) (*Canavalia ensiformis*), hypoglycine A (*Blighia sapida*), 3,4-dihydroxyphenylalanine (DOPA) (*Vicia faba*), mimosine (*Leucaena leucocephala*) and djenkolic acid (*Pithecolobium lobatum*).

sodium and calcium ions into nerve cells that are equipped with the appropriate ionotropic glutamate receptors. Oedematous swelling and degeneration of nerve cells follow. Excitotoxic amino acids include compounds such as cucurbitine (pumpkin seed), α-amino-β-methylaminopropionic acid, also known as β-*N*-methylamino-L-alanine (BMAA, false sago palm), and γ-*N*-oxalyl-L-α,β-diaminopropanoic acid, also known as β-*N*-oxalylamino-L-alanine (BOAA) (Liener, 1980) (Fig. 1.2). Since some of these amino acids chelate metals, it is conceivable that amino acid levels may reflect soil metal characteristics.

BOAA, a pharmacological agonist of a subclass (AMPA, i.e. α-amino-3-hydroxy-5-methylisoxazole propionic acid)

of glutamate receptors on the plasma membrane of nerve cells, is the active neurotoxic principle in the grass pea (*L. sativus*), prolonged ingestion of which causes lathyrism (syn.: neurolathyrism) (Spencer, 1995). β-Aminopropionitrile (BAPN), an amino acid derivative that occurs in *Lathyrus* spp. as β-(γ-L-glutamyl)-aminopropionitrile, is an inhibitor of lysyl oxidase, an enzyme with an important role in collagen and bone development. Whereas experimental administration of BAPN to rodents leads to joint deformities, ligament separation and skeletal deformities (termed 'osteolathyrism'), prolonged treatment of primates with BOAA induces a neurological disorder (experimental neurolathyrism) characterized by myoclonic jerks, extensor hindlimb posturing and hindlimb weakness, a model of the early, reversible form of human lathyrism (Roy and Spencer, 1989). This and other human disorders that arise from ingestion of toxic amino acids are described below.

Grass pea and lathyrism

L. sativus is an environmentally tolerant and protein-rich legume that is eaten on the Indian subcontinent, in northeastern China and the Horn of Africa. Reliance for a few months on a diet of grass pea precipitates lathyrism, a form of spastic paraparesis, characterized by weakness, increased muscle tone and hyper-reflexia in the lower extremities. Continued ingestion results in progressive walking difficulties that eventuate in permanent inability to move the legs. Lathyrism affects all ages, is often seen in several members of an affected family and sometimes in epidemic form, and usually occurs when other edible material is scarce or unavailable (Spencer, 1995). Since the late 1980s, there has been a coordinated worldwide scientific initiative to control lathyrism through the development of grass pea strains that contain little or no BOAA (http://go.to/lathyrus).

Ackee and vomiting sickness

The ackee tree (*Blighia sapida*) synthesizes water-soluble toxic amino acids – known as hypoglycin A (hypoglycine) and hypoglycin B (γ-glutamyl dipeptide) – that cause severe hypoglycaemia and a hepatic encephalopathy comparable with Reye's syndrome (Spencer, 2000b). Hypoglycine (Fig. 1.2) and its lower homologue, methylene*cyclo*propylglycine, are found together in the litchi (*Litchi chinensis*).

Ackee is a native of west Africa; in the 18th century, the plant was imported into the West Indies, including Jamaica, where the arils of the ripe fruit are used as a staple. 'An ackee a day keeps the doctor away', a line from a popular Jamaican song, is a sentiment that conflicts with medical experience. Ingestion of the arils and seed of unripe fruits causes violent vomiting, convulsions, coma and death (Meda *et al*., 1999). In the Caribbean islands, outbreaks have often been familial, affect poorly nourished children, and occur from November to February when mature ackees are scarce. Hypoglycine also induces fetal malformations in rats (Van Veen, 1973). Hypoglycine is metabolized to methylene *cyclo*propylacetyl-coenzyme-A (CoA), which blocks the transport of fatty acids, acyl-CoA dehydrogenases and neoglucogenesis. This causes an energy deficit, which is compensated by markedly increased carbohydrate catabolism and consequent characteristic hypoglycaemia. Both hypoglycaemia and organic acidaemia are thought to contribute to the toxic effects of hypoglycine (Sheratt, 1995).

Canavanine and systemic lupus erythematosus

The arginine analogue L-canavanine, 2-amino-4-(guanidinooxy)butyric acid, is a toxic basic amino acid widespread in seed of *Leguminosae*. Jackbean (*Canavalia ensiformis*) and lucerne (*Medicago sativa*) contain up to 15,000 ppm of canavanine. A human subject developed autoimmune haemolytic anaemia while participating in a research study that required the ingestion of lucerne seeds (Montanaro and Bardana, 1991). Haematological and serological abnormalities similar to those observed in human systemic lupus erythematosus (SLE) developed in cynomolgus macaques fed lucerne sprouts (Malinow *et al*., 1982). Dietary L-canavanine

sulphate reactivated the syndrome in monkeys in which an SLE-like syndrome had been induced previously by the ingestion of these plant materials. Recent work shows that L-canavanine acts on suppressor-inducer T cells to regulate antibody synthesis. Lymphocytes of SLE patients are specifically unresponsive to L-canavanine (Morimoto *et al*., 1990).

Toxic amino acids and other health conditions

Several other health disorders are recognized in humans and animals that consume plants containing non-protein amino acids (Van Veen, 1973; Liener, 1980). For example, renal dysfunction with haematuria is associated with ingestion of seed (djenkol bean) of the leguminous tree, *Pithecolobium lobatum*, which is eaten in certain parts of Sumatra and Thailand (Vachvanichsanong and Lebel, 1997). The seed contains 1–4% of djenkolic acid (Fig. 1.2), a sulphur-containing amino acid that forms needle-like clusters in the urine. *Leucaena leucocephala* (*koa haole* in Hawaii), another legume that is rarely associated with human illness, contains the toxic agent mimosine (Fig. 1.2) (Van Veen, 1973). Hair loss is the characteristic effect in humans and animals, possibly arising from inhibition of the conversion of methionine to cysteine, a major component of hair protein. Mimosine is metabolized to a goitrogenic agent, 3,4-dihydroxypyridine.

Amines and monoamine oxidase inhibitors

Biologically active amines with pressor (vasoconstrictive) properties are present in a number of common foods. Pressor amines of plants include tyramine, tryptamine and substances (serotonin, adrenaline, noradrenaline and dopamine) that serve as chemical neurotransmitters in the human CNS. Significant concentrations of 3,4-dihydroxyphenylalanine (DOPA) occur in the fava bean (*Vicia faba*) (Fig. 1.2) (Liener, 1980). High levels of pressor amines are found in pineapple, avocado, walnut, plantain and banana, wheat, oats, nuts and tomatoes. Ingestion of serotonin-rich bananas results in elevated excretion of adrenaline, noradrenaline, vanillylmandelic acid, metanephrines and 5-hydroxyindolylacetic acid, a measure of circulating serotonin (Heinemann *et al*., 1981).

Pressor amines and hypertensive crisis

A clinically significant adverse health effect in affluent populations is associated with the ingestion of tyramine-rich foods by individuals using prescribed medications that inhibit monoamine oxidase (MAO), the liver enzyme that normally deaminates pressor amines (Merriam, 2000). MAO inhibition results in high circulating levels of tyramine, which triggers the widespread release of the neurotransmitter noradrenaline. This produces a syndrome characterized by hypertension, headache, diaphoresis, mydriasis, excitation and cardiac arrhythmia. Acute hypertension has the potential to eventuate in intracerebral haemorrhage and myocardial infarction. The syndrome may occur for a period of up to 3 weeks following drug discontinuation because MAO levels recover slowly. Prescriptions for these drugs should therefore include instructions to avoid tyramine-rich foods, including aged cheeses, aged meats, herring, concentrated yeast extracts, sauerkraut, broad bean pods, tap beer and red wine. Beans, wheat, nuts and tomatoes have also been reported to trigger headache in individuals treated with MAO inhibitors (Liener, 1980).

Proteins

Some plants used for food harbour proteins that trigger allergic reactions, or bind to cells and disrupt their function, or disrupt the breakdown of proteins. These are considered below.

Proteinase inhibitors

Many raw plant products tend to depress the growth rates of animals, although the significance for human health has yet to be resolved. Reduction of normal growth is

associated with exposure to heat-resistant proteinase inhibitors that serve as highly specific substrates for the respective plant enzymes. These substances are widely distributed in seeds (legumes), fruits (avocado, peach, plum, tomato and aubergine), tubers (potato) and vegetative parts (soybean, lucerne, barley, maize and wheat) of dicotyledons and monocotyledons. Best studied are the inhibitors of serine-type proteinases, including the soybean trypsin inhibitor, the soybean proteinase inihibitor, the potato I and II inhibitor families, the squash inhibitor family and the α-amylase/trypsin inhibitor family of cereal seeds. Sulphydryl proteinase, acid proteinase and metalloproteinase inhibitors are also recognized (Xavier-Filho and Campos, 1989).

Plant proteinase inhibitors that inhibit the action of digestive proteinases can produce adverse health effects. Ingestion of raw soybean reduces proteolysis of dietary protein, causes increased secretion of pancreatic enzymes and impairs body growth of laboratory species. Feeding experimental animals on diets containing isolated soybean trypsin inhibitors (the Kunitz soybean trypsin inhibitor (STI) and the Bowman–Birk trypsin–chymotrypsin inhibitor (BBI)) caused insignificant growth depression in rats and chicks, but induced enlargement of the pancreas in rats, chicks and mice but not in pigs, dogs, calves, monkeys and presumably humans (Birk, 1996). Potatoes contain compounds that inhibit all of the major pancreatic endo- and exopeptidases of the digestive tract of higher animals (Pearce *et al*., 1985). Prolonged feeding of rats and mice with a diet rich in potato and soybean trypsin inhibitor produced short-term pancreatic hyperplasia in both species and long-term nodular hyperplasia and acinar adenoma in rats (Gumbmann *et al*., 1989). Where humans fit on the scale of differential susceptibility to the pre-neoplastic and neoplastic effects of potato trypsin inhibitors on the pancreas is unknown.

Lectins

Lectins are heat- and protease-resistant carbohydrate-binding proteins that bind to red blood cells and cause haemagglutination. Since lectins are widely distributed in the seeds and vegetative parts of plants, especially *Leguminosae* and *Graminaceae*, the human gut is regularly exposed to dietary lectins. One study identified over 50 edible plants with lectin activity, including many in fresh (lettuce and fruit) and processed foods (cereals and nuts) (Nachbar and Openheim, 1980). Lectins may bind to mannose/galactose (concanavalin A from jackbean), *N*-acetylglucosamine (potato and wheat germ lectins) or *N*-acetylgalactosamine/galactose (ricin and kidney bean lectin). It has been stated that 'lectins constitute one of the major antinutritive factors of foods of plant origin, and their presence in food may have very serious consequences for growth and health' (Pusztai, 1989).

Extensive experimental animal studies have been conducted with the lectin of *Phaseolus vulgaris*, which comprises 10–15% of the total protein content of the red kidney bean (Pusztai, 1989). Inclusion of raw kidney bean in the diet of young and mature rats results in rapid weight loss and eventual death. Kidney bean lectins are highly resistant to proteolytic breakdown in the gut, and they bind to and inhibit endo- and exopeptidases that function in food breakdown. They bind to, perturb and damage intestinal enterocytes, and reduce the absorptive surface of the small intestine, which undergoes hypertrophy and hyperplasia. The consequent reduction in the absorption of nutrients from the gut promotes protein catabolism, decreases stores of subcutaneous lipid and hepatic glycogen, greatly amplifies urinary urea, and results in loss of body weight. Additional effects include pancreatic enlargement accompanied by reduced insulin circulation and involution of the thymus. Lectins are also endocytosed by intestinal cells and enter the circulation bound to unidentified blood cells. Lectins interfere with the gut immune system, and animals fed kidney beans develop immunoglobulin (Ig)G- and IgE-mediated hypersensitivity to the specific lectin.

Many other common food plants contain heat-labile lectins that compromise intestinal integrity, interfere with intestinal absorption and have adverse effects on body growth.

These include lectins from soybean, tepary bean (*Phaseolus acutifolius*), runner bean (*Phaseolus coccineus*), lima bean (*Phaseolus lunatus*), jackbean (*Canavalia ensiformis*), winged bean (*Psophocarpus tetragonolobus*), pea (*Pisum sativum*) and red lentil (*Lens culinaris*) (Pusztai, 1989). Ewen and Pusztai (1999) recently claimed that diets containing genetically modified potatoes expressing the snowdrop (*Galanthus nivalis*) agglutinin (GNA) had variable effects on different parts of the rat gastrointestinal tract, including a GNA transgene-associated proliferation of the gastric mucosa.

LECTINS AND COELIAC SPRUE (GLUTEN ENTEROPATHY) Intestinal toxicity triggered by wheat germ agglutinin contaminating gluten in cereal foods has been implicated in an intestinal malabsorption disorder (coeliac sprue) associated with intolerance to gluten. Severity of gluten enteropathy varies with the extent of the loss of jejunal villi. Coeliac sprue has been considered to have a large genetic component, but the rising age of onset and changing clinical pattern and prevalence suggest that diet or other environmental factors make an important contribution to aetiology (Auricchio and Visakorpi, 1992). Changes in infant feeding practices (doubling or tripling of wheat protein intake) that took place in Sweden in the 1980s appear to have played an important role in an unexpected rise in incidence of coeliac disease (Cavell, 1992).

Children from 6 months to 3 years of age may have diarrhoea, projectile vomiting and a bloated abdomen. Behavioural changes, such as irritability and restlessness, characterize children with coeliac sprue. Speech development is often markedly impaired, the vocabulary limited to a few words, and the intonation is soft and whining. Other signs include food craving, retarded growth, weight loss, chronic fatty diarrhoea, abdominal cramping and distension, and myopathy associated with weakness and fatigue. Liver, joint, haematological, dental and neuropsychiatric symptoms may occur. There may be difficulty in concentration, decreased mental alertness and impaired memory. The disease is conservatively estimated to have a prevalence of 0.1% in Europe (Troncone *et al*., 1996). Over 75% of patients with coeliac sprue respond to a gluten-free diet, with symptoms usually improving within weeks. Patients are instructed to avoid food products prepared from wheat, rye, barley and oats. For affected children, the provision of a gluten-free diet may result in a marked decrease of neuropsychological phenomena (Dohan, 1976).

The basis for the neuropsychiatric manifestations of coeliac sprue is not understood. One possibility is that neuroactive peptides produced during digestion of food proteins cross the defective gut barriers and enter the brain via the systemic circulation. This idea has also been advanced to explain the neurobehavioural perturbations of schizophrenia, a disorder that has been related to coeliac disease. Specifically, it has been suggested that schizophrenia may be genetically linked with coeliac disease, and that cereal grain proteins may be pathogenic in individuals with schizophrenia (Dohan, 1969). A recent report described how a gluten-free diet resulted in the regression of both schizophrenic symptoms and an accompanying frontal cortex hypoperfusion (demonstrated by single-photon emission computed tomography) in a 33-year-old patient with coeliac disease (De Santis *et al*., 1997). However, studies of small intestine permeability in 24 schizophrenic patients failed to reveal significant differences from normal subjects (Lambert *et al*., 1989). Attempts to demonstrate links between coeliac disease and childhood autism have also proved unsuccessful (Pavone *et al*., 1997).

Nut protein allergens

Proteins in plant products used for food, especially various types of nuts, may elicit an acute-onset, dose-independent, type-1 immunological reaction. This involves production of IgE antibodies directed toward the plant protein, release of endogenous chemicals from mast cells (e.g. histamine, bradykinin and serotonin) that mediate inflammation, and the rapid development of anaphylaxis, an illness that can prove fatal. Clinical manifestations include oedema of the lip, urticaria, asthma, hypotension, coma and even death (Angus, 1998; Taylor *et al*., 2001).

Possible sources of contact with nut allergens, other than direct ingestion of the plant product, include exposure *in utero* or via breast milk, or through infant formula and vitamin preparations containing nut oils. One cohort study found that, by the age of 4 years, approximately 1% of English children are sensitized to peanuts or treenuts (Tariq *et al*., 1996). Children who suffer from allergic rhinitis or bronchial asthma appear to be at greatest risk for nut allergy. Exposure to only trace amounts of nut protein may be sufficient to trigger an allergic response, and allergy acquired at an early age may persist throughout life (Hourihane, 1998). Fortunately, cross-reactivity among nut proteins is rare, but cross-reactions occur with other allergens in food and other plant materials (latex and grass). Nut allergy is therefore a significant public health problem that will be likely to continue to grow in association with increasing reliance on legumes as abundant sources of cheap protein.

Many allergic responses to nuts are triggered by ingestion of peanuts, the shelled cotyledon pairs of the legume *Arachis hypogaea* (Angus, 1998). The cotyledons are rich in protein (25–28% by weight), including the major (Ara h I, Ara h II) and minor (agglutinin) peanut allergens. In the early 1990s, an estimated 65–85 severe reactions to peanuts occurred annually in the UK (Angus, 1998). Reactions to peanut and treenut allergens accounted for more than 90% of fatalities in a more recent analysis of 32 fatal cases in the US (Bock *et al*., 2001). Allergy to crude peanut oil is also reported, but refined peanut oil reportedly appears to be safe for most people who suffer from peanut allergy.

Ingestion of treenuts, the edible kernels of the seed of several trees, may also cause immunological illness (Taylor *et al*., 2001). Sweet almonds (*Amygdalus communis*) and bitter almonds (*Prunus amygdalus*) comprise 22% protein and contain multiple IgE-binding proteins that may trigger severe allergic reactions. Brazil nuts (*Bertholletia excelsa*, *B. myrtaceae*), ingestion of which has triggered allergic reactions in children and adults, contain 14% protein, including a methionine-rich protein (Ber e 1) that constitutes the major allergen (Bush and Hefle, 1996). Cashews (*Anarcadium occidentale*), like other members of the *Anacardiaceae* (mango, poison ivy and pistachio), have caused contact dermatitis and severe anaphylaxis among asthmatic children, as have hazelnuts (*Corylus avellana*) and pecans (*Carya illinoinensis*). Pistachios (*Pistacia vera*), which likewise cause allergic reactions in sensitized individuals, have a major (mol. wt 34,000 Da) and several minor IgE-binding proteins. Pine nuts (*Pinus edulis*) contain at least three proteins that bind human IgE (Koepke *et al*., 1990). Walnuts (*Juglans regia*) have also been aetiologically implicated in human anaphylaxis (Angus, 1998).

Several nuts and seeds other than treenuts and legumes (groundnuts) may trigger allergic reactions. Sunflower seed (*Helianthus annuus*) has caused anaphylactic reactions after ingestion and dermatitis on skin contact. The seed (and oil) of sesame (*Sesam indicum*), which reportedly contains nine allergens (Malish *et al*., 1981), have caused severe allergic reactions. Ingestion of coconut (*Cocos nucifera*) rarely triggers allergic reactions.

Glycosides

Several plants eaten by humans possess a binary chemical system that presumably is produced as a means for chemical defence. The two chemical elements are innocuous in isolation and, like nerve agent precursors designed by humans for chemical warfare, lethal when mixed together. One component consists of an inactive form of the ultimate toxic agent, inactive because it is bound to sugar molecules (commonly D-glucose) to form a glycoside (e.g. glucoside) (Fig. 1.3). The second component, which is stored in a separate cellular compartment, is a hydrolytic enzyme (e.g. β-glucosidase) that is designed to cleave the glycoside and release the toxic aglycone. Damage to plant cells brings the β-glycosidase in contact with the glycoside, thereby releasing the noxious agent (the aglycone), which itself may be metabolized to other toxic species. Release of the aglycone may occur during insect or

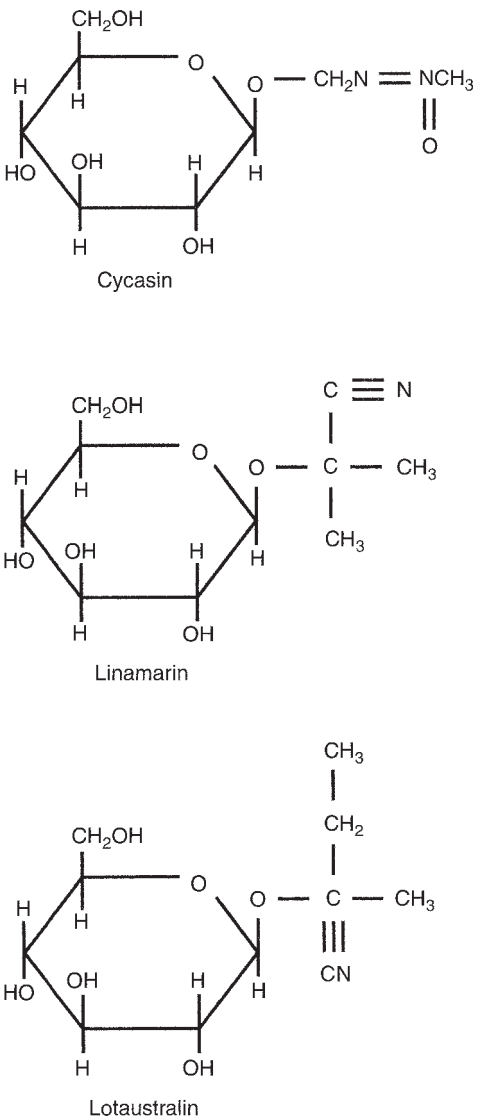

Fig. 1.3. β-Glucosides that harbour toxic agyclones. Cycasin (methylazoxymethanol-β-glucoside) from *Cycas* spp. (top), and cyanide-containing linamarin and lotaustralin from *Manihot esculenta*.

animal attack, through bruising, during food preparation or through the action of intestinal microflora. Some aglycones have strong odours, others have marked toxicity and some are teratogenic. While these properties may be sufficient to ward off attack by many members of the animal kingdom, humans are rarely deterred; in fact, in some cases (e.g. cassava and almonds), the toxic principles may be exploited for their bitter taste!

There is a further, largely unrecognized and uninvestigated potential toxicity of glycosides, in particular those that employ glucose as the carrier for the toxic aglycone. The pancreas continuously monitors blood glucose because the molecule must be available to the nervous system and other organs for normal function. Neurones, pancreatic β-islet and other cells are equipped with glucose transport systems that shuttle required supplies of glucose to intracellular sites of metabolism and energy generation. These glucose transport systems may be unable to discriminate between a glucose molecule and a glucoside. Once inside the cell, the glucoside can be cleaved by a β-glucosidase, thereby generating an intracellular biocide with cytotoxic potential. While little studied, this mechanism may have importance in populations that have a high incidence of conditions such as diabetes mellitus and neurodegenerative disease (Eizirik *et al*., 1996).

Four groups of glycosides present in plant products ingested by humans are considered next: (i) fava glycosides, which harbour substances able to cause red blood cell rupture (haemolysis); (ii) thioglycosides (glucosinolates) in *Brassica* and other widely consumed vegetables, which liberate odoriferous and thyrotoxic substances; (iii) cyanoglycosides in cassava and sorghum, which harbour agents that attack the thyroid, brain and, possibly, the pancreas; and (iv) azoxyglucosides of cycads, the aglycone of which is a mutagen, carcinogen, hepatotoxin and developmental neurotoxin, with strong epidemiological links with amyotrophic lateral sclerosis and parkinsonism–dementia complex (ALS-PDC). Other glycosides (i.e. solanum, isoflavone and β-sitosterol glycosides) are discussed in later sections in this chapter.

Cycads and neurodegeneration

Studies of aboriginal groups in Australia suggest that the poisonous seed of glycoside-containing cycads (e.g. *Cycas* spp.) have been eaten throughout human history. Cycads, the contemporaries of dinosaurs, are

gymnosperms that store the potent alkylating aglycone methylazoxymethanol (MAM) in the form of glycosides such as cycasin (MAM-β-D-glucoside) (Fig. 1.3). Australian aborigines have developed elaborate and thorough detoxification methods that consist of crushing, drying, soaking, fermenting and pulverizing cycad seed contents prior to cooking the resulting paste. Similarly, in the Ryukyu Islands of Japan, residents have employed fermentation processes to render the seed and sago (from the inner parts of the overground stem) free of cycasin (P.S. Spencer, personal observations). Failure to detoxify cycad materials may result in acute illness characterized by liver damage, coma and death. In the southern Marianas Islands, notably Guam, cycad seed may be soaked for only short periods of time and then left to dry in the sun. Flour derived from these incompletely detoxified materials contains varying concentrations of cycasin and other materials such as the neurotoxic amino acid BMAA (Kisby *et al*., 1992). Epidemiological studies on Guam have shown an exceptionally strong correlation between the concentration of MAM (but not of BMAA) in cycad flour and the age-adjusted incidence of ALS-PDC in the Chamorro communities from which the flour was derived (Zhang *et al*., 1996). While a causal relationship between cycad and ALS-PDC has yet to be established, it is well known that ingestion of cycad leaves (*Macrozamia*, *Cycas* spp.) induces neuromuscular disease in grazing animals. Moreover, MAM perturbs brain development by disrupting cell division and migration, resulting in ectopic, multicellular entities that are reminiscent of those seen in Chamorros with ALS-PDC (Spencer, 2000a). This is a key part of the evidence suggesting that this prototypical neurodegenerative disease, which generally appears in the second half of life, may be acquired in the late pre-natal or early post-natal period. Given that the brains of Chamorro people show the hallmarks of brain ageing much earlier than those of other people, the general importance of these observations in understanding brain ageing cannot be overemphasized. More specifically, it should be noted that cycad stems yield the finest quality sago, a product of many plants (notably *Metroxylon* spp.) imported after the Second World War from the Dutch East Indies (Indonesia) that was used to feed British and perhaps other schoolchildren who are now approaching retirement. Additionally, until 1926, a cycad species (*Zamia floridana*) was harvested and processed in Florida, USA, for the production and regional distribution of Florida arrowroot (Spencer, 1990).

Cassava (manioc) and multiorgan disease

Whereas human ingestion of cycad seed is geographically restricted, minimal and declining, consumption of another toxic plant, cassava (*Manihot esculenta*), is widespread, massive and steadily rising. A native of South America, cassava was probably carried by Portuguese explorers in the 16th century first into Africa and then throughout the discovered world (Jones, 1959). The introduction of cassava met with widespread acceptance because its tuber and leaf provide a valuable and reliable source of carbohydrate and protein, respectively. Current estimates cite cassava consumption by 400 million people worldwide, mostly in the tropics and subtropics, a majority of which lives in Africa (Rosling and Tylleskär, 2000). Cassava is currently considered to be a valuable export crop and its penetration now includes Europe and North America.

The root and leaves of cassava harbour linamarin and lotaustralin (Fig. 1.3), two of the more than 50 stable cyanogenic (cyanide-liberating) glucosides that have been isolated from a similar number of plant species, several of which are used by humans for food (Tewe and Iyayi, 1989). Sweet potato or yam, maize, bamboo, chick pea and sorghum are also able to liberate hydrogen cyanide. Cassava and lima beans, a leguminous species that is widely eaten, are documented causes of acute cyanide toxicity (Conn, 1973; Rosling and Tylleskär, 2000). Cassava, sorghum and lima bean stand out because they are likely to be heavily consumed by human populations subject to nutritional shortage resulting from war, civilian disruption or climatic extremes. Since these events occur among populations that rarely attract medical and scientific attention, there is little appreciation of the adverse

health impact associated with cyanogenic plants such as cassava. In brief, reliance on cassava is an established cause of goitre and neurodegeneration, and it may also be an aetiological factor in a tropical form of diabetes mellitus (Bokanga *et al*., 1994).

Cyanogenic plants such as cassava contain a binary chemical defence system consisting of glucosides and an enzyme specific for the β-glucosidic linkage. Degradation of glucoside takes place under enzymatic and base hydrolysis to yield β-D-glucopyranose and acetone cyanohydrin (2-hydroxyisobutyronitrile); the latter dissociates to hydrogen cyanide (HCN) under the action of hydroxynitrile lyase. As with cycad seed on Guam, traditional methods of cassava tuber preparation (soaking, drying and crushing) may leave residual glycoside or cyanohydrin; hence, ingestion may result in acute HCN intoxication. HCN is absorbed rapidly from the gastrointestinal tract and produces recognizable effects in both fatal (0.5–3.5 mg kg^{-1}) and non-fatal dosages as a result of the inhibition of cytochrome oxidase, a key enzyme in energy generation for the brain. Some plant varieties ('bitter cassava') eaten raw induce seizures, coma and death, with the possibility of concomitant brain damage expressed in the form of delayed-onset parkinsonism or dystonia among survivors. Headache and gastrointestinal upset follow ingestion of the less acutely toxic 'sweet' varieties. Both sweet and bitter forms are under widespread cultivation, the latter to promote pest resistance and, after incomplete detoxication, for the quality of their taste.

An important public health problem arises from heavy dietary reliance on incompletely detoxified cassava among protein-poor populations, particularly in western and southern Africa (Rosling and Tylleskär, 2000). In Nigeria, for example, cassava root (38 mg HCN 100 g^{-1}) is eaten as *gari* (1.1 mg HCN 100 g^{-1}) and *purupuru* (4–6 mg HCN 100 g^{-1}) in amounts up to 750 g day^{-1}, which correspond to 8 mg and 32–48 mg HCN, respectively (Osuntokun, 1981). The minimal lethal HCN dose in humans is 35 mg. HCN is metabolized by reaction with sulphane sulphur to thiocyanate (SCN) through the catalytic action of rhodanese, an enzyme that is widely distributed in animal tissues. The thiocyanate ion (SCN^-) inhibits the uptake of iodine by the thyroid gland and may cause goitre when the iodine content of the diet is low (VanEtten and Wolff, 1973). Higher levels of SCN^- inhibit the formation of thyroxine and related compounds even when the iodine supply is marginal. In the 1960s, goitre was widespread in eastern Nigeria where a dry, unfermented form of cassava formed a major component of the diet.

The major concern arising from heavy cassava consumption is its effect on the developing and adult nervous system. While unproven, there is a strong possibility that chronic HCN exposure promotes miscarriage and adversely impacts the developing brain. That this concern has neither been discussed nor investigated in relation to cassava-consuming populations is shocking. There is, however, recognition that cassava dependency is associated with neurodegenerative disease in adults, but this condition appears to be confined to populations that have protein-poor diets associated with heavy or exclusive dependency on cassava (Rosling and Tylleskär, 2000). While SCN^- may have a role in neurotoxicity by increasing binding of glutamate to AMPA-type glutamate receptors on target neurones, under states of sulphur deficiency HCN may be metabolized by a minor pathway to cyanate (OCN^-), an established cause of peripheral neuropathy in humans and spasticity in primates (Spencer, 1999). Minimally nourished children and women who are reliant on poorly detoxified cassava are prone to be stricken with leg weakness and spasticity, which may be accompanied by visual and hearing deficits. Epidemics of cassava-associated spastic paraparesis (*konzo* and *mantakassa*) arising from degeneration of motor nerve cells in the cerebral cortex are reported from cassava-reliant regions of Mozambique, Zaire and the Central African Republic, among others. Affected subjects are left with a persistent crippling disease (Rosling and Tylleskär, 2000).

Related to this disorder is a condition described from west Africa known as tropical ataxic myeloneuropathy, a slowly evolving illness of adults that affects the brain, spinal cord and peripheral nerves (Osuntokun,

1981). A similar condition has been reported among Senegalese who subsist on a diet of millet (sorghum) (Conn, 1973). Also reported among elderly Nigerians, but never confirmed, is a high incidence of a unique neurodegenerative disorder of the elderly that conceivably could represent the effects of prolonged, low-level cassava intoxication.

There are other adverse health effects that may be associated with cassava (Bokanga *et al*., 1994). One is a form of tropical diabetes mellitus (type III) that has been found in cassava-consuming populations. While the association between cassava and diabetes has been questioned on the basis of negative results in animals chronically treated with cyanide (Soto-Blanco *et al*., 2001), this does not exclude the possibility that cyanogenic glucosides enter and destroy β-islet cells. A second potential adverse health effect of cassava can be deduced from advances in cancer research. Thiocyanate, the principal metabolite of HCN, is a particularly effective catalyst for the formation of carcinogenic nitrosamines through the action of sodium nitrite on a secondary amine (Archer, 1984). This concern may be relevant to the reported use of cassava as a meat extender for use in hamburgers, since meat may be treated with sodium nitrite as a preservative.

Glucosinolates and goitre

The more than 100 known glucosinolates are sulphur-containing glycosides found exclusively in cruciferous plants, notably in seed. The highest concentrations are found in *Resedaceae*, *Capparaceae* and *Brassicaceae*. Species containing glucosinolates include mustard, rape, swede, crambe, kale, turnips, cabbage, cauliflower, broccoli, Brussels sprout and radish; the last five comprise the major source of glucosinolates in the human diet. Radishes are an important component of the Japanese diet, whereas glucosinolate-rich Brussels sprouts contribute heavily to the British diet. An estimated 5% of the UK population consumes up to more than 300 mg of glucosinolates daily; in 1975, Japan had a daily estimated consumption of approximately 100 mg (radish, daikon, cabbage plus fermented root and leaf vegetables), while mean daily intake for North Americans is approximately 15 mg. Boiling reduces and fermentation destroys glucosinolates such that, in the UK, mean daily intake calculated for cooked vegetables amounts to approximately 30 mg (Fenwick *et al*., 1989).

Glucosinolates make up one component of a binary chemical system that delivers substances with insecticidal properties and pungent odours. The second component is an endogenous enzyme, myrosinase (thioglucoside glucohydrolase), which is stored in the plant separately from the glucosinolates. Bruising, cutting and chewing the plants activate the chemical defence system through enzymatic cleavage of the glucosinolate to yield an unstable aglycone (thiohydroxymate-*O*-sulphonate). Elimination of sulphur leads to the pH-dependent formation of isothiocyanate, nitrile or thiocyanate. Glucosinolates and isothiocyanates protect against chemical carcinogenesis in rodents although, as noted before, thiocyanates (which survive after cooking) in the presence of nitrite would probably favour the formation of carcinogenic nitrosamines (Archer, 1984).

There is considerable evidence showing that glucosinolate-rich plant components have adverse effects on the health and growth of animals. In several species, rape or crambe seed meal decreases feed intake and growth while enlarging the liver, kidney, thyroid and adrenal glands (Verkerk *et al*., 1998). For humans, the principal concern is possible depression of thyroid function associated with glucinosolate derivatives. Thiocyanate and certain isothiocyanates are goitrogenic in states of iodine deficiency. Other metabolites, notably *S*-5-vinyl-oxazolidine-2-thione (goitrin) from rapeseed, interfere with thyroxine synthesis and therefore promote goitre irrespective of iodine status. Goitre has been attributed to the consumption of large amounts of cabbage or of kale containing thiocyanate, isothiocyanate and goitrin. A 1956 survey of children in Tasmania attributed enlarged thyroid glands to the consumption of milk from dairy cattle fed kale. However, contemporary surveys in England and The Netherlands in areas where crucifer forages were used for dairy cattle gave no

indication that cow's milk was goitrogenic (VanEtten and Wolff, 1973). More recently, consumption of goitrogenic substances (cabbage, kale and sulphonamide) was found to be a major risk factor in a study of thyroid nodules among a population of 430 Serbo-Croatian patients, most of whom were women with a mean age of approximately 50 years (Obradovic, 2000).

Fava beans and favism

Vicine and convicine are glycosides primarily associated with the fava (broad) bean (*V. faba*), an important source of protein for populations in the Mediterranean, North Africa, Middle East and Far East, notably China (Mager *et al*., 1980; Marquardt, 1989). The content of glycoside, which is highest in the seed, varies by maturity, environmental factors and genetic variation. Cooking has little effect on glycoside content. Ingested glycosides (vicine and convicine) are hydrolysed by intestinal microflora to the aglycones divicine and isouramil, respectively, the apparent causes of a potentially fatal haemolytic disorder in susceptible humans known as favism. The oxidized form of isouramil, which has structural relationships with the pancreatic β-islet toxin alloxan, may have diabetogenic effects (Ashcroft *et al*., 1986).

Susceptibility to fava beans is associated with an inherited X-chromosome-linked systemic deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49). This is one of the most common genetic polymorphisms in the human population: over 400 variants have been described and an estimated 200 million people are affected worldwide. G6PD is required by red blood cells for the maintenance of adequate levels of reduced glutathione and nicotinamide adenine dinucleotide phosphate, which serve as cellular antioxidants. Divicine and isouramil (in addition to a number of therapeutic drugs, including certain antimalarials and antimicrobials) serve as free-radical generators that promote formation of hydrogen peroxide. In the absence of adequate antioxidants, peroxide induces the formation of methaemoglobin, protein cross-linking, loss of red cell shape and haemolysis. Acute haemolytic anaemia usually occurs in G6PD-deficient children (especially males) within hours of ingestion. Symptoms result from a reduction in the oxygen-carrying capacity of the bloodstream. Mildly affected individuals experience malaise, headache, nausea, vomiting, chills, shortness of breath, lumbar pain and fever. Severely affected neonates and children may develop jaundice, haemoglobinuria and renal failure (Luisada, 1941).

The prevalence of G6PD deficiency is highest among the Kurds, Iraqis, Sardinians, Cypriot Greeks, African-Americans and certain African populations. A 1979 study in Sicily found more than 10% of male subjects with G6PD deficiency, with most cases of favism arising from ingestion of fresh fava beans but, in addition, cases associated with breast feeding and pollen inhalation (Schiliro *et al*., 1979). Temporal changes in favism incidence have been reported in Sardinia, where G6PD screening and health education began in 1971. In the period 1961–1970, there were 508 cases of favism, of which 76% occurred in boys. In contrast, during the period 1981–1990, there were 144 cases, of which only 52% occurred in boys. The relative increase of favism in girls was attributed to the possible failure of the screening method to detect all subjects with heterozygous G6PD deficiency (Meloni *et al*., 1992).

Alkaloids

Alkaloids are basic nitrogenous compounds in which the nitrogen is usually contained within a heterocyclic ring system. Some affect the nervous system, others perturb fetal development, and pyrrolizidine alkaloids damage the liver.

Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) are found in 13 plant families, including *Compositae* (*Asteraceae*), *Boraginaceae*, *Leguminosae*, *Apocyanaceae*, *Ranunculaceae* and *Scrophulariaceae*. Their structure is based on two fused five-member rings that share a nitrogen

atom, and they exist in plants either as the esterified alkaloid, the corresponding *N*-oxide or both. Human contact occurs through the use of various toxic species as herbs, 'health foods' (Cupp, 2000), food supplements, green vegetables and food contaminants. Comfrey plants (*Symphytum* spp.), which find use as vegetables and tea, repeatedly expose consumers to PAs such as intermedine, lycopsamine, symphytine and others (Bruneton, 1999; Coulombe, 2001), as do a number of plants (*Petasites, Symphytum and Tussilago*) used by the Japanese in food (Crews, 1998). Humans may also be exposed to PAs from plants visited by honeybees and by herbivores that secrete milk.

PAs are important causes of human illness and a significant threat to human health, especially in less developed countries subject to drought and famine (Huxtable, 1989; Crews, 1998). Ingested PAs are bioactivated in the liver to form highly reactive dehydroalkaloid pyrroles that alkylate DNA, RNA and proteins. The principal outcome is liver damage in the form of veno-occlusive disease, hepatic venous thrombosis, ascites, jaundice and, probably, an elevated risk of liver cancer. Secondary targets of pyrroles derived from PAs include the lungs, heart, kidney, stomach, reproductive system and brain (Huxtable, 1989). Monocrotaline is pneumotoxic. Atypically, neurological effects (vertigo, headache, delirium and coma) in the absence of overt liver toxicity occurred among Uzbeks in the 1950s after consumption of grain contaminated with seed of *Trichodesma incanum*.

Herbal use of PA-containing legumes of the genus *Crotalaria*, together with *Senecio* spp., is held responsible for past outbreaks of veno-occlusive disease and ascites in Jamaica (Huxtable, 1989). Seasonal endemic veno-occlusive disease in Madhya Pradesh, India, is attributed to contamination of millet with *Crotalaria nana* pods (Krishnamachari *et al*., 1977). Afghanistan was the setting in 1976 for a large outbreak of veno-occlusive disease resulting from the consumption of bread prepared from grain contaminated with seed of *Heliotropium popovii* (Mohabbat *et al*., 1976). In the 1970s, herbal teas prepared from *Senecio longilobus* caused liver disease, hepatomegaly, jaundice and fatalities among American children (Huxtable, 1980).

Solanum alkaloids

The toxic alkaloids of the potato plant (*Solanum tuberosum*), α-chachonine and α-solanine, are saponin-like alkaloids that exist in the form of β-D-glycosides (Sharma and Salunkhe, 1989). These substances inhibit cholinesterase enzymes: butyrylcholinesterase (BuChE), which is concentrated in liver and lungs and serves as an important defence against toxic substances; and acetylcholinesterase (AChE), which is required to terminate the transmitter action of acetylcholine at the neuromuscular junction. Since BuChE and AChE hydrolyse and inactivate several anaesthetic drugs (cocaine, heroin, esmolol and local ester anaesthetics) and neuromuscular-blocking agents, ingestion of potatoes may impact the metabolism and duration of action of these substances during and following surgery (McGehee *et al*., 2000).

Changes in the glycoalkaloid content of fresh and processed potatoes may occur during storage, under the influence of light and radiation, following mechanical damage and as a result of food processing (Friedman and McDonald, 1999). Human toxicity from ingestion of green potatoes with a high solanum glycoalkaloid content is associated with gastric pain, weakness, nausea, vomiting and laboured breathing.

The potential for teratogenic effects has been a significant public health concern in relation to populations consuming large amounts of potato. The concern arises from studies with Syrian hamsters. Animals treated orally with potato sprouts containing solanidine, the common aglycone of α-chaconine and α-solanine, have litters with craniofacial malformations that result in herniated or exposed brain tissue, defects in the nasal chamber, a single eye and a cleft palate. Salasodine, another teratogenic substance, is present in potato cultivars and in related food plants, namely *S. melongena* (aubergine) and *S. quitoense* (Andean *naranjilla*). A 1972 report suggesting that certain birth defects in humans are caused by ingestion of blighted potatoes (infested with *Phytophthora infestans*)

has not received experimental support (Allen *et al*., 1977). Note that tomatidine, the aglycone of the related glycoalkaloid tomatine, lacks a teratogenic property.

Lupin alkaloids

These substances include a large number of quinolizidine alkaloids with toxic (lupanine > sparteine > lupinine in guinea pigs) or teratogenic (anagyrine) properties found in *Lupinus* spp. Their presence and concentration in the protein-rich seed of these legumes vary with species and environmental factors. Those with high alkaloid content tend to have a bitter taste, are associated with acute toxicity in humans and animals and, in the latter, with a congenital skeletal malformation known as 'crooked calf disease' (Keeler, 1989). No anagyrine was found in several 'bitter' and 'sweet' selections of lupins used as human food (Keeler and Gross, 1980). It has been suggested that sweet lupin flour may be used for the improvement of protein supply if the alkaloid content does not exceed 0.02% and the seed contains no secondary fungi that cause lupinosis (Gross *et al*., 1976). Lupin seed flour has been investigated as a component of infant formula and bread. Those sensitized to groundnut may have allergic responses to lupin flour after ingestion or inhalation exposure (Crespo *et al*., 2001).

Isoquinoline alkaloids

These alkaloids are said to be more numerous and cover a wider range of structural types than those of any other group (Bentley, 1998). Benzyltetrahydroisoquinolines, which are formed from dopamine and phenylacetylaldehyde, are pivotal intermediates in the metabolism of isoquinoline alkaloids. Several isoquinoline alkaloids are active on the nervous system, including tubocurarine (neuromuscular blocker), apomorphine (dopamine D2 receptor agonist), morphine (enkephalin agonist), colchicine (spindle inhibitor and axonal neurotoxin), lycorine and galanthamine (cholinesterase inhibitors). A form of parkinsonism and motor neurone disease in Guadeloupe, French West Indies, has been tentatively linked with the use of herbal teas prepared from *Annonaceae* (custard apple and paw-paw family) (Caparros-Lefebvre and Elbaz, 1999). These plants contain tetrahydroisoquinolines (TIQs) such as reticuline and higenamine, as well as non-TIQ compounds (acetogenins) that block mitochondrial respiration (Bruneton, 1999). In addition to these edible tropical fruits, TIQs are found in a variety of widely consumed food items of plant (banana) and other origins (Nagatsu, 2000).

Carboline alkaloids

β-Carboline indole alkaloids occur in a number of plants and, together with α-, γ- and δ-carbolines, as pyrolysis-induced tryptophan condensations and rearrangements as a consequence of grilling of proteinaceous foods. These compounds form co-mutagenic derivatives and also possess neurotoxic activity (Wakabayashi *et al*., 1997). The β-carbolines of the passion flower (*Passiflora incarnata*), for example, include harman (motor depressant and convulsant) and its 7-oxygenated derivatives harmine and harmaline, both of which are hallucinogenic. β-Carboline analogues of MPP^+ (Fig. 1.1), such as 2-*N*-methyl- and 2,9-*N*,*N*-dimethyl-harminium and harmalinium derivatives, inhibit mitochondrial respiration and are toxic to dopaminergic neurones (Collins and Neafsey, 2000).

Non-nitrogenous Compounds

Phyto-oestrogens and anti-oestrogens

Many plant species contain active principles that act as contraceptives, interceptives, abortifacients, uterine stimulants, antispermatogens, spermicides and phyto-oestrogens. Most are beyond the scope of this chapter; however, the phyto-oestrogens are of considerable current interest because of their significant presence in plants used for food (Helferich *et al*., 2001).

Phyto-oestrogens and anti-oestrogens lack the steroid ring structure of oestrogen, the mammalian steroid hormone that regulates

and maintains female sexual characteristics, but they nevertheless have properties similar to the principal human oestrogen 17β-oestradiol (Aldridge and Tahourdin, 1998). Their ability to disrupt reproductive performance has been recognized in sheep grazing on subterranean clover (*Trifolium subterraneum*) and cattle fed lucerne (*M. sativa*). Feminization of male animals has been reported following ingestion of phyto-oestrogens during critical periods of development. This and other health concerns have resulted in intense scrutiny of the effects of phyto-oestrogens on reproductive health, development and cancer risk. Vegetarians and certain ethnic groups have the highest exposure to phyto-oestrogens.

Isoflavone glycosides

Isoflavones make up the majority of phyto-oestrogens found in food. These compounds are linked to a sugar molecule as *O*-glycosides (genistin, daidzin and glycitin). Hydrolysis to the corresponding biologically active aglycones (genistein, daidzein and glycitein) may occur during fermentation or through the action of microflora in the gut. Soybeans and sprouts are a rich source of isoflavones, and soy foods constitute the main source of phyto-oestrogens in the human diet. Infants are exposed through the use of soy-based infant formula or via breast milk of mothers who ingest large amounts of soya products. The oestrogenic potential of genistein in an *in vivo* assay has been estimated to be two to four orders of magnitude lower than that of 17β-oestradiol.

β-*Sitosterol*

Plant oils, such as groundnut, sunflower and olive oils, contain the highest concentration of this major phytosterol of higher plants. β-Sitosterol (BSS), together with its glycoside, β-sitosterolin (BSSG), has been implicated in the feminization of fish in the vicinity of pulp mill effluents (Bruneton, 1999). Animal studies have demonstrated that BSS and BSSG possess anti-inflammatory, antipyretic, antineoplastic and immune-modulating properties. BSS has been used without adverse health effects for the long-term treatment of prostatic hypertrophy (Klippel *et al*., 1997).

Coumestans, lignans and other

Non-glycosidic plant substances with oestrogenic activity include the coumestans (coumestrol), found in lucerne, mung bean, clover sprouts, soybeans, lima beans and red beans, and the lignans, precursors of which occur in grains, seeds, berries and nuts (Helferich *et al*., 2001). Coumestrol is the most potent of the phyto-oestrogens, with biological activity relative to 17β-oestradiol some five times higher than that of genistein (Aldridge and Tahourdin, 1998). Lignans (enterolactone and enterodiol) form in the gut from plant precursors (matairesinol and secoisolariciresinol, respectively) (Setchell *et al*., 1980). Zearalenone, a myco-oestrogenic substance produced by *Fusarium* spp. growing on mouldy maize (Chapter 4), has been implicated in fertility problems in pigs and cows. Anti-oestrogenic compounds (indole-3-carbinol) occur in cruciferous vegetables, such as cabbage, broccoli and Brussels sprouts. Safe levels for human consumption of oestrogenic and anti-oestrogenic compounds have yet to be established (Helferich *et al*., 2001).

Ptaquiloside

The 'fiddleheads' of bracken fern (*Pteridium aquilinum, P. esculentum*) are consumed as greens and salads in various parts of the world, such as Japan. The plant contains a number of toxic substances and, in particular, a potent alkylating glycoside and carcinogen known as ptaquiloside. Livestock grazing on bracken fern develop bladder cancer, bone marrow depression, leukaemia, thrombocytopenia and a haemorrhagic syndrome. Laboratory rodents fed bracken fern develop malignant tumours of the bladder, lung and intestine, and the milk of cows fed bracken fern is carcinogenic to rats. The high incidence of oesophageal cancer among the Japanese has been attributed to dietary use of bracken fern. Since ptaquiloside is heat labile, the

cooked fronds of bracken fern do not contain detectable amounts of ptaquiloside (Sato *et al*., 1989).

Alkenylbenzenes

Several spices, essential oils, herbs and certain vegetables (parsnips, parsley and sesame seed) contain structurally related alkenylbenzenes; these form epoxy intermediates that develop covalent adducts with guanine and act as weak rodent hepatocarcinogens (Luo and Guenthner, 1996). Alkenylbenzenes present in food include safrole (1-allyl-3,4-methylenedioxybenzene), a component of sassafras tea, oil of sassafras (*Sassafras albidum*) and nutmeg (*Myristica fragrans*). Tarragon, basil and fennel contain the related compound estragole (methylchavicol). Isosafrole, a component of the flavourant oil of ylang-ylang (*Cananga odorata*), and β-asarone, a component of oil of calamus (*Acorus calamus* root), are also rodent carcinogens (Coulombe, 2001). Another alkenylbenzene, myristicin, the major flavour of nutmeg (*M. fragrans*) and also present in black pepper, parsley, dill and carrots, is not thought to be carcinogenic but instead, in large quantities, is allegedly hallucinogenic. Piperine, which is responsible for much of the pungent flavour of black pepper (*Piper nigrum*), forms potentially carcinogenic intermediates (nitrosamines) in the presence of nitrite. Capsaicin, the pungent component of chilli peppers (*Capsicum frutescens* and others), is questionably a weak carcinogen and better known as an experimental neurotoxin selective for substance P-containing nerve cells. D-Limonene, a major constituent of oils obtained from the peel of citrus fruit (orange, lemon and grapefruit), causes renal tumours in rats but is not considered harmful to humans (Coulombe, 2001).

Coumarins

Over 1000 coumarins (2*H*-1-benzopyran-2-ones) have been described, and the simplest among them are widely distributed among plants as water-soluble glycosides (Bruneton, 1999). Coumarin, first isolated from the tonka bean (*Dipteryx odorata*), is found in vegetables (cabbage, radish and spinach) and plants used as flavouring agents or herbs (lavender, sweet woodruff and sweet clover). Coumarin, used in human medicine as an anticoagulant, is metabolized rapidly in the liver to form the hepatotoxin 7-hydroxycoumarin.

Psoralen

Psoralens are straight-chain furanocoumarins that are activated by sunlight to phototoxins with mutagenic and carcinogenic properties. They are found in celery, parsnips, limes, cloves and figs (Coulombe, 2001). 8-Methoxypsoralen (xanthotoxin) is a carcinogenic species used in combination with ultraviolet (UV) irradiation (PUVA) to treat patients with psoriasis and mycosis fungoides. Long-term PUVA treatment has been associated with squamous-cell carcinoma and melanoma many years after onset of treatment (Bruneton, 1999).

Conclusions

Several conclusions emerge from the foregoing selected summary of plant toxins.

1. Many plants manufacture, store and release chemicals with the potential to cause human illness.

2. These chemicals, some of which may be designed for defence, are frequently stored as a binary system. A widely exploited binary system employs a glycoside (a sequestered form of the toxic agent linked to glucose) and a glycosidase (the mechanism by which the toxic agent is released), which are stored in separate parts of the plant. Ingestion of the plant product may result in: (i) release of sequestered toxic substance through the action of microbial or tissue glycosidases; and/or (ii) uptake of intact glycosides by way of cellular glucose transport systems and subsequent intracellular enzymatic release of the agent at a remote site. Cells with plasma

membranes rich in glucose transporters (e.g. nerve cells and β-islet pancreatic cells) theoretically are at greatest risk for toxic damage.

3. Humans consume plants that are incompletely detoxified: while they are equipped to detect and reject acutely toxic materials, plant products containing levels of hazardous chemicals that cause delayed illness (months, years or decades) are sufficiently palatable to be ingested, especially in settings where safer foodstuffs are unavailable.

4. Long-term effects of low-level exposure to phytotoxic chemicals are little studied, even when the material is widely ingested, whether in the setting of poverty or affluence. For example, dried roasted *Coffea arabica* bean is reported to contain atractyloside (17.5–32 mg kg^{-1}), a diterpenoid glycoside that in larger doses (10- to 20-fold) causes fatal renal proximal tubule necrosis and/or centrilobular hepatic necrosis in humans and animals (Obatomi and Bach, 1998). In general, much less scientific attention is directed towards the safety of plant products consumed by populations at greatest risk for plant toxicity, namely protein-poor people who subsist on single staples.

5. In the short term, there is a need to increase public understanding of the true nature of plant materials, namely that they contain substances that are beneficial and others that are hazardous to health; ideally, the latter should be removed prior to ingestion. Of special concern is the ever widening food use of cassava root, which, when consumed after incomplete detoxication, is associated with a range of chronic health disorders.

6. In the long term, the extraordinary complexity of plant chemistry suggests that efforts to develop diets to promote optimal human health and longevity may represent a futile search of vast expense. An alternative approach would be for humans to expand knowledge of their nutritional and related needs, with the long-term goal of developing a synthetic diet that would support life and maintain optimal health. Adoption of this strategy would reduce and ultimately eliminate plant-related human morbidity and mortality; it would also support the species' ambition to colonize outer space and other parts of the universe.

Note Added at Proof Stage

Recent studies show that the toxic agent acrylamide is formed in certain plant products subjected to high cooking temperatures associated with frying, grilling and baking. Formation is proposed to occur through a Maillard reaction between sugars and amino acids, such as asparagine, methionine and cysteine (Stadler *et al.*, 2002). The highest levels of acrylamide (> 1000 μg kg^{-1}) are found in potato crisps; lower levels (150–350 μg kg^{-1}) occur in maize crisps, potato chips, biscuits, toast, cereals and coffee powder. Cooked foods derived from other plant (and animal) products are under scrutiny at the time of writing. The estimated average dietary intake is in the order of 0.5–1 μg kg^{-1} body weight day^{-1}, with two- to threefold higher levels for children.

While acrylamide is known to cause peripheral neuropathy following occupational exposures, with structural damage to both the central and peripheral nervous systems, doses from food are not anticipated to be of sufficient magnitude to induce comparable changes in the general public. Similar considerations diminish concerns in relation to animal studies demonstrating testicular toxicity. However, animal studies demonstrate that acrylamide, and/or its metabolite glycidamide, is genotoxic and able to induce somatic and germ cell damage, with induction of benign and malignant tumours (thyroid, adrenal gland, brain, spinal cord) and heritable damage at the gene and chromosomal level. Since these toxic effects of acrylamide have no known threshold, the substance is classified as a probable human carcinogen. Epidemiological studies of populations with occupational exposure to acrylamide have not revealed increased occurrence of cancer, but the detection sensitivity of these studies has been low. Research recommendations have been made to increase understanding of the public health threat posed by low-level exposure to acrylamide derived from food (WHO/FAO, 2002).

Acknowledgements

Valerie Palmer, Suzanne Spencer and Meg Heaton are thanked for their helpful comments.

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2 Bacterial Pathogens and Toxins in Foodborne Disease

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Introduction

Foodborne disease mediated by pathogenic microorganisms or microbial toxins is an important global public health problem. Foodborne disease has been defined by the World Health Organization (WHO) as 'a disease of infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water' (World Health Organization, 1997). Foodborne disease takes a huge toll on human health and mortality: in the USA alone it has been estimated that microbial foodborne illnesses number in the millions, causing several thousand deaths, with an economic burden of about $5 billion dollars annually (CAST, 1994; Mead *et al*., 1999). Globally, the WHO has estimated that approximately 1.5 billion episodes of diarrhoea and more than 3 million deaths occur in children under 5 years of age, and a significant proportion of these results from consumption of food contaminated with microbial pathogens and toxins (World Health Organization, 1997). These estimates of foodborne illnesses are probably 100–300 times less than the actual occurrence for a variety of reasons (Bryan *et al*., 1997; Lund *et al*., 2000). The annual incidence of foodborne illnesses in industrialized countries has been estimated to affect 5–10% of the population annually, and in many developing countries the incidence is probably considerably higher.

Foodborne diseases or illnesses are commonly classified into two main categories: (i) infections commencing within the gastrointestinal (GI) tract; and (ii) poisonings or intoxications resulting from consumption of pre-formed toxins in foods. This classification, however, is overly simplistic and does not take into account the wide spectrum of foodborne illnesses and intoxications, as well as chronic disease syndromes that can develop following acute foodborne infections. The classification was expanded to encompass five major modes of acute foodborne illness (Granum and Brynestad, 1999): (i) intoxications in which a pre-formed microbial toxin in a food is consumed; (ii) toxicoinfections in which a toxin is produced in the intestinal tract in the absence of adherence to epithelial cells in the GI tract; (iii) illnesses caused by production of an enterotoxin following adherence of pathogens to epithelial cells in the GI tract but without bacterial invasion of intestinal cells; (iv) illnesses caused by bacterial infection of the GI tract with mucosal and intestinal cell penetration and usually production of enterotoxin, but in which the infection does not become

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systemic; and (v) systemic infections following bacterial GI tract colonization and penetration through the intestinal barrier.

The production and maintenance of a safe food supply depends on an understanding of the biological and virulence properties of food-poisoning organisms. Foods and their surroundings can be considered as selective environments that allow growth or survival of certain groups of microorganisms. Some of these microorganisms can be beneficial to the quality and safety of a food, such as many yeasts and lactic acid bacteria, others are innocuous, while still others are pathogens and can present safety hazards in foods. In this chapter, basic principles of bacterial foodborne infections and intoxications are described, which is followed by a description of the aetiological agents and toxins, and strategies for their control in foods.

History of Foodborne Disease and Beginning Concepts

Microorganisms documented to cause food poisoning comprise approximately 50 species of fungi, bacteria and viruses (reviewed in Lund *et al*., 2000). Recognition of associations between food and disease came about long before an understanding of microbiology, and some of the seminal events have been traced in history to Moses, the Romans, Cato (234–194 BC), Pliny the Elder (AD 23–79), the fall of the Roman Empire, the Dark Ages, 17th and 18th century England, and into the modern era (Hutt and Hutt, 1984). Moses spoke of foods that should not be eaten by the Israelites because of their propensity to cause illness, and he also provided advice on food handling practices. Beginning the pre-modern era of epidemiology, the causal association of water and disease was realized in the famous investigations of John Snow, who reported in 1851 that drinking water could spread cholera, and this in turn led to filtration methods to eliminate the unknown agent (Hobbs and Gilbert, 1978). The causative agent, *Vibrio cholerae*, was not discovered until the 1880s by Robert Koch. William Budd demonstrated in the mid-1800s that typhoid fever could be spread by milk. These seminal events clearly showed an association of food and water with infectious diseases.

Knowledge of the actual microbial causes of foodborne disease began when Pasteur and Koch founded the science of microbiology, allowing microbiologists to isolate, characterize and systematically describe microorganisms associated with spoiled or poisonous foods (Brock, 1961; Tannahill, 1973). Up until this time, the organisms causing most GI-mediated diseases were of unknown aetiology. The first description of a documented food-poisoning bacterium was in 1888 by Gaertner, who isolated a bacterium (a *Salmonella* species) from meat and the organs of a man who had died from food poisoning after eating the contaminated food (Hobbs and Gilbert, 1978). Landmark legislation was denoted in the USA in the 1906 Pure Food and Drugs Act and its successor the 1938 Federal Food, Drug, and Cosmetic Act (Middlekauf and Shubik, 1989). It has become apparent that protecting the safety and wholesomeness of foods is an important discipline fulfilled by legislators, industry and researchers.

The documented association of microorganisms and food- and waterborne disease formed a conceptual foundation for hygiene, sanitation and food preservation. It also contributed significantly to the science of epidemiology (Evans and Brachman, 1991). Foodborne disease surveillance began in the USA in the early 1900s as a response to the morbidity and mortality caused by typhoid fever and infantile diarrhoea (Centers for Disease Control and Prevention, 2000). In 1939, a public health bacteriological service was instituted in Great Britain, and in 1950 the Public Health Laboratory and the Department of Health and Social Security pooled and tabulated their reports on food poisoning and the documented disease agents (Hobbs and Gilbert, 1978). These early surveillance systems established the foundation for epidemiological study of foodborne diseases. Guidelines for establishing and evaluating surveillance systems and epidemiological analyses have been described (Evans and Brachman, 1991; Bryan *et al*., 1997; Guzewich *et al*., 1997; Centers for Disease Control and

Prevention, 2000). The availability of several well-designed surveillance studies (Bean and Griffin, 1990; World Health Organization, 1997; Mead *et al*., 1999; Centers for Disease Control and Prevention, 2000) has been very important in documenting foodborne disease agents and elucidating trends, changing patterns and discovery of emerging or re-emerging pathogens. Advances in the fields of surveillance and epidemiology have demonstrated the enormous impact that foodborne disease has on morbidity, mortality and economic losses throughout the world. In developing countries, foodborne disease is among the leading causes of morbidity and mortality, particularly among children, and has been considered to be a leading factor impeding technological progress (Miller and Taylor, 1989).

Virulence and Foodborne Disease

Of several thousand species of bacteria in the microbial world (Holt, 1984–1989; Dworkin, 1999; Fischetti *et al*., 2000; Lund *et al*., 2000; Madigan *et al*., 2000), only about 40 species have been documented to cause foodborne disease. The main taxonomic groups and genera of bacterial pathogens are presented in Fig. 2.1. Similarly, hundreds to thousands of bacterial species are commonly present in human foods, but only a few of these present a hazard to the consumer. The occurrence in foods of certain pathogens is clearly undesirable and may render a food unpalatable or unsafe. Bacteria vary tremendously in their pathogenicity, or their capacity to cause disease.

The quantity of a pathogen or toxin in food required to produce illness is correlated with the virulence of the agent. Virulence is a term that describes the infectivity of the pathogen and the severity of the illness that it produces. Virulence factors are those phenotypic properties of a pathogen that when lost, for example by mutation, decrease the pathogenicity but not the viability under laboratory conditions. The phenotypic characteristics that determine the pathogenicity of microorganisms can be defined by determining the effects of mutations in certain genes. For example, mutants of *Salmonella typhimurium* impaired in their ability to survive within macrophages were no longer virulent when injected intraperitoneally into a mouse (cited in Johnson and Pariza, 1989). These mutant bacteria were shown to have mutations in specific genes. The individual genes and gene products were elucidated, thus defining the specific virulence factors.

The two principal classes of virulence factors in bacteria are toxins and surface molecules, although other classes of molecules can also affect virulence of many pathogens. These two main classes of virulence factors are broad and diverse, and among foodborne pathogens they vary greatly in structure and mode of action. The primary extracellular protein toxins causing true intoxications are botulinum and staphylococcal toxins, which vary markedly in properties including structure, mechanism, and resistance to heat, acid and proteolytic degradation. Similarly, surface molecules also include a number of different molecules that provide various biological functions such as adherence factors, capsules that resist phagocytosis and immune responses, flagella for motility, molecules determining receptor binding and chemotaxis, and so forth. Since virulence factors are traits that are not required for viability of the pathogen, they may be produced in a temporal and variable manner, particularly in response to host factors. Their production can vary markedly from strain to strain, and they may be expressed at certain points in the growth cycle or under specific nutritional conditions. Maintenance and expression of virulence genes depend upon a balanced genome structure in bacterial strains and species (Relman and Falkow, 2000). Virulence is a highly polygenic property of bacteria and has evolved to be compatible with the overall genome structure and physiology of the pathogen. Thus, the insertion of a virulence gene into most distantly related non-pathogenic bacteria would not result in the formation of an effective pathogen. Pathogens have been found to have a clonal population structure, in which they carry specific arrays of virulence-associated genes (see Fischetti *et al*., 2000; Relman and Falkow,

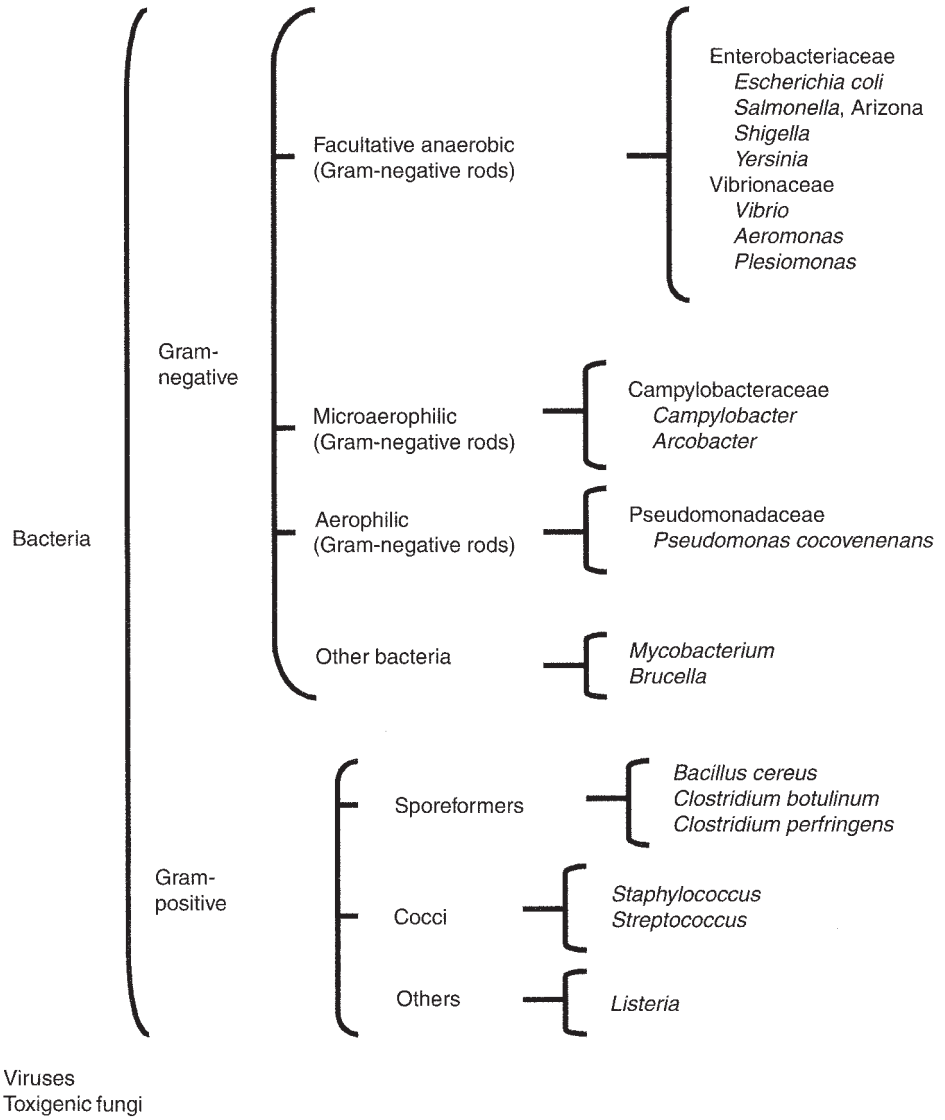

Fig. 2.1. A taxonomic grouping of the principal foodborne pathogens (expanded from Johnson and Pariza, 1989).

2000). For example, although many clonal lineages of *Escherichia coli* persist in the human intestinal tract, only a few lineages such as *E. coli* O157:H7 are able to cause illness.

A variety of powerful methods using molecular biology have become available to identify virulence genes and their expression *in vitro* and *in vivo* on infection within the host (Relman and Falkow, 2000). Study of virulence genes has shown that they frequently are associated with mobile genetic elements such as bacteriophages, transposons and plasmids, and may occur in distinct chromosomal regions called pathogenicity islands. Some of these mobile elements carrying virulence genes can be transferred horizontally to recipient bacteria and, in the proper environment and particularly under genetic selection, can be maintained in the recipient. An excellent example of acquisition of traits beneficial to

pathogenicity is the transfer of genes encoding antibiotic resistance, enabling a normally sensitive organism to gain resistance to the anti-microbial agent. These genes can be maintained in the absence of selection if the recipient contains a genome structure and physiology that enable the productive regulation and expression of the virulence- or resistance-encoding genes. Methods to identify virulence genes, mechanisms governing the expression of virulence factors and horizontal gene transfer are an extremely active area of research, and excellent treatises are available on the subject for both Gram-negative and Gram-positive bacteria (see Fischetti *et al*., 2000; Mandell *et al*., 2000, as examples).

The virulence of a pathogen or toxin, i.e. the infectious dose to cause disease or the potency of a toxin, often is expressed quantitatively as the ID_{50} or LD_{50}. These values represent the dose that infects or causes an infectious or toxic response (e.g. lethality) in 50% of a population of test animals in a designated period of time. The ID_{50} and LD_{50} are chosen to quantify virulence or toxicity because of the nature of the dose–response relationship (Fig. 2.2). The curves in the upper panel demonstrate that the rate of change in mortality (slope of the curve) as a function of dose reaches a maximum at the point of about 50% survival. Curves with greater slopes give a more accurate estimate of toxin concentration or infectious dose. The sigmoid shape of the ID_{50} or LD_{50} results primarily from the chance distributions of lethal events in any given animal, although the heterogeneity of the animal population may also be a factor in certain cases. The type, strain, health and other features of the animal will also influence the shape of the curve and resulting ID_{50} or LD_{50}. For these reasons, determining the ID_{50} and LD_{50} is often the most appropriate method for determining the dose required for illness in experimental animals.

Recognition of Pathogenic Bacteria Causing Foodborne Disease

The recognition of a bacterium as an aetiological agent of foodborne disease usually is first indicated by epidemiological evidence,

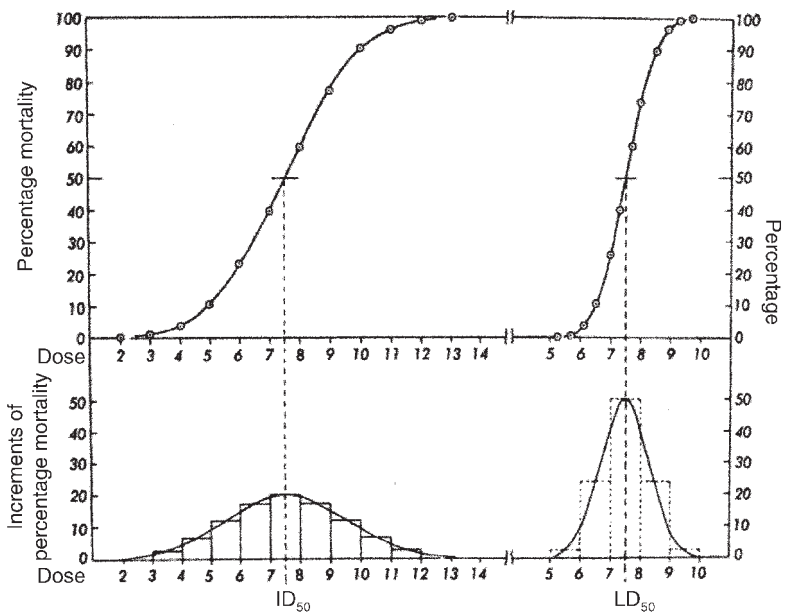

Fig. 2.2. Examples of dose–response curves used to quantitate bacterial virulence and lethality of toxins. The infecting dose is plotted horizontally in logarithmic units. The ID_{50} or LD_{50} is calculated by extrapolating to the dose that causes 50% infection (ID_{50}) or toxicity (usually fatality; LD_{50}). From Wilson and Dick (1983).

in which the occurrence of an illness in a human epidemic is examined and found to correlate with the consumption of a food (Evans and Brachman, 1992). A foodborne illness outbreak occurs when two or more people have a similar illness after eating a common food, and microbiological evidence, as described later in the chapter, implicates the food as the vehicle (an exception is botulism, where one case is considered to be an outbreak). The epidemiological investigation ideally is established by diagnosis of suspected aetiological agents from clinical samples and the causative food.

When a connection between a food consumption and disease is suggested, the investigator tries to satisfy the following criteria to demonstrate the microorganism as the causal agent: (i) the organism is isolated from the food and from the sickened host and cultured on artificial growth media; (ii) the organism is characterized and shown to be identical from the two sources; (iii) inoculation of the organism to an experimental animal model produces a closely similar disease; and (iv) the organism is recovered from the site of infection of the animal and shown to be the same as the pathogen originally inoculated. These criteria, patterned after the famous Koch's postulates, can be extremely useful in establishing an unrecognized pathogen as the aetiological agent of foodborne disease. Unfortunately, the criteria often cannot be satisfied because some organisms cannot be grown on artificial culture media, a suitable animal model for testing of pathogenicity is not available, the disease is caused by more than one pathogen or because the specific cause of disease is due to extracellular products of the organism, such as toxins formed outside the host, rather than the organism itself. In practice, many foodborne disease outbreaks are diagnosed by first examining the onset time of illness and the symptoms, and then isolating the likely aetiological agent(s) or its toxin from the food and clinical samples (e.g. vomitus, faeces, blood or organs) of the victim(s). The successful epidemiological investigation coupled with the aetiological diagnosis can facilitate both short- and long-term control measures.

Surveillance and Epidemiology of Foodborne Disease

Microbial food poisoning is caused by the consumption of a food that is contaminated with harmful levels of pathogenic organisms or microbial toxins. The major taxonomic groups and genera of bacteria that are species that have been documented to cause foodborne disease are portrayed in Fig. 2.1. The recognition of these pathogens has come about through collaborative efforts of scientists in a variety of disciplines including epidemiology, public health, microbiology, medicine and others. Surveillance and epidemiological analysis often initially provide evidence of a causal relationship, and this can lead to isolation and characterization of the suspected aetiological agent. However, some infectious agents such as many viruses and parasites as well as prions are difficult or impossible to culture, and diagnosis will depend on alternative methods of detection.

Surveys of microbial pathogens and toxins transmitted in foods have been published in several useful compilations (Bryan, 1982; Bean and Griffin, 1990; World Health Organization, 1997; Petersen and James, 1998; Mead *et al*., 1999; Centers for Disease Control and Prevention, 2000; Lund *et al*., 2000). Overall, most of the summaries agree in their conclusion that bacterial pathogens are responsible for the majority (> 80%) of outbreaks, cases and deaths. Members of the Enterobacteriaceae, particularly *Salmonella* serovars, enteropathogenic *E. coli* and *Shigella* spp., and members of the Campylobacteraceae, *Campylobacter jejuni* and *C. coli*, are responsible for the majority (> 70%) of foodborne bacterial illnesses. Of secondary importance are toxicoinfections by *Clostridium perfringens* and *Bacillus cereus*, intoxications by staphylococcal enterotoxin, *B. cereus* emetic toxin and botulinum neurotoxin, and infections by *Vibrio* spp., *Streptococcus* spp. and *Listeria monocytogenes*. Less common foodborne pathogens in US and UK surveys include *Aeromonas hydrophila*, various species and strains within genera of the Enterobacteriaceae (*Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Yersinia* and others),

Arcobacter spp., certain *Bacillus* spp., *Brucella* spp. and *Mycobacterium* spp. The two organisms with the highest death to case ratios are *L. monocytogenes* and *Clostridium botulinum*, but *Salmonella* strains (particularly typhoidal serovars and highly virulent non-typhoidal strains), vibrios, such as *Vibrio cholerae* O1 and *V. vulnificus*, and certain other virulent bacterial foodborne pathogens can cause deaths. Severe illnesses and fatalities occur most commonly in persons with underlying infections or diseases, and in individuals suffering from malnutrition or immune deficiency. Infants and the elderly are also more susceptible to foodborne diseases than is the general population. Recent compilations indicate that foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations and 1800 deaths in the USA each year (Mead *et al*., 1999). Of these, 1500 deaths have been attributed to *Salmonella*, *L. monocytogenes* and *Toxoplasma* (Mead *et al*., 1999). Obviously, the magnitude is much greater on a global scale, but the actual incidence is difficult to assess because of the lack of surveillance systems and public health resources in many countries.

Epidemiological investigations in industrialized countries have indicated that the spectrum of foodborne disease agents is changing over time (Altekruse *et al*., 1997; Mead *et al*., 1999; Centers for Disease Control and Prevention, 2000). Formerly, the most commonly recognized foodborne pathogens or toxins were *Salmonella*, *C. perfringens* and staphylococcal enterotoxin (Bryan, 1982), but the incidence of the latter two aetiological agents in causing disease has decreased over time in the USA and UK. Certain pathogens including antibiotic-resistant *Salmonella* serovars (e.g. DT104), *E. coli* O157:H7, *L. monocytogenes*, parasites such as *Giardia*, *Cryptosporidium*, *Cyclospora* and *Toxoplasma*, and human enteric viruses (particularly Norwalk virus) are now among the most frequent food-transmitted pathogens (Mead *et al*., 1999). The changing spectrum is emphasized by the fact that some pathogens of greatest concern, including *C. jejuni*, *E. coli* O157:H7, *L. monocytogenes* and *Cyclospora cayatenensis*, were not recognized as significant causes of foodborne illness only 20 years ago (see compilation of Bryan, 1982). It is unknown in the absence of thorough surveillance if such changes in foodborne disease agents are occurring globally. Primary factors probably contributing to these paradigm shifts in foodborne disease epidemiology are similar to changes in other infectious diseases (Box 2.1) (Altekruse *et al*., 1997; Mossel *et al*., 1999). It has been emphasized that foodborne illness is vastly underreported, not only because many of the illnesses are mild and self-limiting, and that many illnesses have long incubation times and are difficult to associate with foods, but also because a proportion of the illnesses are caused by aetiological agents that cannot be identified using available methods. In addition to the changes in aetiological agents, acute foodborne illnesses are now recognized frequently to trigger long-lasting and sometimes chronic disease syndromes such as reactive arthritis, Reiter's syndrome and Guillain–Barré syndrome (Archer and Kvenberg, 1985; Mossel *et al*., 1999).

Box 2.1. Factors contributing to the global incidence of foodborne disease.

- Crowding and poor sanitary conditons
- Drought and famine
- Malnutrition
- Changing demographics with increasing populations of infants, the elderly and the infirm
- Inadequate public health infrastructure
- Inadequate government involvement and legislation
- Inadequate pathogen surveillance and reporting systems
- Emerging foodborne pathogens
- Acquisition of virulence and antibiotic-resistant genes by non-pathogenic bacteria
- Adaptation and enhanced survival of pathogens in foods
- Low priority of food safety by certain governments and companies
- Inadequate education of consumer

Surveillance and epidemiological analysis of foodborne disease are limited by several factors. Most bacterial foodborne illnesses involve sporadic cases and go unnoticed since they occur as isolated incidences that often are not diagnosed and reported to public health

authorities. Furthermore, chronic diseases associated with ingestion of bacterial pathogens or toxins are poorly recognized because of the long incubation time for the disease process to occur. Thus, the reported number of foodborne illnesses reflects a large underestimation (100- to 300-fold) of the actual occurrences of the food-mediated illnesses in the human population.

Bacterial Hazards in Foods

The primary aetiological agents of bacterial disease are presented in Table 2.1, which lists geographic range and habitats, associated foods, and factors affecting transmission of the various foodborne pathogens. The table is segregated according to the degree of bacterial pathogen or toxic hazard: severe hazards,

Table 2.1. Bacterial pathogens causing foodborne disease in various regions of the world. The agents are listed according to severity of hazard (modified and expanded from National Research Council, 1985).

Pathogen or toxin	Geographical distribution and habitats	Associated foods and conditions contributing to outbreaks
Severe hazards		
Clostridium botulinum; botulinum neurotoxin	Widespread; distribution depends on serotype	Vegetables, fruits, fermented fish, home-canned foods, honey (infant botulism); low acid foods (pH > 4.6); some strains grow under refrigerated conditions; spores extremely resistant to heat and chemicals
Salmonella typhi, S. suis, S. paratyphi, S. cholera-suis (typhoidal salmonellae)	Widespread; mainly problem in developing countries	Water, raw meats, raw milk; cells killed by pasteurization and most disinfectants
Shigella spp.	Central America, Mexico, North and central Africa, Japan, South-east Asia; host adapted to humans and primates	Water, vegetables, many fruits, salads, raw milk; transmission in most foods is by faecal–oral route; resistance properties similar to *Salmonella*
Vibrio cholerae (serogroup O1)	Coastal countries in South America, Central America, endemic in Calcutta, occasionally epidemic in Africa, southern Asia	Water, raw shellfish; spread by faecal–oral route, poor sanitation
Vibrio vulnificus	Coastal waters, South-east USA	Raw or poorly cooked shellfish and finfish; halophilic; individuals with underlying diseases such as cirrhosis highly susceptible; cases more frequent in summer months
Escherichia coli O157:H7 (EHEC) (enterohaemorrhagic)	Distribution unknown; found in intestines of dairy cattle; found on dairy farms and cattle ranches	Undercooked or raw minced beef; vegetables, fruits; lucerne sprouts; unpasteurized fruit juices; raw milk, cheese curds; inactivated by pasteurization and many disinfectants
Escherichia coli (EIEC) (enteroinvasive)	Geographical distribution unknown; found in intestines of many animals	Unknown, probably spread by faecal–oral route; illness similar to shigellosis (bacillary dysentery)
Listeria monocytogenes	Geographical distribution largely unknown; often associated with animals; also isolated from silage, soil, other environmental sources	Raw milk, dairy products made from raw milk, ready-to-eat meats, raw vegetables, raw meat, poultry, fish, smoked fish; minimally processed refrigerated foods
Brucella spp.	Worldwide	Associated with raw milk obtained from infected herds; rare in countries that enforce herd control and adequate pasteurization of milk

Table 2.1. *Continued.*

Pathogen or toxin	Geographical distribution and habitats	Associated foods and conditions contributing to outbreaks
Mycobacterium spp.	Worldwide	Associated with raw milk obtained from infected herds; rare in countries that enforce herd control and adequate pasteurization of milk
Moderate hazards, potentially extensive spread		
Salmonella serovars (non-typhoidal)	Worldwide; frequently associated with animals, particularly poultry and pigs	Raw meats, poultry, fish and shellfish, raw eggs, a variety of other foods where contamination with raw animal products can occur; inactivated by pasteurization and most disinfectants
Miscellaneous Enterobacteriaceae	Worldwide; frequently associated with animals, but many species also found freeliving or associated with plants	Raw milk, raw meats, shellfish, vegetables, fruits
Campylobacter jejuni, *C. coli*	Probably worldwide; associated with animals, particularly poultry, but also cattle, flies, other unknown vectors	Poultry, raw milk; readily inactivated by pasteurization and disinfectants
Escherichia coli (EPEC) (enteropathogenic)	Unknown distribution; isolated from humans, cattle, pigs	Foodborne outbreaks appear to be rare; frequent cause of infantile diarrhoea, particularly in developing countries; potential food vectors are raw beef and poultry, but most outbreaks probably involve faecal–oral transmission
Escherichia coli (ETEC) (enterotoxigenic)	Probably worldwide; seems more prevalent in developing countries; has caused cruise ship diarrhoeal episodes	Frequently causes diarrhoea in infants and also 'traveller's' diarrhoea; large infectious dose needed; food vehicles have included those that contact contaminated water such as salads; also associated with unpasteurized milk and dairy products
Streptococcus pyogenes (group A)	Probably worldwide; main reservoir is the human oral–nasal mucosa, also found in pus, on skin, freeliving in the environment	Virulent strains cause septic pharyngitis and scarlet fever; also can induce moderate to severe inflammatory responses; like most Gram-positives, streptococci are more resistant than are Gram-negatives to heat and disinfectants. Food vehicles have included salads, raw milk, ice cream, custards, eggs and a variety of other foods that were allowed to stand at warm or ambient temperatures for several hours; food handlers frequently have pharyngitis
Moderate hazards, limited spread		
Aeromonas hydrophila	Present in freshwater environments and associated fish, amphibians and animals	Food vehicles have included fish and shellfish; also found in meats and poultry; aetiology not well understood
Vibrio cholerae (serogroup non-O1)	Inhabitant of marine and estuarine waters and sediments	Food vehicles are commonly raw, improperly cooked or recontaminated shellfish

continued

Table 2.1. *Continued.*

Pathogen or toxin	Geographical distribution and habitats	Associated foods and conditions contributing to outbreaks
Vibrio parahaemolyticus	Inhabitant of estuarine and marine environments	Food vehicles are usually raw, improperly cooked or recontaminated shellfish; most outbreaks occur during summer months
Yersinia enterocolitica, *Y. pseudotuberculosis*	Probably worldwide; often isolated from pigs, birds, pets	Commonly associated with meats, especially pork, also beef, lamb, others; outbreaks have occurred in improperly pasteurized milk, tofu; symptoms can mimic appendicitis
Streptococcus spp. (group D)	See description for *S. pyogenes*	Food vehicles have included salads, and a variety or foods that were left at ambient or warm temperatures for several hours
Staphylococcus aureus	Worldwide; diminishing in developed countries; commonly associated with pimples, boils on skin, mucous membranes of humans	Ham, poultry, salads, pastries, other foods that were left at ambient or warm temperatures for several hours; many strains tolerate high osmotic conditions such as relatively high salt and sugar; outbreaks caused by toxin causing emesis
Clostridium perfringens	Worldwide; diminishing in developed countries; spores are widely distributed throughout the world	Beef, poultry, casseroles, foods cooked in bulk and cooled insufficiently; can cause fatalities in elderly individuals
Bacillus cereus; other *Bacillus* spp.	Probably worldwide; spores resistant to environmental conditions	Meats, vegetables, casseroles and other foods cooked in bulk and improperly cooled are associated with diarrhoeal illness; emetic illness nearly always associated with fried rice or rice dishes and less frequently with pasta dishes

moderate hazards with potentially extensive spread, and moderate hazards with limited spread (National Research Council, 1985). This information should be useful in establishing microbiological criteria and for developing HACCP (Hazard Analysis and Critical Control Point) and Food Safety Objective (FSO) programmes.

The taxonomic and biological characteristics of the various bacterial foodborne pathogens recently have been reviewed extensively in several definitive treatises (Holt, 1986; Blaser *et al*., 1995; Mossel *et al*., 1995; International Commission of the Microbiological Specifications for Foods, 1996; Collier *et al*., 1998; Dworkin, 1999; Fischetti *et al*., 2000; Lund *et al*., 2000; Mandell *et al*., 2000; Downes and Ito, 2001). The reader is referred to these treatises for biological descriptions of the known foodborne pathogens. Methods for isolation of bacterial pathogens and determination of toxins have also been published in excellent manuals and compendia (Food and Drug Administration, 1995; Downes and Ito, 2001). These treatises describe necessary sampling plans, sample collection and methods for analysis of pathogenic bacteria in specific foods. Other important aspects, including laboratory quality assurance, molecular typing and differentiation, and rapid methods, are also described. Safety guidelines for working with pathogens and toxins are also available (Fleming and Hunt, 2000). Physicians' guidelines for diagnosis, treatment and reporting of foodborne illnesses were published recently (Centers for Disease Control and Prevention, 2001b).

Foods as selective ecological environments influencing bacterial growth and survival have been aptly described (Mossel and Ingram, 1955; Mossel *et al*., 1995; International Commission of the Microbiological Specifications for Foods, 1996). The factors governing growth and survival of bacterial pathogens in

foods include the physical, chemical and nutritional composition of the food (intrinsic factors) and factors external to the foods (extrinsic factors) (Mossel and Ingram, 1955; Mossel *et al*., 1995). The primary intrinsic factors include hydrogen ion concentration (pH), water activity (a_w), redox potential (Eh), nutrients and antimicrobial constituents. The major extrinsic parameters include temperature, gaseous atmosphere and relative humidity. The effects of intrinsic and extrinsic factors on the growth and survival of foodborne pathogens in a variety of buffers, media and foods have been tabulated extensively (Mitscherlich and Marth, 1984; Mossel *et al*., 1995; International Commission of the Microbiological Specifications for Foods, 1996). An understanding of the effects of intrinsic and extrinsic parameters on bacterial foodborne pathogens has also facilitated the development of predictive models for assessment of growth in various media and foods (summarized in Lund *et al*., 2000). Intrinsic and extrinsic factors interact in their effects on growth and survival of foodborne pathogens, and thus a combination of inhibitory factors at sublethal concentrations is often more practical for control of pathogens in foods than the use of lethal levels of a single parameter. Since foods are complex ecosystems, it is often desired or necessary to conduct actual challenge studies in which the foodborne pathogen is inoculated to the food and growth and survival are monitored. Challenge tests are especially useful in foods that are reformulated or processed by newer preservation techniques (Rahman, 1999; Glass and Johnson, 2001).

Foodborne pathogens vary considerably in their association with certain foods. In recent years, some bacterial pathogens have been linked to foods, including *Campylobacter* (milk and poultry), *E. coli* O157:H7 (ground meats, unpasteurized apple cider and cheese curds), *Salmonella* (eggs, fruits and vegetables), and *L. monocytogenes* (raw milk, minimally processed and ready-to-eat meats). Several foodborne bacterial pathogens such as *C. perfringens* or *B. cereus* must grow in foods to very high numbers ($> 10^8$–10^9) in order to evoke illness, while certain other pathogens such as *E. coli* O157:H7 or *Shigella* spp. can evoke illness through ingestion of only a few cells. The requirement for a high infective dose implies that the pathogen must either be capable of successfully growing to high numbers in the food or is introduced in high numbers to the food by gross contamination prior to consumption in order to cause illness. In the case of those pathogens causing illnesses by only a few cells, limited growth or survival of a small number of contaminants is sufficient to elicit illness. The infectious doses of various foodborne pathogens as well as the onset time to illness, clinical symptoms and duration of the illness have been reviewed in several treatises (Bryan, 1982; Blaser *et al*., 1995; Collier *et al*., 1998; Fischetti *et al*., 2000; Lund *et al*., 2000; Mandell *et al*., 2000; Centers for Disease Control and Prevention, 2001a,b) and are summarized briefly in Table 2.2.

General Strategies for Pathogen Detection in Foods

The microbiological analysis of foods strives for accuracy and reproducibility of pathogen numbers obtained in the food samples. The accuracy of the test depends on analysis of a suitable number of replicate samples and from different lots of the test material. Sampling and validation plans for microbiological testing have been described (National Academy of Sciences, 1985; Mossel *et al*., 1995; Lund *et al*., 2000).

The following section describes general approaches for the isolation of foodborne pathogens from foods, with an emphasis on the Enterobacteriaceae since their isolation has been studied most extensively and they cause the highest incidence of bacterial-mediated foodborne disease. Other groups of foodborne pathogens (Fig. 2.1 and Table 2.1) are isolated using similar strategies. When isolating Enterobacteriaceae and other bacteria from most foods or clinical samples (stools, vomitus, occasionally blood or internal organs), it is usually necessary to use selective media because of the presence of greater numbers of non-pathogenic flora in the food or clinical samples. Culture media are made selective by the inclusion of specific inhibitors

Table 2.2. Properties of the primary food poisoning bacteria. Modified from Granum and Brynestad (1999).

Organism	Incubation time	Infective dose	Symptoms[a]	Duration
A. Intoxications				
Bacillus cereus (emetic)	1–6 h	NA	NV	6–24 h
Clostridium botulinum	12–72 h	~1 μg	Neurological	Weeks to months
Staphylococcus aureus	1–6 h	100–200 ng	NVD	8–24 h
B. Toxicoinfections in which the enterotoxin is produced in the intestine without infection of intestinal cells				
Bacillus cereus (diarrhoeal type)	6–12 h	10^5–10^7	AD	12–24 h
Clostridium perfringens	8–16 h	10^7–10^8	AND (F)	16–24 h
C. Infections in which enterotoxins are produced after bacterial adherence to epithelial cells but without invasion into the cells				
Aeromonas spp.	6–48 h	10^3–10^8	DA (F)	14–30 h
Escherichia coli				
ETEC (ST)	16–48 h	10^5–10^8	D (AVF)	1–2 days
ETEC (LT)	16–48 h	10^5–10^7	D (AVF)	1–3 days
EHEC (O157:H7)	1–7 days	10	DAB (H)	Days–weeks
Vibrio Cholerae	2–5 days	10^8	DA (V)	4–6 days
Vibrio parahaemolyticus	3–76 h	10^5–10^7	DA (NVF)	3–7 days
D. Infections in which bacterial invasion generally is localized to the epithelial cells and intestinal immune system				
Campylobacter jejuni/coli	3–8 days	$\geq 10^3$	FADB	Several days to weeks
Salmonella spp. (non-typhoidal)	6–72 days	10^3–10^6	DAF (VH)	2–7 days
Shigella spp.	1–7 days	10^3–10^4	AFDB (HNV)	Days–weeks
Yersinia enterocolitica	3–5 days	10^3–10^7	FDA (VH)	Weeks
D. Infections that often lead to systemic and organ invasion				
Listeria monocytogenes	Days–weeks	10^3–10^8	Systemic	Weeks
Salmonella typhi	10–21 days	1–10^2	Systemic	Weeks
Salmonella paratyphi	10–21 days	1–10^2	Systemic	Weeks

[a]Symptom abbreviations: A, abdominal pain; H, headache; B, bloody diarrhoea; N, nausea; D, diarrhoea; V, vomiting; F, fever.

such as antibiotics, which inhibit unwanted bacteria but do not inhibit growth of the pathogen. For example, selective media used to recover pathogenic Enterobacteriaceae from stools or foods are designed to inhibit growth of Gram-positive bacteria and to slow the growth of undesired enterobacteria. This is accomplished by taking advantage of the property that the enterobacteria are more resistant than Gram-positive bacteria to inhibition by certain dyes (e.g. brilliant green) and surfactant compounds (e.g. bile salts). Media designed to selectively promote growth of the pathogens greatly facilitate their isolation. Within the enterobacteria, further advantage is taken of the property that the pathogenic genera, such as *Salmonella* and *Shigella*, are more resistant than non-pathogens to the metal chelator citrate; therefore, media containing both citrate and bile salts (*Salmonella/Shigella* agar) can be used for selective isolation of pathogenic species from heavily populated samples such as faeces, sewage, or many raw or minimally processed foods. It may be necessary to use non-selective media in certain analyses where cells may be stressed or injured. Recovery of injured cells often is possible only when the flora in the food or clinical sample is low compared with the target organisms. In order to recover certain pathogens that may be present in low numbers in the stools of carriers, an enrichment broth may be used which preferentially enhances growth of the pathogens present relative to the normal flora. Enrichment media can also facilitate recovery of injured

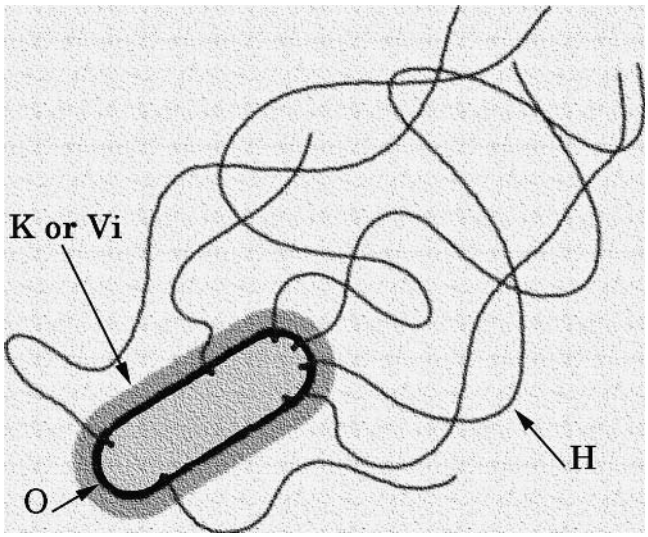

Fig. 2.3. Designation of the major antigens, O, H and K/Vi, used in serotyping enteric pathogens.

or stressed pathogens, which are probably present at higher numbers than healthy cells in many foods, clinical samples and food plant environments. Specific media and techniques for recovery, enrichment and isolation of pathogens are described in several technical manuals (Holt, 1984–1989; Atlas, 1995; Food and Drug Administration, 1995; Downes and Ito, 2001). The choice of media and conditions for recovery and isolation depends on the samples available for testing and on the preference and personal experience of the investigator. Following the primary isolation and purification of the pathogen generally by isolation of single colonies, further characterization of the species is performed to differentiate the isolate from related bacteria (Holt, 1984–1989; Food and Drug Administration, 1995; Downes and Ito, 2001). Differential media and test platforms for characterizing pathogens have been designed to discern important diagnostic characters. The goal of the investigator is to identify the pathogenic organisms from clinical and food samples as accurately and rapidly as possible. The procedures and interpretation of various tests are described in diagnostic manuals (Holt, 1984–1989; Food and Drug Administration, 1995; Downes and Ito, 2001).

An important method for characterization of certain Enterobacteriaceae is serological analysis of cell surface antigens, which is often used as a final method of identification and typing (see Goodfellow and O'Donnell, 1993). Antisera to surface antigens have been used for identification of many species of bacteria for nearly a century, and it has been useful for strain delineation in several Enterobacteriaceae including *Salmonella* and pathogenic *E. coli*. Three classes of surface antigens (H, O, and Vi or K) (Fig. 2.3) have been used as the fundamental serotyping antigens for *Salmonella* and *E. coli* because of considerable variation in their structure, their association with virulence and their strong antigenicity. Motile species of the Enterobacteriaceae possess flagellar (H) antigens, which owe their antigenicity to a heat-labile protein termed flagellin. Certain species also contain O antigens, commonly called the somatic or cell wall antigen, which comprises part of the lipopolysaccharide in the outermost layer of the outer membrane. O antigenic analysis by bacterial agglutination separates the genus *Salmonella* into more than 1000 distinct serotypes, and *E. coli* into 173 distinct serotypes. The O antigens in the Enterobacteriaceae, particularly typhoidal salmonellae, frequently are covered by the Vi (capsular virulence antigen in *Salmonella*) or the K (capsular antigen in *E. coli*).

A serotyping scheme for the O, H and K antigens has been adopted internationally for characterization of *E. coli*. The scheme at present includes O antigens (1–173), K antigens (1–103) and H antigens (1–56). The number of possible combinations of these obviously is enormous. In practice, it is considered necessary only to determine the O and H antigens in order to designate virulent strains of *E. coli* involved in food poisoning. Serotyping is also useful to distinguish virulent strains of other pathogenic Enterobacteriaceae, but certain problems in methodology and interpretation of the results can be encountered, and researchers should refer to comprehensive treatises (Blaser *et al*., 1995; Mandell *et al*., 2000). Analogous strategies are used for characterization of surface antigens of several other genera and species of Gram-negative and Gram-positive foodborne pathogens (Holt, 1984–1989; Fischetti *et al*., 2000; Lund *et al*., 2000).

Rapid Detection of Foodborne Pathogens

The diagnosis and prevention of foodborne disease can be greatly facilitated by rapid methods that allow identification of pathogens within a few hours (for recent reviews see Lund *et al*., 2000; Downes and Ito, 2001). Methods for rapid isolation and identification of pathogens or toxins generally are based on nucleic acid or analogous probes that react with signature regions in the genetic material of the organism (DNA or RNA), or on antibodies that can detect specific protein antigens characteristic of the pathogen (see Lund *et al*., 2000; Downes and Ito, 2001). Polymerase chain reaction (PCR) has been used for the detection of signature sequences of pathogens either following enrichment or directly in clinical or food samples. Obstacles to the use of PCR include inhibition of the amplification reaction by components in foods or clinical samples, and the property that PCR can amplify DNA samples present free in the food or in dead organisms. Thus, PCR and certain other sensitive and rapid methods often can only provide presumptive identification, and confirmation by cultural methods and phenotypic tests is needed. Antibody-based methods, while theoretically not as sensitive as PCR and some other DNA-based methods, can be useful for detection of toxins or other protein antigens produced by the pathogen. Protein detection often is performed by enzyme-linked immunosorbent assay (ELISA), which allows testing of multiple samples. Since biologically inactive antigens can be detected with antibodies, again the test often is only presumptive in its utility.

The field of rapid methods is becoming increasingly important in food microbiology, and is increasing in sophistication and innovation as microbiologists work with molecular biologists and engineers in devising new rapid methods. Newer technologies such as microfluidics, molecular imprinting and receptor-based assays currently are being evaluated as detection methods for bacterial pathogens and toxins. As genomic sequences of foodborne pathogens increasingly become available, novel identification methods based on signature genomic sequences will be developed. Knowledge of genomic sequences will also facilitate epidemiological studies of outbreaks and tracebacks, since the methods used could be simpler, more rapid and more easily interpreted than currently used methods such as pulsed-field gel electrophoresis (PFGE) (Swaminathan *et al*., 2001).

Toxins of Foodborne Pathogens

Most bacterial foodborne pathogens produce toxins that are involved in the disease process (Table 2.3). Certain toxins are produced in foods, such as botulinum neurotoxins, staphylococcal enterotoxins and *B. cereus* emetic toxin. Ingestion of these pre-formed toxins is sufficient to cause symptoms in the absence of the producer organism. Since the direct ingestion can cause symptoms, the onset time can be quite rapid, typically 1–6 h for staphylococcal enterotoxins or *B. cereus* emetic toxin. The onset of botulism symptoms after ingestion of botulinum neurotoxin generally occurs after 12–36 h, but symptoms have

Table 2.3. Properties of the primary foodborne toxins causing intoxications or toxicoinfections[a]. Modified from Granum and Brynestad (1999).

Producer organism of toxin	Toxin nature	Heat labile (L)/or heat stable (S)	Mode of action
Bacillus cereus (emetic)	Cereulide; small peptide, 1.2 kDa	S	Binds to 5-HT_3 cells; causes emesis by action on nervus vagus
Bacillus cereus (diarrhoeal)	Two–three components; structure not fully characterized	L	Receptor unknown; causes haemolysis and/or cytolysis
Clostridium botulinum	Potent neurotoxin (NT); seven serotypes; NT is ~ 150 kDa; forms stable complex with non-toxic proteins in culture and foods	L	Binds to gangliosides and putative protein receptor; enters nerve cells by endocytosis and cleaves neuronal proteins involved in vesicular trafficking and neurotransmitter release
Clostridium perfringens	Protein toxin of ~35.3 kDa	L	Binds to 22 kDa proteins in intestinal cells and causes pore formation
Staphylococcus aureus	Proteins; 26–29 kDa; seven serotypes	S	Binds to TCRVb cells or to T cells causing emetic or potent superantigen responses, respectively

[a]The table covers toxins that are pre-formed in foods or elicited in the gut and does not include toxins that may be formed during intestinal and/or septic infections.

occurred as early as 6–8 h or as late as 1–2 weeks. The longer onset of botulism compared with *S. aureus* and *B. cereus* emetic intoxications reflects the need for trafficking of the toxin across the intestinal barrier, its transport in the blood to nerves, the entry process into the nerves and its proteolytic action on neuronal substrates. Secondly, toxins are produced on entrance into the gut without establishing infection (*C. perfringens* enterotoxin and *B. cereus* diarrhoeal toxin). Thirdly, certain toxins are produced on binding to intestinal cells or during penetration into tissues. In the case of enterotoxin formation by *C. perfringens* and *B. cereus*, since these organisms do not need to establish an infection to elicit toxin, the incubation time is generally less than that of infectious pathogens, and typically symptoms are observed about 10–18 h after ingestion compared with 12–50 h for most infectious pathogens. The properties of toxins from foodborne pathogens are summarized in Table 2.3.

Recognition and Treatment of Foodborne Illnesses

Guidelines for physicians and public health workers for the diagnosis and treatment of foodborne illnesses have been published recently (Centers for Disease Control and Prevention, 2001b). Most patients, but not all, typically present with GI tract symptoms such as vomiting, diarrhoea and abdominal pain. However, somatic symptoms apparently unrelated to GI distress may present in certain patients, including neurological symptoms in cases of botulism caused by ingestion of botulinum toxin. The first recognized patient is referred to as the index case, often with exacerbated symptoms, which may allow the physician to make an early diagnosis enabling rapid treatment of other patients and to prevent the illness from spreading.

Several key features can provide clues in elucidating foodborne illness aetiology: the

incubation period; duration of illness; predominant symptoms; and the population involved in the outbreak (Centers for Disease Control and Prevention, 2001a,b). Also the health care provider should query the index case and later cases as to whether the patients have consumed raw or poorly cooked foods sometimes known to harbour pathogens, such as eggs, meats, shellfish, unpasteurized milk or juices, fresh produce, home-canned foods or soft cheeses from unpasteurized milk (see Table 2.1). Additional questions regarding foreign travel, contact with pets or exotic animals, attendance at picnics or group events, and similar symptoms being experienced by the patient's family or close circle can also provide clues as to the aetiology of the illness. Since certain foodborne illnesses involving neurological symptoms such as botulism and shellfish poisoning can be particularly life-threatening, a diagnosis should ideally be made quickly and life support measures (e.g. respiratory assistance, administration of antitoxin) should be considered.

When a foodborne illness is suspected, appropriate clinical samples including faeces, vomitus (occasionally serum) and likely foods should be submitted to state or local health departments for clinical microbiology testing. The public health authorities often can assist in investigation of the epidemiology of the outbreak, questioning individuals who may have eaten the same food or at the same location, and collection of suspect foods for microbial analysis. Rapid identification of an aetiological agent as a cause of foodborne illness can prevent spread of an outbreak. Sometimes specimens must be submitted to specialized laboratories for special testing of agents such as botulinum toxin, or rapid diagnosis of certain aetiological agents. For example, in the USA, a specialized and highly competent and experienced laboratory is responsible for testing of all clinical specimens or food samples suspected of containing botulinum toxin.

Reporting of foodborne illness outbreaks is an important component of an investigation, as it can detect trends in foodborne illnesses and can also lead to the recognition of previously unrecognized (emerging) or re-emerging pathogens or toxins. In the USA, the local and state health departments are generally responsible for reporting to the Centers for Disease Control and Prevention (CDC) but the physicians should also report suspected foodborne illnesses to the local and state health departments. The data are compiled and disseminated to the public through publication and the Internet (Centers for Disease Control and Prevention, 2001a,b).

The CDC, in cooperation with several state health department laboratories, established and coordinates a national molecular subtyping network called PulseNet for foodborne disease surveillance and epidemiological purposes (Swaminathan *et al*., 2001). The system uses standardized PFGE to characterize restriction fragment length polymorphisms in DNA extracted from clinical and food isolates of various pathogens including *E. coli* O157:H7, non-typhoidal *Salmonella* serotypes, *L. monocytogenes* and *Shigella*. It is anticipated that other bacterial, viral and parasitic organisms will be added to the system in the near future (Swaminathan *et al*., 2001). Subtyping has facilitated the identification of outbreaks and linked the clinical isolates with those in suspect foods, thus providing strong proof for involvement of genetically similar pathogens in foodborne outbreaks, even when the clinical and food isolates are from geographically distinct regions. Since in the majority of foodborne illness cases, most ill persons do not recall a likely food or water source for their infection, and foods can be distributed rapidly among states and countries, the PulseNet system has provided a valuable system to link food vehicles and patients in outbreaks and to identify virulent strains in sporadic and isolated clinical cases. Although molecular subtyping by PulseNet or other methods such as rDNA analyses are valuable in detecting foodborne outbreaks and facilitating investigation and implementation of public health protective measures, the subtyping methods are an adjunct to and not a replacement for more traditional epidemiological investigations (Swaminathan *et al*., 2001).

Control of Bacterial Foodborne Diseases

The prevention of bacterial foodborne diseases relies on proper handling procedures of foods, adequate quality and preventive programmes, good sanitation and hygiene, and many other factors (for reviews, see Lund *et al*., 2000). Raw foods and processed low-acid (equilibrium pH > 4.6) foods that do not reach commercial sterility should be promptly refrigerated to ≤ 5°C (40°F). Foods should be cooked or heated to an internal temperature of at least 72°C (160°F). Raw milk should be pasteurized. Cross-contamination of raw foods and cooked foods must be avoided by separating raw and cooked areas in the home, food processing plants, and in retail and food service operations. Additional precautions include adequate sewage disposal, prevention of water contamination in preharvest and postharvest foodhandling facilities, and rigorous avoidance of carriers as food handlers. The eradication of *Salmonella* from the food supply is not likely to occur in the near future because it is extremely difficult to eliminate the many animal reservoirs of this pathogen. Risk assessment-based approaches such as HACCP and FSO programmes and other preventive programmes involving critical control points and microbiological criteria have proved useful in control of foodborne disease. Good Manufacturing Practices (GMP), quality systems, and adequate sanitation and hygiene are essential in reducing the incidence of foodborne disease (reviewed in Lund *et al*., 2000).

The primary goal of food processing is to improve the microbial safety and quality of foods by destroying pathogenic and spoilage microorganisms and associated toxins. Traditionally, the most common method of cell and spore inactivation involves thermal processing. Pasteurization (≥ 70°C for ≥ 15 s) or its equivalent will destroy most vegetative pathogens but not spores of most species of food-related bacteria such as *C. botulinum*, *C. perfringens* and *B. cereus*. Temperatures exceeding 100°C are necessary to inactivate spores (Downes and Ito, 2001), which can be accomplished in pressurized retorts or other thermal processing systems. Other preservation strategies such as high-pressure treatment of foods, aseptic processing, electropasteurization, irradiation, UV light and other technologies are gradually being evaluated and implemented to enhance quality and safety.

Considerable research has been conducted on the inactivation of vegetative pathogens and endospores by traditional and alternative processes (see Rahman, 1999; Lund *et al*., 2000), but much less information is available on their effects on toxins. Pasteurization will not inactivate heat-resistant toxins including *B. cereus* emetic toxin and staphylococcal enterotoxins. Botulinum neurotoxins are heat labile and are inactivated rapidly at pasteurization or boiling temperatures. The enterotoxins from *Salmonella*, *E. coli* (LT), *Campylobacter*, *C. perfringens* and *B. cereus* are inactivated at temperatures exceeding 70°C, while the enterotoxins from *E. coli* (ST) and *Yersinia enterocolitica* are heat resistant to temperatures exceeding 100°C. However, it is unlikely that the consumption of enterotoxins in foods could lead to human illness in the absence of the toxin-producing pathogen. Research is needed to determine the resistance of bacterial toxins, particularly staphylococcal enterotoxin, *Bacillus* toxins and botulinum neurotoxin, to alternative methods of food processing such as pulsed electric fields, high pressure, γ-irradiation and light (Rahman, 1999).

Future Issues and Perspectives

Since diarrhoeal diseases are not pleasant and perhaps remind us of our vulnerabilities, the impact of foodborne diseases is probably underappreciated (McNeil, 1976), and increased public health and research efforts are not supported to the extent needed for their control. Recommendations have been made for improved control of foodborne disease in the USA (CAST, 1994). Although the recommendations were directed towards control in the USA, they would be applicable for control of foodborne disease in many

other countries. The recommendations were categorized into four areas: (i) goal setting; (ii) research needs; (iii) production control; and (iv) education. In goal setting, it was emphasized that food safety policy and regulations should be based on risk assessment, risk management and risk communication. The risk analysis approach proposed is consistent with those used by the Codex Alimentarius and National Academy of Sciences (National Research Council, 1985). For example, risk analysis can identify the probability of particular foods transmitting foodborne disease, such as *Salmonella enteritidis* in raw shell eggs, *Vibrio* spp. in raw oysters and *L. monocytogenes* in ready-to-eat meats. Facets of risk-base analysis include the severity of the hazards, risks in particular foods, severity of the disease produced and its consequences, dose response of the aetiological agent and management options. Research needs for decreasing foodborne disease include expanding epidemiological and food safety information to provide more complete assessments of the incidence of foodborne disease. As with goal setting, the results from such an analysis will vary depending on cultural and technological practices of various countries. Another research area deemed of high priority was to support studies of chronic illnesses resulting from acute exposure to foodborne agents (Mossel *et al*., 1999).

Basic and applied research areas of microbiology deemed critical for control of foodborne disease included enhanced knowledge in the following areas: (i) microbial ecology of pathogenic bacteria in pre- and postharvest environments; (ii) mechanisms of tolerance of foodborne pathogens to acid, heat, and other processing, sanitation and environmental conditions; (iii) mechanisms of virulence in pathogens, genetic transfer of virulence determinants and the impact of environmental conditions on expression of virulence; (iv) development of innovative procedures and technologies to eliminate or control pathogens and their toxins in pre- and postharvest environments; (v) improvements in strategies and methods to track pathogens in the environment and in epidemiological investigations; and (vi) development of rapid, accurate and sensitive methods to detect pathogens from various sources.

In the quickly developing field of rapid detection, it was realized that genome-based detection methods show tremendous potential for assessment of virulence and detection of pathogens. Rapid detection could be used for online assays of pathogens in process flow for monitoring of pathogen levels in HACCP or other preventive systems. Under the recommendation of production control, it was emphasized that producers should adopt effective intervention strategies, and apply control practices from food source to consumption. The harmonization of international food safety standards was considered to be of increasing importance taking into account increases in global trading of food and differences in food safety standards among countries. Lastly, education of the public and food safety professionals was considered an important area to decrease foodborne disease. In particular, the education of high-risk populations regarding foodborne pathogen risks was considered a high priority.

Conclusions

The provision of a nutritious and safe food supply is an essential goal of society to ensure the health and survival of humankind throughout the world (see Middlekauff and Shubik, 1989). Epidemiological evidence has indicated that certain food-associated bacteria and their toxins are the major source of illness and mortality transmitted by foods. In addition to the importance of pathogenic bacteria in human health, they are also important in the acceptance of foods. Surveys have indicated that most consumers are more concerned about microbiological hazards than any other area, including the presence of pesticide residues in foods and use of antibiotics and hormones in animal production (see World Health Organization, 1997; Lund *et al*., 2000).

Control of microbial foodborne disease is extremely difficult due to a myriad of factors (Box 2.1). Bacterial foodborne pathogens are

constantly changing and elusive to detection and control, as highlighted by the emergence of foodborne pathogens such as *E. coli* O157:H7, antibiotic-resistant *Salmonella* spp. and *L. monocytogenes*. The need for maintaining a safe food supply in the face of adversities has created a need for new technologies such as biotechnology and advanced preservation methods to provide a supply of safe and nutritious food. Certain of these novel preservation technologies (Rahman, 1999) are currently being evaluated as potential adjuncts to or replacements for traditional methods of preservation such as thermal treatments and formulation of foods for safety. Genomics and proteomics of foodborne bacteria appear to have been largely neglected with regard to food safety, but these fields could provide tremendous advances in technologies to enhance food safety.

Although microbial food safety is a major public health issue of increasing importance, many public health authorities in certain countries throughout the world do not adequately appreciate its importance for human health and economic development (World Health Organization, 1997). National and international programmes frequently are considered a low priority within governments. Many countries have not developed legislation and public health infrastructure to control foodborne disease. Even certain food companies do not consider food safety a high priority, and legislation is needed to enforce the production of safe food. On the other hand, certain multinational companies have taken a lead role to enhance the safety of the food supply. Although consumers are integral in the prevention of foodborne disease, many are unaware of their importance in enhancing food safety and do not receive adequate education to prevent illnesses within the home or at community events. As emphasized by the WHO (1997), strategies for decreasing the incidence of foodborne disease, enhancing human well being, and facilitating technology developments will require a shared responsibility among governments, industry, scholarly institutions and consumers to accomplish these goals.

Acknowledgements

This contribution was supported by a grant from the USDA and sponsors of the Food Research Institute, University of Wisconsin, Madison, Wisconsin.

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3 Shellfish Toxins

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Introduction

Marine phytoplankton is being seriously affected by the presence of certain microscopic algae, which are critical food for filter-feeding bivalve shellfish (mussels, clams scallops, oysters, etc.) as well as larvae of crustaceans and finfish. The plankton algae proliferation ('algal blooms') is beneficial for aquaculture; however, algal blooms can also have negative effects, causing important socio-economic damage.

The first written reference to a harmful algal bloom could be in the Bible (Exodus 7: 20–21): '. . . all the waters that were in the river were turned to blood, fishes died, Egyptians could not drink the water of the river'.

One of the first fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins was reported in 1793 (Poison Cove, British Columbia). At that time, local Indian tribes were not allowed to eat shellfish when the seawater became phosphorescent due to dinoflagellate blooms; these were related to certain alkaloid toxins, now called paralytic shellfish poisoning (PSP) toxins. Since then, more cases have been reported and, on a global scale, close to 2000 cases of human poisoning by toxins through fish or shellfish consumption are reported each year. For this reason, there is a need to strictly control the compounds responsible in order to ensure seafood safety.

Where toxic algal species are present, shellfish can be rendered unfit for human consumption. In this way, filter-feeding shellfish can act as vectors of various seafood poisoning syndromes such as PSP, diarrhoetic shellfish poisoning (DSP) and amnesic shellfish poisoning (ASP) in human consumers. The existence of the phenomenon of toxic phytoplankton blooms has given rise to many international scientific meetings concerned with the environmental and health impacts of these potentially catastrophic incidents.

Paralytic Shellfish Poisoning Toxins

PSP is the neurotoxic syndrome that is the result of human consumption of contaminated seafood. Toxins associated with this syndrome were referred to as saxitoxins since saxitoxin initially was thought to be the only agent responsible for this contamination.

The incidence of PSP has significantly increased since the 1970s, and this poisoning at present is appearing in regions of the world where it has never been known. The poisoning is sporadic and unpredictable. At present, PSP must be considered as a global problem

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that requires better professional and public awareness.

This poisoning has long been known to native Americans (Kao, 1993). Captain George Vancouver aboard the *Discovery* described experiences with PSP intoxications on a trip to British Columbia in 1793. After eating mussels, some members of his crew were sick with neurotoxic symptoms (Vancouver, 1798). Until 1970, about 1600 cases of human intoxication had been recorded worldwide, especially in North America and Europe (Prakash *et al*., 1971). Since then, almost 1000 cases have been reported, many occurring in regions where PSP had been known (World Health Organization, 1984).

Source organisms

The organisms considered as primary sources of PSP include three morphologically distinct genera of dinoflagellates as well as one species of blue-green algae present in freshwater. *Aphanizomenon flos-aquae*, which was long suspected to contain saxitoxin-like compounds, has been an important tool in the elucidation of saxitoxin biosynthesis as well as being responsible for poisonings occurring among terrestrial animals drinking algal-infested freshwater supplies. Marine animals were also affected by this poisoning (Carmichael and Falconer, 1993). The link between shellfish toxicity and dinoflagellates was first established in 1927 after an outbreak of PSP in San Francisco Bay. The toxic dinoflagellate was assigned to the genus *Gonyaulax* and named *G. catenella*. Several dinoflagellates of similar morphology were found later to be responsible for the PSP toxicity. These organisms usually have been assigned to the genus *Gonyaulax*; taxonomic revisions have been recently carried out and these dinoflagellates are now considered as *Alexandrium* (Balech, 1985). *Pyrodinium bahamense*, a dinoflagellate, was also found to be responsible for a PSP outbreak in Papua New Guinea. Figure 3.1 shows some structures of dinoflagellates responsible for PSP toxicity.

Chemistry

PSP toxins represent a group of highly polar water-soluble compounds whose structure is shown in Fig. 3.2. More than 20 analogues of saxitoxin, considered in the past as the main agent responsible for this syndrome, have been reported to occur naturally. The saxitoxin molecule is a tetrahydropurine composed of two guanidinium functions fused together in a stable azaketal linkage. At C-11, saxitoxin possesses a geminal diol. Traditionally, the PSP toxins, heterocyclic guanidines, were divided into three groups – carbamates, sulphocarbamoyls and decarbamoyls (Oshima *et al*., 1989) – with six toxins in each. Subsequently, a few deoxycarbamoyl compounds have been added to this group. The structural relationships among these compounds suggested the

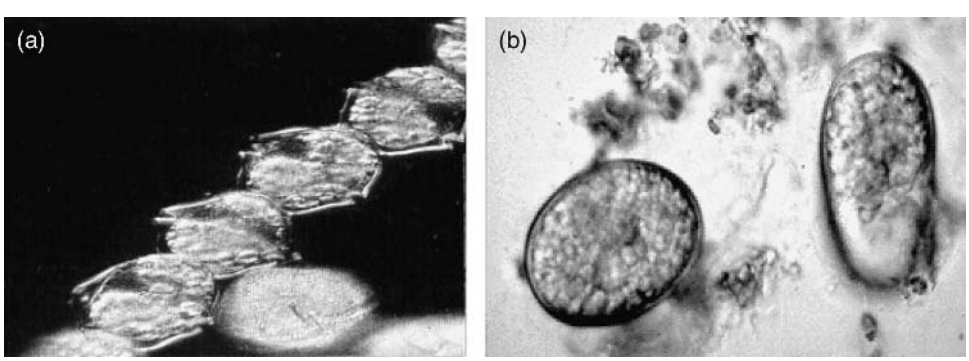
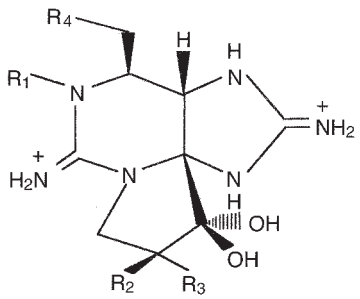


Fig. 3.1. Dinoflagellate species producing PSP toxins: (a) *Gymnodinium catenatum*; (b) *Alexandrium tamarense*.



			Carbamoyl toxins	*N*-Sulphocarbamoyl toxins	Decarbamoyl toxins
R_1	**R_2**	**R_3**	**R_4 = -OONH$_2$**	**-OONHSO$_3^-$**	**-OH**
H	H	H	STX	GTX5	dcSTX
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	H	H	NEO	GTX6	dcNEO
OH	H	OSO_3^-	GRX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Fig. 3.2. Chemical structures of PSP toxins.

possibility of multiple bioconversions into these PSP analogues.

Toxicology

The *in vitro* effects of saxitoxins have been studied carefully (Kao *et al*., 1971). Saxitoxin exhibits a relaxant action on vascular smooth muscle, and the action of the cardiac muscle is depressed as it also has a physiological channel-blocking effect. Guanidinium groups are key structural features of toxins involved in blockage of Na^+ conductance through nerve membranes.

The main symptoms of this intoxication include tingling and numbness of the mouth and lips, appearing shortly after intake of seafood containing PSP toxins. The symptoms spread to the rest of the face and the neck. A sensation of prickling in the fingers and toes is experienced, followed by headache and dizziness. In some cases, symptoms of nausea and vomiting can occur in the early stages of the PSP intoxication. In cases of moderate to severe intoxications, paraesthesia spreads to the arms and legs. After that, the patients can speak only incoherently, and a feeling of weakness is also experienced. In addition, respiratory difficulties can appear and patients with severe intoxication may experience paralysis of muscles and, finally, death as a consequence of the progressive respiratory problems (Prakash *et al*., 1971).

The main source of the intoxication is the consumption of bivalves, including mussels, clams, oysters, etc. Nevertheless, some other seafood such as crabs, several fish, etc. can also be responsible for this poisoning.

These toxins are absorbed rapidly from the gastrointestinal tract due to the fact that they are positively charged, since the two guanidinium functions have an alkaline pK_a and are protonated with a net cationic charge in the human body pH of 7.4. The elimination of PSP toxins takes about 90 min, and clinical studies have shown that patients who survive the first 24 h usually recover with no apparent late effects (Kao, 1993). In terms of toxicity, sulphocarbamoyl compounds are considered as the less toxic PSP compounds; nevertheless,

these compounds can be converted into carbamates, which are the most toxic PSPs under acidic conditions (Hall *et al*., 1990). The levels of PSP toxins reported to cause intoxications vary considerably, possibly due to interindividual differences in sensitivity as well as the precision of the methods used for quantification. Prakash defines mild poisonings in adults at doses of PSP toxins between 304 and 4128 µg per person, while severe poisonings are caused by doses between 576 and 8272 µg (Prakash *et al*., 1971). Other sources report mild symptoms from doses between 144 and 1660 µg saxitoxin equivalents per person, and fatal intoxications from doses between 456 and 12,400 µg saxitoxin equivalents (Acres and Gray, 1978).

There is no specific antidote for PSP toxins. The clinical management of patients intoxicated with these toxins is entirely supportive; if vomiting does not occur spontaneously, induced emesis or gastric lavage are required. The toxins can be adsorbed effectively by activated charcoal.

In moderately severe cases, maintenance of adequate ventilation is the primary concern. Periodic monitoring of blood pH and blood gases to ensure adequate oxygenation is important. Because of the toxin interference with respiratory functions, the acidosis cannot be compensed by hyperventilation. Fluid therapy is essential to correct any possible acidosis and, additionally, the renal excretion of the toxin must be facilitated.

There is no rational basis for the use of anticholinesterase agents to improve muscular performance, even if the practical effect appears beneficial, since this does not involve a reversal of the sodium channel blockage caused by these toxins, Similarly, there is no rational basis for any beneficial effects of vigorous exercise; this would only increase the production and accumulation of lactate, to add to the pathophysiological derangement. Since the half-time of elimination of the PSP toxins from the body is around 90 min, as mentioned above, this should be adequate for a physiological reduction of the toxin concentration to harmless levels, except in those cases where the toxin concentration is very high or victims have damage to renal function.

Regulatory levels

Today, most countries apply a tolerance level for PSP toxins at 0.8 mg saxitoxin equivalents kg^{-1} mussel meat (equivalent to 400 mouse units). If the consumption of mussels is estimated at 100 g, this indicates a safety factor of about 2–4 for the risk of developing mild symptoms among the most susceptible, and, more importantly, a minimum safety factor of about 6–7 for serious intoxication or death.

Analytical methods

The common method used for the control of PSP toxin is the mouse bioassay (Association of Official Analytical Chemists, 1990). This bioassay is still the official method in most countries. It measures the total toxicity of shellfish extracts and can monitor shellfish safety efficiently. This method poses some limitations regarding its selectivity, sensitivity and variability of results, as well as the constant supply of mice and maintenance facilities not available in most analytical chemistry laboratories. High-performance liquid chromatography (HPLC) is the method widely used as an alternative to this mouse bioassay for the detection and quantification of PSP toxins. Fluorescence detection has been selected as the more sensitive approach, and derivatization oxidation reactions are required for converting the PSP toxins into the corresponding fluorescent analogues. Post- and pre-column techniques have been developed for this purpose, and the oxidation reaction is based on earlier work (Bates and Rapoport, 1975) where PSP toxins were oxidized with peroxide to yield fluorescent products and the total amount of fluorescence produced was used as an estimate of PSP concentration. It was found, however, that the N-1 hydroxy compounds are poorly oxidized with peroxide, so the use of this reagent can seriously underestimate the true PSP concentration in unknown extracts. With the post-column approach, individual PSP analogues are separated using gradient elution ion-pair chromatography, the toxins being detected by fluorescence after conversion to purine derivatives

with periodate. Periodate was found to produce fluorescent products with all PSP toxins. The advantage of using a chemical method is the ability to separate the different analogues and quantitate them individually. The post-column method is much more suited to monitoring PSP contamination on an on-going basis rather than being set up for determinations on an occasional basis. Oshima *et al*. (1989) have modified the post-column methods developed by Sullivan and Iwaoka (1983). The significant changes made were in the chromatography, using three isocratic ion-pair mobile phases instead of a gradient elution, resulting in a separate determination of the three PSP groups of toxins, as well as in an improvement in the detection limits for individual toxins because of the higher efficiency separations.

The pre-chromatographic mode has emerged to overcome problems related to time and special equipment required for setting up the post-column oxidation mode. With this approach, the oxidation reaction is carried out prior to the chromatographic separation; the oxidation products are then separated by HPLC and quantitated directly with no post-column equipment. The reaction is simple, requiring only peroxide or periodate at weakly basic pH. The pre-chromatographic oxidation method was studied extensively and optimized further by Lawrence *et al*. (1995), who evaluated both peroxide and periodate under a variety of reaction conditions. Optimal conditions for the oxidation reaction have been evaluated recently (Gago-Martínez *et al*., 2001).

Although HPLC techniques are promising, capillary electrophoresis (CE) is emerging as an analytical alternative for such toxins (Piñeiro *et al*., 1999). HPLC has also been used coupled with mass spectrometry (MS). FAB ionization MS has provided useful data on a variety of individual PSP analogues. Electrospray MS coupled with CE has also been used for the analysis of PSP toxins (Locke and Thibault, 1994; Gago-Martínez *et al*., 1996). These techniques are not particularly suited for routine analysis, but nevertheless can offer useful information about the PSP toxins present in contaminated samples.

Differences in toxicity between the sulphocarbamoyls and the other groups of PSP toxins present a problem, since they undergo hydrolysis under acidic conditions, being transformed into the more toxic carbamates (Hall *et al*., 1990). The degree of hydrolysis depends on the acidity. The acidity applied in the traditional extraction procedure, at about pH 3, is insufficient for total hydrolysis. Consequently, analysis of PSP toxins extracted from seafood may underestimate the total toxicity if the sulphocarbamoyls present in the seafood are transformed to a greater degree in the human stomach, and if they constitute a significant amount.

Several biochemical assays have also been developed. Among the most interesting are enzyme-linked immunosorbent assay (ELISA) methods. Use of ELISA methods is hampered by the lack of sensitivity towards many of the toxins making up the PSP toxin complex; however, fast screening methods for PSP toxins that show good correlation with the mouse bioassay are being developed. Some other analytical methods such as the neuroblastoma assay have also been developed (Gallacher and Birbek, 1992) and applied for the determination of PSP toxins in Portuguese samples (Alvito, 2001).

The main obstacle to the development of analytical methods is associated with the problem in obtaining standards and reference materials, although this situation is improving currently.

Diarrhoetic Shellfish Poisoning

DSP is an illness in humans that can occur as a result of consuming shellfish contaminated with toxic dinoflagellates. The first evidence of the presence of this new type of gastrointestinal illness associated with the consumption of mussels that had ingested dinoflagellates was reported in The Netherlands in the 1960s (Kat, 1979). Another toxic incident due to the consumption of scallops was reported in Japan in 1976–1977, when a large group of people suffered from gastrointestinal symptoms. These symptoms have now become typical in cases of intoxication due to

the consumption of shellfish that have become contaminated with okadaic acid (OA) and related compounds (Yasumoto *et al*., 1978). Although it was believed that similar episodes occurred in Scandinavia during the 1960s, it was not until the 1980s that an episode of DSP was confirmed (Kumagai *et al*., 1986).

The discovery of this type of shellfish poisoning is attributed to Yasumoto and his research team. These workers found a close correlation between the dinoflagellate *Dinophysis fortii* and this contamination; consequently, the toxin was named dinophysistoxin (DTX) and, because of the diarrhoetic symptoms, the syndrome was named 'diarrhetic shellfish poisoning' (Yasumoto *et al*., 1980).

DSP toxins can be divided into three groups, OA and its analogues the DTXs, pectenotoxins (PTXs) and yessotoxins (YTXs); these last two groups of toxins initially were included in this group despite having different toxicological effects. While PTXs are clearly hepatotoxic, YTXs show a cardiotoxic symptomatology.

OA and analogues are now considered as the typical DSP toxins and are widely distributed. For this reason, the study of this group is emphasized in this chapter; nevertheless, a brief description of PTX and YTX will also follow.

PTXs are polyether lactones. PTX-2 is the only PTX found in phytoplankton, while a whole range of structurally closely similar compounds are found in shellfish, probably as a result of transformations. The PTXs have not been associated with the typical DSP symptoms in humans; however, they are acutely toxic to mice. The mechanism of toxicity of the PTXs is not clear. According to Quilliam *et al*. (2000), PTX-2 seco acids may have contributed to gastrointestinal symptoms, vomiting or diarrhoea in humans, after consumption of a bivalve mollusc in New South Wales, Australia.

The YTXs are polyethers closely resembling the brevetoxins. In addition to YTX, several derivatives are identified (among others 45-OH-YTX, homo-YTX and 45-OH-homo-YTX; Satake *et al*., 1997). The target organ for YTX is the myocardium, while the small intestine is unaffected (Murata *et al*., 1987). According to recent studies, the oral toxicity of YTX is at least one order of magnitude lower compared with its intraperitoneal (i.p.) toxicity (Aune *et al*., 2000, oral communication). In contrast to YTX, the desulphated derivative displays no toxicity in the heart muscle, but it exerts toxicity in the liver and pancreas at 300 μg kg^{-1} upon i.p. injection. Mice treated orally with desulphated YTX at 500 μg kg^{-1} body weight developed fatty degeneration of the liver.

Source organisms

Dinoflagellates belonging to the genus *Dinophysis* were implicated in outbreaks of DSP toxicity The confirmation of their toxigenicity has been difficult because of the difficulty in culturing these dinoflagellates. *Prorocentrum lima* has also been proved to be a producer of OA and related compounds (Lee *et al*., 1987).

In outbreaks of DSP in Japan in 1976 and 1977, DTX-1 was the major toxin present in mussels and the causative organism was *Dinophysis fortii*. DTX-3 was also found as the predominant toxin in scallops collected in 1982; nevertheless, DTX-3 was not found in *Dinophysis* species, so the origin of DTX-3 was suggested to be in the acylation of DTX-1 in the hepatopancreas of scallops (Murata *et al*., 1982).

From outbreaks of DSP in France, Spain, Portugal, Italy and Sweden, it was reported that OA was the major toxin, *D. acuminata* and *D. acuta* being the species responsible for these toxins. DSP outbreaks in The Netherlands were mostly due to high concentrations of *Prorocentrum* species. Important DSP outbreaks in Norway and Sweden in 1985 and 1986 were attributed to the presence of *D. acuta* (Aune and Yndestad, 1993). While the dominant toxin in Europe is OA, DTX-1 was the major DSP toxin in Japan; this difference is attributed to the presence of different dinoflagellates in European and Japanese waters. However, DTX-1 was also the major toxin found in a toxic episode in Norway in 1986 in mussels harvested in Songdal. In contrast, OA was the main toxin responsible

for DSP toxicity in another part of Norway; *D. acuta* and *D. norvegica* were responsible for these toxins. Although OA was considered the main toxin responsible for DSP toxicity in Ireland, DTX-2 has been found together with OA during routine monitoring for DSP toxins (Hu *et al*., 1992). This toxin was also found to be responsible for DSP toxicity in Galician waters, being on occasions the predominant DSP toxin in mussels (Gago-Martínez *et al*., 1996).

DSP outbreaks in America are associated with the presence of OA and DTX-1, *Prorocentrum* spp. being responsible for this toxic profile in Canada, while *Dinophysis* species are responsible for DSP toxicity in the USA and Chile. The structures of some species capable of producing DSP toxins are shown in Fig. 3.3.

Chemistry

The DSP toxins, as mentioned above, are considered as three main groups of toxins: OA and derivatives (DTXs), PTXs and YTXs. Among all these toxins, OA and the DTXs are most commonly distributed worldwide. The group of OA and DTXs initially was composed of OA and DTX-1. A new dinophysistoxin (DTX-2) was isolated in Ireland (Hu *et al*., 1992) and this toxin was later found in dinoflagellates and mussels from the Galician Rias (north-west of Spain) (Gago-Martínez *et al*., 1996). The chemical structures of some of these compounds are shown in Fig. 3.4. More DTXs have been discovered recently and included in the DSP group, such as DTX-3, where saturated or unsaturated fatty acyl groups are attached (Yasumoto *et al*., 1985). These compounds also possess toxic activity, but were only found in shellfish tissues, suggesting a probable metabolic origin. Recently, OA analogues have been identified in shellfish (DTX-2B and DTX-2C). The first evidence of the existence of diol esters of OA came from the isolation of a mixture of such esters from *P. lima* (Yasumoto *et al*., 1989). Subsequently, esters of OA such as OA-DE1 have also been isolated from many types of *Prorocentrum* spp. such as *P. lima* and *P. maculosum* (Hu *et al*., 1992). These diol esters were also found in Spanish strains of *P. lima* (Norte *et al*., 1994).

A water-soluble DSP toxin that has been named DTX-4 has been discovered recently (Hu *et al*., 1995). This new toxin was discovered after observing that the mouse toxicity was not representative of the concentrations of known DSP toxins present. Other sulphated esters of OA were isolated from *P. maculosum*, namely DTX-5a and DTX-5b, which were found to hydrolyse rapidly to OA by the action of esterases.

The DSP toxins are lipid-soluble long-chain compounds containing cyclic polyether

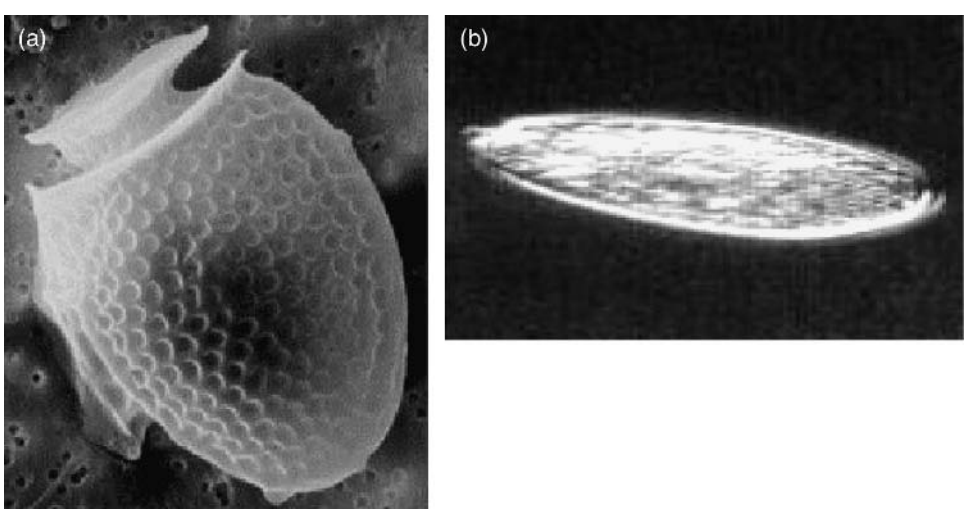
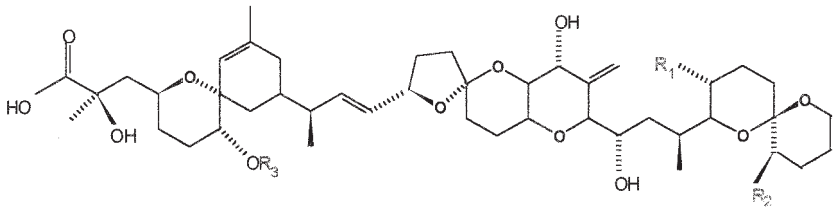


Fig. 3.3. Dinoflagellate species producing DSP toxins: (a) *Dinophysis* spp.; (b) *Prorocentrum* spp.



R_1	R_2	R_3	
CH_3	H	H	Okadaic acid (OA)
CH_3	CH_3	H	Dinophysistoxin-1 (DTX-1)
H	CH_3	H	Dinophysistoxin-2 (DTX-2)
H/CH_3	H/CH_3	Acyl	Dinophysistoxin-3 (DTX-3)

Fig. 3.4. Chemical structure of DSP toxins (okadaic acid group).

rings. They are soluble in acetone, chloroform, methylene chloride, methanol and dimethylsulphoxide (DMSO).

Toxicology

The toxic effects exerted by the OA derivatives have been well documented since the first clinical reports in Japan in 1978, when 42 people experienced severe vomiting and diarrhoea. From the results obtained with the mouse bioassay, a correlation between toxicity of these toxins in humans and physical effects in mice was obtained. The amount of toxin required to produce illness in humans was defined by mouse units: 1 mouse unit (MU) is defined as the amount of toxin required to cause death to a 20 g mouse over a specific time period (48 h). The amount of toxin needed to cause mild poisoning in an adult was determined to be 12 MU. In later years, more information about the toxic effects has been reported from a variety of research teams. From these studies, it was concluded that DSPs also present chronic effects; these toxins have been shown to possess the ability to induce tumour promotion (Fujiki and Suganuma, 1999), These toxins have also been reported to strongly inhibit protein phosphatases, thereby disrupting normal eucaryotic cell functions. Concerning the mechanism of action, OA and DTX-1 are potent inhibitors of protein phosphatase 1 and 2A (PP1 and PP2A, respectively). PP2A is about 50–100 times more strongly inhibited than PP1 by OA/DTX-1 (Fujiki and Suganuma, 1993). There are also some reports on mutagenic and genotoxic effects of OA and DTX-1. According to Aonuma *et al*. (1991), the mutagenic effects were due to inhibition of protein phosphatases involved in DNA repair, and not formation of DNA adducts.

The health hazard associated with exposure to toxins from the DSP complex is related to the toxic effects of the individual compounds. DSP symptoms start after intake of OA or DTXs above 40–50 µg per person (adult). Experience from a whole range of DSP episodes indicates that the patients recover after a few days. Since the effects in question are diarrhoea, vomiting, headache and general discomfort, but no serious and irreversible adverse health effects, a lower uncertainty factor may be tolerated, compared with toxins producing more severe effects. However, human health associated with the chronic toxicity of DSP as tumour promoters and mutagenic compounds cannot be estimated yet.

Regulatory levels

Today the European Union applies a tolerance level of 0.16 µg OA equivalents kg^{-1}

mussel meat. Depending upon the amount of shellfish consumed, this indicates a minimum safety factor of ≥ 2 before symptoms appear (Aune and Yndestad, 1993).

Depending on the amount of toxin ingested, the intensity of the symptoms can be different. Patients intoxicated with DSP toxins are not usually hospitalized. Intravenous injection of an electrolyte mixture can be used for a fast recovery and the symptoms will disappear in a few days.

Analytical methods

The most commonly used method for detection of the DSP toxins is the mouse bioassay (Yasumoto *et al*., 1978). This method has many disadvantages; one of the major objections is the use of animals for research purposes. A lack of selectivity is also observed since other toxins or fatty acids present in mussels or seafood can come into the lipid fraction causing interference, which may make difficult the identification of the studied toxins or cause false-positive results. The mouse bioassay cannot discern reliably between different types of toxins but provides information on the overall toxicity present in samples.

Cytotoxicity assays were developed after discovering that DSP toxins were responsible for morphological changes in some cells. These are sensitive, rapid and more ethically satisfactory than live animal assays. Effective use was made of the fact that DSP toxins inhibit protein phosphatases. Assays based on the inhibitory power of DSP toxins have been developed and provide sensitive detection of DSP toxins; however, the response is non-specific and, like the mouse bioassay, gives information regarding the total toxicity. Inmunoassays can also be used to detect OA and some of its analogues; these assays show poor reactivity, especially for DTX-3.

Physico-chemical approaches have been developed for a sensitive determination of DSP toxins. Methods based on HPLC are the most widely used, coupled with various detection modes. Fluorescence detection (FLD) provides a highly sensitive response, and this alternative has been widely used as a routine monitoring tool. Different derivatization reagents have been used for converting the DSP analogues into the correspondent fluorescent derivatives by mean of the derivatization of the carboxylic acid moiety of the compounds to form highly fluorescent esters, which are then separated by reverse-phase chromatography. The method which has received most attention up to the present is that developed by Lee *et al*. (1987). This method uses 9-anthryldiazomethane (ADAM) as a derivatization reagent. A number of modifications of the method of Lee *et al*. have been reported, especially focused on clean-up improvements. Since the ADAM reagent is relatively expensive and of limited stability, a method for synthesizing it immediately before use has been described (Quilliam *et al*., 1998). Other reagents, such as coumarin, luminarine-3, 9-chloromethylanthracene, etc., have also been evaluated for OA and DTX-1.

The only reports to date on the direct determination of OA and DTXs in shellfish have involved HPLC combined with ion-spray MS (Quilliam, 1998). With this approach, extracts of shellfish can be analysed directly without derivatization and clean-up, resulting in a fast and sensitive technique for the determination and confirmation of DSP toxins present in contaminated samples. This technique is also useful to determine DSP toxins when standards are not available.

Amnesic Shellfish Poisoning

A new type of shellfish intoxication named ASP was first discovered in Prince Edward Island, Canada, in 1987, after a serious outbreak of shellfish poisoning (Quilliam and Wright, 1989).

The ASP toxin domoic acid (DA) originally had been isolated from a red microalga *Chondria armata* by Japanese researchers studying the insecticidal properties of algal extracts (Takemoto and Daigo, 1958).

In the Canadian episode, due to the consumption of blue mussels, none of the known toxins was implicated in this incident and eventually DA was identified as the toxic

agent (Wright *el al*., 1989). The toxin was present at levels as high as 1 g kg^{-1} of edible tissue, and this was the first report of DA as a shellfish toxin (Wright and Quilliam, 1995) The victims were reported to have suffered neurotoxic and gastrointestinal symptoms but also an acute loss of memory; these symptoms were observed within 24 h.

Source organisms

The source of DA in the eastern Canadian incident was the diatom *Pseudonitzschia pungens* f. *multiseries* (Subba Rao *et al*., 1988). Until this toxic event, it was believed that phycotoxins were only produced by dinoflagellates, and diatoms were not considered as a possible source of toxins. Other species belonging to the genus *Pseudonitzschia*, such as *P. pseudodelicatissima* and *P. australis*, were responsible for deaths of pelicans and cormorants in Monterey Bay, California (Fritz *et al*., 1992), after the ingestion of anchovies containing domoic acid at concentrations as high as 0.1 g kg^{-1}. In addition, DA has also been found in other bivalve molluscs (scallops, clams, oysters, etc.) as well as gastropods, crabs and lobsters. An example of a species of *Pseudonitzschia* is shown in Fig. 3.5.

Chemistry

DA is a naturally occurring analogue of glutamic acid and belongs to the kainod class of compounds, which have been isolated from marine microalgae. The chemical structures of DA and isomers, which were discovered after investigations in the red alga *Chondria armata*, are shown in Fig. 3.6. DA seems to be the dominating toxin associated with ASP in both plankton and contaminated shellfish. Some of these isomers such as isodomoic A, C and D or domoilactones A and B were not even found in shellfish tissue or plankton extracts. DA is a crystalline water-soluble compound with typical acidic amino acid properties. The structure is clearly pH-dependent, and five protonated forms of the toxin are possible.

Toxicology

The toxic effects of DA were established after studies were carried out using mice, rats or monkeys. After i.p. injection in mice, this toxin induces a very peculiar symptomatology, known as 'scratching syndrome'. The animals scratch their shoulders using the hind leg, followed by convulsions and often death. Subtle effects such as hypoactivity rigidity, tremors, etc. have also been reported (Tasker *et al*., 1991). The toxic effects in humans have been reported in the Canadian incident when 107 people had to be hospitalized; 14 of them displayed severe neurological poisoning and four among the oldest persons intoxicated by the mussels died after 11–24 days. Severe damage to the hippocampus and other parts of the brain was found (Todd, 1993). The human symptoms were related mainly to gastrointestinal disorders, but

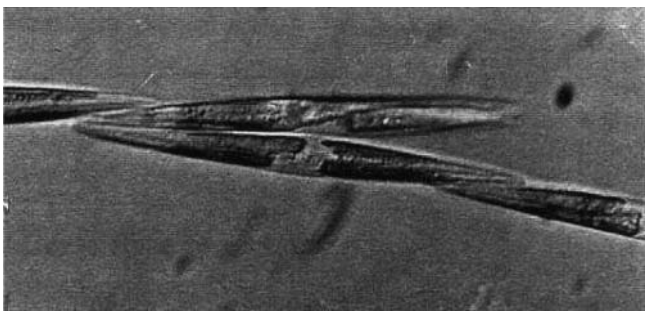
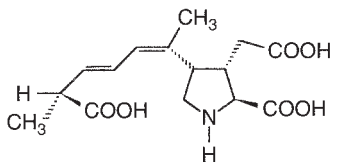


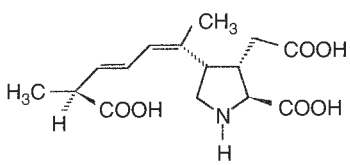
Fig. 3.5. Species of diatoms producing ASP toxins: *Pseudonitzschia* spp.

neurological symptoms were also observed, a permanent short-term memory deficit being one of the most characteristic symptoms associated with this intoxication. The pharmacokinetics and mechanism of action of DA show that, upon oral exposure, most of the toxin is excreted in the faeces of mice and rats. In the bloodstream, DA is cleared very easily by the kidneys (Suzuki and Hierlihy, 1993).

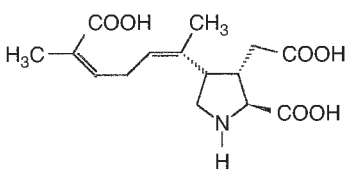
The mechanism of action of DA is as an agonist of the glutamate receptor (Takemoto, 1978). Domoic and kainic acids can be regarded as conformationally restricted forms of glutamic acid, both acting as high-affinity glutamate receptors of the quisqualate type. The glutamate receptor conducts Na^+ ion channels in the postsynaptic membrane so then DA acts to open the Na^+ channels, leading to Na^+ influx, inducing depolarization. As



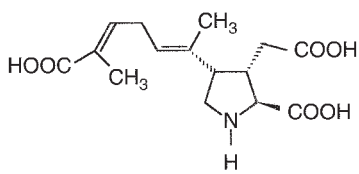
Domoic acid



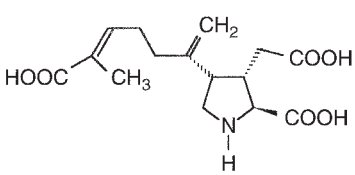
Isodomoic acid G



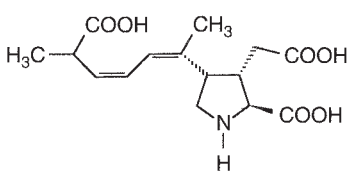
Isodomoic acid A



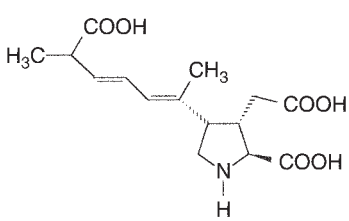
Isodomoic acid B



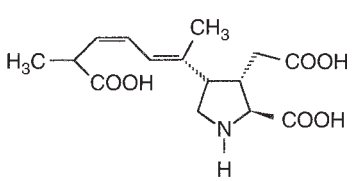
Isodomoic acid C



Isodomoic acid D



Isodomoic acid



Isodomoic acid F

Fig. 3.6. Chemical structures of domoic acid and isomers.

a result of this, the Ca^{+} ion influx is increased and may lead to cell death. DA is a 2–3 times stronger neuroexcitator than kainic acid, and about 100 times more potent than glutamate.

Regulatory levels

After the Canadian incident, a safe limit for DA was established; this limit was set at 20 mg kg^{-1} shellfish tissue (Iverson and Truelove, 1994). This level has been adopted by most countries as the regulatory level for this toxin. Recently, the action level for DA in crab viscera has been modified and increased to 80 mg kg^{-1}. Data reported from the Canadian incident estimated that the concentration of DA in shellfish was in the range of 300–1000 mg kg^{-1} and the intoxicated individuals may have ingested 1–2 mg kg^{-1} of the toxin.

Consumption of 250 g of mussel meat at maximum tolerance level will give an intake of about 0.1 mg DA kg^{-1} body weight for an adult.

The rates of accumulation of DA in shellfish and the speed of its elimination vary, both within different species and between different organs. In most shellfish, DA accumulates in the digestive organs.

Analytical methods

DA can be detected by the mouse bioassay for PSP if the observation time is extended to more than 4 h. The toxin is detected in mice by means of a unique syndrome, the above mentioned 'scratching syndrome'. The success of this biological assay in the Canadian incident was due in part to the high levels of toxin present in contaminated shellfish (300–1000 mg kg^{-1} tissue). However, the sensitivity of the bioassay is inadequate for the action level of 20 mg kg^{-1} tissue established as the regulatory level. Symptoms such as scratching are observed in mice with extracts containing more than 40 mg kg^{-1}.

Several alternatives have been developed for the analysis of ASP toxins. The first chemical approach involves the use of reverse-phase HPLC analysis with UV detection of the underivatized compound at its absorption maximum of 242 nm (Quilliam *et al*., 1989a).

Since then, several alternatives using different extraction procedures or different detection methods, such as fluorescence after derivatization using different reagents (Pocklington *et al*., 1990; James *et al*., 2000), have been developed, all with detection limits at 1 mg kg^{-1} or lower. CE, a very promising analytical technique, has also been investigated and applied to the analysis of DA (Nguyen *et al*., 1990; Zhao *et al*., 1997; Piñeiro *et al*., 1999). Gas chromatography (GC)-MS and liquid chromatography (LC)-MS techniques have also been proposed for the determination of these compounds. GC-MS is applicable to concentrations of DA in contaminated shellfish ranging from 1 to 500 mg kg^{-1}; nevertheless, a derivatization reaction is required to convert the ASP compounds into the *N*-trifluoroacetyl-*O*-silyl derivatives, requiring an intensive clean-up to facilitate this derivatization (Pleasance *et al*., 1990). HPLC combined with ion-spray MS has been shown to be particularly useful for confirmation of DA in shellfish (Quilliam *et al*., 1989b).

Among biochemical assays, several ELISAs have been developed. According to Garthwaite *et al*. (1998), a robust and highly sensitive ELISA method is now available which should be suitable for routine testing of shellfish for regulatory purposes.

Among all these analytical alternatives for the control of ASP toxins, HPLC-UV is the preferred method and has been used by most regulatory agencies worldwide for preventing incidents of ASP (Lawrence *et al*., 1989, 1991; Quilliam *et al*., 1989a; Association of Official Analytical Chemists, 1991). This method is suitable for detecting contamination levels greater than 20 mg kg^{-1}, nevertheless, interferences commonly present in such complex matrices can cause false positives with crude extracts. It has been shown that tryptophan and some of its derivatives are often present in shellfish tissues, eluting close to DA and isomers, and it is necessary to use efficient clean-up procedures to remove such interferences and consequently obtain an accurate control of these toxic compounds. Although intensive work

in developing selective clean-up methods, by means of solid phase extraction using C18 or anion exchange as stationary phases, has been carried out recently, enormous variability has been found (Piñeiro, 2001). Improvements are still required, and thus the use of confirmatory techniques such as MS is highly recommended to ensure the presence or absence of ASP toxins in seafood.

New and Emerging Toxins

The progress in the development of new analytical techniques has led to the discovery of new toxins including pinnatoxins, azaspiracids, gymnodimine and spirolides.

Pinnatoxins

Pinnatoxins are a group of potent marine toxins implicated in human food poisoning resulting from the ingestion of shellfish belonging to the *Pinna* genus. This bivalve is a common seafood in China and Japan, and human intoxication is a regular occurrence (Twohig, 2001). The symptoms associated with this intoxication involve diarrhoea with typical neurological symptoms. Pinnatoxins are thought to be Ca^{2+} activators.

Azaspiracids

A toxic incident occurred in The Netherlands where eight people became ill after having eaten mussels originally from Killary harbour. Symptoms were typical of DSP including nausea, vomiting, diarrhoea and abdominal cramps. High mouse toxicity was not proportional to the low levels of OA and DTX-2 found in the same mussels. The structure of the original azaspiracid found in mussels taken from Killary harbour has been determined (Satake *et al*., 1998), and several isomers described. Toxicological studies of azaspiracid show that, in addition to causing damage to the small intestine, the toxin also causes damage in both liver and spleen (Ito *et al*., 2000). Both the target organs and mode of action of azaspiracid are distinctly different from those of DSP, PSP and ASP toxins.

Gymnodimine

In 1994, oysters from South Island, New Zealand, were analysed and gave rise to mouse toxicity levels that could not be attributed to known toxins. After multiple chromatographic steps using UV diode array detection, and mouse bioassays, the potent compound responsible was isolated. This compound was named gymnodimine, since the causative organism was *Gymnodinium* sp. The minimum lethal dose in mice was 450 mg kg^{-1}. Mice injected died within 5–15 min. Gymnodimine also showed potent ichthyotoxicity at levels of 250–500 ppb. The structure of gymnodimine was characterized using nuclear magnetic resonance.

Spirolides

A family of macrocyclic toxins was isolated from the digestive glands of shellfish which were collected from the eastern shore of Nova Scotia, Canada. These compounds were named spirolides as they possess an unusual seven-membered cyclic imine moiety that is spiro-linked to a cyclohexene ring. The pharmacological activity of the spirolides may be the activation of Ca channels. Four spirolides were initially isolated and structurally elucidated (Hu *et al*., 1995). Two minor components were isolated later, which were inactive in mice and did not possess a cyclic imine moiety, suggesting that this group is essential for pharmacological activity.

Conclusions

A number of seafood toxins are now known following the development of new analytical methods. Most of these toxins are naturally occurring substances that can negatively affect seafood safety and, consequently, human health at very low levels. The search for sensitive analytical approaches is

necessary for an accurate risk assessment. Toxicological studies are still required for a better understanding of the toxicity of these compounds. The lack of standards and reference materials clearly compromises advances in this area. Nevertheless, interest in the study of these compounds is increasing considerably, due to their enormous socio-economic impact. New analytical methods are being developed by research teams involved in this field, which will result in a better knowledge of the toxic compounds involved in such poisonings, thereby reducing health risks to consumers.

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4 Mycotoxins in Cereal Grains, Nuts and Other Plant Products

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Introduction

Mycotoxins are a diverse and ubiquitous group of fungal compounds specifically associated with the precipitation of deleterious effects in humans and animals. Viewed globally, food safety is regularly compromised by the presence of mycotoxins occurring in cereal grains, nuts, fruit and green coffee beans. If feeds are contaminated with mycotoxins, associated residues and metabolites may appear in animal products. The mycotoxins of major concern in human health emanate from the secondary metabolism of *Claviceps*, *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. Mycotoxins may be categorized and, indeed, named on the basis of their fungal origin. Mycotoxins may also be classified on the basis of their biosynthetic origin from key primary intermediates. Thus, the polyketide mycotoxins are derived from acetyl coenzyme A, while the terpene mycotoxins are synthesized from mevalonic acid. Amino acids are incorporated in the formation of a third group of mycotoxins comprising cyclic polypeptides and their derivatives. It is salutary to note, however, that mycotoxin production may be strain specific. Thus both toxigenic and atoxigenic strains exist within the *Aspergillus flavus* species. It is conventional to subdivide toxigenic fungi into 'field' (or plant pathogenic) and 'storage' (or saprophytic/spoilage) organisms. *Claviceps*, *Fusarium* and *Alternaria* are classical representatives of field fungi, while *Aspergillus* and *Penicillium* exemplify storage organisms. This distinction is academic since the inoculum for postharvest spoilage of grain and fruit, for example, frequently originates from field sources such as soil or plant debris. Furthermore, mycotoxins from storage fungi frequently are detected on grain, nuts and fruit prior to harvest. Mycotoxigenic species may be distinguished further on the basis of geographical prevalence, reflecting specific environmental requirements for growth and secondary metabolism. Thus, *A. flavus*, *A. parasiticus* and *A. ochraceus* readily proliferate under warm, humid conditions, whereas *Penicillium expansum* and *P. verrucosum* are essentially temperate fungi. Consequently, the *Aspergillus* mycotoxins predominate in plant products emanating from the tropics and other warm regions, while the *Penicillium* mycotoxins occur widely in temperate foods, particularly cereal grains and infected fruit. *Fusarium* fungi are more ubiquitous, but even

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this genus contains toxigenic species which are associated almost exclusively with cereals from warm countries.

The diverse ill effects caused by these compounds are incorporated within the generic term 'mycotoxicosis', including distinct conditions and syndromes which may add to or occur concurrently with existing disorders such as kwashiorkor and gastroenteritis. In this chapter, the mycotoxins likely to prejudice human health are reviewed in terms of origin and chemical nature, distribution in foods, toxicology and risk management. Particular emphasis is placed on recent evidence indicating continuing human exposure to these fungal toxins.

Origin and Nature of Compounds

The foodborne mycotoxins most frequently implicated in human disorders are presented in Table 4.1, which also indicates the fungal origin of these compounds. The pathways of biosynthesis are summarized in Table 4.2. In historical terms, the ergot alkaloids, synthesized by *Claviceps purpurea*, have occupied a central position by virtue of their assumed role in widespread gangrenous and convulsive manifestations in Europe during the Middle Ages. Current concerns relate to the aflatoxins, ochratoxins, fumonisins and patulin. However, the trichothecenes and zearalenone have emerged recently as global

Table 4.1. Principal foodborne mycotoxins of confirmed or potential relevance in human health.

Mycotoxins	Fungal species	Foods
Ergot alkaloids	*Claviceps purpurea*	Cereal grains
Aflatoxins	*Aspergillus flavus*; *A. parasiticus*	Nuts; maize kernels; dried fruits
Cyclopiazonic acid	*A. flavus*	Nuts
Ochratoxin A	*A. ochraceus*; *Penicillium viridicatum*; *P. cyclopium*	Cereal grains and products; pig products; raw coffee
Citrinin	*P. citrinum*; *P. expansum*	Cereal grains
Patulin	*P. expansum*	Apple products
Citreoviridin	*P. citreo-viride*	Rice
T-2 toxin (type A trichothecene)	*Fusarium sporotrichioides*; *F. poae*	Cereal grains
Diacetoxyscirpenol (type A trichothecene)	*F. sporotrichioides*; *F. poae*	Cereal grains
Deoxynivalenol (type B trichothecene)	*F. culmorum*; *F. graminearum*	Cereal grains
Zearalenone	*F. culmorum*; *F. graminearum*; *F. sporotrichioides*	Cereal grains
Fumonisins; moniliformin; fusaric acid	*F. moniliforme*	Maize kernels
Tenuazonic acid; alternariol; alternariol methyl ether; altenuene	*Alternaria alternata*	Fruit; vegetables; cereal grains

Table 4.2. Biosynthesis of the major foodborne mycotoxins.

Primary metabolite	Pathway	Mycotoxins
Acetyl coenzyme A	Polyketide	Patulin, citrinin, ochratoxins, zearalenone, moniliformin, aflatoxins, fumonisins
Mevalonic acid	Isoprenoid	Trichothecenes: deoxynivalenol, nivalenol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol
Amino acids	Peptide synthesis	Ergot alkaloids

contaminants of the major cereal grains, and the human health implications need to be addressed.

Ergot alkaloids

The major ergot alkaloids comprise the lysergic acid derivatives ergocristine and ergotamine (Fig. 4.1), although ergosine, ergocornine and ergometrine may also occur in contaminated cereal grains (Flannigan, 1991).

Aflatoxins and cyclopiazonic acid

Aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) are the secondary products of *A. flavus* and *A. parasiticus* (Smith, 1997). In addition, aflatoxin M_1 (AFM_1) has been identified in the milk of dairy cows and in women consuming and metabolizing AFB_1 from contaminated diets. The aflatoxins are a group of structurally related fluorescent heterocyclic compounds characterized by dihydrofuran or tetrahydrofuran residues fused to a substituted coumarin moiety. The AFG molecules differ from the AFB structures in possessing a δ-lactone ring in place of a cyclopentenone ring. As explained later, the presence of a double bond in the terminal furan ring of AFB_1 (Fig. 4.2) and AFG_1, but not in AFB_2 or AFG_2, confers distinct biological properties to the former two aflatoxins. It is now generally acknowledged that *A. flavus* only synthesizes AFB_1 but is also capable of yielding cyclopiazonic acid, a mycotoxin recently confirmed as a co-contaminant in a batch of groundnuts associated with mass mortality in turkey poults in 1960. On the other hand, *A. parasiticus* often produces all four aflatoxins. However, in both species of *Aspergillus*, there are strains which are non-aflatoxigenic. The two species develop when conditions such as temperature and humidity/water activity favour their proliferation. In the case of *A. parasiticus*, temperatures of 25 to 30°C are optimal for maximizing aflatoxin synthesis. However, both temperature and water activity may interact in the promotion of aflatoxin synthesis, and the risk of contamination is, therefore, much greater in foods produced in warm and humid regions.

Ochratoxins and citrinin

The ochratoxins, produced by several species of *Aspergillus* and *Penicillium*, are a family of structurally related compounds based on an isocoumarin molecule linked to L-phenylalanine (Abramson, 1997). Ochratoxin A (OTA; Fig. 4.3) and ochratoxin B (OTB) are the only forms to occur naturally in contaminated

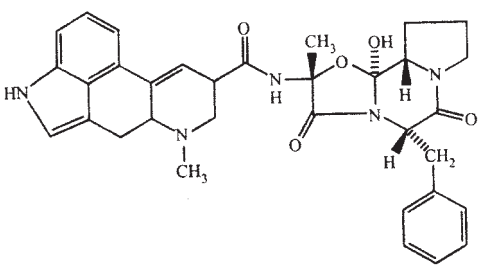

Fig. 4.1. Ergotamine (Moss, 1996; reproduced with permission from *Mycological Research*).

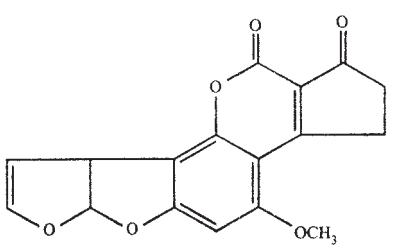

Fig. 4.2. Aflatoxin B_1 (Moss, 1996; reproduced with permission from *Mycological Research*).

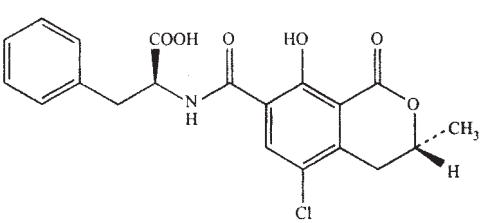

Fig. 4.3. Ochratoxin A (Moss, 1996; reproduced with permission from *Mycological Research*).

foods and, of the two, OTA is more ubiquitous, often occurring with another pentaketide mycotoxin, citrinin, in cereals and associated products. Citrinin is synthesized by a number of *Penicillium* species.

Patulin and citreoviridin

Several *Penicillium* species are also capable of synthesizing patulin (Fig. 4.4), a low molecular weight hemiacetal lactone with antibiotic properties. *Penicillum expansum* is of particular relevance since it is commonly associated with storage rot of apples and a wide variety of other fruits. The occurrence of patulin in apple juice has been attributed to the use of mouldy fruit. Other species of *Penicillium* contaminating rice from Italy, Spain, Thailand, Burma and other countries are now recognized as producers of an open-chain nonaketide derivative known as citreoviridin.

Fusarium mycotoxins

The natural occurrence of mycotoxins from *Fusarium* species is generally associated with temperate countries, since many of these fungi require somewhat lower temperatures for growth and mycotoxin production than the aflatoxigenic *Aspergillus* species. However, extensive data exist to indicate the global scale of contamination of cereal grains with a number of *Fusarium* mycotoxins. Indeed, *F. moniliforme* and its mycotoxins are associated primarily with foods from tropical and subtropical regions. *Fusarium* species are important pathogens of cereal plants, causing diseases such as fusarium head blight (FHB). The very same species may also synthesize a wide range of mycotoxins, of which the most important from the point of view of human health are the trichothecenes, zearalenone, moniliformin and the fumonisins (D'Mello *et al*., 1997). Following episodes of FHB, residues of these mycotoxins may contaminate harvested grain. The co-occurrence of *Fusarium* mycotoxins in cereal grains has now emerged as an intractable issue with regard to risk assessment and establishment of regulatory or advisory directives.

Trichothecenes

The trichothecenes comprise four basic groups, with types A and B representing the most important mycotoxins. Type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol (DAS), while type B trichothecenes include deoxynivalenol (DON, also known as vomitoxin) and its 3-acetyl and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol (NIV) and fusarenon-X. All trichothecenes possess a basic tetracyclic sesquiterpene structure with a 6-membered oxygen-containing ring and an epoxide group. These features are illustrated in the structure for DON (Fig. 4.5). The synthesis of the two types of trichothecenes appears to

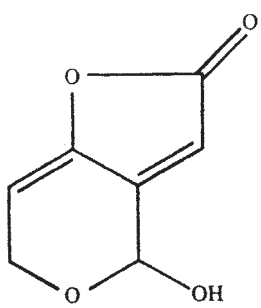

Fig. 4.4. Patulin (Moss, 1996; reproduced with permission from *Mycological Research*).

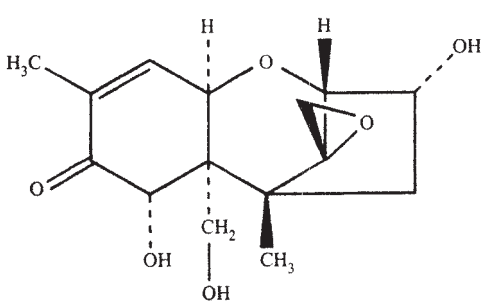

Fig. 4.5. Deoxynivalenol (Moss, 1996; reproduced with permission from *Mycological Research*).

be characteristic for a particular *Fusarium* species. Thus, for example, production of type A trichothecenes predominates in *F. sporotrichioides* and possibly also *F. poae*, whereas synthesis of type B trichothecenes occurs principally in *F. culmorum* and *F. graminearum*.

Zearalenone

A common feature of many *Fusarium* species is their ability to synthesize zearalenone (ZEN), and its co-occurrence with certain trichothecenes raises important issues regarding additivity and/or synergism in the aetiology of mycotoxicoses in humans. ZEN (also known as F-2 toxin) is a phenolic resorcyclic lactone (Fig. 4.6), which also occurs as a hydroxy derivative in the form of α-zearalenol. The presence of appropriate reductases in animal tissues implies that α-zearalenol may be the active form of ZEN in animals.

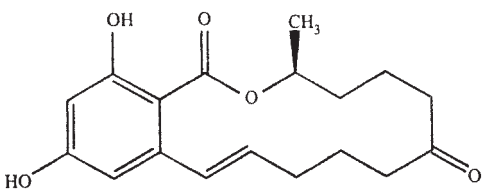

Fig. 4.6. Zearalenone (Moss, 1996; reproduced with permission from *Mycological Research*).

Fumonisins and moniliformin

With respect to the co-occurrence of mycotoxins, the secondary metabolism of *F. moniliforme* is of particular significance since it is capable of producing at least three mycotoxins: the fumonisins, moniliformin and fusarin C. The fumonisins are relatively recent additions to the list of mycotoxins, but their significance as major contaminants of maize has already been established and linked with the incidence of cancer in humans. Several structurally related forms of fumonisins (FBs) have been characterized, with FB_1, FB_2 and FB_3 occurring regularly in maize from different geographical sources. FB_1 (Fig. 4.7) is 2-amino-12,16-dimethyl-3, 5,10,14,15-pentahydroxyicosane with a propane-1,2,3-tricarboxylate substituent at C-14 and C-15, whereas FB_2 and FB_3 are, respectively, the C-10 and C-5 deoxy analogues of FB_1. In addition, FB_1 is structurally similar to sphinganine and sphingosine, intermediates in the biosynthesis and degradation of sphingolipids. Moniliformin occurs as the Na or K salt of 1-hydroxycyclobut-1-ene-3,

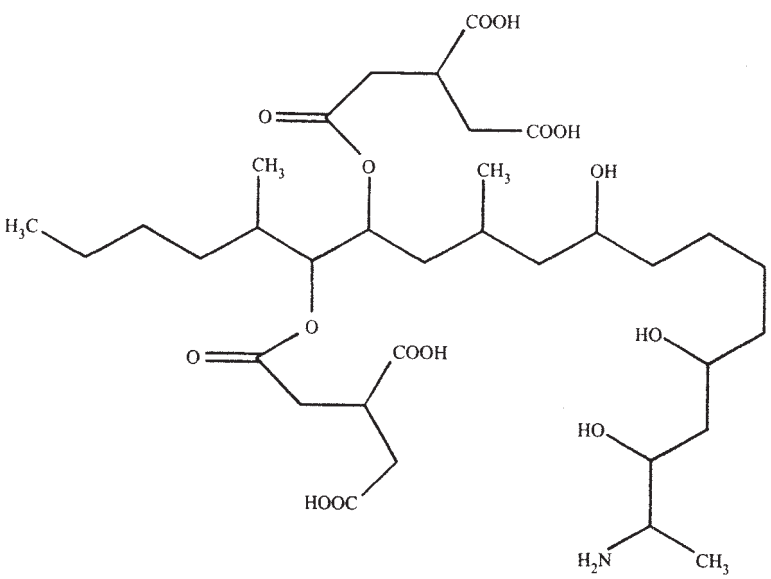

Fig. 4.7. Fumonisin B_1 (Moss, 1996; reproduced with permission from *Mycological Research*).

4-dione and, like the fumonisins, has been detected in maize.

Alternaria mycotoxins

A wide range of *Alternaria* species are capable of synthesizing mycotoxins of diverse chemistry. The dibenzo-α-pyrone group includes alternariol, alternariol methyl ether and altenuene. The nitrogen-containing group includes tenuazonic acid and the cyclic polypeptide tentoxin. In addition, *Alternaria* spp. produce a number of metabolites of varied structure, including altertoxin I, an unusual partially saturated perylene.

Distribution in Foods

The ubiquitous distribution of toxigenic fungi as plant pathogens (e.g. in FHB) and as spoilage organisms implies that contamination of primary and processed foods is almost inevitable when appropriate environmental or storage conditions prevail. Mycotoxins have been detected in such diverse commodities as cereal grains, nuts and fruit, often at levels that exceed legal or advisory limits. Considerable data already exist to demonstrate the global scale of mycotoxin contamination of these foods. The evidence has been presented elsewhere (D'Mello and Macdonald, 1998), but there is scope for reviewing more recent data.

Ergot alkaloids

Historically, rye and other cereals intended for breadmaking have been linked with ergot contamination (Flannigan, 1991). The incidence of contamination is now considered to be negligible due to surveillance and legislation as well as the global decline in the production of rye. However, some modern cultivars of malting barley appear to be prone to infection with *C. purpurea*, resulting in the rejection of grain for brewing. For example, in 1999, large consignments of barley harvested in Scotland were rejected due to detectable quantities of ergot in the grain. Ergot contamination of sorghum grain is an emerging issue in some developing countries, and vigilance is, therefore, still necessary.

Aflatoxins

Predictably, aflatoxin contamination of peanuts continues to attract worldwide attention. However, surveillance has extended to other foods and products. A selection of recent data is presented in Table 4.3. It is clear that diverse foods contain levels of aflatoxin that exceed current statutory limits. The outstanding feature is the high level of AFB_1 contamination of Indonesian maize. Of equal concern are the relatively high concentrations in maize-based gruels used as weaning food for children in Nigeria (Oyelami *et al*., 1996). Samples of peanut butter analysed in the UK in 1986 and 1991 showed that 'crunchy' types continued to contain more aflatoxin than 'smooth' varieties (Ministry of Agriculture, Fisheries and Food, 1993). The maximum concentrations of total aflatoxin found in the two surveys were similar, at 53 μg kg^{-1} in a sample of crunchy peanut butter obtained in 1986 and 51 μg kg^{-1} in a smooth sample collected in 1991. The UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) expressed concern that there had been no decrease in these levels since the previous report, but anticipated reductions with the implementation of new regulations (Ministry of Agriculture, Fisheries and Food, 1993).

Reports in 1990 drew attention to aflatoxin contamination of imported pistachio nuts. UK surveillance conducted between March 1990 and April 1991 and between May 1991 and April 1992 indicated that 52 and 28%, respectively, of samples exceeded the 4 μg kg^{-1} statutory limit (total aflatoxins) for finished products. In addition, 38 and 25%, respectively, exceeded the 10 μg kg^{-1} limit in products destined for further processing. Elsewhere, there are similar reports of contamination of pistachio nuts, particularly small pistachio 'scalpers' in California, which

Table 4.3. Aflatoxin contamination of foods.

Food	Aflatoxin	Incidence of contamination (%)	Mean/range (μg kg^{-1})	Country
Maize	Total	19	17	Zambia
	Total		0–76	Costa Rica
	B_1	81	0–70	
	B_2	56	0–6	
	B_1		0–428	Indonesia[a]
	B_2		0–160	
Maize-based gruels	Total	25	0.002–19.7	Nigeria
Peanuts	B_1		0.8–16	Botswana
	B_2		1.6–16	
	G_1		1.6–8	
	G_2		1.6–16	
	Total	52	3–48	
	B_1		0.8–10.9	Japan
	B_2		0.2–1.7	
	G_1		0.1–21.8	
	G_2		0.4–4.1	
Pistachio nuts	Total	28–52	4.1–224	UK
	Total		up to 149	California, USA
	B_1		up to 165	The Netherlands
	B_1		0.8–128	Japan
Peanut butter:				
'smooth'	Total	11	4.1–10	UK
'crunchy'	Total	28	4.1–10	
	B_1		3.2–16	Botswana
	B_2		1.6–20	
	G_1		3.2–20	
	G_2		1.6–20	
	Total	71	1.6–64	
Dried figs	Total	64	4–227	UK
Fig paste	Total	24	4.1–165	UK
Date fruits	B_1		113	United Arab Emirates
	G_1		133	
Spices	B_1	40	25	Egypt

[a]See also Table 4.6.

may contain total aflatoxin concentrations of up to 149 μg kg^{-1}. In The Netherlands, AFB_1 levels as high as 165 μg kg^{-1} have been reported for pistachio nuts, with much lower concentrations in shells (up to 8 μg kg^{-1}). In whole dried figs, UK data (Ministry of Agriculture, Fisheries and Food, 1993) showed that between December 1988 and April 1992, the percentage contaminated with aflatoxins (total) at levels above 4 μg kg^{-1} fell from 26 to 16%. However, samples containing up to 427 μg kg^{-1} were found. The incidence of aflatoxins in fig paste samples above the 4 μg kg^{-1} level also fell during this period from 50 to 14%. The maximum concentration of total aflatoxins found in fig paste also declined from 165 to 15 μg kg^{-1}. These findings attracted comment by COT, who were clearly concerned by the high levels of contamination of pistachio nuts, dried figs and fig pastes but were satisfied that consignments exceeding the 10 μg kg^{-1} limit were refused entry by the UK port health authorities. The results of a recent survey of Egyptian foods indicated high incidence and unacceptable levels of AFB_1 in spices, herbs and medicinal plants. As will be seen later, contamination of spices is the subject of scrutiny by EC authorities, but

there may be a case for surveillance of other imported foods not currently controlled by legislation.

Ochratoxin A

Ochratoxin A is ubiquitous in foods (Table 4.4; see also D'Mello and Macdonald, 1998), occurring principally in cereal grains (Vrabcheva *et al*., 2000), dried vine fruit (MacDonald *et al*., 1999) and green coffee beans (Blanc *et al*., 1998). The relatively high values in Bulgarian cereals were associated with grain samples taken from villages with a high incidence of Balkan endemic nephropathy. A recent study in France indicated consistent contamination of cereals and oilseeds with OTA, the values ranging from 0.6 to 12.8 μg kg^{-1} in positive samples. In the UK, OTA analyses of dried vine fruit imported from Greece and other countries (Table 4.4) indicated that 88% were contaminated with levels in the range 0.2–53.6 μg kg^{-1}. The OTA data for green coffee beans shown in Table 4.4 are at the lower end of a range of other published values, which included a maximum of 360 μg kg^{-1} (Blanc *et al*., 1998).

Use of contaminated grain in brewing and as animal feed regularly results in transfer of residues into beer and offal. Some OTA contamination of porcine organs has been reported in a survey conducted in the UK (Ministry of Agriculture, Fisheries and Food, 1993). Of 104 samples of kidney, 12% were contaminated with OTA at 1–5 μg kg^{-1}, while 3% had concentrations of up to 10 μg kg^{-1}. Of the black pudding samples analysed, 13% were contaminated with OTA in the range 1–5 μg kg^{-1}.

Citrinin often occurs with OTA in cereal grains. In naturally contaminated samples of Bulgarian wheat, citrinin levels up to 420 μg kg^{-1} were detected (Table 4.4).

Patulin

The occurrence of patulin in fruit juice has been a cause of concern in the UK and elsewhere in Europe (D'Mello and Macdonald, 1998). In recent years, there has been a marked increase in the production of cloudy apple juices prepared by pressing the fruit and stabilizing with vitamin C prior to pasteurization of the juice. The reduction in processing steps, as compared with the procedure for production of clear juices, means that patulin losses during fining and filtration are restricted, with higher residual levels of the mycotoxin in the cloudy juices. A comparison of the patulin concentrations in the two types of juices has been published recently for samples from the UK and Spain. Although the UK data were derived from a relatively small number of samples, it was apparent that the incidence of patulin contamination was higher in cloudy juices, with a median value of 28 μg kg^{-1}, compared with 0–10 μg kg^{-1} for clear juices. Four cloudy samples had patulin concentrations in excess of 50 μg kg^{-1}, compared with only one of the clear juice samples. In two cloudy samples,

Table 4.4. Ochratoxin A and citrinin contamination of foods (μg kg^{-1}).

Source	Food	Ochratoxin A (range/mean)	Citrinin
Bulgaria	Wheat	< 0.5–39	< 5–420
	Oats	0.9–140	< 5
	Bran	< 0.5–3.4	< 5–230
UK	Wheat	0.3	
	Barley	0.7	
	Oats	0.2	
Greece	Currants	< 0.2–54	
	Sultanas	< 0.2–18	
Several countries	Raisins	< 0.2–20	
Thailand	Green coffee beans	4.1–22.1	

patulin concentrations exceeded 151 μg kg^{-1}. In a more extensive study in Spain, patulin was detected in 82% of commercial samples (assumed to be clear for this comparison). However, 70% of these juices contained levels of less than 10 μg l^{-1}, although 22% had levels of 10–50 μg l^{-1} and, in two samples, concentrations of 164 and 170 μg l^{-1} were recorded. On a more reassuring note, patulin was absent in all 12 tested samples of apple food for children.

Trichothecenes and zearalenone

D'Mello and Macdonald (1998) provided an exhaustive survey of the global contamination of cereal grains with trichothecenes and ZEN. Recent data confirm the widespread distribution of these mycotoxins, particularly with respect to DON, NIV and ZEN (Table 4.5). The levels of DON in Polish wheat and maize and in Japanese barley are striking, but it will be noted that some samples from the USA exceeded advisory limits. Within-country variation in DON contamination of wheat has also been observed. Highest levels in the 1991 harvest in the USA were seen in Missouri, North Dakota and Tennessee. In the 1993 harvest, 86% of samples from Minnesota and up to 78% of samples from North and South Dakota had levels in excess of 2 mg kg^{-1}. A comprehensive review of trichothecene levels in Canadian grain is now available (Scott, 1997), indicating higher values for DON in cereal grains (Table 4.5) than those previously reported (see D'Mello and Macdonald, 1998). In Ontario, DON incidence was consistently higher for maize than for soft wheat over a 15-year period (Scott, 1997). Of particular note are the lower levels of DON in soft spring wheats over this period (Table 4.5). It may be concluded that DON is a frequent contaminant of Canadian cereals.

The predominant feature of ZEN distribution in cereal grains is its co-occurrence with other *Fusarium* mycotoxins, including trichothecenes (Table 4.5). This observation is consistent with the confirmed production of ZEN by virtually all toxigenic and plant pathogenic species of *Fusarium* (D'Mello *et al*.,

Table 4.5. Natural occurrence of deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEN) in cereal grains (mg kg^{-1}).

Country	Cereal grains	DON	NIV	ZEN
Germany	Barley	0.032–0.44		0.005–0.006
	Wheat	0.036–0.37		0.005–0.012
Poland	Wheat	2–40	0.01	0.01–2
	Maize kernels	4–320		
Finland	Oats	1.3–2.6		
The Netherlands	Wheat	0.020–0.231	0.007–0.203	0.002–0.174
	Barley	0.004–0.152	0.030–0.145	0.004–0.009
	Oats	0.056–0.147	0.017–0.039	0.016–0.029
	Rye	0.008–0.384	0.010–0.034	0.011
Nepal	Maize	1.2–6.5		
Japan	Wheat	0.029–11.7	0.01–4.4	0.053–0.51
	Barley	61–71	14–26	11–15
USA	Wheat (winter), 1991	< 0.1–4.9		
	Wheat (spring), 1991	< 0.1–0.9		
	Wheat, 1993	< 0.5–18		
	Barley, 1993	< 0.5–26		
Canada	Wheat (hard)	0.01–10.5		
	Wheat (soft, winter)	0.01–5.67		
	Wheat (soft, spring)	0.01–1.51		
	Maize	0.02–4.09		
Argentina	Wheat	0.10–9.25		
Brazil	Wheat	0.47–0.59	0.16–0.40	0.04–0.21

1997). The highest values for ZEN in Table 4.5 (11 and 15 mg kg^{-1}) relate to two barley samples from the Fukuoka region of Japan (Yoshizawa, 1997).

Fumonisins

The widespread contamination of maize with fumonisins is unmistakable and likely to remain an issue of overriding concern. Recent surveillance has confirmed the extensive distribution of fumonisins, particularly in maize produced in the tropics (Table 4.6, adapted from D'Mello and Macdonald, 1998). In most instances, the predominant fumonisin is FB_1. Outstanding features include high FB_1 concentrations in samples from Thailand (Yamashita *et al*., 1995), China (Wang *et al*., 1995a) South Africa (cited by Shephard *et al*., 2000) and Kenya (Kedera *et al*., 1999). Highest levels of FB_2 were reported in Argentinian (Chulze *et al*., 1996) and South African samples. In the Philippines, Thailand and Indonesia, FB_1 and FB_2 occurred in over 50% of maize samples, while incidence rates of 82–100% were recorded for samples from Italy, Portugal, Zambia and Benin. In Honduras, Julian *et al*. (1995) detected FB_1 in all 24 samples of maize tested. In Costa Rica, significant regional differences were observed in contamination of maize with FB_1, while in Mexico concern has been

Table 4.6. Worldwide contamination of maize kernels and products with fumonisins B_1, B_2 and B_3, (μg kg^{-1}). Data for maize products are identified by footnotes.

Country	FB_1	FB_2	FB_3	Total
Benin	n.d.[a]–2,630	n.d.–680		
Botswana	35–255	n.d.–75	n.d.–30	35–305
Mozambique	240–295	75–110	25–50	340–395
South Africa (Transkei)	< 50–46,900	< 50–16,300		
Malawi	n.d.–115	n.d.–30	n.d.	n.d.–145
Zambia	20–1,420	n.d.–290		
Zimbabwe	55–1,910	n.d.–620	n.d.–205	55–2,735
Tanzania	n.d.–160	n.d.–60	n.d.	n.d.–220
Kenya	110–12,000			
Honduras	68–6,555			
Mexico	1,000–1,800[b]			
Argentina	85–8,791	n.d.–11,300	n.d.–3,537	85–16,760
Costa Rica	1,700–4,780			
Italy	10–2,330	n.d.–520		10–2,850
Portugal	90–3,370	n.d.–1,080		90–4,450
USA	n.d.–350			
Vietnam	268–1,516	155–401	101–268	524–2,185
China	160–25,970	160–6,770	110–4,130	430–36,870
China	< 500–8,800[c]			
	< 500–5,700[d]			
	< 500–7,200[e]			
Taiwan	0–1,148	0–255		
The Philippines	57–1,820	58–1,210		
Thailand	63–18,800	50–1,400		
Indonesia	226–1,780	231–556		
Nepal				110–8,400

[a]Not detectable.
[b]Masa and tortillas.
[c]Maize (corn) meal.
[d]Unfermented batter.
[e]Fermented batter.

expressed at the higher levels in masa and tortillas compared with similar products imported from the USA. The data for maize meal and batter (Groves *et al*., 1999) prepared in the Shandong Province of China may also be viewed with disquiet. As with ZEN, a disturbing feature is the co-occurrence of fumonisins with other mycotoxins.

Co-occurrence

The co-occurrence of several mycotoxins in the same sample of cereal grains has provoked worldwide concern. Of considerable significance are consistent reports of co-occurrence of *Fusarium* mycotoxins (D'Mello and Macdonald, 1998). In the Lublin region of south-eastern Poland, type A trichothecene contamination of barley grain was linked with the natural incidence of FHB, in which the predominating organism was *F. sporotrichioides* (Perkowski *et al*., 1997). Of 24 barley grain samples, 12 were positive for T-2 toxin with a range of 0.02–2.4 mg kg^{-1}. In five of these samples, co-contamination with HT-2 toxin occurred with a range of 0.01–0.37 mg kg^{-1}. The findings of another study in Poland indicated that infection with *F. graminearum* can result in contamination of cobs simultaneously with DON and 15-ADON. Concentrations of DON and 15-ADON in *Fusarium*-damaged kernels ranged from 4 to 320 mg kg^{-1} (Table 4.5) and from 3 to 86 mg kg^{-1}, respectively, but the axial stems of the cobs were more heavily contaminated at 9–927 mg kg^{-1} and 6–606 mg kg^{-1}, respectively. A study of Japanese barley samples confirmed the co-occurrence of DON with NIV (Table 4.5; Yoshizawa, 1997). In addition, an appreciable number of the barley samples were found with 3-ADON at levels of up to 19 mg kg^{-1}. In highly contaminated grains, a positive correlation occurred between levels of DON and its acetyl derivatives. DON levels were always higher than those of 3-ADON and 15-ADON, with ratios ranging from 3 to 155. Regional differences were also observed in that DON was the major contaminant in grain from northern districts of Japan, whereas in central districts NIV was the predominant trichothecene. These differences were correlated with chemotype variants of *Fusarium* species. Furthermore, Yoshizawa (1997) and Lauren *et al*. (1996) revealed the occurrence of relatively high levels of ZEN with DON and NIV in cereal samples from Japan and New Zealand, respectively (Table 4.5). Of the 29 cereal samples tested in The Netherlands, 90 and 79% were positive for DON and NIV, respectively, with 76% containing both mycotoxins together, while ZEN occurred as a third contaminant, albeit at low levels (Table 4.5).

In China, FB_1 and AFB_1 co-occurred in 85% of maize samples (Wang *et al*., 1995a), while, in the Philippines, Thailand and Indonesia, FB_1 and FB_2 co-occurred with aflatoxins in 48% of maize samples. These fumonisins also co-occurred with NIV and ZEN (Yamashita *et al*., 1995). Multiple contamination of maize with fumonisins, DON, NIV and AFB_1 was also observed in north Vietnam (Wang *et al*., 1995b). Of additional concern is the co-occurrence of FB_1, fusaproliferin and beauvericin in Italian samples of maize.

Uptake and Disposition

The principal route of exposure to mycotoxins in humans is through consumption of contaminated diets. Uptake of foodborne mycotoxins is implied from the appearance of these compounds and associated derivatives (e.g. adducts) in body fluids. A wide array of factors may affect absorption. For example, FB_1 absorption is greater in fasted than in fed rats, with potentially profound implications for undernourished humans. Uptake of mycotoxins may also be affected by the onset of other conditions such as gastrointestinal disorders. Mycotoxin form can influence both uptake and disposal. Thus, studies with animal models indicate that hydrolysed FB_1 is absorbed more readily than FB_1 itself, and urinary excretion is also greater.

Mycotoxin metabolism is an important feature preceding events such as carcino-

genesis and hepatotoxicity. The hepatic metabolism of AFB_1 exemplifies the diverse reactions involving mixed-function oxidases and cytosolic enzymes. In the case of AFB_1, a variety of metabolites are produced, including AFM_1, aflatoxicol and AFB_1-8,9-epoxide. This epoxide form is a key intermediate which covalently binds to DNA to initiate carcinogenesis (Smith, 1997). Alternatively, the epoxide may bind to proteins. Conjugation with glutathione represents a detoxification route, whereas adduct formation with other proteins results in hepatotoxic effects. The major protein adduct in blood is AFB_1–albumin and its level in humans is indicative of exposure to the mycotoxin.

Disposition of mycotoxins occurs primarily via the faeces and urine. However, in humans, considerable quantities of aflatoxins must be ingested before they are detected in the urine. Furthermore, energy–protein malnutrition may determine aflatoxin disposition (de Vries *et al*., 1990). Thus, in children with kwashiorkor, aflatoxins in urine disappeared 2 days after rehabilitation on an aflatoxin-free diet, whereas, in those with marasmic kwashiorkor, excretion continued for up to 4 days. In contrast, faecal disposition of aflatoxin was observed up to the 9th day in kwashiorkor but had ceased by the 6th day in children with marasmic kwashiorkor. AFB_1 and aflatoxicol were the most frequently found form of aflatoxin in children with kwashiorkor, while AFB_1 occurred least frequently in stools from subjects with marasmic kwashiorkor, which also contained no aflatoxicol.

Toxicology

The classical assessment of toxicity of any compound inevitably centres on the acquisition of LD_{50} data (D'Mello and Macdonald, 1998). These values of acute toxicity are subject to wide variation, depending, for example, on age, sex and size of animals. There are also distinct species differences in sensitivity to a particular mycotoxin (Table 4.7). Thus day-old ducklings are more susceptible to AFB_1 than laboratory animals. OTA is also acutely toxic, but its effects (together with that of citrinin) in the kidney are of greater relevance to human health. On the other hand, ZEN is much less toxic but exerts profound effects on mammalian reproduction.

Table 4.7. Deleterious properties of mycotoxins as determined with animal models.[a]

Mycotoxins	LD_{50} data	Other properties
Aflatoxin B_1	1–17.9 mg kg^{-1} BW (laboratory animals), 0.5 mg kg^{-1} BW (ducklings)	Hepatotoxin; teratogen; immunotoxin; carcinogen
Aflatoxin M_1	12–16 μg per duckling (newly hatched)	Hepatotoxin; nephrotoxin; carcinogen
Ochratoxin A	<6 mg kg^{-1} BW (pigs)	Nephrotoxin; teratogen; carcinogen; immunosuppressor
Citrinin	105–112 mg kg^{-1} BW (mice)	Nephrotoxin
Deoxynivalenol	70 mg kg^{-1} (mice)	Feed intake inhibitor; teratogen; immunosuppressor
Diacetoxyscirpenol (DAS) and T-2 toxin	23 and 5 mg kg^{-1} BW, respectively (mice)	Induces oral lesions
Zearalenone	2–10 g kg^{-1} BW (rodents)	Inhibitor of reproduction; endocrine disruptor
Fumonisins	No data	Hepatotoxin; causes lesions in lungs and brain
Tenuazonic acid	125–225 mg kg^{-1} BW (mice)	Causes emesis and circulatory failure

[a]LD_{50} = median lethal dose; BW = body weight.

Table 4.8. Mycotoxins implicated in human disease.

Mycotoxin	Disease	Food/source	Countries
Ergot alkaloids	Ergotism (St Anthony's Fire)	Rye	Europe
Aflatoxins	Liver cancer; kwashiorkor, cirrhosis; acute hepatitis; Reye's syndrome	Peanuts; maize	East and West Africa; India; Taiwan; Thailand; the Philippines
Cyclopiazonic acid	'Kodua poisoning'	Millet	India
Ochratoxins	Balkan (and possible Tunisian) endemic nephropathy	Cereal grains	Bulgaria; Romania; former Yugoslavia; Tunisia
Citreoviridin	'Shoshin-kakke'	Rice	Japan
T-2 toxin	Alimentary toxic aleukia	Cereal grains	Former USSR
Fumonisins	Oesophageal cancer	Maize	South Africa
	Primary liver cancer	Maize	China
Moniliformin	'Keshan disease'	Maize	China

Human Disorders

Mycotoxins have long been implicated in specific human conditions (Table 4.8). However, conclusive evidence for such an association has yet to emerge for several of these disorders.

Ergotism

One of the ancient European episodes of mycotoxicoses in humans relates to ergotism (St Anthony's Fire) caused by the bioactive alkaloids produced in the sclerotia of *C. purpurea*. The alkaloids cause constriction of peripheral blood capillaries leading to oxygen starvation and gangrene of the limbs (Flannigan, 1991). Occasional cases of ergotism still occur to the present day.

Aflatoxicosis

Aflatoxins can induce acute effects in humans, and field cases continue to occur despite worldwide awareness of the toxicological and health implications. Thus, in 1974, an outbreak of liver disease occurred in India following the consumption of mouldy grain containing aflatoxins (see D'Mello and Macdonald, 1998). Of 997 subjects, 97 were reported to have died during this episode. Principal pathological features in the liver included destruction of centrilobular zones, thickening of central veins and cirrhosis. Chronic aflatoxin exposure may modulate immune function, thereby increasing susceptibility to infection. Indeed, it has been suggested that aflatoxins are the major cause of kwashiorkor in children.

Ochratoxicosis

Balkan endemic nephropathy is a chronic disease occurring in rural populations of Bulgaria, Romania and the former state of Yugoslavia (see D'Mello and Macdonald, 1998). In affected subjects, the kidneys are markedly reduced in size and, histologically, the disease is characterized by tubular degeneration, interstitial fibrosis and glomerular defects. Tubular function is also impaired. The similarities to porcine nephropathy are striking and have led to the conclusion that OTA is also the causative agent in Balkan endemic nephropathy. However, the co-occurrence of OTA with citrinin in cereals from Bulgarian villages with a history of Balkan endemic nephropathy suggests an interaction between the two mycotoxins in the aetiology of this condition (Vrabcheva

et al., 2000). It will be noted that the two mycotoxins have similar properties in animal models (Table 4.7). A possible endemic ochratoxin-related nephropathy has also been suggested to occur in Tunisia. Affected subjects were classified into those with chronic interstitial nephropathy, chronic glomerular nephropathy and chronic vascular nephropathy.

Cancer

Current concern over mycotoxins centres on their carcinogenic potential in humans. In tropical countries, particularly East and West Africa, India, Thailand, the Philippines and China, aflatoxin exposure is a continuing health issue among the indigenous populations. There is now good epidemiological evidence linking aflatoxin exposure with the incidence of liver cancer (Smith, 1997). In one study, it was possible to demonstrate that men were more sensitive than women to the carcinogenic effects of aflatoxins but that in both cases there was a linear effect of dose on the development of liver cancer. The epidemiological data should be interpreted with caution, as other factors such as malnutrition and disease may have contributed to the incidence of liver cancer. Furthermore, interactions may occur. Thus, Wang *et al*. (1996) indicated that aflatoxin exposure may enhance the carcinogenic potential of hepatitis B virus. The generally accepted order of carcinogenicity is AFB_1 > AFG_1 > AFB_2 > AFG_2. In toxicological classification, AFB_1 has been designated as a group 1 carcinogen (i.e. sufficient evidence in humans for carcinogenicity), whereas AFM_1 falls in the group 2B category (i.e. probable human carcinogen).

Epidemiological evidence has also been presented to link human oesophageal cancer in South Africa with dietary exposure to the fumonisins. In addition, it has been suggested that, in China, fumonisins may promote primary liver cancer initiated by AFB_1 and/or hepatitis B virus (Ueno *et al*., 1997).

Continuing Human Exposure to Foodborne Mycotoxins

Despite enhanced awareness and the adoption of legal or advisory guidelines, human exposure to foodborne mycotoxins continues on a global scale, even in developed countries (see D'Mello and Macdonald, 1998). Recent evidence is summarized in Table 4.9 for aflatoxins and in Table 4.10 for OTA. The tables are not designed to be exhaustive but rather illustrative of widespread exposure to these mycotoxins.

The evidence of exposure generally is based on mycotoxin residues in body fluids, mother's milk and tissue specimens. In addition, the association between mycotoxin exposure and cancer relies on presumptive intake of contaminated foods, rather than direct determinations of metabolites or DNA adducts. However, efforts are now focusing on measurements of the major adducts in tissues and fluids.

Aflatoxins

The widespread contamination of maize and peanuts with aflatoxin is reflected in the analyses of faeces, urine, blood and breast milk samples of people in different parts of Africa (Table 4.9). In addition to the four forms of aflatoxin, metabolites such as aflatoxicol, AFM_1 and AFM_2 may appear in body fluids and tissues. One study showed widespread fetal exposure to aflatoxins in East and West Africa, as demonstrated by analysis of cord and maternal blood samples (Maxwell, 1998). Aflatoxins were also detected in breast milk samples of mothers. Thus, there is widespread pre- and post-natal exposure of infants to aflatoxins, which may predispose children to infection. Indeed, a hypothesis has been advanced implicating aflatoxin exposure with the pathogenesis of kwashiorkor in African children. It will be noted that despite stringent EU regulations, detectable levels of AFB_1–albumin adducts have been recorded for individuals in the UK (Table 4.9; Turner *et al*., 1998). Detection of adducts is clearly a highly sensitive means of

Table 4.9. Continuing human exposure to aflatoxins.

Region/country	Basis of evidence	Observations
Kenya	Analysis of urine and stools	Following feeding of aflatoxin-free diet, children with kwashiorkor continued to excrete aflatoxins in urine for 2 days; but those with marasmus excreted aflatoxins for up to 4 days. Differences also seen in the type of aflatoxins discharged in faeces
East and West Africa	Analysis of body fluids	Up to 7 ng of AFM_1 and 65 μg of AFB_1 l^{-1} in cord blood; AFM_1 and AFM_2 detected at 12–1689 ng l^{-1} in maternal blood
Sierra Leone	Analysis of cord blood and maternal sera	Aflatoxins[a] detected in 91% of cord blood and 75% of maternal blood samples. Highest values in cord blood recorded for AFB_1, AFG_1, AFG_2, AFM_1 and AFM_2; in maternal blood, AFG_2 detected most frequently
Sierra Leone	Analysis of serum, urine and breast milk	Major aflatoxins and metabolites detected in serum and urine of children of varying nutritional status; 95% of breast milk samples contaminated mainly with aflatoxins[a]
Nigeria	Analysis of autopsy kidney samples	Concentrations ranged from 6 pg g^{-1} for AFB_2 to 42,452 pg g^{-1} for AFG_1 in kidneys of children who died from kwashiorkor; comparable values in kidneys of children who died from miscellaneous diseases were 1843 and 23,626 pg g^{-1}, respectively. Aflatoxicol detected in kidneys from both groups
UK	Serum albumin adducts	Detectable levels of AFB_1–albumin adducts observed in the UK population
Worldwide	Hepatocellular carcinoma *p53* G→T transversions at codon 249	Increased proportions of *p53* mutations in hepatocellular carcinoma probably attributable to aflatoxin exposure. Further studies required to confirm that *p53* mutations are the fingerprint of aflatoxin exposure

[a]See also Table 4.10.

assessing human exposure to aflatoxins, and it is likely that the UK results will be replicated in other developed countries. The UK data suggest problems with sampling and monitoring of regulated imported foodstuffs. Alternatively, or in addition, aflatoxin exposure may arise from the consumption of other imported foods not currently controlled by legislation. Spices, breakfast cereals and ethnic foods might fall in this category.

Ochratoxin A

OTA exposure in humans is also widespread, as indicated by analyses of physiological fluids (Table 4.10). However, geographical differences may exist. Thus it has been concluded that levels of exposure are lower in Japan than in Europe (Ueno, 1998; Table 4.10). Regional variations are also apparent within Europe. In Croatia, the highest blood levels of OTA were reported for inhabitants living in villages noted for the incidence of endemic nephropathy (Radic *et al.*, 1997). This observation is consistent with the incidence of Balkan endemic nephropathy in the region. Levels in some Norwegian and Italian breast milk samples give cause for concern in that they imply infant OTA exposure exceeding the tolerable daily intake (TDI) of 5 ng kg^{-1} bodyweight. In Tunisia, an endemic

Table 4.10. Continuing human exposure to ochratoxins.

Region/country	Basis of evidence	Observations
Sweden	Blood analysis	OTA levels below 0.3 μg l^{-1}
Norway	Breast milk analysis	OTA detected in 33% of samples at levels of 10–130 ng l^{-1}; 12% of samples contained > 40 ng l^{-1}
UK	Blood and urine analysis	All blood and 92% of urine samples contained OTA
Croatia	Blood analysis	OTA levels 2–50 ng ml^{-1}
Italy	Breast milk analysis	Significant exposure of babies to OTA at levels exceeding tolerable daily intakes estimated from animal models
Hungary	Blood and colostrum analysis	52% of random blood samples with 0.2–12.9 ng ml^{-1}; 41% of colostrum samples with 0.2–7.3 ng ml^{-1}
Spain	Blood analysis	53% of healthy donors and 78% of patients undergoing haemodialysis positive for OTA; mean concentrations 0.7 and 2 ng ml^{-1}, respectively
Egypt	Blood and urine analysis	OTA levels of 0–10 and 0–8 ng ml^{-1}, respectively, in blood and urine of patients with nephrotic syndrome; OTA levels of 0–3.4 and 0–0.3 ng ml^{-1}, respectively, in urine of potential kidney donors and healthy volunteers
Tunisia	Blood analysis	Chronic forms of interstitial, glomerular and vascular nephropathy; OTA levels of 25–59 μg l^{-1} in patients with interstitial nephropathy, 6–18 μg l^{-1} in other groups and 0.7–7.8 μg l^{-1} in the general population
Sierra Leone	Blood and urine analysis	OTA detected in 25% of cord blood samples at levels of 0.2–3.5 ng ml^{-1}; 24% of urine samples contained OTA; 20% of urine samples contained OTB
Sierra Leone	Breast milk analysis	Confirmed exposure of infants to combinations of OTA and various aflatoxins
Canada	Blood analysis	OTA levels 0.6–1.4 ng ml^{-1} depending on geographical location
Japan	Blood analysis	OTA levels 0.004–0.28 ng ml^{-1}

OTA-related nephropathy is thought to occur with similarities to the Balkan syndrome. Three subsets were identified in affected subjects: those with chronic interstitial nephropathy, chronic glomerular nephropathy and chronic vascular nephropathy. Patients with chronic interstitial nephropathy had the highest blood OTA levels in comparison with the other subgroups or with the general population. However, even the latter group had overall blood OTA levels which were in excess of those seen in Sweden. In Sierra Leone, monitoring of breast milk samples showed that only 9% were mycotoxin-free, with 35% containing OTA. It is clear that infants and mothers in Sierra Leone are exposed to OTA at levels greater than the current allowances of TDI (Table 4.10). The urinary excretion of OTB by infants in Sierra Leone was quantitatively similar to that of OTA (Jonsyn, 1999).

Other individuals at risk may be patients with renal disorders. Although OTA and citrinin are established nephrotoxins, any association with conditions such as the Balkan and Tunisian endemic nephropathies still remains tentative. Thus, the higher incidence and concentrations of OTA in blood of patients requiring haemodialysis and in those with urothelial cancer await elucidation to distinguish between cause and effect (Table 4.10; Jimenez *et al*., 1998; Wafa *et al*., 1998).

Combinations

The pre-natal and neonatal exposure of children in Sierra Leone to combinations of aflatoxins and OTA is noteworthy (Jonsyn, 1998). Of 64 cord blood samples analysed, 94% contained either OTA, aflatoxins or both (Tables 4.9 and 4.10). It is suggested that pre-natal exposure to such combinations may have resulted in low birth weights and premature mortality of infants. Continued exposure post-natally is likely in view of contamination of breast milk and cereal grains with combinations of OTA and aflatoxins. Of particular concern, however, is the apparent absence of direct determinations of human exposure to combinations of aflatoxins and fumonisins in countries where peanuts and maize constitute the staple foods (Tables 4.3 and 4.6).

Tolerable daily intakes

The foregoing account demonstrates that mycotoxin intake is inevitable even in countries with stringent regulatory and process controls. In instances where there are adequate toxicological data, TDI have been estimated for humans (Table 4.11). As previously stated, the actual intakes in many countries may exceed the TDI allowances. In the case of AFB_1, the TDI estimates have been based on studies conducted in certain tropical countries where infection with hepatitis B virus is an additional carcinogenic factor. In countries where this virus is not a major risk, the TDI for AFB_1 may be set considerably higher. It will be noted that, for the fumonisin carcinogens, TDI limits have yet to be established.

Table 4.11. Tolerable daily intakes (TDIs) of major mycotoxins (kg^{-1} body weight).

Mycotoxin	TDI
Aflatoxin B_1	0.11–0.19 ng
Ochratoxin A	1.5–5 ng
Deoxynivalenol	1.5 μg (infants) 3.0 μg (adults)
Zearalenone	100 ng
Fumonisins	Inadequate data

Regulatory Control

The ubiquitous distribution, acute effects and carcinogenic potential of mycotoxins have resulted in the imposition or adoption of regulations for maximum permitted levels of these contaminants in primary foods and associated products. Regulations also apply to feedingstuffs in order to reduce transmission of mycotoxins to edible animal products. Van Egmond and Dekker (1995) indicated that 90 countries had regulations relating to maximum permissible levels of mycotoxins in various commodities. However, 13 countries were known to have no regulations and, for some 50 countries, mostly in Africa, no data were available. It is unlikely that the situation has changed significantly since 1995. Virtually all developed countries have statutory regulations for the aflatoxins and advisory directives for a limited number of the other mycotoxins. Of particular concern, however, is the lack of statutory or advisory regulations for control of fumonisins in foods.

Rationale

With the aflatoxins, the underlying rationale is based on the need to reduce contamination to 'irreducible levels', defined as the concentration which cannot be eliminated from a food without involving the complete rejection of the food, thereby severely limiting the ultimate availability of major food supplies. However, in the evolution of statutory regulations for aflatoxins, the guiding principle has remained unaltered, which is to reduce contamination to the lowest level that is 'technologically achievable', taking into account advances in analytical methodologies. In the preparation of proposals, comments received through groups such as the World Trade Organization are taken into account. The resulting regulations, therefore, represent a compromise between avoidance of international trade disputes with producer countries and maintenance of consumer protection.

Statutory instruments

On a worldwide basis, statutory control only exists for the aflatoxins, and current regulations reflect evolution over time (see D'Mello and Macdonald, 1998). For example, in the UK, port authorities had applied a 10 μg kg^{-1} total aflatoxin limit to imported nuts and dried figs. Consignments exceeding this value have been rejected since implementation of regulations in 1988. The statutory limits were amended and extended in 1992 to reflect recommendations that aflatoxin concentrations in susceptible commodities be reduced to the lowest level 'that is technologically achievable', and to take account of improvements in analytical methodology. The regulations were extended to dried fig products, which were also considered to be susceptible to aflatoxin contamination. The limits were reduced to 4 μg kg^{-1} for nuts, dried figs and their products for sale or for incorporation in any compound food or for import for direct human consumption; for such imports intended for further processing before sale or incorporation in any compound food for human consumption, the limit for total aflatoxin was set at 10 μg kg^{-1}. In instances where aflatoxin levels between 4 and 10 μg kg^{-1} were found, the importer was required to give a written undertaking to process the batch so that it complied with the 4 μg kg^{-1} limit. Alternatively, the consignment could be returned to the consignor, or used for a purpose other than human consumption, or destroyed. Schedules for food sampling and analysis of aflatoxins were also provided. The latter included performance parameters for the aflatoxin tests. For example, a detection limit of ≤ 2 μg kg^{-1} was set for foods intended for direct human consumption. The statutory instruments also included regulations concerning importation procedure, authorized places of entry and duties of authorized officers.

In the UK, new regulations for aflatoxins were introduced on 30 June 1999, bringing into force an EC regulation setting maximum limits for the foods most commonly contaminated with aflatoxins, namely cereals, milk, nuts, dried fruits and any products derived from these commodities. The new regulations contain separate maximum limits for AFB_1 as well as total aflatoxins. Higher limits are designated for foods which will undergo further processing. As before, the new regulations prescribe methods of sampling and analysis of aflatoxins for use by law enforcement bodies. The 1992 UK regulations for aflatoxins were revoked on introduction of the new measures. A comparison of food regulations for aflatoxins in force in selected countries is presented in Table 4.12.

Since human exposure to aflatoxins is determined partly by intake via milk and since animal health and productivity may be compromised by these compounds, statutory regulations also apply to feedingstuffs (Table 4.13). Higher limits are allowed for animal feeds than for human foods. It will be noted that, in parts of Asia, permitted levels of aflatoxins in human foods (Table 4.12) equal or exceed current norms for animal feeds in EU countries (Table 4.13).

Draft EU regulations for ochratoxin A and deoxynivalenol

Proposed EU regulations for OTA limits in human foods and beverages are at an advanced stage of preparation. In addition, action levels have been intimated for DON in cereals and flour. Measures under discussion within European Commission expert committees are presented in Table 4.14. There appears to be general agreement for statutory limits for cereals, but no consensus has yet emerged on the precise levels. Discussion necessarily has focused on limits for cereals since these food items account for 50–70% of OTA intakes in Europe. The position with derived cereal products such as bran warrants further consideration. Recently, attention has turned to limits for dried vine fruit and spices. It is not clear how the proposed action levels for DON will be interpreted and used in the absence of sufficient data on which to base regulatory limits.

Table 4.12. Examples of worldwide regulations for control of aflatoxins in human foods (μg kg^{-1}).

		Aflatoxins: maximum levels		
Country	Foods	B_1	$B_1+B_2+G_1+G_2$	M_1
European Union	Groundnuts, nuts and dried fruit, and processed products thereof intended for direct human consumption or as an ingredient in foodstuffs	2	4	
	Groundnuts to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	8	15	
	Nuts and dried fruit to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5	10	
	Cereals and processed products thereof intended for direct human consumption or as an ingredient in foodstuffs	2	4	
	Spices[a]	5	10	
	Milk			0.05
South Africa	All foods	5	10	
Taiwan	Cereals		50	
Thailand	All foods		20	
Japan	All foods	10		
USA	All foods		20	
	Milk			0.5

[a]Proposals under consideration.

Table 4.13. Examples of worldwide regulations for aflatoxins in animal feedingstuffs (μg kg^{-1}).

Country	Aflatoxins	Feedingstuffs	Maximum levels	Status
Indonesia	$B_1+B_2+G_1+G_2$	Copra	1000	Proposal
		Groundnut	200	
		Sunflower seed meal	90	
European Union	B_1	Straight feedingstuffs except: groundnut, copra, palm kernel, cottonseed, babassu, maize and products derived from the processing thereof	50 20	Statutory
		Complete feedingstuffs for cattle, sheep and goats (with the exception of complete feedingstuffs for calves, lambs and kids)	50	
		Complete feedingstuffs for pigs and poultry (except those for young animals)	20	
		Other complete feedingstuffs	10	
Taiwan	$B_1+B_2+G_1+G_2$	Feed, oilseed meals for feed under 4% of mixed feed	1000	Statutory
USA	$B_1+B_2+G_1+G_2$	Cottonseed meal	300	Statutory
		Maize and groundnut products intended for breeding beef cattle/pigs or mature poultry	100	
		Maize and groundnut products intended for finishing beef cattle	300	

Table 4.14. Permitted levels (μg kg^{-1}) for ochratoxin A (OTA) in foods and beverages and action levels for deoxynivalenol (DON) in cereals: measures under discussion within the European Commission.

		Suggested/action limits	
Food/beverages	Categories	OTA	DON
Cereals	To be subjected to sorting or other physical treatment prior to human consumption or use as an ingredient in foodstuffs	5	750[a]
	Cereals and processed products thereof intended for direct human consumption or use as an ingredient in foodstuffs	3	500
Coffee	Green beans	8	
	Roasted beans and coffee products	4	
Dried vine fruit	Currants, raisins and sultanas	10	
Spices		10	
Beer		0.2	
Wine		0.2–1.0	

[a]Flour used as raw material in food products; monitoring level for raw cereals.

Advisory directives

In several countries, advisory directives exist which are not enforceable by law. However, the limits suggested have been used to reduce human exposure to mycotoxins. In the USA and Canada, the advisory level for DON is 1000 μg kg^{-1} in finished wheat products such as flour and bran. For apple products including juice, cider and puree, the advisory level for patulin in the UK is set at 50 ppb.

Methodologies

Specific methodologies are prescribed for the aflatoxins, OTA and DON. Of particular relevance are the protocols for the legally controlled aflatoxins.

Sampling

Due to the heterogeneous distribution of mycotoxins in foods, adequate sampling is a primary consideration. In the official control of aflatoxins in the EU, samples are taken according to prescribed methods. Three types of samples are identified. An incremental sample is the quantity of food taken from a single position in a lot or sublot. An aggregate sample represents the combined total of all the incremental samples taken from the particular lot. Laboratory samples are derived from the mixed aggregate sample. The number and size of incremental samples are laid down in the provisions, and specific protocols are prescribed for nuts and dried fruit, milk and derived products. Treatment of laboratory samples is also described in detail.

Analytical

Specific methods for the determination of aflatoxins are not prescribed at the EC level, and laboratories may select any method provided that it is consistent with a number of criteria based on recoveries and precision parameters such as repeatability and reproducibility. However, adequate laboratory standards must be demonstrated through participation in proficiency testing and internal quality control schemes. In practice three principal methods are employed in aflatoxin analysis worldwide. Thin-layer chromatography (TLC) remains the method of choice in many countries and its efficacy has been enhanced by new technology including the use of immunoaffinity columns in clean-up and the application of densitometry for quantification. Other methods include liquid chromatography and enzyme-linked immunosorbent assay. For DON, the official methods used for regulatory purposes in the USA and Canada are TLC and gas chromatography.

Compliance

Surveillance of food consignments at ports of entry and from retail outlets has resulted in a number of actions to ensure compliance with directives. It is instructive to consider recent measures concerning mycotoxin contamination of nuts, fruit and apple juice (Table 4.15). The EC proposed and implemented actions listed in Table 4.15 were conducted under Article 10 of the Food Hygiene Directive (93/43/EEC). It is clear that despite increased awareness and a protracted history, aflatoxin contamination of nuts and products is still a formidable issue resulting in temporary suspension of imports into EU Member States. Following the EU mission to Iran in 1998, improvements in pistachio production were noted but it was recommended that the suspension of imports be extended for an additional period of 12 months to allow for further investigations. The specific points at issue were the development of an effective traceability system and improvements in sampling methods. It should be noted that, in the case of the peanut butter contamination (Table 4.15), no action was taken because samples were not taken for enforcement

Table 4.15. Actions resulting from mycotoxin surveillance of foods.

Commodity	Year	Country instigating action	Issue	Outcome
Peanuts (Egyptian)	1999	EU Member States	Unacceptable incidence and levels of AF.[a] No assurances given by Egyptian authorities of measures to reduce contamination	Temporary suspension (initially for 4 months) of imports into EU states. EC mission to visit Egypt to conduct further investigations
Peanuts (Indian)	1999	EU Member States	Unacceptable incidence and levels of AFB_1 (up to 400 μg kg^{-1})	Proposal for temporary suspension of imports into EU states not implemented. Indian authorities provided assurances of improvements in production practices. Since then no further reports of contaminated peanuts from India
Peanut butter (retail own-brand samples, UK)	1994	UK	Five samples with AF levels in excess of 4 μg kg^{-1} (one sample with 20 μg AF kg^{-1})	Batch with AF at 20 μg kg^{-1} withdrawn from sale; no action taken over other batches as sampling did not comply with official regulations
Pistachio nuts (Iranian)	1997	EU Member States	Unacceptable incidence and levels of AFB_1	Temporary suspension of imports into EU countries
Dried vine fruits	1997	UK	High-level consumers calculated to exceed the tolerable intake of OTA[b]	Industry to implement test procedures for OTA both in producing countries and on import into the UK; code of practice suggested; surveillance to verify efficacy of new measures
Apple juice	1998	UK	Four samples of freshly pressed juices contained patulin in the range 73–171 ppb	Relevant local authorities informed of results. Producers contacted to discuss findings and to identify strategy for reducing contamination

[a]Aflatoxin.
[b]Ochratoxin A.

purposes; neither were the samples taken in accordance with the relevant Regulations. Compliance can also be secured with non-statutory directives. For example, in 1997, it was calculated that high-level consumers of dried vine fruits in the UK were at risk due to potentially widespread contamination of these food items with OTA. The dried-fruit industry has responded with plans, as outlined in Table 4.15. Patulin contamination of apple juices in the UK has clearly declined since 1995 when 6% of samples contained levels above the advisory limit. In 1995, action was taken to remove affected batches from sale and to name those brands with unacceptable levels of contamination.

Preventive Strategies

The abiding principle in food safety must be the prevention of contamination, as curative methods are of limited efficacy. When fungicides are used effectively to control fungal diseases of crop plants, then the risk may be minimized. However, under certain conditions, fungicides may enhance mycotoxin production (see D'Mello *et al*., 1998). In the case of FHB of cereals, it is generally accepted that fungicide control is only partially effective and the potential exists for mycotoxin contamination of harvested grain (Table 4.16). There is growing optimism that, in terms of an environmentally acceptable solution, plant selection and breeding offer considerable potential.

Experimental studies show that breeding maize plants that are resistant to colonization and ear rot caused by *A. flavus* generally results in lower contamination of grain with AFB_1. Similarly, exploitation of genetic resistance to FHB in wheat has been used successfully to reduce DON levels in the grain. Selection of Chinese cultivars of wheat which are resistant to FHB can also result in lower levels of DON in kernels compared with those of grain from susceptible Canadian cultivars.

Adequate storage of harvested grain, nuts and fruit is fundamental in the prevention of mycotoxins from storage fungi. Grain moisture content and temperature are critical factors during storage. In addition, insect and rodent invasion should be minimized as these pests adversely affect the microclimate within grain silos and also act as important vectors for transmission of fungal inoculum.

Prevention of aflatoxin-induced cancers is one strategy which may be advocated for subjects at particular risk in Africa and Asia. Experimentally, it has been shown that

Table 4.16. Fungicide efficacy: a tentative classification for trichothecene control.[a]

Class	Descriptor	Examples of fungicides	Trichothecene[b] affected	Conditions
I	Effective	None	—	—
IIA	Partially effective (growth-dependent inhibition; mycotoxin residues possible)	Tebuconazole	DON	Field trial
		Thiophanate-methyl	DON and NIV	Field trial
		Prochloraz	3-ADON	*In vitro*
IIB	Partially effective (direct inhibition of mycotoxin synthesis; disease/infection/ fungal growth possible)	Thiabendazole	DON	Field trial
		Dicloran	DAS	*In vitro*
IIIA	Ineffective	Propiconazole	DON	Field trial
		Morpholines	3-ADON	*In vitro*
IIIB	Stimulatory and/or inducing resistance	Iprodione	DON	Field trial
		Trademorph	T-2 toxin	*In vitro*
		Difenoconazole	3-ADON	*In vitro*
		Carbendazim	T-2 toxin	*In vitro*
		Azoxystrobin	T-2 toxin, DAS and NEO	*In vitro*

[a]D'Mello *et al.* (2001).
[b]DON = deoxynivalenol; NIV = nivalenol; 3-ADON = 3-acetyl deoxynivalenol; DAS = diacetoxyscirpenol; NEO = neosolaniol.

antioxidants, when administered during aflatoxin exposure, significantly reduced the incidence of hepatic cancers in rats. Aflatoxin–DNA adducts formed in the liver were also reduced substantially by antioxidant provision. In other studies with rats, antioxidants provided protection against free radical-mediated lipid peroxidation induced by DON or T-2 toxin. Other rat studies point to the potential of dithiocarbamates in the chemoprevention of liver carcinogenesis induced by AFB_1. In trials with humans showing detectable serum aflatoxin–albumin adduct levels, administration of the anti-schistosomal drug oltipraz may be beneficial. At intermittent high doses, oltipraz inhibited activation of aflatoxin, while at sustained low doses the drug increased elimination of the mycotoxin as the aflatoxin–mercapturic acid conjugate. At the practical level, prevention of aflatoxin-induced liver cancer may be feasible through consumption of brassica vegetables. Thus, rats given a diet with freeze-dried cauliflower showed reduced toxic effects of AFB_1. Epidemiological evidence strongly indicates that consumption of brassica vegetables is associated with reductions in the incidence of cancer at several sites in humans, possibly through provision of natural sulphur-containing compounds such as glucosinolates and *S*-methylcysteine sulphoxide.

Remedial Measures

Mycotoxin contamination of foods is unavoidable even with implementation of good agronomic practices. Once mycotoxin contamination of primary foods has occurred, a number of remedial options, of varying efficacy, may be considered.

Efficacy of processing technologies

Processing is an acceptable method of reducing mycotoxin contamination, and current legislation and advisory directives (Tables 4.12 and 4.14) distinguish between foods intended for direct consumption and those likely to warrant some kind of treatment. Conventional physical methods range from basic to sophisticated. For example, sorting of susceptible foods such as nuts and cereals has been advocated. It was observed that, when pistachio nuts were sorted on the basis of quality, a set of process streams with differing aflatoxin levels were obtained. These levels were correlated with preharvest physical damage, such as that caused by hull splitting and insect invasion. Hull discoloration was also linked with high aflatoxin content. In developing countries, hand sorting of visibly diseased maize kernels is an effective method of reducing exposure to mycotoxins such as DON and fumonisins, but some prior training of personnel may be advisable. Methods to remove DON from contaminated cereal grains primarily depend upon physical separation from the more heavily contaminated outer layers of the kernels. Milling of grain to produce flour and extrusion to yield products for direct consumption are other examples with potential to reduce contamination. The efficacy of decontamination varies with the procedures used, but none of these has been shown to be completely effective.

Processing of green coffee beans in the manufacture of soluble powder can markedly reduce final OTA residues. Thus, Blanc *et al*. (1998) demonstrated that cleaning of beans by density segregation and air suction removed some OTA, but the most significant reduction occurred during roasting. Soluble coffee powder contained only 16% of the OTA originally present in the beans.

Other treatments are known to be ineffective. Thus in the preparation of maize-based products, baking or frying has little effect on fumonisin contamination. However, fumonisin–sugar molecules may form during food processing, with the result that toxicity may be reduced. Brewing is another ineffective process for reducing mycotoxin contamination of beer.

Ammoniation is a highly effective commercial process for detoxifying aflatoxins in animal feed. As a result, AFM_1 residues in milk of dairy cows offered feeds decontaminated in this manner are substantially reduced or eliminated altogether.

Conclusions

Current surveillance indicates unavoidable, widespread and continuing mycotoxin contamination of basic plant products, with global implications for human health. Concentrations of aflatoxins in maize and peanut kernels regularly exceed safety threshold limits. At particular risk are consumers in warm and humid countries where these foods constitute a significant proportion of the diet. OTA and trichothecenes are ubiquitous, occurring primarily in the major cereal grains. However, use of grain contaminated with OTA in brewing and as animal feed regularly results in transfer of residues into beer and offal. In addition, the occurrence of OTA in dried vine fruits and green coffee beans is an emerging issue currently under review in several EU countries. Of considerable concern is the widespread contamination of maize and associated products with fumonisins. Humans in the tropics and southern hemisphere countries, for example, are frequently exposed to various combinations of foodborne mycotoxins.

Contamination of foods with the major mycotoxins continues unabated even in specific regions where the incidence of hepatocellular and oespohageal cancers and nephropathy have been linked epidemiologically with consumption of, respectively, aflatoxins, fumonisins and ochratoxins. However, there is evidence of chronic dietary exposure in a wider context, possibly associated with an array of other human disorders. Thus, foodborne aflatoxins may enhance the carcinogenic potential of hepatitis B virus. It has also been proposed that kwashiorkor in African children may be a primary manifestation of aflatoxicosis. Moreover, it has been suggested that pre-natal exposure to combinations of aflatoxins and ochratoxins result in low birth weights and premature mortality of infants in Sierra Leone and elsewhere. Post-natal exposure is inevitable in view of contamination of breast milk and cereal grains with combinations of aflatoxins and ochratoxins. Of particular concern, however, is the absence of direct assessments of human exposure to combinations of aflatoxins and fumonisins in countries where peanuts and maize constitute the staple foods.

In Europe and elsewhere, legal and advisory regulations exist with the aim of reducing mycotoxin contamination of foods to the lowest level that is technologically achievable. However, even in these countries, there is evidence of general chronic exposure to particular mycotoxins. The detection of specific aflatoxin–albumin adducts in the serum of UK individuals suggests problems with sampling and monitoring of regulated imported foodstuffs. Alternatively, or in addition, aflatoxin exposure may arise from the consumption of other imported foods, such as breakfast cereals and spices, not currently controlled by legislation. The occurrence of OTA in the blood and breast milk of donors in several European countries underlines the need for statutory control with respect to contamination of cereals, dried vine fruits and coffee. The lack of any legislative measures for aflatoxins and ochratoxins in countries at greatest risk is an issue of considerable concern. As regards the fumonisins, there is an urgent need to introduce guidelines for its control in maize-based foods and to monitor exposure to this group of contaminants in vulnerable populations.

Acknowledgements

This work was partly funded by the Scottish Executive Rural Affairs Department.

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5 Pesticides: Toxicology and Residues in Food

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Introduction

The term pesticides includes all chemical, natural or synthetic substances used to fight parasites on crops. Though pesticides are used mainly for this purpose, they can also be used to fight the carriers of illnesses such as malaria, yellow fever, typhoid fever, etc., or even against domestic insects. About 20% of the production of insecticides is used for this purpose. The first organic pesticide to be introduced on the market by Geigy in 1939 was dichlorodiphenyltrichloroethane (DDT) as a result of systematic research on its insect killing activity by the Swiss entomologist Paul Müller. Till then, the substances available to fight crop parasites were very limited, and almost all inorganic, e.g. sulphur (reported by Homer in 1000 BC), arsenic (recommended by Pliny in 50 BC to kill insects) and a few natural insect-killing substances, such as pyrethrum, rotenone and nicotine. A few catastrophes, such as the destruction of a harvest of potatoes by *Phytophthora infestans* in Ireland in 1845, causing a million deaths and driving a 1,500,000 people to emigrate, and the destruction of French vineyards by downy mildew, which was imported from the USA in 1878, led to the development of significant research in mineral chemistry for plant protection. The fungitoxic activity of copper was discovered casually by Millardet in 1882. He observed that the rows of grapevine along roads that were treated with copper sulphate and lime to discourage trespassers were protected from *Plasmopara viticola* and, based on this information, he developed the Bordeaux mixture, containing calcium hydroxide and copper sulphate, with which he managed to control this pathogen. DDT, on the other hand, was the result of systematic research that opened up a new methodology in the research on pesticides. Before being used in agriculture, DDT was applied extensively against the carriers of diseases during and after the Second World War. Diseases such as malaria and typhoid fever were eliminated in many areas where they had been endemic. Paul Müller was awarded the Nobel Prize for Medicine in 1948 for this discovery, which saved millions of lives.

The extraordinary success of DDT against a host of insects harmful both to agriculture and to human health led to the development of other synthetic products. At present the number of compounds marketed around the world as pesticides is about 1300 (Tomlin, 1997). Due to their heterogeneous nature, these compounds are difficult to classify; they are normally classified, according to

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their target, as insecticides, fungicides and herbicides. Other less important typologies (4.7% of the world market in 1998; Wood Mackenzie, 1999), such as nematocides, fumigants, growth regulators, etc., come under the general classification of 'others'. The term insecticides also includes acaricides.

Insecticides

The world market for insecticides in 1998 was US$6930 million or 23.9% of the total market value (Table 5.1). With 37.1% in the 1960s and 1970s, insecticides had the largest market share among the pesticides. This was later reduced progressively to 34.7% in 1980, 29.0% in 1990 and 23.9% in 1998 (Wood Mackenzie, 1999).

Most insecticides come under one of the following five chemical classes: organochlorine compounds, organic phosphorus compounds, carbamates, pyrethroids and benzoylureas. The term 'others' includes stannic organic compounds such as fenbutatin oxide, growth regulators such as cyromazine, etc.

As can be seen from the data reported in Table 5.2, from a commercial point of view organophosphorus compounds are the most important class with 37.2% of the market, followed by pyrethroids and carbamates with 18.3 and 13.9%, respectively. Organochlorine compounds and benzoylureas are less important. The decline in the use of organochlorine compounds can be attributed to the fact that some of them, such as DDT, aldrin, dieldrin, eldrin, etc., have been banned all over the world, while the benzoylureas have only been introduced recently. Fruit and vegetables are the crops that take up most of the pesticides with 38.9%, followed by cotton, rice and maize with 22.8, 16.1 and 9.4%, respectively. Ninety-three per cent of the demand for pesticides for rice is located in Asia, which accounts for the fact that Asia has the largest world consumption of pesticides (Table 5.2).

Organochlorines

DDT is the historic predecessor of synthetic pesticides and organochlorine compounds. It was followed in fast succession by other molecules belonging to the same chemical family, such as lindane (1942), aldrin (1948), dieldrin (1949) and endrin (1951). The characteristics shared by this chemical class of pesticides is their effectiveness towards numerous insect

Table 5.1. The development of the pesticide market.

Year	1970	1980	1990	1998
Value ($ billion)	2.6	11.4	26.1	29.0
Insecticides (%)	37.1	34.7	29.0	23.9
Fungicides (%)	22.2	18.8	21.0	19.5
Herbicides (%)	34.8	41.0	44.0	51.9
Others (%)	5.9	5.5	6.0	4.7

Table 5.2. World insecticide market in 1998.

Areas	%	Crops	%	Classes	%
Western Europe	15.8	Rape	1.3	Organophosphates	37.2
Eastern Europe	3.9	Sugarbeet	3.4	Pyrethroids	18.3
North America	23.9	Cotton	22.8	Carbamates	13.9
Far East	29.6	Rice	16.1	Organochlorines	2.5
Latin America	13.8	Fruit and vegetables	38.9	Benzoylureas	2.7
Rest of the world	13.0	Cereals	5.3	Others	25.4
		Soybean	2.7		
		Maize	9.4		

species, their high persistence and their lipophilicity. Though these characteristics were considered ideal for an insecticide initially, they were soon found to be negative because of their persistence in the environment, and their tendency to accumulate in the food chain. Though not lethal, they directly or indirectly affected the fertility and reproduction of many wild species. For this reason, DDT and organochlorine compounds have been banned in agriculture since 1973 and heavily limited in the fight against the carriers of diseases of mankind. Since the mid-1980s, the use of DDT has been banned in agriculture in all countries of the world.

Chlorinated insecticides are a heterogeneous group of compounds belonging to three different chemical classes: the diphenylethanes, the cyclodienes and the cyclohexanes (Fig. 5.1). The diphenylethanes include DDT, dicofol and methoxychlor.

Synthesized DDT was a mixture of the isomers *pp´* (75–80%) and *op´* (15–20%), and could contain up to 4% 4,4′-dichlorodiphenyl acetic acid (*pp´* DDA) as an impurity. The generally accepted main metabolic route includes three main processes: (i) dehydrochlorination to 1,1-dichloro-2,2-*bis*(4-chlorophenyl)ethylene (DDE); (ii) reductive dechlorination to 1,1-dichloro-2,2-*bis*(4-chlorophenyl)ethane (DDD); and (iii) oxidation of DDD to DDA (Fig. 5.2). DDT and its major metabolites DDE and DDD are lipophilic compounds and tend to accumulate in body fats. DDT tends to degrade very slowly in the environment. A half-life of 4.3–5.3 years has been calculated in soil (Woodwell *et al.*, 1971) and of 15 years in seawater (Edwards, 1973). The metabolite DDE is also very persistent. In DDT, the substitution of a hydrogen atom in position 1 with a hydroxy group, with formation of dicofol, radically changes the stability of the molecule, which tends to degrade very rapidly, with formation of 4,4´-dichlorobenzophenone (Roberts and Hutson, 1999). For this reason, dicofol is still

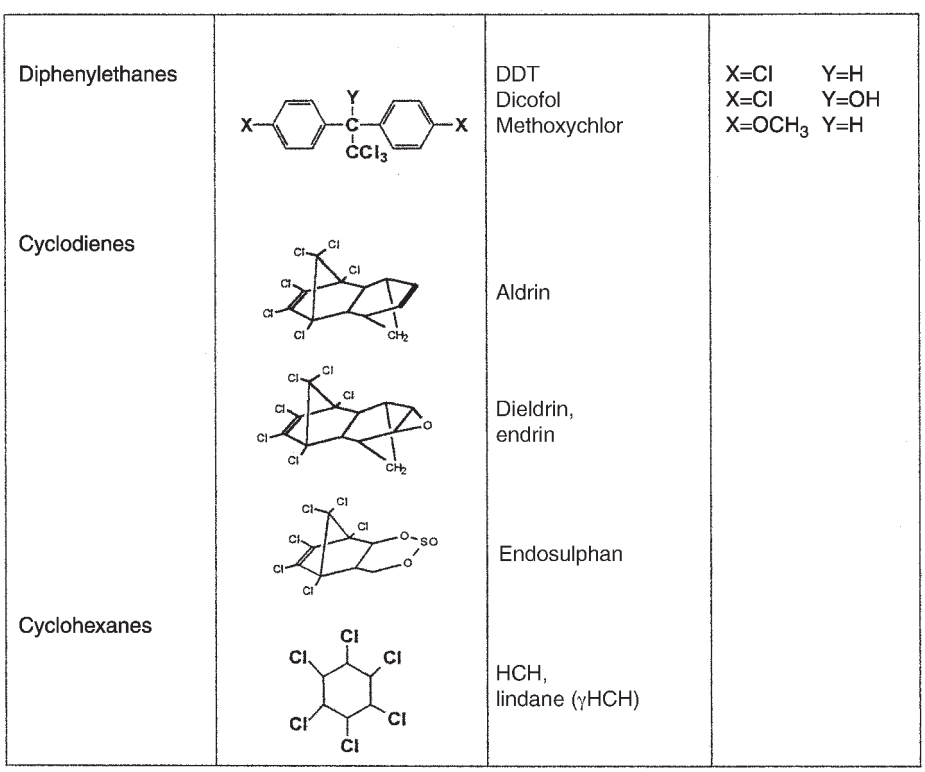


Fig. 5.1. Structure of organochlorine insecticides.

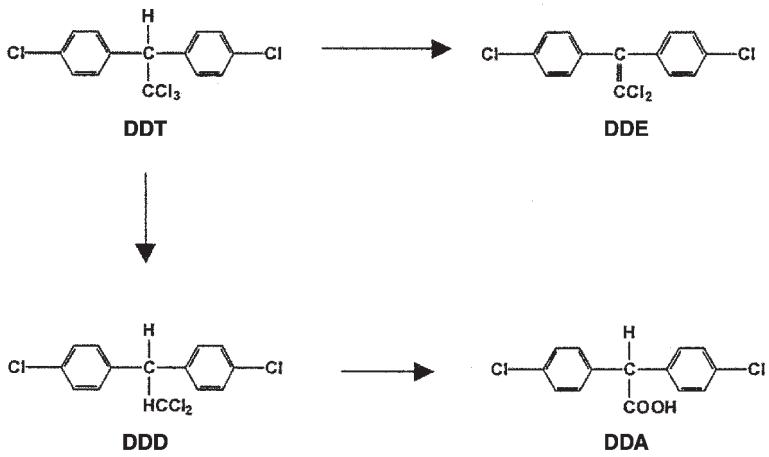


Fig. 5.2. Metabolism of DDT.

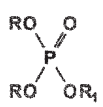
commonly used as an insecticide in agrarian cultures.

Aldrin, which degrades rapidly and forms its epoxide dieldrin by hydroxylation, is very stable in the environment. A half-life of 5 years in the soil has been calculated for dieldrin. Endrin is a stereoisomer of dieldrin. They are now used only in a very few special cases such as the control of termites. Unlike the other cyclodienes, endosulphan shows moderate stability; in fruit and vegetables it tends to degrade and form the corresponding sulphate with half-lives mostly ranging between 3 and 7 days.

Hexachlorocyclohexane (HCH) mainly contains four isomers (α, β, γ and δ). The isomer γ, lindane, which is the active isomer, has been isolated by crystallization from this product. Lindane is the least persistent among the organochlorine compounds.

Organophosphates

Organophosphorus insecticides (OPs) were first synthesized at Bayer in Germany in 1937. Due to their high toxicity, they were developed during the Second World War as chemical weapons. In 1944, the insecticide activity of parathion, the first marketed organophosphorus insecticide, was discovered. Thanks to its remarkable efficacy, wide range of action and fast degradation in the environment, it rapidly became widespread notwithstanding its high toxicity. The number of OPs registered in various parts of the world has increased rapidly, and has now reached about 250. At present, they are the most widely marketed insecticides (37.2%). The general structure of an OP is represented by the following scheme:



where R and R_1 are alkyl groups which could be bonded directly to phosphorus or through atoms of S, O and N (Fig. 5.3).

Further in-depth reading on the chemistry and biochemistry of OPs is available in the book by Fest and Schmidt (1982).

Carbamates

The first carbamate insecticide, carbaryl, was developed by Union Carbide in the USA in 1953. Within a few years, a number of insecticides of the same class followed. These compounds generally present low toxicity for mammals, and many of them are systemic. Thanks to the latter property, insects that develop in the roots can be controlled. Chemically they are divided into three classes: *N*-methylcarbamate (carbaryl),

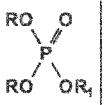
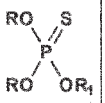
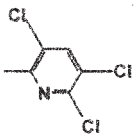
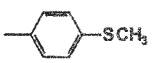
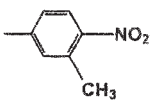
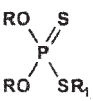
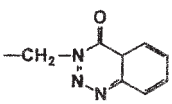
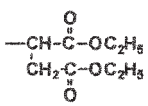
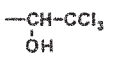
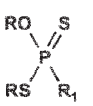
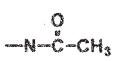
		R	R_1	
Phosphates		CH_3		Dichlorvos
Thionophosphates		CH_3		Chlorpyrifos
		CH_3		Fenitrothion
		CH_3		Fenthion
		C_2H_5		Parathion
Dithiophosphates		CH_3		Azinphos methyl
		CH_3		Dimethoate
		CH_3		Malathion
Phosphonates		CH_3		Trichlorfon
Phosphoroamidates		CH_3		Acephate
		CH_3	NH_2	Methamidophos

Fig. 5.3. Structure of organophosphorus insecticides.

N,N-dimethylcarbamate (pirimicarb) and oxime-carbamate (methomyl) (Fig. 5.4).

Pyrethroids

Pyrethroids are the synthetic analogues of pyrethins, which are natural constituents of the flowers of *Tanacetum cinerariae-folium*. Since the natural insecticide, pyrethrum, is extremely labile to light, it was unsuitable for use in the field. However, when the structure of pyrethrin II, the most effective component of pyrethrum, was modified, and its stability to light improved, these compounds became suitable to be used in the field. The first synthetic pyrethroid, fenvalerate, was put on the market in 1978, and today the class includes 42 active ingredients. Thanks to the discovery of the importance of the stereochemistry of molecules for the bioactivity and toxicity in mammals, it became possible to use these compounds in the field at very low doses (deltamethrin, which is the isomer 1R*cis*αS, is used at 12 g ha^{-1}, since the LD_{50} for the fly is 0.0003 μg). Another very important aspect is their low toxicity in mammals. For these reasons, after the OPs, pyrethroids are the most widely used insecticides (18.3% of the insecticide market). They can be grouped into two classes containing 3-phenoxybenzylic alcohol (permethrin) and α-cyano-3-phenoxybenzylic alcohol (cypermethrin, deltamethrin) (Fig. 5.5). Since pyrethroids cannot penetrate the plant, their action is mainly by contact, which is favoured by their

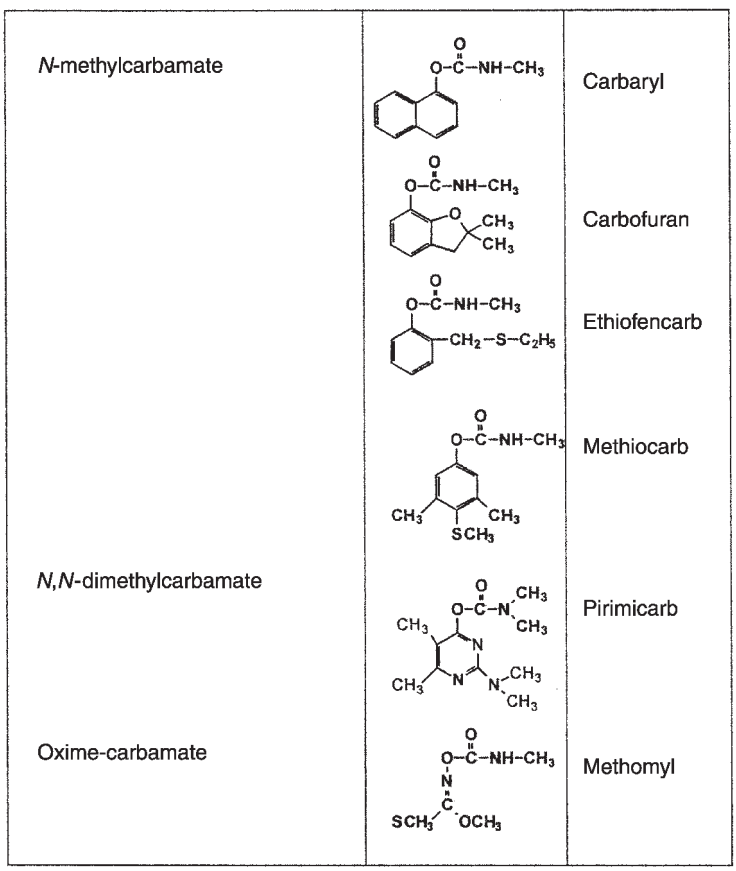


Fig. 5.4. Structure of carbamate insecticides.

liposolubility, which allows them to penetrate the layer of epicuticular waxes.

Benzoylureas

This class of compounds was discovered by chance in the 1970s. During a programmed synthesis between dichlobenil derivatives and fenuron, a product without any herbicidal activity but with a very high insecticidal activity was obtained. The first compound of this class to be out on the market was diflubenzuron in 1975. At present, there are ten benzoylureas on the market (Fig. 5.6). The mechanism of action of the benzoylureas is completely different from that of the other known chemical classes. The compounds of this class act on the formation of chitin, hindering the development of larvae during moult (by causing the imperfect formation of the new cuticle) and causing their death. For this reason, they are classified as insect growth modulators. These pesticides are not systemic and they exert their action mainly by ingestion.

Toxicology

Most insecticides are neurotoxic and act by poisoning the nervous system of the target organisms. Moreover, since they are not selective, they also act on non-target species. The central nervous system (CNS) of insects is highly developed and not very different

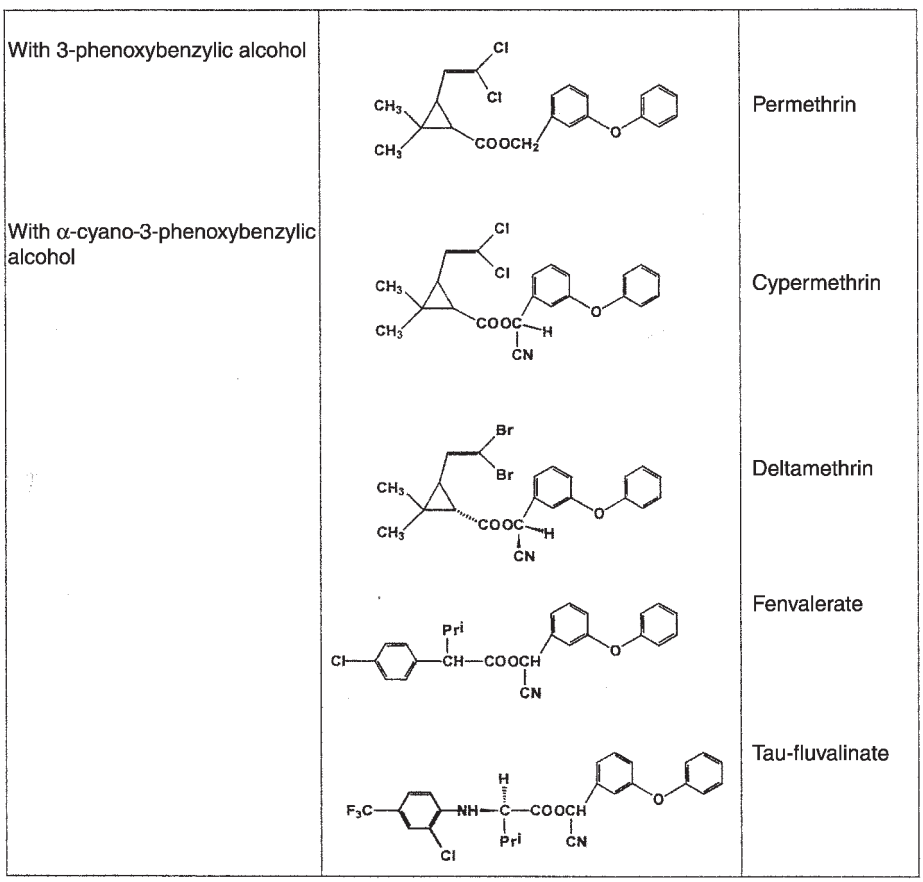


Fig. 5.5. Structure of pyrethroid insecticides.

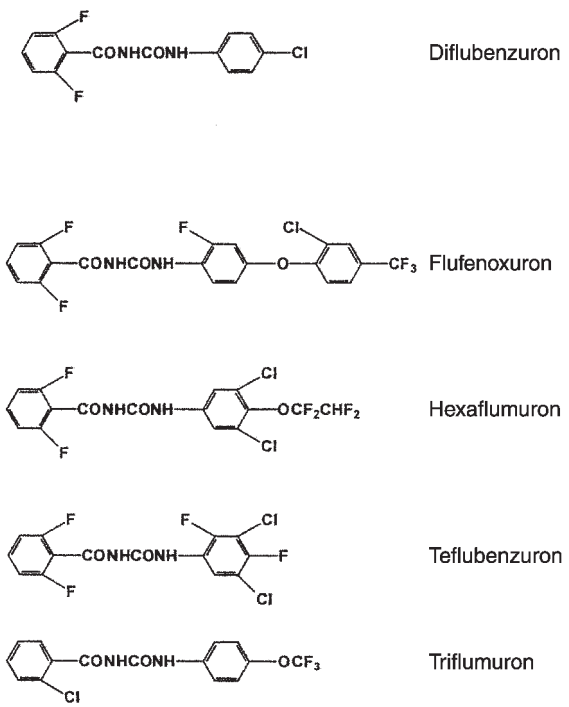


Fig. 5.6. Structure of benzoylurea insecticides.

from that of mammals. Therefore, chemical compounds that act on the nervous system of insects also have similar effects on man. DDT acts by causing a disturbance in the sodium balance of the nerve membranes. Because of its high persistence in the environment, DDT tends to bioaccumulate in the food chains. In mammals, it undergoes slow biotransformation, forming a very stable metabolite, DDE. Due to its lipophilic properties, DDT tends to accumulate in lipid-rich tissues (liver, kidney, nervous and fatty tissue). In the 1950s and 1960s, when DDT was used extensively, the accumulated levels in the fatty tissues were of the order of 5 and 15 mg kg^{-1} for DDT and its metabolites, respectively (Morgan and Roan, 1970). Today, only traces of DDT (2 mg kg^{-1} of its metabolites) are observed in human fatty tissue (Stevens *et al.*, 1993). Studies carried out on wild species have shown that organochlorine insecticides interfere directly or indirectly with their fertility and reproduction, in particular birds and fish (Stickel, 1968; Longcore *et al.*, 1971). Among organochlorine pesticides, the most toxic are the cyclodienes, with extremely low acceptable daily intakes (ADIs) (0.0001–0.0002 mg kg^{-1} body weight (BW), while the least toxic is DDT, with one of the highest ADIs of all insecticides. Cyclodienes are a major hazard to professionally exposed individuals, since, unlike DDT, they are easily absorbed through the skin.

Though very different structurally, phosphoric and carbamic acid esters have the same mechanism of action. They inhibit the enzyme acetylcholinesterase, which degrades the neurotransmitter, acetylcholine, causing the latter to accumulate, leading to manifestations of intoxication.

In OPs, metabolism is a very important factor for toxicity. A prerequisite of its toxic action is the oxidation of the thionates to the corresponding phosphates. Thus parathion is oxidized to paraoxon, which exerts toxic action. The toxicity of organophosphorus compounds and carbamates

Table 5.3. Mammalian toxicology of insecticides.[a]

Class	LD_{50} (mg kg^{-1} rats)	NOEL (mg kg^{-1} rats)	ADI (mg kg^{-1} BW[b])	Toxicity class
Organochlorine compounds				
Aldrin	38–67		0.0001[c]	
DDT	113–118	1	0.02	II
Dicofol	578	5	0.002	III
Dieldrin	37–87		0.0001[c]	
Endosulphan	70	15	0.006	II
Endrin	10–40		0.0002	
γ-HCH (lindane)	88–270	25	0.008	II
Organophosphorus compounds				
Azinphos methyl	9	5	0.005	Ib
Chlorpyrifos	135–163	—	0.01	II
Dimethoate	387	5	0.002	II
Fenitrothion	250	10	0.005	II
Fenthion	250	< 5	0.007	II
Malathion	1375–2800	100	0.02	III
Methamidophos	20	2	0.004	Ib
Parathion	2	2	0.004	Ia
Quinalphos	1750	3	—	II
Tetrachlorvinphos	4000–5000	125	—	III
Carbamates				
Carbaryl	850	200	0.01	II
Carbofuran	8	20	0.002	Ib
Ethiofencarb	200	330	0.1	II
Methiocarb	20	67	0.001	II
Pirimicarb	147	250	0.02	II
Propoxur	50	200	0.02	II
Pyrethroids				
Cypermethrin	250–4150	7.5	0.05	II
Deltamethrin	135–5000	1	0.01	II
Fenvalerate	451	250	0.02	II
Tau-fluvalinate	261	1	0.01	II
Permethrin	430–4000	100	0.05	II
Benzoylureas				
Diflubenzuron	> 4640	40	0.02	III
Flufenoxuron	> 3000	50	—	III
Hexaflumuron	> 5000	75	—	III
Teflubenzuron	> 5000	8	0.01	III
Triflumuron	> 5000	20	0.007	III

[a]Tomlin (1997).
[b]Body weight.
[c]Addition of aldrin + dieldrin.

varies depending on their structure (Table 5.3). In fact, in the same class, there are highly toxic compounds (e.g. carbofuran and aldicarb representing the carbamates; parathion and azinphos methyl representing the OPs), and poorly toxic compounds (carbaryl for the carbamates; malathion for the OPs).

The mechanism of action of pyrethroids is different from that of the OPs and carbamates, but very similar to that of DDT. They too close the sodium channels. Benzoylureas do not have a toxic mechanism towards insects, but they act on them as inhibitors of the biosynthesis of chitin. They are therefore poorly toxic towards mammals. Further

details on the toxicology of insecticides may be found in Ecobichon (1997).

Fungicides

The expenditure on fungicides in 1998 was US$5640 million, or 19.5% of the world market value. This market share has been constant since the 1970s, fluctuating around 20% (Table 5.1). The most important market is the fruit and vegetable market, which on its own accounts for almost 50%, followed by cereals and rice. Europe is the main consumer of fungicides, since its crops are mainly fruit and vegetables (Table 5.4). The most widely used synthetic fungicides, apart from the traditional inorganic compounds (7.3%), belong to the chemical classes of triazoles (19.5%), dithiocarbamates (14.1%), anilinopyrimidines (8.4%), strobilurines (7.4%) and benzimidazoles (5.9%).

Inorganic fungicides

This group of fungicides includes sulphur and copper salts. Sulphur has been used since the time of Homer. The sulphurs available on the market are extremely pure (99.5–100%) since they must be free from selenium, which is harmful to man and animals. The fungicidal power of sulphur depends on temperature, the fineness of the particles and relative humidity. Fungicidal activity starts at 10–12°C with the finest sulphurs and at 18–20°C with the coarser ones, and progressively increases up to 40°C. Their action decreases on increasing the humidity. It should be mentioned that at high temperatures sulphurs are toxic to plants and therefore applications should be made early in the morning in summer.

Copper is included in fungicidal formulations as oxychloride ($Cu_2Cl(OH)_3$), sulphate ($CuSO_4{\cdot}5H_2O$) or hydroxide ($Cu(OH)_2$). Its continued use has led to a significant increase in copper levels in the soil, which have caused ecotoxicological problems. Copper is currently being monitored with a view to limiting its use.

Dithiocarbamates

Zineb, which appeared on the market in 1948, was the first synthetic organic compound to be used in the control of cryptogamic diseases. This active ingredient was followed by other compounds derived from dithiocarbamic acid that belong to two groups: the EBDCs (ethylenebisdithiocarbamates) (maneb and mancozeb) and the dialkyl dithiocarbamates (thiram and ziram) (Fig. 5.7). These compounds are not systemic and act by leaf contact. One of the degradation products of the EBDCs is ethylene thiourea (ETU), a potentially carcinogenic product that forms during normal storage conditions, especially with increased humidity. This product is also found in the formulation as an impurity. In order to limit its presence as a residue in

Table 5.4. World fungicide market in 1998.

Areas	%	Crops	%	Classes	%
Western Europe	42.2	Colza	0.7	Benzimidazoles	5.9
Eastern Europe	2.8	Sugarbeet	0.9	Triazoles	19.5
North America	12.3	Cotton	1.8	Substituted anilides	8.4
Far East	28.1	Rice	16.5	Organophosphorus compounds	3.8
Latin America	11.6	Fruit and vegetables	49.6	Morpholines	2.4
Rest of the world	3.0	Cereals	27.5	Strobilurines	7.4
		Soybean	0.9	Other systemic compounds	15.2
		Maize	1.8	Dithiocarbamates	14.1
				Inorganic compounds	7.3
				Other non-systemic compounds	16.1

Table 5.5. Mammalian toxicology of fungicides.[a]

Class	LD_{50} (mg kg^{-1} rats)	NOEL (mg kg^{-1} rats)	ADI (mg kg^{-1} BW[b])	Toxicity class[a]
Dithiocarbamates				
Maneb	> 5,000	250	0.03	III
Mancozeb	> 5,000	—	0.03	III
Thiram	2,600	1.5	0.01	III
Zineb	> 5,200	—	0.03	III
Ziram	320	—	0.02	III
Benzimidazoles				
Benomyl	> 5,000	> 2,500	0.1	III
Carbendazim	> 15,000	—	0.03	III
Thiabendazole	3,600	40	0.1	III
Thiophanate methyl				
Dicarboxamides				
Chlozolinate	> 5,000	200	—	III
Iprodione	> 2,000	150	0.06	III
Procymidone	6,800	1,000	0.1	III
Vinclozolin	> 15,000	1.4	0.01	III
Triazoles				
Bitertanol	> 5,000	100	0.01	III
Cyproconazole	1,020	1	—	II
Hexaconazole	2,189	2.5	0.005	III
Propiconazole	1,517	3.6	0.02	II
Tebuconazole				
Anilinopyrimidines				
Cyprodinil	> 2,000	3	0.03	III
Mepanipyrim	> 5,000	2.45	0.024	III
Pyrimethanil	> 4,150	20	0.2	III
Strobilurines				
Azoxystrobin	> 5,000	18	0.2	—
Kresoxin-methyl	> 5,000	800	0.4	—

[a]Tomlin (1997).
[b]According to WHO.

food, a maximum limit of 0.5% has been established in the technical active ingredient when marketed.

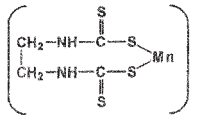
Benzimidazoles

The fungicidal activity of benzimidazoles was first described in 1964 for thiabendazole. Benzimidazoles are systemic fungicides that penetrate through the cuticle into the plant, where they exert their fungicidal activity. Benomyl and thiophanate methyl are transformed into carbendazim and it is this metabolite that exerts the fungicidal action.

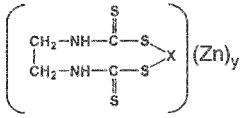
Dicarboximides

Chlozolinate, iprodione, procymidone and vinclozolin are fungicides that belong to this chemical class. Procymidone and chlozolinate are systemic, while iprodione and vinclozolin are mainly contact fungicides with both preventive and curative activity. They were the most widely used fungicides in the 1980s but, when resistance phenomena developed, their efficacy was diminished. With the appearance of the new molecules belonging to the class of the anilinopyrimidines and strobilurines, their use has been reduced greatly.

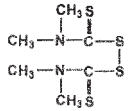
Dithiocarbamates



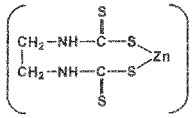
Maneb



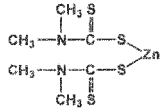
Mancozeb



Thiram

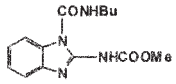


Zineb

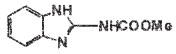


Ziram

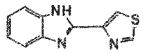
Benzimidazoles



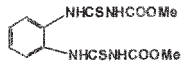
Benomyl



Carbendazim

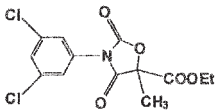


Thiabendazole

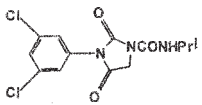


Thiophanate methyl

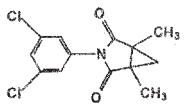
Dicarboxamides



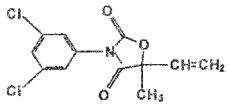
Chlozolinate



Iprodione

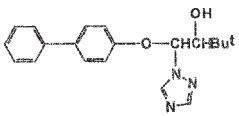


Procymidone

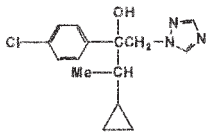


Vinclozolin

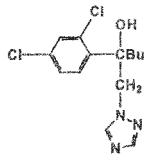
Triazoles



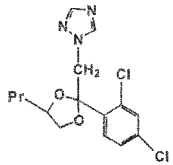
Bitertanol



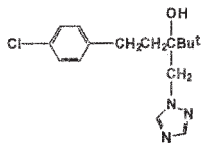
Cyprocazonazole



Hexaconazole



Propiconazole



Tebuconazole

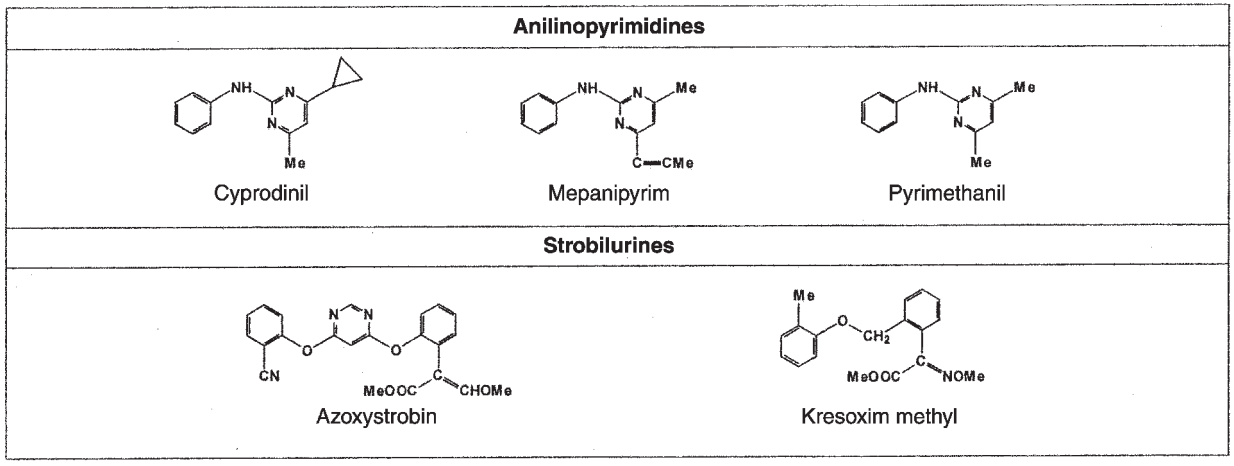


Fig. 5.7. Fungicide structures.

Triazoles

Triazoles account for almost 20% of the fungicide market. This class includes a number of compounds (Tomlin, 1997). Triazoles are systemic fungicides that enter the plant and spread from the site of application to untreated or newly grown areas, uprooting existing fungi or protecting the plant from future attacks. The mechanism of action of these fungicides is due to their ability to interfere with the biosynthesis of biosteroids or to inhibit the biosynthesis of ergosterol. They are used at very low doses and generally have a very low toxicity to mammals.

Anilinopyrimidines

In the early 1990s, three fungicides belonging to the class of the anilinopyrimidines appeared on the market. They were cyprodinil, mepanipyrim and pyrimethanil. These anilinopyrimidines are systemic fungicides that act on the biosynthesis of amino acids and key cell enzymes. They are mildly toxic to man.

Strobilurines

Azoxystrobin and kresoxim-methyl belong to the class of strobilurines, whose name comes from the fact that these molecules were synthesized as a development of the natural product, strobilurine. This class of compounds was put on the market in the 1990s and they are marginally toxic to man.

Toxicology

Most fungicides are minimally toxic to mammals since they have an oral LD_{50} in rats ranging between 800 and > 15,000 mg kg^{-1} (Table 5.5). Nevertheless, many give positive results in current mutagenetic tests. Among the first-generation fungicides, a few compounds, such as hexachlorobenzene, a few organomercurial fungicides and pentachlorophenol, caused such large-scale poisoning and intoxication that they were banned. In the case of the EBCDs, ETU, a mutagenic, carcinogenic and teratogenic product, can be formed from their degradation. Recent studies have not provided proof of the existence of hazards to human health.

Herbicides

At present, herbicides have the largest market share (Table 5.1). In past years, herbicide use has increased significantly from 34.8% in 1970 to 51.9% in 2001. One of the main reasons for this increase is related to developing countries, where there has been a change to more intensive production due to a shortage of cheap labour. New chemical compounds have been developed to fight against a larger number of weeds. First-generation herbicides (non-systemic) were characterized by a wide range of action, low cost and rather high doses. The newer products are more selective and can be applied in doses of the order of a few tens of grams per hectare. Cereals, rice, maize and soybean are the crops that most require herbicides, with consumptions of about 20% for each crop, and it is especially in North America that herbicides are used very extensively (Table 5.6).

Herbicides can be classified not only according to their chemical class, but also according to their selectivity, nature of action and application characteristics (region and time).

1. Selectivity. Herbicides that destroy all vegetation are classified as total or non-selective, while those that control some weeds without damaging other agricultural cultures are defined as selective. Non-selective pesticides, e.g. paraquat, are used to weed orchards, industrial farmyards, wheel tracks, embankments, etc. 2,4-dichlorophenoxyacetic acid (2,4-D), which is selective, is used with the *Gramineae* (wheat, barley, rice, oats) to control infesting annual (papaver, etc.) and perennial (convolvulus, etc.) dicotyledons.

2. Nature of action. Contact herbicides exert their action only on the part of the plant where they have been deposited (e.g. paraquat). Systemic herbicides, on the other hand, penetrate the plant and reach regions that are far from

Table 5.6. World herbicide market in 1998.

Areas	%	Crops	%	Classes	%
Western Europe	22.6	Colza	3.2	Triazines	7.0
Eastern Europe	3.1	Sugarbeet	5.6	Amides	11.3
North America	44.0	Cotton	5.6	Carbamates	3.8
Far East	12.8	Rice	11.1	Ureas	8.6
Latin America	14.9	Fruit and vegetables	15.0	Toluidines	4.1
		Cereals			
Rest of the world	2.6	Soybean	20.6	Hormones	2.9
		Maize	19.1	Diazines	3.5
			19.8	Diphenyl ethers	2.2
				Sulphonylureas	7.6
				Imidazolinones	5.9
				Bipyridyls	3.5
				Amino acid derivatives	19.9
				Arylphenoxypropionates	5.1
				Cyclohexanediones	1.2
				Pyridines	3.0
				Benzonitriles	1.5
				Others	12.7

the point of application. Translocation within the plant occurs via the phloem and the xylem. A few herbicides may also be absorbed by the roots.

3. Region of application. Herbicides may be applied to the foliage or to the soil. Foliage-applied herbicides, such as a few s-triazines, normally have low solubility in water, and are absorbed by the roots and translocated into the plant via the xylem. Soil-applied herbicides may be subject to degradation in the soil and only part of the applied dose may be available to be absorbed by the plant. Foliage-applied herbicides penetrate the cuticular membrane and translocate into the plant via the phloem system.

4. Timing of application. Application timing normally is correlated to the developmental stage of the culture. We therefore have herbicides used in pre-sowing, pre-emergence, and post-emergence. Pre-sowing treatments are made with non-selective herbicides when selective elimination of weeds is difficult. Pre-emergence herbicides are used to control annual weeds whose germination competes with the culture. Post-emergence compounds are used to control the weeds that compete with the culture during its development.

The best known and most commonly used herbicides belong chemically to the following categories: phenoxy derivatives (phenoxyalkanoic acids), dipyridilic compounds, amides, dinitroanilines, ureas, triazines, sulphonylureas and amino acid derivatives (Fig. 5.8).

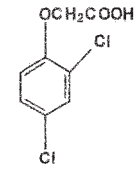
Phenoxyalkanoic acids

The phenoxy derivatives make up a historical group of herbicides, since, with the introduction of MCPA in 1942 followed shortly after by 2,4-D, they marked the start of the modern practice of chemical weeding. Chemically they are very similar to natural auxins, hormones that regulate the physiological processes underlying plant growth. By substituting themselves to natural auxins, they interfere with plant growth. They are selective, systemic herbicides that, applied to the foliage in post-emergence, can exert their herbicidal action at very low doses (0.1%).

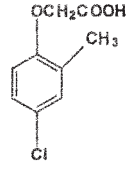
Bipyridyls

The herbicidal activities of diquat and paraquat, the two compounds that belong to this chemical class, were discovered in 1956.

Phenoxyalkanoic acids

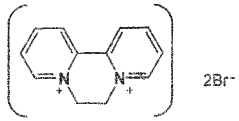


2,4-D

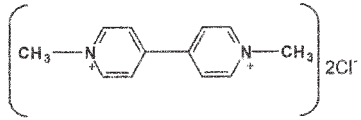


MCPA

Bipyridyls

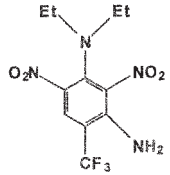


Diquat

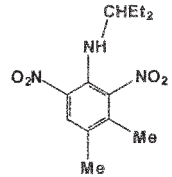


Paraquat

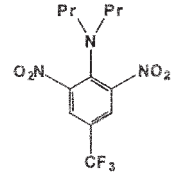
Dinitroanilines



Dinitramine



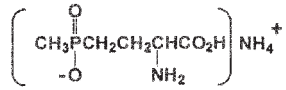
Pendimethanil



Trifluralin

Amino acid derivatives

Glyphosate



Glufosinate NH_4

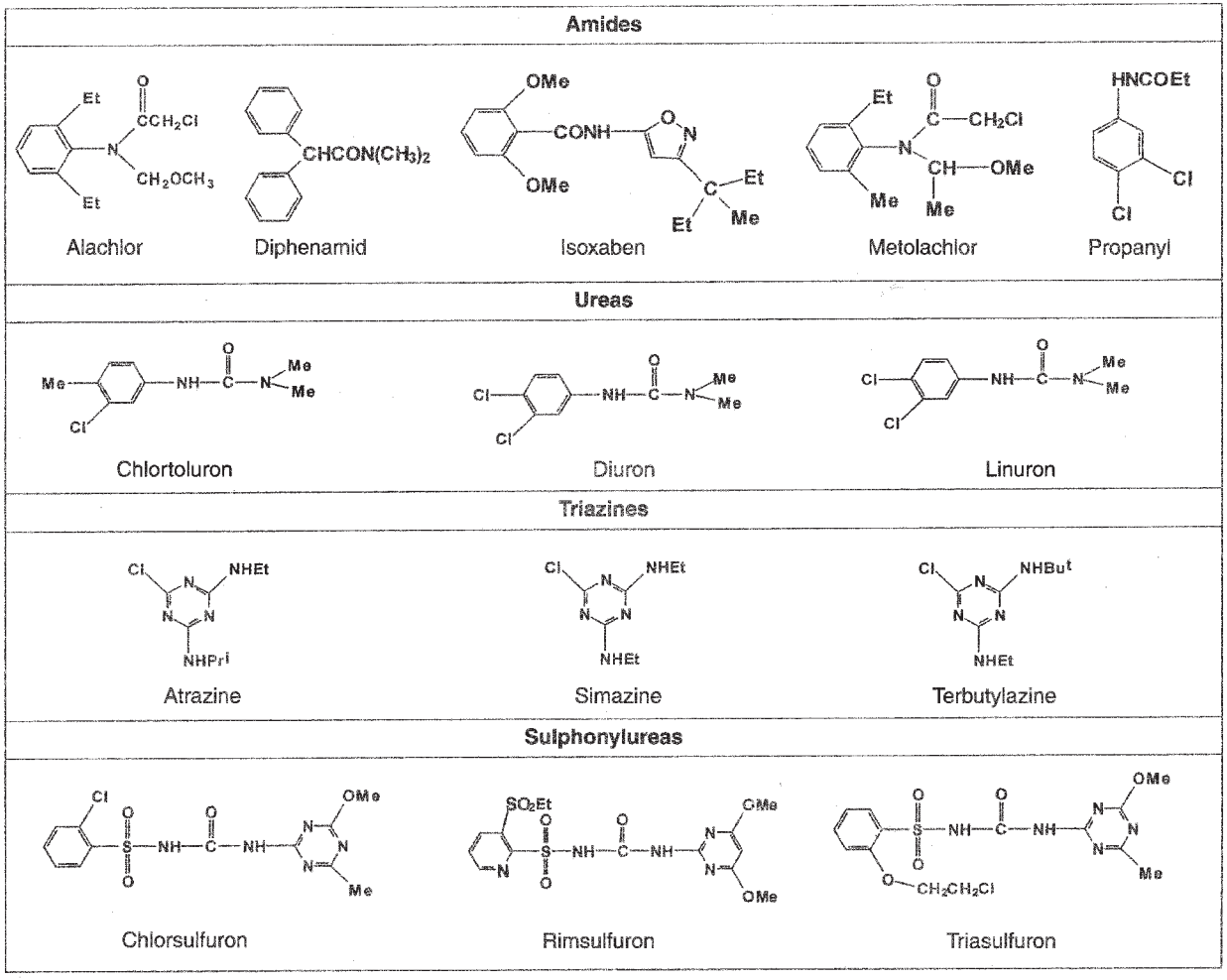


Fig. 5.8. Structure of herbicides.

These compounds, chemically ammonium quaternary salts, are not selective and act rapidly by contact on the green parts of the plants, but not on its ligneous parts. The herbicidal mechanism of action is the inhibition of chlorophyll photosynthesis. Since these compounds are irreversibly adsorbed by the colloids of the ground, where they remain sunk in the superficial layers, they are not biologically active.

Amides

The compounds belonging to this family are divided into three groups: acetamides (e.g. diphenamide), anilides (e.g. alachlor, propanyl) and benzamides (e.g. isoxaben). They are widely used (11.3%), especially on products such as rice, maize, wheat and soybean. They generally have antigerminative activity, but also act via the roots since they can also be adsorbed by the young roots.

Dinitroanilines

Trifluralin was the first herbicide of this class to be introduced on the market in 1960, and was followed by several other compounds. These compounds are applied to the soil, where they inhibit seed germination by root absorption and block the development of young plantlets. Since these products are unstable to light and volatile, they have to be incorporated immediately into the soil. They are selective herbicides used in pre-emergence.

Ureas

This is one of the chemical classes with the largest number of marketed compounds (Tomlin, 1997) (e.g. diuron, linuron). They have been on the market since 1950. Since these herbicides are absorbed mainly through the roots, they generally are administered to the soil during pre-emergence. They are selective systemic herbicides that act as inhibitors of photosynthesis. According to modern standards, these compounds are used at high doses (0.4–4 kg ha^{-1}).

Triazines

Simazine appeared on the market in 1956 and was followed by a number of other compounds. At present, there are 14 triazines on the market. These compounds are inhibitors of photosynthesis that are adsorbed by the leaves and roots. They are chemically very stable and therefore persist in the environment. They are selective for a limited number of cultures (maize, sorghum, chard, etc.). Among the best-known compounds is atrazine on account of problems related to the pollution of the water table.

Sulphonylureas

Chlorsolfuron, which appeared on the market in 1980, was the first herbicide of this class. The success of sulphonylureas was due to their low dose of application (10–20 g ha^{-1}) and reduced toxicity for man and the environment. At present, there are 25 sulphonylureas on the market. Their mechanism of action is the inhibition of the biosynthesis of essential amino acids. They are selective systemic herbicides that can be absorbed by both foliage and roots.

Amino acid derivatives

This class of compounds (also classified as organophosphorus) has the largest market share at 19.9%. The first herbicide of this group, glyphosate, appeared in 1971 and is characterized by high systemicity and a wide range of action. It acts by inhibiting the synthesis of aromatic amino acids. It is also marketed as a salt. The great success of this herbicide is related to its lack of residues in the soil and its low toxicity.

Toxicology

Among the pesticides, herbicides are generally the least toxic compounds for vertebrates. It has often been said that, since the mechanism of action of herbicides is an interaction with the biochemical processes of vegetables, they have no toxicity for animals. As can be deduced from the data reported in Table 5.7, almost all herbicides belong to the toxicological class III (according to WHO). In general, since the main absorption pathway is the skin, the most widespread toxic effects are contact dermatitis. Now that formulation impurities have been greatly reduced, especially in very toxic compounds, as the technical product must be more than 95% pure, some of their attributed toxic effects have been removed. A case in point is the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), where the presence of a dioxin (TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was the real cause of the toxicity. Paraquat is very toxic to the lungs, but most intoxications with paraquat are due to ingestion of the product. Normally, herbicides are administered on the soil and not directly on the culture; moreover, since they are administered when no edible parts are present, they do not present particular contamination problems.

Table 5.7. Mammalian toxicology of herbicides.[a]

Class	LD_{50} (mg kg^{-1} rats)	NOEL (mg kg^{-1} rats)	ADI (mg kg^{-1} BW)	Toxicity class[b]
Phenoxyalkanoic acids				
2,4-D	639–764	5	0.3	II
MCPA	900–1,160	20	—	III
Bipyridyls				
Diquat	231	0.25	0.002	II
Paraquat	157	170	0.004	II
Amides				
Alachlor	930–1,350	2.5	—	III
Diphenamid	1,050	> 2,000	—	III
Isoxaben	> 10,000	5.6	0.056	III
Metoalachlor	580	—	—	II
Propanyl	> 2,500	400	0.005	III
Dinitroanilines				
Dinitramine	3,000	> 2,000	—	III
Pendimethanil	1,250	> 100	—	III
Trifluralin	> 5,000	813	0.024	III
Ureas				
Chlortoluron	> 5,000	100	—	III
Diuron	3,400	250	0.002	III
Linuron	1,500–4,000	—	0.008	III
Triazines				
Atrazine	1,869–3,090	10	0.005	III
Simazine	> 5,000	0.5	0.005	III
Terbutylazine	1,590–2,000	0.22	0.002	III
Sulphonylureas				
Chlorsulfuron	5,545	100	0.05	III
Rimsulfuron	> 5,000	300	—	III
Triasulfuron	> 5,000	32.1	0.012	III
Amino acid derivatives				
Glyphosate	5,600	> 410	1.75	III
Glufosinate NH_4	2,000	2	0.02	III

[a]Tomlin (1997).
[b]According to WHO.

One of the main problems related to the use of herbicides is the fact that some molecules may percolate into the ground and pollute the water table.

Formulation of Pesticides

Active ingredients show activity at particularly low doses. Therefore, since they have to be distributed evenly on large areas, and at times in concentrations of a few grams per hectare, formulations that allow a homogeneous protection of the vegetable area with a precise amount of parasiticide are used. Dose precision is very important to avoid environmental pollution in the event of overdosing, and lack of efficacy in the event of underdosing.

The formulation is made up of the active ingredient added to other compounds to develop maximum biological activity, allow easy and safe distribution and give sufficient adhesion to the treated surfaces for the time needed to exert its biological activity. The active ingredient included in the formulation is of a technical grade. The early pesticides were of poor purity, e.g. in the synthesis of lindane, only 13% of the γ isomer of hexachlorocyclohexane, which has insecticide activity, was obtained, while other isomers that were environmental pollutants were present. The synthesis of malathion, which has low toxicity, produced isomalathion as an impurity, which is highly toxic. Its use in 1976 in the fight against malaria in Pakistan caused a poisoning epidemic among 7500 workers (Baker *et al*., 1978). In the production of the herbicide 2,4,5-T, a product contaminated by TCDD was obtained. This compound is highly toxic for man and other mammals and causes a form of acne in the workers involved in its production (Kimmig and Schultz, 1957). For this reason, severe restrictions were imposed on the presence of contaminants more toxic than the active ingredient generated in the production process or as degradation products. Today, the technical products of pesticides have a content of about 95% and do not contain any contaminants hazardous to health or the environment.

The formulations can be considered distributed in the field in liquid or solid form. Those distributed in liquid form are diluted in water before use and are subdivided as follows:

1. *Wettable powders*, in which a suspension of the solid phase, finely subdivided and dispersed, is obtained in the liquid phase.
2. *Emulsifiable concentrates*, in which the active ingredient, which is insoluble in water, is dissolved in an appropriate organic solvent. Thanks to the action of a surfactant, the active ingredient forms an emulsion when added to water at a ratio of 1:1000–10,000.
3. *Granules*, in which the active ingredient together with the various adjuvants, all finely ground, are fixed on to round mineral granules.
4. *Flowable powders*, in which the active ingredient is micronized and added to appropriate adjuvants and water to obtain a smooth flowing paste.
5. *Microcapsules*, in which the active ingredient is enclosed in nylon microcapsules of a diameter of a few micrometres (7–30) preserved in aqueous suspensions. The characteristic of this formulation is that after treatment, once the water has evaporated, the active ingredient is released progressively and flows outwards through the pores of the capsule walls.

The formulations distributed in solid form are made up of dry powders and must be submitted to forced grinding, since their adhesiveness is inversely proportional to the particle diameter. These formulations are pumped forcefully on to the plants by an air jet.

The components of the formulation may be subdivided as follows:

1. *Adhesive agents*, made up of carboxymethylcelluloses and paraffin-type mineral oils, which increase the adhesiveness of the active ingredients, since adhesiveness is generally insufficient to guarantee an adequate deposit on the treated vegetable surface.
2. *Anti-evaporating agents*, made up of mixtures of aliphatic hydrocarbons, used to contain the fast evaporation of molecules at a high vapour tension within limits sufficient to allow adequate biological activity.

3. *Diluents for solids*, made up of SiO_2, carbonates, silicates, montmorillonites, bentonites, talc, etc., and *diluents for liquids*, made up of aliphatic and/or aromatic hydrocarbons.
4. *Dispersants*, made up of oligo- and polysaccharides, gelatins, bentonites, etc., used to avoid the sedimentation of dispersed particles.
5. *Penetrants*, made up of ethoxylated amines and fatty acid amines that favour the penetration of the active ingredient inside the plant.
6. *Solvents*, mainly made up of xylenes, alkylnaphthalines, cyclohexanones, etc., used to dissolve solid or liquid active ingredients. These solutions may be absorbed on inert powders (granular formulations) or diluted further in liquid by adding a surfactant (emulsifiable concentrates).
7. *Surfactants* are subdivided into non-ionic or apolar (alkyl phenols, fatty acids and alcohols condensed with ethylene oxide) and ionic or polar (organic acid salts such as alkyl- and aryl-sulphonates, dodecyl benzenesulphonates, lauryl sulphonates, etc.). Their function is to reduce the surface tension of water, thus making the droplets completely wettable on all contact surfaces, with regard both to parasites and vegetables (Martelli, 1992).

The main objective of the formulation is to improve the efficacy of the active ingredient, which is obtained with co-formulants that prolong its presence on the culture over time. From the point of view of public health, however, the pesticide residue should disappear as rapidly as possible. The correct balance between these two contrasting needs is the aim of adequate formulations.

Registration

A company that intends to market a pesticide in a country must register the product with the appropriate authorities, before putting it on the market. The authority that issues the authorization to market a pesticide is usually the Ministry of Agriculture, less often the Ministry of Health, and in some cases a state agency such as the EPA in the USA, or the BCE in Germany. In order to register a product, the company must present studies showing that the pesticide is efficient against the target parasite and does not cause harmful effects on human and animal health or on the environment. The documentation that every company must present is established by law and must be carried out according to the criteria of good laboratory practice. The adoption of guidelines and principles of good laboratory practice has allowed procedures to be standardized and has guaranteed the quality of the data produced. At present, legislation is very similar in developed countries; in Europe, the European Community is harmonizing the national regulations into one system. Even in developing countries similar laws have been issued, but they are often not applied due to the lack of the necessary competences; moreover, some products that have been banned by richer countries are still marketed in these countries. The studies that must be presented by the companies applying for registration can be divided into the following categories: toxicological, agronomic and environmental.

The toxicological studies include the following: (i) acute toxicity; (ii) short-term toxicity (at least 90 days); (iii) long-term toxicity (2 years); (iv) toxicity on reproduction; and (v) late neurotoxicity. The studies are carried out for all possible forms of contamination with the active ingredient: oral intake, cutaneous intake or intake by inhalation. Based on the toxicological data the dose causing no observed effect is determined, the NOEL (no observed effect level), i.e. the dose at which no toxic effects are observed in animal studies. Relating to toxic effects, the NOEL is extrapolated from long-term studies on the most sensitive species and on species similar to man. The ADI is obtained from the NOEL by dividing it by a safety factor of between 10 and 1000. The factor 100 is generally used. The aim of this factor is to provide the consumer with a sufficient safety margin, supposing that humans are 10 times more sensitive than a laboratory animal and that differences in sensitivity within a human population are between one and ten. The NOEL and the ADI

are expressed in mg kg^{-1} BW day^{-1}. In order to maintain the concentration of the pesticide in the food at levels of exposure of the ADI, the tolerance level (TL) is calculated according to the following formula:

$$TL = \frac{ADI}{DFI}$$

where DFI = daily food intake (kg).

The TL depends on the daily food intake and will therefore vary according to the diet in each country. The TL is the maximum residue allowed in the food and serves as a basis to establish the legal MRL (maximum residue level). This is decided on the basis of residues actually found in the food as a result of practical supervised tests. If the amount of residue found in these tests is lower than the TL, it will be the limit that will be chosen for legal purposes; if it is higher, the product will not be registered. Therefore, the MRL is the result of toxicological and agronomic studies. Since the quantity of residue in the food depends strongly on the number of treatments and on the environmental conditions, the quantity of pesticide residue in the food may vary depending on the existing environmental conditions in the country. This explains why different MRLs are established in different countries for the same active ingredient (Table 5.8).

On registering a pesticide, the following elements are indicated for each active ingredient: culture for which the ingredient is authorized, dose, safety interval (days that must elapse from last treatment to harvest), MRLs and toxicological class. When a pesticide is not authorized on a culture, the residue must be less than 0.01 mg kg^{-1}, which is the legal zero. This limit is also established for baby foods.

As can be seen in Table 5.8, the differences between the lowest and the highest MRLs for the same culture may be a factor of 30. This indicates that the MRLs are often very different from the TL, and that to surpass an MRL does not necessarily mean a hazard to man, but simply that the conditions of use provided for and indicated on the label have not been met: admitted cultures, doses and safety interval. Implementation of these practices is known as good agricultural practice.

The toxicological classes are intended to show the level of danger to the consumer and are based on acute toxicity. The four classes of toxicity classification by the WHO based on active ingredients are reported in Table 5.9,

Table 5.8. National maximum residue limits (MRLs) for folpet.

	MRL (mg kg^{-1})					
Country	Apple	Cucumber	Grape	Lettuce	Strawberry	Tomato
Brazil	10	2	15	15	20	—
Chile	25	—	25	—	25	25
Greece	—	3	3	2	3	3
Israel	10	0.5	—	—	—	—
Italy	3	0.1	3	2	0.1	3
USA	25	15	25	50	25	25

— = not registered.

Table 5.9. WHO toxicity classification.

		LD_{50} for the rat (mg kg^{-1} BW)			
		Oral		Dermal	
	Class	Solids	Liquids	Solids	Liquids
Extremely hazardous	Ia	≤ 5	≤ 20	≤ 10	≤ 40
Highly hazardous	Ib	5–50	20–200	10–100	40–400
Moderately hazardous	II	50–500	200–2000	100–1000	400–4000
Slightly hazardous	III	≥ 501	≥ 2001	≥ 1001	≥ 4001

Table 5.10. EU toxicity classification.

	LD_{50} oral for the rat (mg kg^{-1} BW)	
	Liquids	Solids
Extremely hazardous	≤ 25	≤ 5
Hazardous	25–200	5–50
Noxious	200–2000	50–500

while Table 5.10 reports the more simplified classification by the European Union based on the formulations.

The TL and, consequently, the ADI and the MRL can be changed as further data about the toxicology and residue levels become available.

In the past, registration used to be for an indefinite period, but it has been decided recently to limit its duration (10 years in the EU) and reassess each molecule with updated toxicological and environmental studies at the date of expiry. This new approach of limiting the registration validity will result in the banning of a number of pesticides, among the most toxic and with a larger environmental impact, from the market. It is expected that not more than 250 active ingredients, 174 of which will be newly introduced, will be on the market after the year 2005.

Residues in Food

After the pesticides are used to treat cultures, they are deposited on them and, in order to be marketed, they must be lower than the legal limit at harvest. From a legal point of view, by residue we not only mean the active ingredient on the food, but also its metabolites and/or degradation products and toxicological impurities in the formulation. The amount of pesticide residues present on fruit and vegetables at harvest depends on the initial deposit and on the residue reduction rate.

Initial deposit

A number of factors determine the level of the initial deposit; some depend on the pesticide (rate, formulation, application methods), others on the culture (surface/weight ratio, shape).

Table 5.11. Pesticide residues (mg kg^{-1}) on grapevine immediately after treatment at the application doses recommended by the manufacturers and at double the doses.

Pesticide	Dose (g ha^{-1})	Residues (mg kg^{-1})
Deltamethrin	12.5	0.13
Benalaxyl	200	0.80
	400	1.61
Vinclozolin	1000	1.37
	2000	2.53

Application rate

With first-generation pesticides, the amount of active ingredient used per hectare was of the order of 1 kg. Subsequently, with second-generation pesticides, it was reduced to a few hundred grams, while with last-generation pesticides it is of the order of a few tens of grams. As can be seen from the data on the residues on grapevine reported in Table 5.11, the lower the dose of application, the smaller the initial deposit (Cabras *et al*., 1984a,b, 1991). There is a direct proportion between dose and residue but only with the same active ingredient, and not if the active ingredient is different. This depends mainly on the characteristics of the formulation and on the physical–chemical properties of the active ingredient.

Formulation

A few systemic pesticides can be administered to the soil in granular formulation. There they are absorbed by the plants through the roots. This creates a progressive absorption and a distribution effect of the

active ingredient throughout the plant, with consequent dilution and the presence of residues at low levels. Experiments carried out with carbofuran on lettuce (Table 5.12) have shown that absorption occurs progressively; the residues were still undetectable 4 days after administration and reached a maximum value after 11 days, though lower than 0.1 mg kg^{-1} (Cabras *et al*., 1988).

Application techniques

In the past year, there has been a great deal of interest in pesticide application techniques, in particular with low volumes (300 l ha^{-1}). Thanks to the high micronization of drops obtained with this technique, a greater distribution uniformity and smaller losses of liquid are possible. Experiments carried out on celery (Table 5.13) have shown that only by associating this technique with an electrostatic system was an increase in the residue deposit obtained (Cabras *et al*., 1993).

Influence of cultivar

Since residues are expressed in mg kg^{-1}, the surface/weight ratio of a fruit will strongly affect the amount of residue. On treating two cultivars of Yacouti and Koroneik olives, the latter with very small fruits, therefore with a greater surface/weight ratio, with the same amount of formulation greater amounts of residue were found on the latter cultivar (Table 5.14) (Cabras *et al*., 1997c).

Shape of the cultivar

In some cultures, such as artichokes, the edible part (the head) is different in shape in different cultivars. Since in a few cultivars (e.g. Masedu) the head is shaped like a calyx with open bracts, the sprayed pesticide may deposit even inside, while with other cultivars (e.g. Spinoso Sardo) whose external bracts tend to close the inner parts are protected and the sprayed pesticide does not enter the artichoke. This causes remarkably different deposits among the different cultivars (Table 5.15). Analogous considerations can be made for the Roman type of lettuce (calyx-shaped) and the Iceberg lettuce (ball-shaped); it should be remembered that in this case, the lettuce is marketed after removing the outer leaves (Cabras *et al*., 1988, 1996).

Table 5.12. Residues (mg kg^{-1}) on lettuce foliage treated with carbofuran in granules at a dose of 750 g a.i.[a] ha^{-1}

	Days after treatment			
	4	11	18	32
Carbofuran	< 0.001	0.063	0.023	0.027

[a]Active ingredient.

Table 5.13. Residues (mg kg^{-1}) of cyromazine on celery after treatment at doses of 270 g a.i. ha^{-1} with different distribution volumes.

	Distribution volume (l ha^{-1})			
	1500	900	300	300 with electrostatic
Cyromazine	1.01	1.35	0.95	1.90

Table 5.14. Pesticide residues (mg kg^{-1}) on the olives of two different cultivars after treatment.

Cultivar	Azinphos methyl	Diazinon	Dimethoate	Metidathion	Parathion methyl	Quinalphos
Yacouti	1.82	1.34	1.60	3.01	1.40	1.84
Koroneik	3.02	3.46	4.71	4.25	4.26	3.56

Table 5.15. Residues (mg kg^{-1}) on different cultivars of artichokes and lettuce after treatment.

Culture	Cultivar	Shape	Dimethoate	Parathion	Pyrazophos
Artichoke	Masedu		2.20	4.02	1.16
	Spinoso Sardo		1.40	1.96	0.53
			Chlozolinate	Parathion	
Lettuce	Roman		6.18	1.19	
	Iceberg		1.75	0.36	

Disappearance rate

Pesticides are mostly lipophilic and exert their activity by contact or systemically, depending on whether they penetrate the plant or not. On account of these properties, after treatment, the residues on the surface of the plant spread in the epicuticular waxy layer and in the cuticle in the case of contact products, while systemic products continue to penetrate inside the plant.

If the residue penetrates inside the plant, it degrades with different mechanisms by way of its enzymes, while, if it remains on the surface layers, it will undergo mainly reduction processes related to environmental conditions such as washing, evaporation, co-distillation during evaporation of the water from the fruit or vegetable, and photodegradation. These degradative processes determine a 'real' decrease of the residue, while during the growth phase the increase in the weight of the fruit will produce an 'apparent' reduction of the residue by way of dilution.

Disappearance of the residue will depend on the combined effect of these factors. The rate of disappearance normally follows first-order kinetics. Below is an assessment of how the single factors may affect the disappearance of the initial deposit.

Fruit growth

Experiments on peaches have shown that the residues of two pesticides, fenbutatin oxide (Cabras *et al*., 1992) and pirimicarb (Cabras *et al*., 1995b), diminish after exclusive treatment by dilution effect due to fruit growth when grown in greenhouses, while in the open field the reduction by growth is only about one-third of the initial residue (Table 5.16). The lack of residue degradation in greenhouses has been attributed to the fact that the glass absorbs the radiation that causes their photodegradation. The high stability of these compounds will cause residue increases in the event of repeated treatment.

Crops

Experiments carried out using the same active ingredient on different cultures show that the disappearance rates are different. From the data reported in Table 5.17, it can be seen that, after 1 week's treatment, dimethoate disappeared almost completely on plums (Cabras *et al*., 1998); on grapes, an 80% reduction was observed after 1 week's treatment but the residue was constant during the following 3 weeks (Cabras *et al*., 1994). The disappearance rates on apricots, oranges and peaches are similar, with half-lives of the order of 10 days (Cabras *et al*., 1995a,c, 1997d; Minelli *et al*., 1996).

Enzymatic degradation

When a pesticide enters the plant, it can be transformed rapidly by enzymatic action. This is the case of the insecticide ethiofencarb

administered on lettuce. Immediately after the treatment, when the plant is dry (after ~1 h), besides the active ingredient, significant amounts of three metabolites, sulphoxide phenol, sulphoxide and sulphone, were observed (Table 5.18). After only 1 day, though present in significant amounts initially, the sulphoxide phenol was completely degraded. Three days after the treatment, the amount of active ingredient was not determinable. At harvest, only the metabolites sulphoxide and sulphone were present. In Italy, a legal limit is established only for the active ingredient, while in other European countries, such as Spain, the limit includes the sum of active ingredient and metabolites.

Table 5.16. Residues (mg kg^{-1}) of fenbutatin oxide and pirimicarb after treatment on peaches.

	Fenbutatin oxide			Pirimicarb	
Days after treatment	Weight (g)	Residues	Days after treatment	Weight (g)	Residues
Greenhouse					
0	50	1.80	0	24	1.31
8	79	1.36	4	29	1.11
15	113	1.06	8	31	0.86
22	138	0.58	14	52	0.59
28	156	0.59	26	92	0.36
—	—	(1.84)[a]	—	—	(1.38)[a]
Field					
0	45	1.76	0	51	0.62
7	64	1.12	3	66	0.47
14	95	0.63	7	83	0.40
21	124	0.34	14	107	0.17
28	140	0.22	21	115	0.10
—	—	(0.68)[a]	—	—	(0.23)[a]

[a]( ) = residues corrected by dilution effect.

Table 5.17. Residues (mg kg^{-1}) of dimethoate on different fruits after treatment.

Days after treatment	Apricots	Oranges	Olives	Peaches	Plums	Grapes
0	1.51	0.41	1.60	0.97	1.08	1.13
7	0.79	0.22	1.08	0.31	0.05	0.21
14	0.45	0.17	0.17	0.22	n.d.[a]	0.26
21	0.22	0.17	—	0.12	—	0.28
28	0.13	—	—	—	—	0.28
35	0.12	—	—	—	—	—

[a]n.d. = not detectable.

Table 5.18. Residues (mg kg^{-1}) of ethiofencarb and its metabolites on lettuce after treatment.

Days after treatment	Weight (g)	E. sulphoxide	E. sulphoxide phenol	E. sulphone	Ethiofencarb
0	96	9.21	8.77	1.66	6.02
1	86	12.79	n.d.[a]	1.73	1.62
3	136	4.60	n.d.	0.71	n.d.
8	371	0.71	n.d.	0.22	n.d.

[a]n.d. = not detectable.

Different systems of assessment of residues could create problems with the circulation of goods, since, depending on the system used, the residues could be legal when referring to the active ingredient alone and illegal when referring to the sum of active ingredient and metabolites (Cabras *et al*., 1988).

Washing

The removal of the active ingredient from the surface of the plant by water (rain, washing or irrigation) is not easy to interpret, since the results obtained in experiments are often contradictory. Experiments carried out on tomatoes irrigated by drop or sprayer have not shown significant differences that could be correlated with the irrigation system (Cabras *et al*., 1986a). Also, in washing trials with plums, there was no reduction in residue before the drying process. In contrast, in washing trials with olives, there was a residue reduction in some cases, while in other cases the residue was unchanged (Table 5.19). In all cases, however, there were no further residue reductions on submitting the olives to a second washing that was even longer than the first. This shows that residue reduction after first washing is not related to a solubilization process. These apparently contradictory behaviours can be explained as follows.

At the time of treatment, since there could be dust on the fruits, the active ingredient will deposit both on the waxy layer of the fruit surface and on the grains of dust. The pesticide deposited on the waxy layer will tend to spread over the layer and over the underlying cuticle, and will therefore be protected from the action of water. Since the dust is removed from the fruit during washing, the greater the amount of residue bonded to the dust the greater the residue removal. Therefore, if there is no dust on the fruit at the time of treatment or at harvest because it has been washed away by the rain, as must have happened to samples 5 and 6 in Table 5.19, washing will not remove any dust and therefore there will be no related residue reduction (Cabras *et al*., 1997c).

Residues in Processing of Foods

Some foods, such as olive oil and wine, are the result of food transformation processes; others, such as dried fruit, undergo a concentration process by removal of water. Since on average 1 l of wine is obtained from 1.5 kg of grapes, 1 l of olive oil from 5 kg of olives, and 1 kg of dried prunes from 3 kg of plums, if the technological process of transformation did

Table 5.19. Effect of washing on residues in olives.

Pesticides	Solubility in water (mg l^{-1})	Treatment[a]	Residues (mg kg^{-1}) Sample 1	2	3	4	5	6
Azinphos methyl	28	C	3.02	2.73	2.15	2.12	1.01	0.72
		W	1.85	2.49	1.40	1.28	0.92	0.79
Diazinone	60	C	3.46	2.63	1.74	1.53	1.46	1.15
		W	2.29	1.72	1.73	0.91	1.53	1.27
Dimethoate	23,300	C	4.71	3.43	2.35	2.30	0.91	0.76
		W	4.02	2.47	1.70	1.98	0.85	0.82
Metidathion	200	C	4.25	3.81	2.89	2.63	2.51	1.67
		W	3.59	2.88	2.51	2.36	2.55	1.74
Parathion methyl	55	C	4.26	4.58	2.29	2.29	1.69	1.35
		W	3.03	4.67	1.51	2.36	1.71	1.40
Quinalphos	18	C	3.56	1.90	1.75	1.46	0.88	1.06
		W	2.38	1.70	1.28	0.81	0.93	1.09

[a]C = control, W = samples washed in water.

not cause a reduction in residues, the final products would contain a higher residue concentration factor than the initial fruits. A number of studies have been carried out to assess the incidence of the technological process of transformation on the residue content.

Dried fruit

Some fruits, such as apricots and plums, are consumed both fresh and dried. The industrial drying process is carried out in ovens with programmes that reach temperatures of 95°C for plums and 100°C for apricots. In drying experiments with industrial processes carried out on these fruits, it was shown that there is a change in residue after drying peculiar to each active ingredient (Cabras *et al*., 1997d, 1998). From the data reported in Table 5.20, it can be seen that the residue dimethoate was unchanged. Therefore, considering a concentration factor of 5.3, the residue was reduced by this factor.

Analogous considerations can be made for ziram, while fenitrothion and vinclozolin, on apricots and plums, respectively, are completely removed during the drying process.

Olive oil

Olive oil is obtained by pressing the fruit. Therefore, by pressing olives with a yield in oil of between 14 and 16%, 6–7 kg of fruit will be needed to obtain 1 l of oil. From experiments carried out with olives with these characteristics aimed at assessing the amount of residues transferred from the olives to the oil, the results reported in Table 5.21 were

Table 5.20. Changes in the residues (mg kg^{-1}) of some pesticides during the drying process of apricots and plums.

Fruit	Weight (g)	Dimethoate	Fenitrothion	Ziram
Apricots				
Fresh	46	0.12	0.03	0.12
Dried	8.6	0.14	n.d.[a]	0.27
Rehydrated	10.5	0.09	n.d.	0.22
Plums				
Fresh	32.1	—	0.14	—
Dried	9.1	—	n.d.	—
Rehydrated	10.2	—	n.d.	—

[a]n.d. = not detectable.

Table 5.21. Residues (mg kg^{-1}) of a few insecticides on olives and olive oil.

Pesticide	Olive	Yield %	Oil	Concentration factor in oil
Azinphos methyl	1.03	16	3.10	3.0
	0.69	16	1.62	2.3
Diazinon	1.11	16	3.78	3.4
	0.35	14	1.95	5.6
Dimethoate	1.08	16	0.24	0.22
	0.17	16	n.d.[a]	0
Metidathion	3.01	15		2.3
	1.28	16	3.37	2.6
Parathion methyl	0.61	16	2.91	4.8
	0.19	14	1.33	7.0
Quinalphos	0.68	16	2.13	3.1
	0.20	14	0.80	4.0

[a]n.d. = not detectable.

obtained for a number of pesticides (Cabras *et al*., 1997c).

From the data reported in Table 5.21, it can be seen that the residues are always greater in the oil than in the olives, except for dimethoate, which, due to its high solubility, tends to be distributed preferably in the vegetable water.

The amount of residue present in the oil is also a function of the residue in the fruit; generally speaking, the concentration factor in the oil is greater for smaller concentrations. In the case of parathion methyl, at the lowest concentration, the residue is completely transferred from the olive to the oil. With the other insecticides, on average, about 50% of the residue passes from the olive to the oil.

Wine

To obtain 1 l of wine, an average of about 1.5 kg of fruit is needed. This means that if the entire residue present in the grapes passed into the wine, we would always have residue increases in the wine. From the data reported in Table 5.22, which were obtained from a number of experiments (Cabras *et al*., 1986b, 1997a,b; Farris *et al*., 1992; Cabras and Angioni, 2000), it can be seen that in the transformation from grapes to wine, each pesticide has its own peculiar behaviour. Dimethoate, fenthion, metalaxyl and pyrimethanil do not undergo significant reductions, while with the other compounds there is a residue decrease that can be complete for some pesticides. In no case were higher residues found in the wine than in the grapes.

Must clarification by centrifugation often causes a marked residue decrease; this shows that the residues tend to be adsorbed on the solid fraction of the must. For this reason, by fermenting 'clean' musts, wines with fewer residues are obtained.

The examples reported above show that the quantity of residues present in foods depends on a number of variables. Since no

Table 5.22. Residues (mg kg^{-1}) of fungicides in grapes, must and wine.

		Must		Wine	
Pesticide	Grapes	Not centrifuged	Centrifuged	Without maceration	With maceration
Azoxystrobin	0.19	0.13	0.13	0.13	0.09
Benalaxyl	0.89	0.43	0.24	0.36	0.12
Cyprodinil	5.54	4.01	0.18	0.70	0.74
	1.03	0.36	n.d.[a]	0.18	0.21
Dimethoate	1.13	0.90	0.91	0.92	0.90
	0.28	0.15	0.15	0.14	0.14
Fenthion	0.28	0.22	0.20	0.21	0.24
Folpet	1.08	1.11	n.d.	n.d.	n.d.
Fluazinam	1.21	0.30	0.08	n.d.	n.d.
Fludioxonil	1.86	1.79	1.20	0.71	0.50
	0.78	0.39	n.d.	0.23	n.d.
Kresoxim methyl	0.15	0.13	0.05	0.18	0.09
Iprodione	3.00	1.40	0.80	0.60	—
Metalaxyl	—	1.69	—	1.30	—
	1.09	1.04	—	—	—
Parathion methyl	0.56	0.26	0.25	0.21	0.21
Pyrimethanil	1.62	1.66	1.29	1.04	1.56
	1.11	1.03	0.94	1.02	1.01
Quinalphos	0.18	0.06	0.02	n.d.	n.d.
Tebuconazole	3.16	3.13	1.35	0.96	0.98
	0.42	0.20	n.d.	0.16	0.22
Vinclozolin	4.30	1.50	0.20	0.10	—
	0.80	0.06	0.03	0.01	

[a]n.d. = not detectable.

mathematical models can foretell their behaviour a priori, it is always necessary to carry out experiments in real conditions to evaluate the amount of residues at harvest and, on this basis, to indicate legal limits.

Monitoring Programmes on Pesticide Residues in Food

As already discussed, each country establishes a maximum residue limit (MRL) for each pesticide for the cultures for which it has been authorized. If this limit is exceeded or if this active ingredient is used on cultures that have not been authorized, the obtained foods are considered irregular and therefore not marketable. Though the MRL is not a toxicological limit, exceeding it means that the pesticides are not being used correctly and, by comparing the values with the ADI, the toxicological risk to the consumer can be assessed. For this reason, in the most developed countries, programmes for the monitoring of pesticide residues have been promoted by official agencies for the past few years. The results obtained in European Union countries in 1998 and in the USA in 1999 are reported in Table 5.23. Those referring to the European Union are focused on fruit, vegetables and cereals, while the American results also include fish, milk and dairy products, and eggs. The US data are also available on the Internet at the following website: www.cfsan.fda.gov

The number of samples analysed in Europe is remarkable and indicates the special attention paid to food health problems these days. The results obtained indicate that the number of irregular samples is small both in the EU and in the USA. Moreover, on comparing these data with those of the two previous years, it can be seen that the values are not significantly different. It should be mentioned that the data obtained in the different countries are not comparable since the national MRLs differ both in entity and in number of cultures for which each pesticide is registered. Moreover, when planning samplings, the cultures are usually chosen according to the national diet and the pesticides to be analysed, and selected according to their use, the frequency observed in previous years and the

Table 5.23. Results of the monitoring programmes for pesticide residues in 15 countries of the EU in 1998 and of the USA in 1999.

Countries	Samples (*n*)	Pesticides		Samples without residues (%)	Samples with residues < MRL (%)	Samples with residues > MRL (%)
		Analysed (*n*)	Found (*n*)			
Belgium	1,947	122	46	65.5	28	6.5
Denmark	2,164	131	76	69.1	28	2.9
Germany	6,696	—	—	61.6	34	4.4
Greece	1,164	93	41	76.5	19	4.5
Spain	3,202	169	—	61.8	36	2.2
France	4,058	224	106	40.2	53	6.8
Ireland	329	—	—	43	53	4.0
Italy	8,779	—	—	67.8	31	1.2
Luxemburg	230	94	31	67.5	29	3.5
Netherlands	4,976	275	108	56.1	38	5.9
Austria	322	83	41	55.9	41	3.1
Portugal	455	100	28	61.5	35	3.5
Finland	2,359	173	97	54.2	43	2.8
Sweden	34,999	—	—	65	33	2.0
UK	976	151	51	57	40	3.0
EU	41,336	147	63	60.8	36	3.2
USA						
Domestic	3,426	400	—	60.2	39	0.8
Imported	6,012	400	—	64.8	31.3	3.9

danger they represent. In addition, there are technical problems such as the analytical methods used and the analytical expertise of the laboratory.

Besides the official checks, many other structures (producers' cooperatives, agricultural farms, large distribution chains) carry out checks on their products before putting them on the market. In 1999 in Italy, for example, the National Residue Observatory, a private organization that collects unofficial control data every year, published the results of a survey of 18,972 samples, 51.4% of which were without residues, 46.7% with regular residues and 1.9% with irregular residues. The data referred to an analysis of 132 types of food with 276 active ingredients, 137 of which had left determinable residues.

Thanks to these checks, it is possible to determine for each country the pesticides that are used mainly in each culture, their levels and those that most often exceed the MRL. Moreover, the data obtained are used as a basis for subsequent checks with particular attention to pesticides that are most frequently used and are the most hazardous for the consumers' health.

Such frequent pesticide checks in food are a good tool to calculate the consumers' real exposure to toxic compounds.

Risk Assessment for Pesticide Residues in Food

Risk assessment is an estimate of the likelihood of harmful effects on the health of the population as a result of exposure. The best guarantee that the exposure to pesticide residues will be contained within safety limits is obtained from dietary ingestion studies. Using the data from toxicological studies, it is possible to assess the quantity of pesticide, in reference to body weight, that may be ingested in a lifetime without appreciable risks to one's health. This quantity is the ADI value. If the quantity of pesticide ingested daily is lower than the ADI value, the probability of harmful effects for health theoretically is zero. Risk assessment due to the presence of pesticide residues is subdivided into the following phases:

- pesticide residue estimate
- national diet estimate
- dietary pesticide exposure

Residue estimate

Exposure estimates may be carried out on theoretical and analytical data. The theoretical data are used in the pesticide registration process. In this case, it is assumed that the residues are present in the food at the maximum legal limit (MRL). In fact, these values are difficult to reach even in extreme conditions when the maximum doses are used in the treatment, with the largest number of applications and the shortest time intervals between treatment and harvest. If the amount of residue ingested with this theoretical value in the foods making up the diet and for which an authorization is requested exceeds the ADI, the product may not be registered.

In fact, the residues present in the cultures for which they are authorized are often very far from these limit values, because they are not always used, because a large number of treatments is rarely carried out and because the time interval between the treatment and the harvest is often longer than the preharvest interval.

A true assessment of the residues in the foods may be carried out by analysing the samples when they are marketed. Thanks to monitoring programmes, it is possible to know the residue level of each pesticide in different foods. The larger the number of samples analysed, the better the knowledge of their pollution level and, therefore, the more reliable the risk assessment.

National diet estimate

Data on food consumption are essential for an assessment of the risk related to food safety. Food consumption varies from country to country and often also within the same country. For this reason, each country must

assess its own standard diet taking into account the food habits of the different categories of people by age, sex, place, etc. Particular attention must be paid to sensitive groups such as the newly born. The food consumption indices that are used are many, namely: mean daily consumption, size of portion and the average consumption of the population. Generally these data are easily available since there are institutions interested in the national food diet in every developed country. For an assessment at the world level, the data contained in the FAO 'Food Balance Sheets' are the most reliable source.

Dietary pesticide exposure

Risk assessment relating to pesticide residues in food has been tackled by the Codex Alimentarius with the special Joint FAO/WHO Expert Committee on Pesticide Residues (JMPR) made up of groups of independent experts. This commission carries out toxicological assessments on pesticides, estimating an ADI value, and proposing MRLs and models to be used to assess the population exposure. The most realistic assessments may be made at the national level since they are based on the most reliable data of food consumption. In order to assess the dietary pesticide exposure, the residues of each pesticide are multiplied by food consumption. These data are expressed in μg kg^{-1} BW day^{-1}, and make up the daily intake (NEDI = national estimated daily intake). The data are then looked at in conjunction with the values of the ADI. The risk to human health starts when the NEDI/ADI % ratio is greater than 100. In calculating the residue intake, account must be taken of factors that may alter their concentration in the actual food intake, such as the part of the agricultural product that is actually eaten (e.g. oranges without peel), the effects of processing the raw product (e.g. wheat → flour), or transforming it (e.g. olives → oil), and those of cooking or preparing it (e.g. potatoes → chips). These correction factors are obtained from literature data on each specific active ingredient. In calculating exposure, an important problem is that of samples that contain residues lower than the limit of detection (LOD) and therefore considered absent. In this case, using data from samples of an unknown treatment history, as in the case of official monitoring, we assign zero or we assign the value of ½ LOD to a certain percentage and zero to the remaining part. As an example, we report some exposure data relating to Italian consumers calculated in 1999 by the National Residue Observatory (Table 5.24). These data have been chosen from those of the highest residue intake

Table 5.24. Exposure of the average Italian consumer to pesticides in 1999.

Pesticide	ADI[a] (μg kg^{-1} BW[c])	NEDI[b] (μg kg^{-1} BW[c])	NEDI/ADI %
Acephate	30	0.0129	0.04
Azinphos methyl	5	0.0403	0.8
Buprofezin	10	0.0032	0.03
Chlorthalonil	30	0.0374	0.1
Chlorpyrifos methyl	10	0.0290	0.3
Cyprodinil	30	0.0331	0.1
Dimethoate	2	0.0204	1.0
Omethoate	0.3	0.0067	2.2
Parathion methyl	3	0.0187	0.6
Pyrimethanil	170	0.0950	0.06
Quinalphos	30	0.0012	< 0.01
Teflubenzuron	10	0.0041	0.04
Vinclozolin	10	0.0222	0.2

[a]Acceptable daily intake.
[b]National estimated daily intake.
[c]Body weight.

and show that dietary residues are so much less than the safety threshold (NEDI/ADI% = 100) that even the most restrictive assessments that take into account particularly sensitive groups (newborns, adolescents, the elderly, etc.) would lead to 100% safe results. The data on monitoring carried out in other countries (Table 5.23) show that even here assessments of the exposure to pesticide residues in food are not very different from those made in Italy.

Conclusions

Pesticide toxicology and residues in food and wine have been reviewed in the context of human exposure to specific insecticides, fungicides and herbicides. Factors affecting residues in fruit and vegetables depend upon initial deposits and disappearance rates. Risk assessments carried out on selected pesticides show that human exposure is well below safety threshold values for consumers in Italy. In the UK, results of a 2001 survey of milk, honey, canned salmon, kiwi fruit, grapes, lemons, breakfast cereals and other foods have just been published by the Pesticide Residues Committee. The results indicated that 29% of the 450 samples tested contained residues of pesticides, with about 10% of all samples containing multiple residues. The health implications of multiple pesticide residues and the significance of any interactions with other types of food contaminants remain unresolved.

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6 Polychlorinated Biphenyls

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Introduction

Polychlorinated biphenyls (PCBs) are synthetic chemical mixtures that theoretically could contain up to 209 chlorinated congeners of the biphenyl moiety (Ballschmiter and Zell, 1980). While about 130 congeners have been identified in commercial products (Fig. 6.1), most commercial PCB mixtures only contain 50–90 different congeners (Nicholson and Landrigan, 1994). Due to their physical and chemical properties, PCBs had a multitude of industrial applications: dielectric fluids in capacitors and transformers, heat transfer agents, plasticizers in paints, flame retardants, pesticide extenders, adhesives, coatings, cutting oils and hydraulic lubricants, and inclusion in inks, carbonless copy paper, sealants and caulking compounds (Safe, 1994; Eisler and Belisle, 1996). Generally, mixtures of PCB congeners were marketed based on their percentage of chlorine. For example, Monsanto Chemical Company sold the following PCB mixtures: Aroclor 1221, 1232, 1242, 1248, 1254, 1260 and 1268. The 12 indicated that the mixture was a biphenyl and the last two digits indicated the percentage of chlorine by weight (i.e. 21, 32, 42%, etc.). One exception to this generality was Aroclor 1016, a distillation product of Aroclor 1242, containing 41% chlorine by weight but with only 1% of the congeners containing five or more chlorine atoms. Other companies marketed their PCB mixtures under such trade names as Clophens (Bayer, Germany), Delor, Delorene and Hydelor (Chemko, Czechoslovakia), Fenclors and Apirolio (Caffaro, Italy), Kanechlors (Kanegafuchi Chemical Co., Japan), Orophene (Deutsche Soda Werkrn-VEB, Germany), Phenochlor and Pyralène (Prodelec, France), Santotherm (Mitsubishi-Monsanto Co., Japan) and Soval (Sovol, USSR) (De Voogt and Brinkman, 1989). The numbering system for the latter mixtures indicates an approximation of the mean number of chlorine atoms per congener. For example, Clophen A60, Phenochlor DP6 and Kanechlor 600 all have an average of six chlorine atoms per molecule, i.e. 59% chlorine by weight. The congener composition of commercial PCB mixtures varies from batch to batch since the extent of their chlorination ranges from 21 to 68% (w/w). Consequently, commercial PCBs are not sold based upon composition *per se* but on the batch's physical properties. In addition, the composition and amount of impurities also vary from batch to batch and among manufacturers. For example, most

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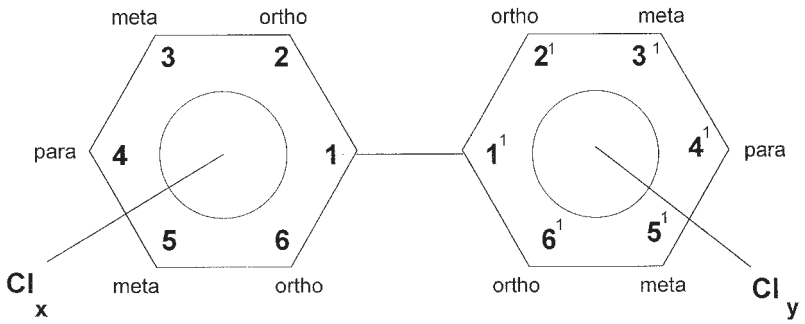

Fig. 6.1. Polychlorinated biphenyls (PCBs), x, y ≤ 5.

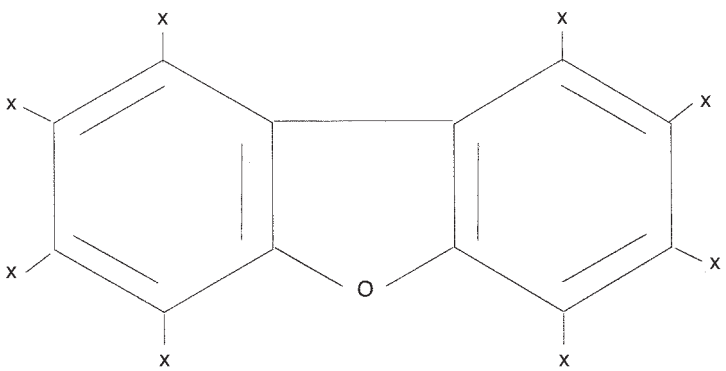

Fig. 6.2. Polychlorinated dibenzofurans (PCDFs), x ≤ 8.

commercial PCBs, except Aroclor 1016, contain polychlorinated dibenzofurans (PCDFs; Fig. 6.2), and some commercial mixtures may contain polychlorinated naphthalenes and polychlorinated quaterphenyls (PCQs; IARC, 1978; De Voogt and Brinkman, 1989; Nicholson and Landrigan, 1994; ATSDR, 2000).

From a historical perspective, PCBs were first synthesized by Griefs in 1867. Monsanto Chemical Company did not start manufacturing PCBs in the USA until 1929, while commercial production of PCBs in Japan did not start until 1954 (IARC, 1978). The manufacture of specific Aroclors by Monsanto tended to occur during specific periods. For example, Aroclor 1254 and 1260 were predominantly used prior to 1950, while Aroclor 1242 was the dominant mixture in the 1950s and 1960s. Starting in 1971, Aroclor 1016 replaced Aroclor 1242, and the sale of PCBs in the USA was limited to capacitor and transformer manufacturers, with Monsanto voluntarily limiting production of Aroclors to those containing less than 60% chlorine. In 1974, most domestic uses for PCBs were restricted to closed applications. In 1976, all new usages of PCBs were banned, and the first effluent standards for PCBs were issued by the US Environmental Protection Agency (EPA) in 1977. Manufacturing and import limitations were issued in 1979, and subsequent amendments to this regulation banned the production of PCBs in the USA (Nicholson and Landrigan, 1994; Eisler and Belisle, 1996; Danse *et al*., 1997; ATSDR, 2000).

In an attempt to conceptualize the extent to which PCBs were manufactured/used and their potential for contamination of the environment, Kannan (2000) reported that production of PCBs by industrialized western nations totalled an estimated 1,054,800 t, while the former USSR produced another 100,000 t. Kannan also reported that a total of 370,000 t may have escaped into the environment while the remainder is still in use, primarily in electrical equipment.

The physical and chemical properties which made PCBs such a useful industrial commodity have resulted in their contaminating every component of the global ecosystem as winds and water currents have dispersed PCBs to parts of the globe where they have never been used (Macdonald *et al*., 2000). It has been reported that biphenyls with one or no chlorine atoms remain in the atmosphere, while those with one to four chlorine atoms migrate towards the polar latitudes; those with four to eight chlorine atoms remain in the mid-latitudes, and those with eight or nine chlorine atoms remain close to the source of contamination (Wania and Mackay, 1996). Over time, the more stable PCB congeners, generally those with a greater degree of chlorination, have found their way into the food chain. Whether such contamination has health implications for humans is still a debatable issue, since the potential health effects of PCBs cannot easily be distinguished from those of other environmentally persistent anthropogenic chemicals (Danse *et al*., 1997; Johnson *et al*., 1998).

Simplistically, there are three general groupings into which humans can be placed regarding their exposure to PCBs. There are those who were exposed to PCBs in an industrial setting, where dermal absorption and/or inhalation were the major routes of exposure. A second grouping includes the people in Japan and Taiwan who ingested rice oil that was inadvertently contaminated with PCBs which were being used as a heat transfer fluid. The Japanese accident occurred in 1968 and became known as the Yusho ('oil disease' in Japanese) incident, affecting about 1800 people (Kuratsune and Shapiro, 1984; Kuratsune *et al*., 1996), while the Taiwan accident occurred in 1979 and became known as the Yu-Cheng ('oil disease' in Chinese) incident, affecting approximately 2000 people (Kuratsune and Shapiro, 1984). While the heat transfer fluid originally consisted of Kanechlor 400, which contained 48% chlorine by weight, heating the Kanechlor under reduced pressure resulted in the loss of some of the lower chlorinated congeners as well as the conversion of other congeners into PCDFs and PCQs (Masuda, 1996). The latter two entities are generally considered to be more toxic than PCBs (Danse *et al*., 1997; Longnecker *et al*., 1997). The third exposure group comprises the rest of the world population, who are primarily exposed to PCBs via their diet, although some additional exposure via drinking water and inhalation occurs. This simplistic grouping does not recognize various subpopulations who may be at risk for higher exposure to PCBs: for example, recreational fishers and hunters who consume contamined fish and game; native populations who are subsistence hunters and fishers; breast-fed infants whose mothers consume significant amounts of PCB-contaminated fish and/or wild game; farm families whose food was exposed to PCB-contaminated silos when PCBs were used as a silo sealant; those living in proximity to waste storage or disposal sites; and other analogous populations (Kimbrough, 1995; Johnson *et al*., 1998). As the theme of this book is food safety, the emphasis of the following discussion will be on the ingestion of PCBs via food, which is the most important route of human exposure (Hu and Bunce, 1999). Dermal and inhalation exposure to PCBs are only of importance in the workplace (Nicholson and Landrigan, 1994).

Nature of PCBs – Chemical and Physical Properties

PCBs are thermally stable; they are resistant to acids, bases and oxidation; at room temperature, they have a low volatility, which increases dramatically with small increases in temperature; they have a high dielectric constant; and they are practically fire resistant because of their high flash point (170–380°C). PCB vapours, while heavier than air, are not explosive. They have low electrical conductivity, high thermal conductivity and they are resistant to thermal degradation. Consequently, PCBs are inert, being stable to hydrolysis and oxidation by conditions encountered during industrial use.

While individual PCB congeners are colourless crystals when isolated in pure form by recrystallization, commercial PCB mixtures can range in colour from clear, through light yellow to dark brown. Their physical

state can range from an oil to a viscous liquid or a sticky resin; however, they do not crystallize, even at low temperatures. PCBs are relatively insoluble in water, with the more highly chlorinated congeners being the least soluble, but they are soluble in oils, non-polar organic solvents and biological lipids (IARC, 1978; Eisler and Belisle, 1996; ATSDR, 2000).

Low levels of PCBs can be found throughout the ecosystem. As they are no longer manufactured in significant quantities, PCBs are continually redistributing among environmental compartments, i.e. soil, water, sediments and air. The fate of PCBs in aquatic systems and soil depends upon their sorption and retention, both of which are markedly influenced by the number of chlorine atoms. Generally, the greater the number of chlorine atoms, the greater the retention. In an aqueous environment and on soil surfaces, PCBs can evaporate and return to earth via rain or snow or by settling on dust particles. The major source of PCBs in surface water is from atmospheric deposition. As PCBs absorb strongly to soil particles, significant leaching from soil and translocation to ground water or plants is unlikely. While there is no known abiotic process that will significantly degrade PCBs contaminating soil, photodegradation on soil surfaces may occur. Aerobic and anaerobic biodegradation are the major degradation processes, but they occur very slowly. Aerobic degradation of PCB congeners depends upon such factors as initial concentration, moisture, temperature (warmer temperatures enhance degradation), inhibitory compounds (e.g. chlorobenzoates) and the availability of such bacterial nutrients as carbon sources (e.g. acetate); however, biodegradation is slowed in soils with a high organic carbon content. Interestingly, anaerobic biodegradation appears to have a greater effect on the more highly chlorinated congeners while aerobic biodegradation is more effective on the lower chlorinated congeners (ATSDR, 2000).

Generally, biological entities do not metabolize the more highly chlorinated PCB congeners nor are they readily excreted. Since PCBs are soluble in body lipids, a biological system's inability to excrete PCBs to any meaningful extent, save for their secretion/excretion in breast milk (Feeley and Brouwer, 2000), results in PCBs being biomagnified in the food chain. Bioaccumulation/biomagnification of PCBs is largely dependent upon a congener's octanol–water partitioning coefficient (Eisler and Belisle, 1996).

Distribution in Foods

As an anthropogenic chemical, PCBs have contaminated the environment solely as a consequence of human activity. While their manufacture and use in new products are minuscule on a worldwide basis, PCBs continue to redistribute themselves among environmental compartments. In addition, some PCBs are still released into the environment from waste sites, incineration, leakage from electrical equipment, improper disposal, spills and leachates from sewage sludge. However, it should be noted that the levels of PCBs in all environmental compartments appear to have decreased significantly in recent years (Duarte-Davidson and Jones, 1994; Johnson *et al*., 1998; ATSDR, 2000; Dougherty *et al*., 2000).

Any attempt to longitudinally study and quantitate the contamination of human foods by PCBs is difficult because the instrumentation has changed dramatically since PCBs were first detected in the food chain in 1966 (Danse *et al*., 1997). Initially, PCB analyses consisted of comparing the PCB chromatographic pattern in the sample of interest with that of various commercial mixtures. The chromatographic patterns found when North American food samples from the 1960s and 1970s were analysed for PCBs were similar to those of Aroclor 1254 and 1260 (Zitko *et al*., 1972; Veith, 1975; Walker, 1976; Veith *et al*., 1981), although contaminant patterns resembling those of Aroclor 1242 and 1016 were also reported (Veith *et al*., 1981). In recent years, congener-specific analyses have been undertaken. McFarland and Clarke (1989) have suggested that approximately half of the 209 potential PCB congeners account for nearly all of the environmental contamination attributed to PCBs. Even fewer congeners are both environmentally prevalent and potentially toxic. Using the criteria of potential toxicity,

environmental prevalence and relative abundance in animal tissues, McFarland and Clarke concluded that only 36 congeners were of environmental concern, and 25 of these congeners accounted for 50–75% of all the PCB congeners found in tissue samples from fish, invertebrates, birds and mammals.

Concurrent with the advances in analytical technology, there was an interest in undertaking toxicological testing with specific PCB congeners. It was found that there was a marked difference among PCB congeners regarding their effects in various *in vivo* and *in vitro* toxicity assays, with the least potent congeners being those that had a non-planar stearic configuration. As biphenyl rings can rotate at the 1,1′ positions, they can exist in a planar orientation. When chlorine substitution occurs at the *meta* and *para* ring positions, but not at the *ortho* position, the rotational barriers are lowered sufficiently for a small number of the *meta*- and *para*-substituted congeners to assume a planar configuration. These congeners (i.e. 77, 81, 126, 169; numbering system of Ballschmiter and Zell, 1980) are referred to using such terms as non-*ortho*, planar or 'dioxin-like' congeners (Fig. 6.3) due to their stearic configuration and toxicological properties being analogous to those of tetrachlorodibenzo-*p*-dioxin (TCDD). Other congeners (i.e. 105, 114, 118, 123, 157, 157, 167 and 189) having only one chlorine atom at the *ortho* position can also exist in a planar conformation but, while these mono-*ortho* congeners can have a dioxin-like configuration, they are less toxic than the non-*ortho* PCBs (Kannan, 2000).

Human populations are exposed to PCBs primarily via the consumption of fish, meat and poultry, although fish has been the major source of PCB exposure in the USA for the past 25 years (ATSDR, 2000). Dougherty *et al*. (2000) also reported that fish consumption, particularly saltwater fish, accounts for a majority of the PCBs ingested by Americans, including children aged 1–5 years. Freshwater fish and shellfish were also significant contributors to PCB ingestion, while milk and beef contributed less that 5% to total average exposure. The most commonly occurring congeners in fish are 95, 101, 110, 118 (pentachlorobiphenyls), 138, 153 (hexachlorobiphenyls) and 180 (heptachlorobiphenyl). Due to their low probability for degradation, these congeners are the major contaminants in most biological tissues (ATSDR, 2000).

For the contemporary UK population, Duarte-Davidson and Jones (1994) estimated that 97% of their total PCB exposure came from food, 3.4% from air and 0.04% from water. The contributions from fish, milk and dairy products; vegetables; meat; and animal fat were estimated to account for 32, 26, 18 and 16% of the exposure, respectively. Vegetables accounted for the majority of the lower chlorinated congeners, while meat, dairy and fish contained most of the higher chlorinated congeners. For example, vegetables accounted for 78% of the total dietary content of congener 28 (trichlorobiphenyl) and 0.2% of congener 180 (heptachlorobiphenyl), while freshwater fish accounted for 1.2% of the dietary content of congener 28 and 27% of

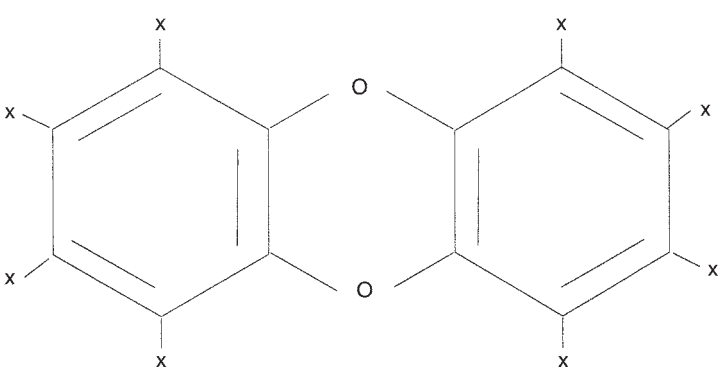

Fig. 6.3. Polychlorinated dibenzodioxins (PCDDs), x ≤ 8.

congener 180. The authors estimated that the average daily PCB exposure in the UK was 0.53 μg per person.

The ingestion of PCBs has only been studied in a few other countries, and the findings are thought to reflect that particular country's dietary habits. For example, 69% of the PCBs ingested by Vietnamese individuals was from cereals and vegetables. In India, 70% of the PCBs consumed was from cereals, vegetables and dairy products. The primary source of dietary PCBs in Germany and The Netherlands was dairy products; in Canada it was meat; in Finland, the Nordic countries and Japan it was fish. It should also be noted that the Vietnamese, Indian and Dutch data were based on raw foodstuffs; it appears that the results for Germany, Finland and the Nordic countries were also for uncooked food items; and the Japanese data were based on cooked foods. It is known that the PCB concentrations in foodstuffs decrease during cooking. While cooking alters the PCB concentration in food, the values obtained from dietary surveys of this type are also affected by how the surveyors chose to handle the data regarding each individual's diet, whether there were or were not any data for certain food products (bread, preserves, fruit, beverages, etc.) and whether values which were below the detection limits were assumed to contain some (i.e. 0.5 of the detection limit) or no PCBs (Ahlborg *et al*., 1992; Duarte-Davidson and Jones, 1994; Boersma *et al*., 2000).

Absorption, Metabolism and Excretion

Studies with laboratory animals have found that gastrointestinal absorption of commercial PCB mixtures, as well as that of individual PCB congeners, has often exceeded 90% (Arnold *et al*., 1993; ATSDR, 2000). Gastrointestinal absorption has been found to occur on a congener-specific basis by passive diffusion, but absorption is enhanced by increased ring chlorination and when the concentration of PCBs in the gut contents is much greater than the concentration in serum lipids. However, it does appear that, similarly to fats and other fat-soluble chemicals, PCBs are absorbed from the gut via the lymphatic circulatory system. PCBs in human plasma are found predominantly attached to the lipoprotein fraction. As the more chlorinated PCB congeners are lipophilic, they tend to accumulate in adipose and lipid-rich tissues, which can then be transferred via breast milk to the nursing infant. It is well known that PCB congeners cross the placental barrier and accumulate in fetal tissue, but the tissue levels of PCBs in the fetus are usually lower than those of its mother at parturition. This observation has been attributed to the lower concentration of lipids in cord blood when the comparison is done on a whole-weight basis; but, when compared on a lipid basis, the difference is not appreciably large (Ahlborg *et al*., 1992; Danse *et al*., 1997; ATSDR, 2000; Feeley and Brouwer, 2000). There are several factors that affect the mother's accumulation of PCBs: her age; number of pregnancies and lactations; place of residence/exposure to PCBs; and changes in her weight during pregnancy. While lactation is a major route of PCB excretion, i.e. approximately 20% of the mother's body content of PCBs (Duarte-Davidson and Jones, 1994), current data suggest that age may be more of a factor than the number of deliveries regarding the amount of PCBs accumulated by the mother. While most studies have found that breast milk from women living in industrial areas has greater amounts of PCBs than from those living in rural areas, breast milk from native women living in Arctic Québec contained even greater amounts of PCBs (Dewailly *et al*., 1992; ATSDR, 2000). It has been estimated that if an infant is breast fed for 6 months, the child will accumulate 6.8–12% of his/her lifetime body burden of PCBs (Kimbrough, 1995; Patandin *et al*., 1999). In addition to the obvious exposure of developing infants to PCBs *in utero* and via breast milk while nursing, there are a number of other intrinsic factors which differentiate children and adults regarding their exposure to and accumulation of PCBs; for a detailed discussion, see ATSDR (2000).

As PCBs are persistent and biodegrade slowly, age and the concentration of PCBs in biological systems are highly correlated. However, the concentration/pattern of PCB

congeners accumulated within the various tissues from the same individual are often dissimilar. For humans, when the concentration of PCBs is determined on a lipid weight basis, the highest concentrations are usually found in adipose (omental/subcutaneous fat), skin and liver tissue, while brain tissue contained the least amount of PCBs (Ahlborg *et al*., 1992; ATSDR, 2000).

Metabolism is not a prerequisite for PCBs to exert many of their biochemical and toxicological effects (Safe, 1992, 1994; ATSDR, 2000), but there are exceptions to this generality (Sipes and Schnellmann, 1987; Ahlborg *et al*., 1994; Safe, 1994; Koga and Yoshimura, 1996). Therefore, the metabolism of PCBs generally represents a detoxification process, with the retention or accumulation of congeners being correlated with its biological stability (ATSDR, 2000). Generally, the presence of fewer chlorine atoms on the biphenyl rings, coupled with the lack of one or more chlorine atoms at the *para* position, appears to facilitate metabolism and excretion (Kimbrough, 1995). The elimination of PCB congeners is largely dependent upon its metabolism, generally to a more polar compound; however, excretion of unmetabolized congeners does occur to a limited degree. Since PCBs are a mixture of congeners that have different stearic configurations, they are metabolized via several enzymatic pathways whose activity is markedly different among species. In general, however, PCBs are metabolized poorly and are eliminated slowly (Sipes and Schnellmann, 1987; Ahlborg *et al*., 1992; Koga and Yoshimura, 1996; ATSDR, 2000).

Commercial PCB mixtures are capable of inducing microsomal cytochrome P_{450} (CYP)-dependent monooxygenases, or phase I enzymes, in a variety of species. This in turn increases the oxidative metabolism/biotransformation of some PCB congeners in addition to a diverse group of exogenous and endogenous aromatic ring substrates (Safe, 1994). The CYP enzymes frequently are termed 'hepatic drug-metabolizing enzymes'. These enzymes frequently are characterized in comparison with the two 'classic' CYP inducers phenobarbital (PB) and 3-methylcholanthrene (MC). As commercial PCBs induce enzymes which possess catalytic properties similar to PB and MC, they are referred to as a mixed-type inducer. Various inducers catalyse the insertion of oxygen in different locations on the biphenyl ring to form reactive arene oxide intermediates, which are often conjugated with such endogenous substrates as glutathione, glutamic acid or sulphate (McFarland and Clarke, 1989). PCBs also induce some of the enzymes associated with the latter conjugations, which are referred to as phase II enzymes and include such enzymes as glutathione *S*-transferase, epoxide hydrolase and glucuronosyl transferases (Safe, 1994). Consequently, the major PCB metabolites are hydroxylated moieties, although some methyl sulphonyl and methyl ether metabolites have been reported (Hu and Bunce, 1999). Many of these reactions increase the polarity of the PCB congeners to facilitate their elimination, primarily via the bile and faeces. However, some of the hydroxylated and sulphonated metabolites also have toxicological effects (Sipes and Schnellmann, 1987; Safe, 1994). It should also be noted that some of the lower chlorinated congeners may be excreted via the urine, but this is often highly species dependent (Ahlborg *et al*., 1992, 1994).

Further work with commercial PCB mixtures in rodent models has found that the mixed-type inducers induce both the PB-induced CYP isozymes of 2A1, 2B1, 2B2, and the MC-induced CYP isozymes of 2A1, 1A1 and 1A2 (Safe, 1994). The metabolism of PCB congeners has been found to be isozyme specific, being governed by the location of the chlorine substitution on the biphenyl rings. For example, the CYP 1A isozymes are induced by MC-like inducers and preferentially oxidize co-planar (non-*ortho)* congeners which also have a chlorine substitution at the *para* position of the least chlorinated ring, i.e. congeners 77, 81, 126 and 169. However, congener 81 is the least active of these four congeners and it also exhibits PB-type induction. The CYP 2B isozymes are induced by and preferentially oxidize *ortho*-substituted, non-planar PCBs at an open *meta* position. As the CYP 1A and CYP 2B isozymes can be induced by MC and PB, respectively, co-planar PCBs are often referred to as MC-type inducers and *ortho*-

substituted PCBs are referred to as PB-type inducers (Hu and Bunce, 1999), while all of the mono-*ortho* (i.e. 105, 114, 118, 123, 156, 157, 167 and 189) and several of the di-*ortho* congeners (i.e. 128, 138, 158, 166, 168 and 170) have both MC- and PB-like inducing properties (Safe *et al*., 1985; Ahlborg *et al*., 1992). Some congeners can induce the CYP 3A and 4A family of isozymes, but the structure–activity relationships for these isozymes are less well characterized. However, it appears that the higher chlorinated PCBs that are biologically persistent congeners are those that have adjacent *meta*- and *para*-unsubstituted carbons (ATSDR, 2000).

The mechanism by which the planar and mono-*ortho* planar PCBs exert their toxicological effects is in large part linked to their affinity for the cytosol aryl hydrocarbon receptor (AhR). Congeners that bind to the AhR are referred to as AhR agonists. While a number of PCB congeners are PB-type inducers, congeners that are AhR agonists result in an MC-type induction of CYP isozymes. The chemical having the greatest affinity for the AhR is 2,3,7,8-TCDD. Once a ligand, such as a mono-*ortho* planar PCB congener, enters the cell via passive diffusion through the cell membrane and binds with the AhR, the resulting complex undergoes transformation and then nuclear translocation, where it binds to a specific genomic sequence prior to the induction of gene transcription (Safe, 1994). Once the ligand complex has been formed, two different toxicological pathways have been identified. For more details, see Hu and Bunce (1999). The best-characterized interaction that is mediated directly by the AhR is the induction of CYP 1A1.

Moore and Peterson (1996) have pointed out that a majority of PCBs apparently have no toxicological effect upon mammalian systems, and that much of PCBs' toxicity is attributable primarily to 13 congeners which have TCDD-like toxicological effects, i.e. AhR agonists. Three of the congeners have no *ortho* chlorines (i.e. the co-planar congeners 77, 126 and 169) due to their stearic configuration, while another co-planar congener, number 81, has comparable activity regarding its ability to induce microsomal enzymes (Safe, 1994). Eight have a single *ortho* chlorine (105, 114, 118, 123, 156, 157, 167 and 189) and two congeners have two *ortho* chlorines (congeners 170 and 180). While there are other di-*ortho* congeners which are AhR agonists, they have been found to be less toxic (Ahlborg *et al*., 1994).

Toxicity and Clinical Effects

Any discussion of the toxicological effects of PCBs, like that of their metabolism, is complicated by the fact that PCBs are a mixture of congeners whose mechanism of action is relatively well known for those congeners that are AhR agonists versus a less certain mechanism for the remaining congeners that have toxicological effects. In addition, when commercial mixtures of PCBs are tested for toxicity in laboratory animals, they contain varying amounts of contaminants, which may have an unquantifiable role in the toxicological outcomes. Only recently have some individual PCB congeners become available for *in vitro* and *in vivo* toxicological evaluation. While the results from such studies will be illuminating for the congeners in question, such studies are generally poor models for human exposure since humans are exposed to a variety of PCB congeners concurrently with several different environmental contaminants which have toxicological properties of their own. Unfortunately, sufficient data do not exist to indicate whether such exposure scenarios result in additive, synergistic or antagonistic effects. Similarly, epidemiological studies have limitations. For example, it is impossible to find a cohort to serve as the 'control' group that would not have a background level of PCBs in their tissues and body fluids. In addition, such studies are also plagued by the presence of other persistent environmental contaminants (methyl mercury, pesticides, etc.), and various lifestyle considerations which have been shown to impact health (alcohol, smoking, etc.) and the parameter being evaluated (i.e. intellectual development, which is affected by environmental, social, economic and genetic factors; reproduction, which is affected by the mother's age and length of pregnancy). While mathematical procedures which are deemed

to be valid by the scientific community are available for 'controlling' such circumstances, the findings of any epidemiology study must be viewed with a degree of caution (Kimbrough, 1995; Seegal, 1996; Danse *et al*., 1997). In addition, there are very limited data available regarding how adverse health and/or toxicological effects are impacted by multiple chemical exposures (Johnson *et al*., 1998).

McFarland and Clarke (1989) concluded that the toxic potential of PCB congeners is correlated with its ability to induce CYP enzymes, and they have suggested three groupings. Specifically, congeners that demonstrate MC-type and mixed-type induction have the greatest potential toxicity. A larger group of congeners has PB-like induction capabilities with less potential toxicity, while weak inducers and non-inducers have the least potential toxicity. While Moore and Peterson (1996) have concluded that PCB congeners with PB-like CYP effects are not overtly toxic, they do point out that such congeners have the potential to disrupt endocrine homoeostasis by accelerating the metabolism of endogenous steroids.

Laboratory studies

An acute effect reported in most laboratory animal studies in which relatively high dosages of PCBs were administered was a wasting syndrome, which resulted in non-thrifty animals continuing to eat and drink while losing weight and subsequently dying (McConnell, 1989; Ahlborg *et al*., 1992). While commercial PCB mixtures have been found to elicit a broad range of toxic responses, their potential to elicit such a response depends upon such factors as: (i) the mixtures' chlorine and contaminant content; (ii) the species and strain of laboratory animal; (iii) the animal's age and sex; and (iv) the route and duration of administration (Safe, 1994).

Reproductive effects

In a study where rhesus monkeys (*Macaca mulatta*) ingested Aroclor 1248, some of the reported toxicological effects included decreased birth weights and other developmental effects. However, the results were confounded due to maternal toxicity. In another study, a dose level of approximately 0.04 mg of Aroclor 1016 kg^{-1} body weight (BW) day^{-1} produced decreased birth weights in rhesus monkeys. Further complicating the interpretation of these studies was the fact that the monkey chow was found to be contaminated with polybrominated biphenyls (Kimbrough, 1995). In a subsequent study, where Aroclor 1254 was fed to female rhesus monkeys, there was a statistically significant ($P = 0.017$) decrease in the conception rate and a significant ($P = 0.04$) increasing trend in fetal mortality with increasing dose. The lowest dosed group ingested 5 μg kg^{-1} BW day^{-1}, and this dose was not considered to be a no-effect level (Arnold *et al*., 1995). Reproductive aberrations have also been found for mink ingesting approximately 0.4 mg kg^{-1} BW day^{-1} (Ahlborg *et al*., 1992).

Teratogenic effects

While not as thoroughly studied, teratogenic effects have been found for mice and chick embryos (Ahlborg *et al*., 1992).

Endocrine system

Several commercial PCB mixtures have been found to be oestrogenic, while co-planar congeners that are AhR agonists have been found to be anti-oestrogenic. Research with individual congeners has found that congeners 1, 4, 18, 21, 48, 52, 61, 75, 101, 136 and 155 have oestrogenic activity in a variety of *in vivo* and *in vitro* systems, while congener 153 is oestrogenic at intermediate levels but not at high or low dosages. It should be noted that none of these congeners are AhR agonists, and it has been demonstrated that the congeners with the strongest bonding to the oestrogen receptor have at least two *ortho* chlorines (Moore and Peterson, 1996). In addition, hydroxylated PCB congeners have also been found to have oestrogenic activity (Sipes and Schnellmann, 1987).

Commercial PCB mixtures are known to reduce plasma thyroxine (T_4) concentrations,

increase circulating thyroid-stimulating hormone (TSH) levels, and alter thyroid histological features, but little is known as to whether these effects arise due to AhR binding. While the AhR agonist congeners 77, 126 and 169, and the mono-*ortho* congeners of 118 and 156 have all been found to reduce plasma T_4 levels, congener 28, a PB-type inducer, does not. These observations suggest that PCBs may affect T_4 levels via AhR agonist and AhR-independent mechanisms (Moore and Peterson, 1996; Seegal, 1996).

Neurological development

Commercial PCB mixtures have been found to cause alterations in active avoidance learning and retention of a visual discrimination task when rats were exposed pre-natally, but no detectable behavioural changes were found when rats were exposed to the same PCB mixture post-natally (Ahlborg *et al*., 1992; Safe, 1994). Behavioural testing with rhesus monkeys whose dams ingested Aroclor 1016 or 1248 showed hyperactivity, retarded learning ability and significant alterations in cognitive behaviour. Many of these changes were long lasting and possibly permanent (Ahlborg *et al*., 1992; Seegal, 1996). While most of the data have indicated that the observed behavioural effects were associated primarily with pre-natal exposure, significant behaviour alterations have also been observed when non-human primates were exposed to PCB congeners post-natally. It was concluded that the structure of the PCB congener (i.e. *ortho*-substituted vs. co-planar) and the animal's age when it was exposed to the PCBs influence the toxicological response (Seegal, 1996). In addition, commercial PCB mixtures also cause regional alterations in neurotransmitter levels in the brains of some laboratory animals (Ahlborg *et al*., 1992; Safe, 1994; Moore and Peterson, 1996).

Immunological changes

Data are available from a variety of studies wherein commercial PCB mixtures have been administered orally to laboratory animals, and the elicited immunological changes vary among commercial PCB mixtures and among species (ATSDR, 2000). It has been observed in mice that the order of potency regarding the Aroclor-induced immunotoxicity was Aroclor 1260 > 1254 > 1248 > 1242 > 1016 > 1232 (Safe, 1994).

The findings with non-human primates tend to be emphasized by some agencies since the monkey appears to be a sensitive species, and has biological and phylogenetic similarities to humans (ATSDR, 2000). In this regard, changes in the immune function of adult female rhesus monkeys and their offspring have been reported when they were exposed to Aroclor 1254 dosages as low as 5 $\mu g\ kg^{-1}$ BW day^{-1}. The suppression of the antibody response to sheep red blood cells (SRBCs) was the parameter most consistently affected in both groups of monkeys. However, the significance and/or relevance of these data have been viewed quite differently (Kimbrough, 1995; ATSDR, 2000).

Carcinogenicity studies

Several chronic bioassays had reported tumours of the liver when PCBs containing 60% chlorine were fed to rats (Ahlborg *et al*., 1992). In one study, diets containing 100 ppm Clophen A60 or Clophen A30 resulted in incidence rates of 61 and 3% hepatocellular carcinomas, while the incidence rate in the controls was 2%. In another study, Aroclor 1260 was chronically fed to male and female Sprague–Dawley rats. The histopathological evaluation revealed that the incidence of hepatocellular adenocarcinomas and trabecular carcinomas was 51 and 40%, while in males the incidence was 4 and 0%, respectively. When male and female Fischer F344 rats were fed Aroclor 1254, the incidence of gastric intestinal metaplasia and adenocarcinoma was similar in both sexes (Safe, 1994). Thus the carcinogenic potential of commercial PCBs is dependent upon its composition, and the sex and strain of the rats being tested. Following a review of such data, it was decided to revise the criteria for the classification system used for various liver lesions. The revised classification reaffirmed that chronic dietary exposure of rats to PCBs containing 60% chlorine did result in the development of benign and malignant liver lesions. However,

chronic exposure of rats to PCB formulations containing 54 or 42% chlorine did not result in a statistically significant increase in benign or malignant liver tumours (Kimbrough, 1995). A subsequent comprehensive chronic toxicity and carcinogenicity study with Aroclors 1016, 1242, 1254 and 1260, using dose levels of 25–200 ppm, resulted in a highly sex-dependent increase in the incidence of hepatocellular neoplasms. For the males, only those in the 100 ppm Aroclor 1260 group, the highest dose group for this mixture, had a significant increase in the number of liver neoplasms. There was also a slight non-dose-related increase in the incidence of thyroid gland follicular cell adenomas for males receiving diets containing Aroclor 1242, 1254 and 1260. A significant and generally dose-related increase in the incidence of hepatic adenomas was found for the females in all treatments except for the 50 ppm group receiving Aroclor 1016 in their diet. The magnitude of the increase was greatest for Aroclor 1254 > 1260 ≈ 1242 > 1016. No increase in thyroid neoplasms was found for the females (Mayes *et al*., 1998). While the latter study suggests that a greater range of chlorinated biphenyls may be able to induce a carcinogenic response in laboratory rats, it still supports the conclusion that different PCB mixtures do not have equal potency regarding their ability to cause cancer (Kimbrough, 1995).

While a variety of commercial PCB mixtures have been found to induce carcinogenic responses in laboratory rats, a substantial amount of evidence is available to suggest that PCB mixtures are not complete carcinogens, but may only be tumour promoters (Ahlborg *et al*., 1992; Safe, 1994). Simplistically, the carcinogenic process can be thought of as encompassing two steps: tumour initiation and tumour promotion. Tumour initiation involves the interaction of the chemical with DNA resulting in a critical DNA lesion, which will evolve into a tumour given sufficient time. Such chemicals are referred to as complete carcinogens or genotoxic carcinogens. Some chemicals have the ability to 'promote' an initiated DNA lesion but are incapable of producing tumours by themselves. These chemicals have been referred to as promoters or epigenetic carcinogens.

Many of the clinical findings attributed to PCBs resemble those of a vitamin A deficiency, and it is known that several PCB mixtures reduce the storage levels of vitamin A in several species (Ahlborg *et al*., 1992). In a review of the toxicity induced by commercial PCB mixtures, Safe (1994) concluded that the PCB-induced lethality was not dependent solely upon the mixture's degree of chlorination. Since the toxicity of PCBs was due to the individual congeners contained in a mixture, it was possible that one or more structural subclasses of congeners were responsible for the toxicities elicited by PCB mixtures. He concluded that there was no consistent structure-dependent effect that was responsible the specific types of toxicological responses observed.

Epidemiology studies – non-cancerous outcomes

Dermatological

Occupational exposure to PCBs appears to be related to hyperpigmentation in addition to chloracne. In the Yusho and Yu-Cheng incidents, chloracne and hyperpigmentation of the skin, gingiva and nails were frequently observed and, while these lesions have diminished in the intervening years, they were still evident 10–14 years later (Masuda, 1996; Guo *et al*., 1999). Ocular manifestations such as hypersecretion and swelling of the sebaceous glands of the eyelids were also common (Kimbrough, 1995; Longnecker *et al*., 1997).

Reproduction

Findings show that women in a number of PCB exposure situations above background levels of PCBs – such as industrial, Yusho and Yu-Cheng, sport fish consumption and native populations – have given birth to children with slightly lower birth weights or shorter birth body lengths and/or smaller head circumferences. In some situations, the lower birth weights were partially attributable to

a shorter gestation period. However, there were a number of methodological problems with these studies: many did not control for influencing factors; there was concern as to the presence of other persistent contaminants; and there was some question as to what PCB standard was used for the analyses. The findings were not consistent among these studies and, therefore, have been deemed by some to be inconclusive with regard to the effect of PCBs *per se*. However, PCB exposure has not been found to affect the rates of spontaneous abortions or stillbirths (Ahlborg *et al*., 1992; Kimbrough, 1995; Longnecker *et al*., 1997; Johnson *et al*., 1998; Yu *et al*., 2000).

Neurological development

Data from three different types of studies, one with a population cross-section in North Carolina and in The Netherlands; another with mothers who were frequent consumers of Lake Michigan fish; and a third with mothers who ingested contaminated rice oil in Japan or Taiwan have suggested that pre-natal exposure to PCBs and other persistent toxic substances may be adversely affecting the neurological development of children (Chen and Hsu, 1994; Johnson *et al*., 1998; ATSDR, 2000; Boersma *et al*., 2000). While the Japanese and Taiwanese mothers showed signs of toxicity which were attributed primarily to the PCB contaminants, the mothers in the other three studies did not exhibit signs of toxicity. However, like the Japanese and Taiwanese incidents, the findings from the Dutch study potentially were compromised regarding the effects of PCBs *per se* due to the presence of dioxin. In the North Carolina study, the mothers' PCB and DDE (1,1-dichloro-2,2-*bis*(*p*-phenyl) ethylene) exposure was limited to background levels of both entities. While some early neurodevelopment deficits were reported for the most highly exposed members of this population, the deficits were not apparent when the infants were 3, 4 or 5 years of age. These results suggest that *in utero* exposure to PCBs is potentially more deleterious to an infant than exposure via breast milk (Seegal, 1996). When the Dutch cohorts were 2 weeks of age, an adverse effect of PCBs, polychlorinated dibenzodioxins (PCDDs) and PCDFs on neurological performance was evident; at 3.5 years of age, they showed an adverse effect of pre-natal PCB exposure on cognitive, but not neurological development. However, at 18 months of age, scores for cognitive development were not related to pre- or post-natal exposure to PCBs. In the Lake Michigan study, a number of effects on the developing nervous system and deficits in intellectual performance were found during the first testing period and were also apparent at subsequent evaluations, but there was no strong correlation between maternal PCB consumption and lower infant birth weights. Some reviewers have concluded that the levels of PCB exposure for the mothers in the Lake Michigan and North Carolina studies were within the range of PCB blood levels reported for the entire North American population and, therefore, have questioned whether the Lake Michigan findings should be attributed to PCBs *per se* since there is no conclusive evidence that the PCB levels in the general population have resulted in intellectual deterioration in children exposed to PCBs *in utero*. It has also been reasoned that, since the findings from the Lake Michigan and North Carolina studies were dissimilar, it is possible that some other chemical entity may be responsible for the Lake Michigan findings (Ahlborg *et al*., 1992; Safe, 1994; Danse *et al*., 1997; Longnecker *et al*., 1997; Boersma *et al*., 2000).

In the Yusho and Yu-Cheng incidents, slow nerve conduction, especially of the sensory nerves, was documented in many cases (Kuratsune and Shapiro, 1984). In addition, infants of the exposed mothers exhibited a range of neurobehavioural deficits which persisted for several years (Chen and Hsu, 1994), but this may be due to the presence of PCDFs and PCQs as contaminants in the PCBs. The fact that the PCB blood levels for Japanese and Taiwanese capacitor workers were greater than those determined for the Yusho and Yu-Cheng victims is cited as support for the effects of PCDFs and PCQs (Danse *et al*., 1997; Johnson *et al*., 1998).

In summary, there appears to be a divergence of opinion as to the weight of the

evidence regarding the neurodevelopment effects on cognitive impairment associated with PCBs, dioxins and other persistent toxic substances in all of the cohorts, except for the Yu-Cheng children (Chen and Hsu, 1994; Danse *et al*., 1997; Johnson *et al*., 1998; ATSDR, 2000). In this group, there is evidence for a shift downward in the IQ distribution curve which does not appear to be reversible (Chen and Hsu, 1994; Johnson *et al*., 1998).

Liver abnormalities

Studies with industrially exposed personnel, while having shortcomings analogous to the reproduction studies, suggested that PCB exposure can lead to increased levels of some hepatic enzymes, but the results were not uniform nor have there been any reports of increased incidents of liver cirrhosis (Kimbrough, 1995; Longnecker *et al*., 1997). However, the Yu-Cheng victims have experienced a substantial elevation in the mortality rates for cirrhosis and chronic liver disease (Yu *et al*., 1997).

Thyroid effects

For a cohort of industrially exposed men, no relationship was found between PCB exposure and thyroid hormone levels. In the Dutch study, background levels of PCBs in breast milk were associated with lower maternal tri-iodothyronine (T_3) and T_4 levels, but both levels were within normal limits; their infants, however, had higher plasma levels of TSH but lower plasma levels of T_3 and T_4. The investigators concluded that elevated levels of dioxins and PCBs can alter thyroid status. This group has also reported contradictory findings among subsets as to whether an association between altered thyroid status and decreased neurological optimality scores exists. Consequently, the pathophysiological meaning of these observations currently is uncertain (Seegal, 1996; Longnecker *et al*., 1997; Johnson *et al*., 1998; Feeley and Brouwer, 2000). In a 14-year follow-up of the Yu-Cheng patients, who were at least 30 years old at the time of the follow-up, an increased incidence of goitre was reported for both the men and the women (Guo *et al*., 1999).

Immunological effects

Several studies were evaluated and the findings were inconsistent and may be confounded by the presence of dioxin (Kimbrough, 1995; Longnecker *et al*., 1997). Immune changes were demonstrated in the Yusho and Yu-Cheng populations, but some were reversible. It was concluded that 16 years after the Yusho incident, the children who were exposed *in utero* did not have suppressed immunity. In the Dutch study, PCBs and dioxin were found to influence fetal and neonatal immune systems, but this was not reflected in an increased incidence of respiratory symptoms (Johnson *et al*., 1998; Yu *et al*., 1998).

Respiratory function

There have been suggestions that industrial PCB exposure may be associated with chronic bronchitis, upper respiratory irritation, abnormal forced vital capacity, etc., but the findings were deemed to be inconclusive due to the study's many confounding factors (Kimbrough, 1995). Some Yusho and Yu-Cheng patients suffered from a chronic bronchitis-like syndrome for several years, marked by a large amount of expectorant during the early stages. Pathophysiological findings revealed that the disease was localized in the small airways. However, this bronchitis-like syndrome has not been detected in studies with people who have been exposed to dioxin (Ahlborg *et al*., 1992).

Miscellaneous health effects

In one study, there was an association between high levels of PCB-contaminated-fish consumption and increased blood pressure, but this finding has not been replicated and the study had several confounding factors (Kimbrough, 1995; Longnecker *et al*., 1997; Johnson *et al*., 1998).

In conclusion, many of the responses observed regarding PCB exposure, particularly for industrial exposure, were reversible, and often there was not a significant correlation between response and PCB levels in fat

and blood. For the Yu-Cheng and Yusho incidents, the symptomatology included severe and persistent chloracne, dark brown pigmentation of nails, skin thickening, a variety of ocular problems and numerous subjective complaints. The offspring, particularly those of the Yu-Cheng mothers, were smaller in stature, were found to have a modest learning deficit and displayed many of the toxic symptoms observed in their mothers. However, it is generally agreed that these symptoms were not due to PCBs *per se*, but were more attributable to the contaminants in the PCBs used to cool the rice oil (Safe, 1994; Feeley and Brouwer, 2000).

Epidemiology studies – cancerous outcomes

Most of the studies have examined industrial exposure to PCBs or there has been an attempt to correlate PCB blood levels with various types of cancer. A number of shortcomings can be found with these studies, including the number of cohorts, the limited length of follow-up, the minimal time of exposure (i.e. 1 day to 6 months), the variable level of exposure, which has limited their usefulness, and the possibly that the PCB mixtures were contaminated. However, in several studies, there were increased incidences of specific cancers such as liver and biliary tract but there were no consistent increases in one or more types of cancer. Therefore, no conclusive evidence of a link between PCB exposure and a human cancer risk has been found for industrial exposure (Ahlborg *et al*., 1992; Safe, 1994; Kimbrough, 1995; Danse *et al*., 1997; Longnecker *et al*., 1997).

The data from the Yusho study have shown a significant increase in the incidence of death attributable to cancer of the liver and respiratory system in males but not females (Ahlborg *et al*., 1992). Thirteen years after the Yu-Cheng incident, a substantial increase in the mortality rate for chronic liver disease and cirrhosis was evident. However, the mortality rate from malignant neoplasms was not significantly different from that of the general population (Yu *et al*., 1997).

Risk Assessment Strategy

While toxicological effects of commercial mixtures of PCBs can be studied in various *in vivo* and *in vitro* assays, the data from such studies have significant shortcomings with regard to their risk assessment value in the regulatory context. Such shortcomings relate to the fact that humans are not exposed to commercial PCB mixtures *per se* or to single congeners, but to a variety of PCB congeners and other environmental pollutants, some of which may be structurally related to PCBs, such as PCDDs and PCDFs. The pragmatic approach developed to deal with this scenario, known as toxicity equivalency factors (TEFs), occurred in the 1980s and was initially used to assess the risks associated with emissions of PCDDs and PCDFs formed during high-temperature incineration of various wastes. PCDDs and PCDFs are also produced as by-products during various industrial chlorination processes, during the smelting of metallic ores and during pulp and paper production (Safe, 1994; Kimbrough, 1995).

TEFs were developed to assess the potency of various polyhalogenated aromatic hydrocarbons against that of 2,3,7,8-TCDD, the most toxic of the dibenzo-*p*-dioxin congeners. Simplistically, TEFs can be determined for any *in vivo* or *in vitro* test/assay, but the relative rankings among tests and assays may not always be similar as they may be species specific and/or affected by pharmacokinetics and exposure time. Generally, TEFs can be developed for such *in vivo* assays as enzyme induction, thymic atrophy, body weight gain, teratogenicity/developmental toxicity, immunotoxicity, carcinogenicity and lethality, as well as for a host of *in vitro* assays. While 2,3,7,8-TCDD has been assigned a TEF value of 1, it has been found that test congeners have a relative potency that was 1–5 orders of magnitude less than that of 2,3,7,8-TCDD (Clemons *et al*., 1997; Van den Berg *et al*., 1998, 2000). However, it should be noted that the TEFs are only estimates of a congener's relative potency.

In the first decade after the TEF concept was introduced, several TEF schemes were developed which led to a number of criticisms

before a harmonized approach was developed (Kimbrough, 1995; Van den Berg *et al*., 2000). For a compound to have a harmonized TEF value, there was agreement that it must be structurally similar to PCDDs and PCDFs; it must bind to the AhR; it must elicit AhR-mediated biochemical and toxic responses; it must be environmentally persistent; and it must accumulate in the food chain. When deriving a TEF value, *in vivo* studies would be given more weight than *in vitro* studies, with chronic *in vivo* studies being given more weight than subchronic > subacute > acute, and the AhR toxic responses would receive more weight than biochemical responses. Enhanced acceptance of the TEF concept, and the resulting TEF values, is attributable to the finding that TEF values that were based on AhR-mediated responses were generally additive. However, there are still three major criticisms of the TEF approach: (i) non-additive interactions when mixtures of dioxin-like and non-dioxin-like congeners are tested; (ii) differences in species' responsiveness; and (iii) differences in the shapes of the dose–response curves among AhR agonists. Even with these acknowledged shortcomings, it has been suggested that the use of TEFs is pragmatically the most feasible approach for human risk assessment. However, their use severely underestimates the risk to humans exposed to PCBs, PCDDs and PCDFs since only dioxin-like congeners are included in the TEF calculation (Van den Berg *et al*., 1998, 2000).

After the TEF values have been determined, they can be combined with the chemical residue data for the calculation of toxic equivalents (TEQs) in an environmental sample, animal tissues, food, soil, etc. TEQ concentrations for samples are calculated using the following equation:

$$\mathrm{TEQ} = \sum n1[\mathrm{PCDD}_i \times \mathrm{TEF}_i] + \sum n2[\mathrm{PCDF}_i \times \mathrm{TEF}_i] + \sum n3[\mathrm{PCB}_i \times \mathrm{TEF}_i]$$

In short, this equation allows one to calculate TEQs for complex mixtures of chemicals for which TEFs are known, thereby reducing a complex mixture of congeners to a single value which represents the amount of TCDD equivalents in the sample. Recently, agreement has been reached whereby TEQs can be calculated not only for samples containing PCDDs, PCDFs and planar PCBs but also for a large number of other halogenated compounds that meet the criteria for inclusion in the TEF concept; this could enhance the usefulness of TEQs derived for environmental samples (Safe, 1994; Van den Berg *et al*., 1998).

Risk Management Issues

While production of PCBs may have started in 1929, it has been estimated that, in recent years, over 1 million kg of PCBs have entered the environment annually from worldwide mobile reserves, due to accidental release, leaching/volatilization from hazardous waste sites, illegal dumping, etc. PCB risk management plans, initially developed by a number of industrialized countries, included policies designed to prevent future releases of PCBs into the environment (Kannan, 2000). For example, the European Union Council Directive 96/59/EC outlined steps to control the release of PCBs into the environment by outlining measures designed to address the disposal of PCBs and the decontamination/disposal of equipment known to contain PCBs. The ultimate goal was the complete cessation of further environmental contamination by PCBs. As an initial step, the directive required EC member states to compile inventories of all equipment containing more than 5 dm^3 of PCBs. (It appeared as if the initial endeavours associated with this directive were to decontaminate and properly dispose of the larger PCB 'reservoirs' without concern for the PCB concentration *per se*.) The inventories were to be completed by 1999 with planned regular updates. Once the inventories were completed, the objective was to have all inventories, depending on the percentage PCB content, decontaminated and/or disposed of by 2010. For example, decontamination of electrical equipment containing PCBs is aimed at reducing the PCB concentrations to less than 0.05% by weight, with the ultimate goal of having a concentration of less than 0.005%. Recent

amendments to this directive, suggested as part of the final draft of the Stockholm Convention on Persistent Organic Pollutants (2001), would permit PCB-containing equipment to remain in use until 2025 but no later than 2028. Also, additional effort should be made to identify and inventory other articles containing greater than 0.005% PCBs.

Similar PCB management/disposal options have been developed in the USA. The manufacture of PCBs has been prohibited under the Toxic Substances Control Act since 1977, and any material containing greater than 50 ppm PCBs is considered to be hazardous waste and treated accordingly. The use of products, such as hydraulic fluids, paper products, etc., which contain less than 50 ppm PCBs is still allowed provided the EPA (2000) has determined that the products in question do not present an environmental or human health risk. Additional regulations deal with the proper storage of PCBs along with the import and export of PCBs for disposal.

Until 1977, PCBs were imported into Canada mainly for use in electrical transformers and capacitors. As part of the Chlorobiphenyl Regulations under the Canadian Environmental Protection Act (CEPA, 2000), a variety of guidelines have been promulgated to deal with the use, inventory, transportation, storage and disposal of PCBs. Based on a comprehensive survey, approximately 16 kt of PCBs were estimated to have been dispersed into the environment. National inventories of PCBs-in-use and PCB-containing materials in regulated storage facilities have been conducted on an annual basis in Canada since 1989. There currently are over 130 kt of PCB waste in regulated storage facilities. Proposed draft amendments to the Chlorobiphenyl Regulations include the following requirements: the use of PCBs in any equipment be discontinued by 2010; any PCB material currently in storage be disposed of by 2015; and the environmental release of any liquid containing PCBs be restricted when the concentration is greater than 0.1 ppb for aqueous mixtures and 400 ppb for oils and non-aqueous liquids.

Regulations enacted to control the further releases of PCBs from closed or partially closed applications as well as remediation of identified hazardous waste sites have been shown to reduce the levels of PCBs in the environment. For example, in the North American Great Lakes ecosystem, concentrations of PCBs found in predatory fish species, such as lake trout, have declined from mid-1970 values of 8 ppm to less than 1 ppm by 1994 (Scheider *et al*., 1998). However, PCBs still account for 47% of the fish consumption advisories issued in Canadian waters; these advisories are issued by various levels of government to sport anglers warning them not to consume specific species of fish from specific waters due to contamination with various persistent chemicals. Further indications of the overall decline of PCBs in the environment can be found in human specimens – for example, PCB residues in breast milk. From 1972 to 1992, the average concentration of PCBs in breast milk samples from Swedish women declined by approximately 70% (from 1.09 to 0.324 ppm) (Norén and Meironyté, 2000), while similar declines have been observed in Canadian breast milk samples from 1982 to 1992 (from 0.68 to 0.21 ppm) (Newsome *et al*., 1995).

With a number of persistent organic contaminants known to be present in the food supply, such as PCBs, government agencies attempt to monitor their presence in foods directly so as to limit the population's exposure to them. They conduct national dietary monitoring surveys to identify those food commodities that contain the greatest concentrations of persistent contaminants so that changes over time can be monitored and future data collections prioritized. Congener-specific PCB analysis of 138 prepared food composites, collected from across Canada between 1992 and 1996, estimated that the average person consumed 342 ng PCBs day^{-1} (Newsome *et al*., 1998) compared with a 1980 estimate of 3.9 µg day^{-1}. The dairy (40%), meats (26%) and fish (16%) food groups combined accounted for approximately 80% of the total ingestion. In comparison, recent food surveys from the UK have estimated their dietary PCB ingestion at approximately 512 ng day^{-1}, with the dairy, meat and fish composites accounting for 32, 16 and 26% of the total exposure, respectively

(Duarte-Davidson and Jones, 1994). The average dietary intake of PCBs in the UK for 1982 was estimated at 1.0 μg day^{-1}, indicating a decrease of 66% between 1982 and 1992 (MAFF, 1997). Total diet studies from the USA indicated that between 1991 and 1997, the mean daily intake of PCBs by all segments of the population ranged from approximately 10 ng for infants to 324 ng for adults (ATSDR, 2000). From the available data, with the exception of infants and children up to 2 years of age, PCB ingestion by all other segments of the population declined by over 50% between 1991 and 1997.

Risk assessment

International perspectives

The importance of a safe food supply from the perspective of health and international trade was well recognized prior to the United Nations establishing the Food and Agriculture Organization (FAO) in 1945, whose initial mandate was to improve nutritional standards and agricultural productivity. The FAO combined with the World Health Organization (WHO) in 1962 to form the FAO/WHO Food Standards Programme, and designated the Codex Alimentarius Commission (CAC) as the authoritative body responsible for establishing international food standards designed to protect consumers from unsafe food. The subsidiary of the CAC given the responsibility for developing guidelines and standards related to food contaminants was the Codex Committee on Food Additives and Contaminants (CCFAC), which, in turn, is supported by the risk assessment activities of the FAO/WHO Joint Expert Committee on Food Additives (JECFA). The task of JECFA is to provide recommendations regarding the maximum tolerable intake of specific contaminants; these serve as the basis for any related guideline decisions by CCFAC. Prior to the ratification of any contaminant guideline by CCFAC and its recognition by the World Trade Organization, member countries are consulted at least twice during the development process in accordance with the step-wise procedure outlined in the CAC General Standard for Contaminants and Toxins in Foods (GSCTF) (FAO/WHO, 1995). After ratification, it is then the responsibility of each member country to introduce the ratified guideline into their national legislation. While the use of Codex standards is still at the discretion of individual countries, the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) of 1995 formally recognizes these guidelines as the international standard and provides legal 'encouragement' for their use. Currently, CAC has 165 member nations, representing more than 98% of the world's population. Additional details regarding the international aspects of the development of food contaminant standards can be found in a recent publication by Rees and Watson (2000).

The WHO previously has conducted formal reviews of PCBs that resulted in published Environmental Health Criteria documents in 1976 and 1993, and JECFA initially considered PCBs in 1989. The final conclusion reached by the latter group was that it would not be possible to suggest a precise tolerable intake level for PCBs regarding human consumption; however, attempts should be made to set guidelines or standards for those nutritionally essential food commodities in which PCBs occur, such as fish, milk, meat and dairy products (WHO, 1990). The Committee did state, however, that dietary intake levels of PCBs up to 0.2 μg kg^{-1} BW day^{-1} 'did not involve any long term hazard'. The Committee also reported that, although breast-fed infants may ingest levels of PCBs up to 12.0 μg kg^{-1} BW day^{-1}, they considered that the known benefits of breast feeding outweighed any potential health hazard associated with PCB ingestion. Since that time, a discussion paper on dioxins and PCBs was presented by The Netherlands to the 27th session of CCFAC in 1995 (as outlined in the GSCTF guideline for the development of a Codex standard). The discussion paper put forward the opinion that there were potential health concerns related to the dietary intake of PCBs and that, in certain instances, international trade had already been affected by PCB-contaminated foodstuffs. The paper included recommendations

that CCFAC should develop a maximum level guideline for PCBs in foods involved in international trade and that JECFA should maintain PCBs on its priority assessment list.

Risk assessment process

Assessing the potential risk to human health posed by environmental contaminants such as PCBs initially involves the identification of the hazardous substances, followed by the description or definition of the risk associated with any potential exposures to the hazardous substances. Identification usually is accomplished using *in vivo* and/or *in vitro* toxicity assays and/or epidemiological findings.

Hazard identification involves determining whether an agent or chemical causes toxic effects, the nature of these effects and whether the effects are likely to occur in humans, i.e. potential relevance to human health. The second stage, exposure assessment, is the process of actually measuring or estimating the intensity, frequency and duration of human exposure to the agent in question. For persistent organic contaminants such as PCBs, where the majority of the exposure will be from the diet, a number of countries have participated in the WHO Global Environment Monitoring System (GEMS) – Food Contamination Monitoring and Assessment Programme, which was established in 1976 (Weigert *et al*., 1997). The third stage, hazard characterization or dose–response analysis, generates estimates of a no observable adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) doses, usually from *in vivo* toxicology data. This stage can also involve deriving the best mechanism/procedure by which to extrapolate experimental findings to humans. The final stage, risk characterization, compares the exposure data with the dose–response analysis in order to develop risk estimations of an adverse health effect for a particular exposure. Various models of this risk assessment paradigm are employed by international (World Health Organization) and national (Health Canada (CEPA, 2000); US Environmental Protection Agency (EPA, 2000)) regulatory agencies.

Through the process of scientific evaluation of all pertinent toxicological data, dose–response relationships for contaminating agents/chemicals can be established, i.e. identifying those exposure levels known to cause and, more importantly, not to cause toxic effects. The latter values commonly are referred to as the NOAELs and are defined as the dose at which no biologically significant adverse effects are observed in the study population compared with the controls. While epidemiological studies with documented exposure assessments are preferred, experimental animal bioassays with internationally accepted study protocols are generally used for identification of NOAELs. While animal bioassays have certain advantages such as controlled exposures and the thorough quantification of toxic responses, the results require extrapolation to humans. This extrapolation process is an inexact exercise at best, due to such things as pharmacokinetic/toxicokinetic differences between species; the use of high dosages in animal bioassays versus the low dosage human exposures; the species-specific mechanism of actions; the difficulty in ascertaining the dose–response curve, etc. Typically, regulatory agencies have employed uncertainty or safety factors to compensate or adjust for the known physiological and biological differences between experimental animals and humans. Initially, the US Food and Drug Administration (FDA) suggested, as a default, that a 100-fold safety factor be applied to NOAELs derived from chronic animal bioassays – other than chronic cancer bioassays – to estimate a safe exposure level for food additives which could be present in the diet (Lehman and Fitzhugh, 1954). The defined rationale for the 100-fold safety factor is that humans are potentially tenfold more sensitive to the toxic effects of chemicals compared with experimental animals, and a tenfold difference in human susceptibility exists within a population, i.e. $10 \times 10 = 100$ (Waltner-Toews and McEwen, 1994). Based, in part, on human responses to a wide range of environmental contaminants, it has been estimated that this tenfold human variability factor would provide protection for up to

95% of a population (Calabrese, 1985). A variety of additional factors have been considered regarding extrapolation methodologies, including the overall adequacy of the scientific database and the severity of the toxicological end point (Vermeire *et al*., 1998). Using such additional information, regulatory agencies have been known to deviate from the standard 100-fold uncertainty factor, especially in the risk assessment for food contaminants. Conversely, the lack of sound epidemiological studies and/or deficiencies in the experimental animal database have resulted in higher degrees of uncertainty and forced risk assessors to adopt a more conservative approach, i.e. the use of an uncertainty or 'safety' factor greater than 100-fold.

After identification of the appropriate NOAELs, based on the available toxicological data, and after deciding on the appropriate extrapolation factor, exposure regulations, such as tolerable daily or weekly intakes (TDIs/TWIs), can be set. However, the terminology used to describe essentially safe exposure levels can vary among organizations; for example, WHO and Health Canada use TDIs, the EPA uses oral reference dose (RfD), while the US Department of Human Health Services (of which the FDA is a component) uses minimal risk level. Regardless of the nomenclature used, these intakes, when expressed on a body weight basis averaged over an entire human lifetime, are thought to represent an exposure level that is without appreciable risk of an adverse effect to human health.

A major part of the overall hazard characterization process includes the determination of the genotoxic/carcinogenic potential of the chemical. For chemicals thought to be genotoxic carcinogens, a more conservative risk assessment approach is taken since any exposure to the chemical is considered to be a potential risk to human health. This approach is referred to as the non-threshold concept and its tenet is that exposure to as little as one molecule of the chemical poses a risk to health. Therefore, no safety or uncertainty factor is usually given for a genotoxic carcinogen. As analytical technology has not progressed to the point where a single molecule of an undesirable chemical (i.e. a genotoxic carcinogen) can be analysed for in a foodstuff, the exposure level is defined alternatively as the average daily dose during a lifetime that would be associated with a negligible or background cancer risk, i.e. one additional cancer per 10^5–10^7 lifetimes. This mathematically extrapolated value is derived from the available scientific data via the use of probabilistic models, such as the linearized multistage model or the Moolgavkar–Venzon–Knudson model. Both models provide estimates of a potential cancer risk in the low or environmental dose range as compared with the high dose experimental animal studies. Currently, the International Agency for Research on Cancer (IARC, 1979) places chemicals or agents which may cause cancer in humans into two groups: in group 1, the agent is carcinogenic to humans; in group 2A, the agent is probably carcinogenic to humans; and, in group 2B, the agent is possibly carcinogenic to humans. To date, the data supporting IARC's classification of an agent or chemical as a group 1 or 2 human carcinogen have been obtained solely from occupational exposure studies. Conversely, IARC has not found sufficient evidence to conclude that environmental exposure to any chemical or agent on the group 1 or 2 list has been associated with any increase in human cancers.

The final step in the process involves a risk management strategy. These decisions are implemented when the intake of a particular contaminant exceeds the TDI. Such a scenario would depend on the duration of exposure, i.e. how often the TDI is or may be exceeded during the average lifespan; the nature and severity of the known toxicological effects in humans; and the known benefits associated with the exposure venues, i.e. breast feeding, social/cultural and nutritional aspects of foods. A more complete description of the risk assessment process and the major uncertainties involved with it can be found in a National Research Council publication (1994).

Risk assessment – PCBs

Initial risk assessments for PCBs were undertaken after the 1968 rice oil poisoning episode

in Japan and after it was known that PCBs were widely prevalent in the environment as well as in the food supply to the extent that PCBs were found in human breast milk samples throughout the world. The Yusho incident originally was attributed to the rice oil being contaminated with PCBs, but subsequent analysis of the rice oil indicated the presence of substantial amounts of PCBs' thermal degradation products, which were either known or subsequently found to be more toxic than PCBs. A variety of experimental studies conducted with rodents and non-human primates, using commercial PCB mixtures, have determined that PCBs can: cause immune system effects; function as endocrine disruptors; induce adverse neurobehavioural and developmental effects, especially in infants; and cause cancer. Any attempt to extrapolate these experimental results with commercial PCBs to humans should consider a variety of issues, including:

- Commercial PCBs were manufactured by various techniques to a specific weight per cent of chlorine and were known to be subject to lot-to-lot variability, especially for Aroclors 1248 and 1254. In addition, certain production techniques for PCBs resulted in higher concentrations of dioxin-like PCB congeners and contaminating dibenzofurans (Frame, 1999).

  For example, a number of adverse effects induced when rhesus monkeys were chronically exposed to Aroclor 1254 were similar to effects seen when non-human primates had been fed diets containing low levels of TCDD. These effects included ocular and dermatological lesions, nail bed deformities, reduced fecundability and increased fetal mortality. On the basis of the relative concentrations of PCB congeners with dioxin-like activity, and the known levels of dibenzofuran contaminants in the Aroclor mixtures, an estimated TCDD toxic equivalent (TEQ) dose calculated for rhesus monkeys consuming 80 µg Aroclor 1254 kg^{-1} BW day^{-1} could be as high as 2400 pg TCDD TEQ kg^{-1} BW day^{-1}. For comparison purposes, rhesus monkeys exposed to doses of 750 pg TCDD kg^{-1} BW day^{-1} or greater were found to have reduced fertility, increased incidence of absorptions/resorptions and overall lower reproductive success (Bowman *et al*., 1989). In addition, rhesus monkeys chronically exposed to TCDD at dosages as low as 150 pg kg^{-1} BW day^{-1} have also been found to have an increased frequency and severity of endometriosis, a disease thought to be associated with immunosuppression. Subsequent immunological testing of these same monkeys 3 years after cessation of their TCDD exposure revealed only a decreased mixed lymphocyte response. Offspring of these same animals did, however, exhibit an increased antibody response to T-cell-dependent tetanus toxoid immunization (Hong *et al*., 1989).
- Once released into the environment, commercial PCBs will be altered in terms of their congener pattern due to various physical, chemical and biological transformation processes. This environmental 'weathering' of commercial PCBs results in the contamination of biota, human foodstuffs, etc. with congener patterns that are not representative of any commercial PCB (Schwartz *et al*., 1987; Draper *et al*., 1991).

  Significant differences exist in the number of PCB congeners detected in surveys of Canadian foodstuffs and breast milk when compared with the congener content of Aroclor 1254 (Fig. 6.4). Whereas the congeners indicated in Fig. 6.4 accounted for 83–93% of the PCB congeners present in the food samples, they account for only 49% of the congeners in Aroclor 1254.
- Reductive dechlorination of commercial PCBs by anaerobic sediment bacteria changes the congener proportions relative to the congener composition of commercial mixtures, while concurrently altering aspects of their toxicological effects (Mousa *et al*., 1998). For example, exposure of pregnant rats to a reconstituted PCB mixture based on human breast milk was more effective at altering behaviour and endocrine-

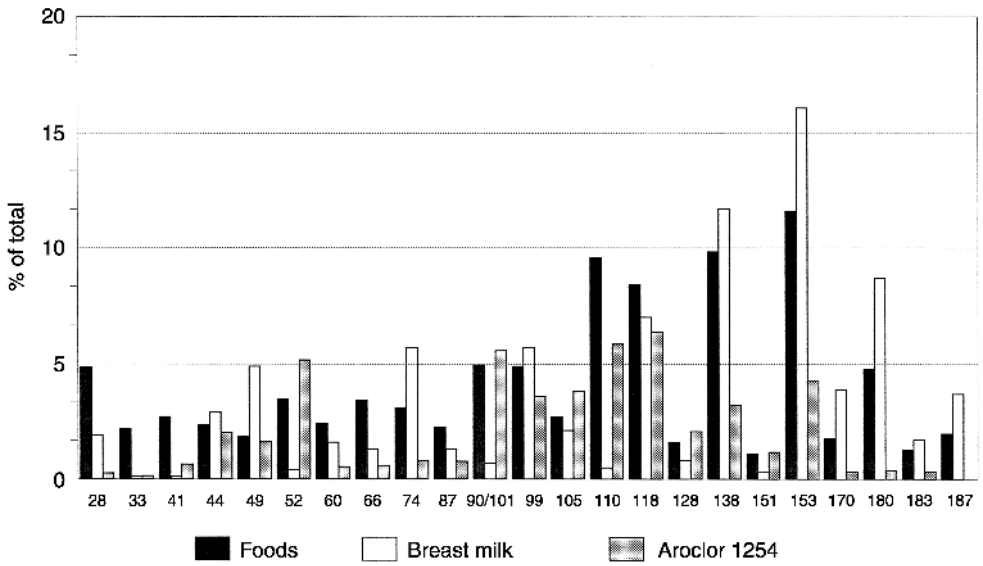

Fig. 6.4. PCB congener distribution.

related functions in the offspring than an equivalent dose of Aroclor 1254 (Hany *et al*., 1999).

- Occupational exposure to commercial PCBs results in the bioaccumulation of a PCB congener profile different from that found in the general population, and the congeners present due to the industrial exposure constitute a higher percentage of the total PCB body burden (Kannan *et al*., 1994).
- The percentage contribution that any PCB congener would make to the total PCB body burden depends on the major source of PCB exposure. For example, Great Lakes fish eaters have elevated cord blood levels of those PCB congeners found in fish when compared with the cord blood congener content of the general population (Stewart *et al*., 1999). Certain PCB congeners, particularly those that bioaccumulate in fish and that are not readily excreted by humans consuming such fish, have been shown to contribute a greater percentage to the total PCB content found in human plasma samples as a consequence of the amount of contaminated fish consumed (Asplund *et al*., 1994).

A number of epidemiological studies, other than the Japanese and Taiwanese rice oil poisonings, are also available for PCB risk assessment consideration. In the Lake Michigan and North Carolina studies initiated in the late 1970s, subtle developmental and neurobehavioural deficits were observed in infants born to women with breast milk PCB concentrations of 1.25–1.7 μg g^{-1} lipid. A conservative estimate of the human PCB intake required to achieve these breast milk levels would be 2 μg day^{-1}, compared with an average consumption of approximately 0.34 μg day^{-1} for that era (Tilson *et al*., 1990). However, high-end consumers from the 1992 UK total diet study were estimated to be ingesting up to 1.9 μg PCB day^{-1}, which could result in PCB tissue burdens and breast milk levels near the potential effect level for subtle behavioural and developmental deficits. In the Dutch PCB/dioxin study, pre-natal PCB exposure was negatively associated with overall cognitive abilities of 42-month-old children (Patandin *et al*., 1999). At maternal plasma PCB levels of ≥ 3.0 μg PCBs l^{-1}, or approximately 0.89 μg g^{-1} lipid, children scored lower in a series of tests designed to assess intellectual functioning when compared with children with pre-natal exposure of < 1.5 μg

PCB l^{-1} plasma or 0.45 μg g^{-1} lipid, although all of the scores were within the normal range for the Dutch population. Approximately 16% of the total study population of 415 mother–infant pairs had maternal PCB levels ≥ 3.0 μg PCB l^{-1}, which, by the previous estimates, would be achieved following chronic ingestion of 1.5 μg PCB day^{-1}. While the exact significance of these observations is unknown, available evidence does suggest that, for certain subpopulations, PCB exposure via the mother's diet can be sufficient to induce subtle developmental alterations in their pre-natally exposed infants. In almost all epidemiological studies to date involving dietary sources of PCBs, maternal body burdens (i.e. pre-natal exposure of the developing fetus) and not lactational exposures have been associated with the observed effects.

Legislation/regulatory issues

PCBs, based on their resistance to degradation and metabolism, have been shown to bioaccumulate readily in all ecosystem trophic levels, leading to human exposures primarily from food consumption. In addition, due to their vapour pressures and partitioning coefficients, PCBs are subject to long-range atmospheric transport, resulting in global redistribution from areas of past or current use to colder climates (cold condensation). While the control measures developed by a variety of nations to prevent further environmental releases have been partially successful, further emphasis should be placed on reducing the PCB dietary intake of potentially susceptible populations, particularly high-end consumers of fatty foods and women of reproductive age. This can be accomplished most effectively by identification and remediation of open hazardous waste sites and closer scrutiny of rendering practices used in the production of animal feeds, as illustrated by the 1999 Belgian PCB/dioxin incident. In the latter episode, 40–50 kg of PCB-contaminated mineral oil, originating from a waste recycling centre, was inadvertently mixed with rendering fat delivered to ten feed plants. The result was that approximately 500 t of animal feed was produced, containing elevated concentrations of PCBs and dioxins, which was used by over 1800 farms (Van Larebeke *et al*., 2001). Following disclosure, the initial public health response was to remove from the market all poultry and associated products as well as all meat with a fat content greater than 25%. As the original source of the PCBs was thought to be discarded transformers, proper inventory control could have prevented further use of their contents.

International harmonization of PCB risk assessment activities, including the development of food tolerances through the Codex Alimentarius Commission, would assist the overall legislative process.

National perspectives

While there is currently a lack of internationally recognized food standards for PCB contamination in foods, the lack of such standards does not preclude individual countries from developing their own national risk assessment guidelines in an attempt to provide adequate human health protection for its citizens. A variety of food commodity-specific PCB guidelines have been developed in Canada under the authority of the Canada Food and Drug Regulations, which state that '. . . no person shall sell an article of food that has in or upon it any poisonous or harmful substance' (Part 1, Section 4(a)). The USA has also developed tolerances for PCBs in foods under provisions of their Federal Food, Drug and Cosmetic Act, which deals with the interstate commerce of foods and to what extent they can be contaminated/adulterated without any adverse effect upon human health (Sections 402(a)(1) and 402 (1)(2)). These tolerances also take into consideration the extent to which contamination/adulteration cannot be avoided even when good manufacturing practices are employed.

US Food and Drug Administration

When the FDA initially started to formulate its regulatory response regarding PCB

contamination of foodstuffs, it was known that there was widespread contamination of freshwater fish and there had been reported incidences of contaminated cattle feed resulting in PCB residues being detected in dairy products. The FDA's response involved the development of a total dietary tolerable intake value, and guidelines or action levels for specific food commodities (Table 6.1). Food commodities found to exceed the Table 6.1 values could theoretically be excluded from the retail market.

In 1968, the Yusho incident occurred when rice oil became accidentally contaminated with Kanechlor 400. By the end of 1982, 1788 Yusho patients were identified as exhibiting such symptoms of poisoning as abnormal skin pigmentation, dermatological effects and neurological complaints (Masuda, 1985). Initial analysis of the contaminated rice oil indicated PCB contamination at levels of 2000–3000 ppm. It was determined subsequently that an average estimated cumulative dose of 2000 mg of PCBs was required before disease symptoms were observed. Therefore, this dose could be regarded as a LOAEL. A tenfold safety factor was used to estimate a NOAEL of 200 mg; this was deemed to be divisible by 1000, the number of days that the victims were exposed to the contaminated rice oil, to determine a crudely estimated average daily intake of PCBs (200 μg). Consequently, an adult should not ingest more than 200 μg of PCBs day^{-1} or approximately 3 μg kg^{-1} BW day^{-1} for the average 65 kg adult (i.e. 200 μg 65 kg^{-1} ≈3 μg kg^{-1} BW day^{-1}). It was also realized that infants/young children could represent a more sensitive subpopulation; therefore, in a similar manner, the lowest minimal cumulative PCB dose associated with toxic effects in infants was determined to be 500 mg. The estimated tolerable daily exposure value was calculated not to exceed 1 μg of PCBs kg^{-1} BW day^{-1} (Cordle and Kolbye, 1979). These values were supported by reports by the Michigan Department of Public Health in 1983, which indicated that consumers of Lake Michigan fish could be ingesting up to 4 μg PCBs kg^{-1} BW day^{-1} with no apparent adverse health effects noted (Boyer *et al.*, 1991). Additional experimental results available at the time with the commercial PCB thought to most closely resemble the chromatographic pattern of PCB congeners found in food residues, i.e. Aroclor 1254, did not suggest a cancer risk at doses up to 100 ppm in the diet (~7 mg kg^{-1} BW day^{-1}).

Health Canada

An analysis of breast milk samples from across Canada during the early 1970s indicated that PCBs were detectable in almost all of the samples. Partially in response to the human health concerns associated with these findings, a toxicological evaluation for PCBs was conducted. Examination of the experimental results with Aroclors 1242, 1254 and 1260 using rodents and dogs indicated a dietary NOEL (no observed effect level) of 10 ppm following chronic exposure which, on a body weight basis, was approximately

Table 6.1. Canadian and US food tolerances for PCBs.[a]

	Tolerance or maximum residue limit (mg kg^{-1})	
Type of food	US FDA	Health Canada
Milk (fat basis)	1.5	0.2
Manufactured dairy products (fat basis)	1.5	0.2
Poultry (fat basis)	3.0	0.5
Eggs	0.3	0.1
Meat, beef (fat basis)	—	0.2
Fish and shellfish (edible portion)	2.0	2.0
Infant and junior foods	0.2	—

[a]Adapted from D'Itri and Kamrin (1983).

0.5 and 0.25 mg kg^{-1} BW day^{-1}, respectively. Application of a 100-fold safety factor resulted in a tolerable exposure range of 2.5–5.0 µg kg^{-1} BW day^{-1}, which was similar to the values derived by the FDA as a consequence of their safety evaluation using the Yusho data.

Subsequent re-analysis of the contaminated Yusho rice oil revealed the presence of other related halogenated aromatic contaminants such as PCQs and PCDFs at concentrations of approximately 866 and 5 ppm, respectively. This ratio of PCQs/PCDFs (866/5) was deemed to be a somewhat unique toxic mixture since it was approximately 100-fold greater than the ratio of PCQs/PCDFs found in the original Kanechlor 400. This ratio also suggested that dibenzofurans were one of the principal aetiological agents responsible for Yusho (Masuda and Yoshimura, 1984). Consequently, Health Canada decided not to base its PCB hazard characterization solely on human data. At the time, additional laboratory experiments were being conducted with non-human primates – rhesus monkeys, a species which appeared to be appreciably more sensitive to the toxic effects of a variety of halogenated aromatics. In these studies, the rhesus monkeys were fed diets containing 2.5 or 5.0 ppm Aroclor 1248, which, on a body weight basis, were calculated to be approximately 100 to 200 µg kg^{-1} BW day^{-1}. The ingestion of Aroclor 1248 resulted in a variety of reproduction-related effects, i.e. reduced fecundability, increased spontaneous abortion rates and reduced birth weights (Allen *et al*., 1979). Based on these data, the initial recommendation that PCB-contaminated foodstuffs should not result in the ingestion of more than 5 µg kg^{-1} BW day^{-1} was revised to a temporary tolerable exposure level of 1 µg kg^{-1} BW day^{-1} (i.e. the 2.5 ppm dietary level in the monkey study – deemed to be the LOAEL – plus the 100-fold 'safety' factor) (Grant, 1983). Following the compilation of detailed Canada-wide market basket surveys for PCB contamination, a variety of maximum residue limits was established for Canadian food commodities, taking into consideration the above tolerable exposure level (Table 6.1).

Conclusions

PCBs are persistent pollutants which were not readily degraded during their industrial applications. They are not readily degraded once they have contaminated the environment nor are they readily metabolized in biological systems. Due to their persistence, PCBs have contaminated all components of the global ecosystem and are readily found in areas of the globe where they were never used. However, the level of PCBs in the ecosystem has decreased dramatically in the last two decades due to legislation which has banned their manufacture and mandated their safe disposal.

PCBs have been found to result in a myriad of toxicological effects, in a variety of *in vivo* and *in vitro* systems, but their potential health implications for humans are less clear due to various factors that have compromised the interpretation of epidemiological studies. While the individual epidemiological study findings, using cohorts exposed to background levels of PCBs, are not always persuasive regarding the effect of PCBs upon human health, when the data are viewed as a whole, there is a suggestion that some subpopulations may be experiencing subtle health effects from the ingestion of PCBs. Therefore, further research into the effective and efficient disposal of equipment containing PCBs and the effective clean-up of dump sites appears warranted if the potential health threat from chronic exposure to PCBs is to be minimized further.

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7 Dioxins in Milk, Meat, Eggs and Fish

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Introduction

Contamination of food with chemicals plays an important role especially for persistent and bioaccumulating substances where dietary intake is the major pathway of exposure for humans. For the general population and some compounds, such as polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDDs/PCDFs), ingestion of food accounts for approximately 95% of the body burden. To guarantee safe and high quality food for human consumption, international regulation such as the Codex Alimentarius of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) has been established. This food code is followed in terms of harmonizing national food regulation, food additives, hygiene and processing as well as facilitating international trade (Codex Alimentarius, n.d.).

The occurrence of unintentional contamination with chemicals but also with bacteria and viruses needs special attention and surveillance to protect humans from consuming unsafe food. Within the chemical contaminants, a major concern is associated with dioxins and furans for several reasons: some of the PCDD/PCDF congeners are highly toxic, they are persistent and bioaccumulate in the food chain and thus can cause chronic effects due to long-term low exposure and, finally, dioxins and furans have been associated with accidents and severe food contaminations.

Nature of the Compounds

Dioxins (PCDDs) and furans (PCDFs) are two groups of planar, tricyclic ethers which have up to eight chlorine atoms attached at carbon atoms 1–4 and 6–9. In total, there are 75 possible PCDD congeners and 135 possible PCDF congeners, giving a total of 210 congeners (see Chapter 6). PCDDs and PCDFs are generally very insoluble in water, are lipophilic and are persistent. Dioxins and furans have never been produced intentionally but are unwanted by-products of many chemical industrial processes and of all combustion processes. The sources and activities that lead to the formation of PCDDs/PCDFs, and subsequently to the release of these contaminants into air and water, with products and residues, have been subject to intensive research, and today the most important dioxin sources seem to be identified. In the past, the chemical industry, with its production of organochlorine chemicals, was the major

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source of PCDDs/PCDFs: chemicals with high concentrations of dioxins and furans were pentachlorophenol (PCP), 2,4,5-trichloroacetic acid (2,4,5-T), polychlorinated biphenyls (PCBs; note that they contain PCDFs only, not PCDDs) (Fiedler *et al*., 1990). In 1977, PCDDs/PCDFs were identified in the emissions of a municipal waste incinerator in Amsterdam (Olie *et al*., 1977) and in 1980 the trace chemistry of fire was established, which states that, in thermal processes and in the presence of organic carbon, oxygen and chlorine, dioxins and furans can be formed (Bumb *et al*., 1980). Today, in industrialized countries, the major sources of dioxin and furan release are combustion processes. Among these sources are the incineration of municipal and hospital waste, the production of iron and steel and other non-ferrous metals, e.g. copper, aluminium, lead and zinc (especially in recycling processes), and all types of uncontrolled burning, e.g. landfill fires, trash burning on soil, forest and bush fires (especially when chlorinated herbicides have been applied).

Lastly, natural formation of PCDDs/PCDFs has been shown on different occasions. Peroxidases are capable of synthesizing PCDDs/PCDFs from precursors such as chlorophenols. The formation of especially. Cl_7DD and Cl_8DD during the composting process has been proven where it was found that the international toxic equivalent (I-TEQ) increases by about 1–2 parts per trillion (ppt) during the composting process. Recent studies provide a strong indication that PCDDs/PCDFs may have been present in the environment for considerably longer than the onset of the chlorine industry, and that they may be formed through non-anthropogenic activities. High concentrations of mainly PCDDs were found in mined ball clay from the USA, kaolinitic clay from Germany, deep soil samples from Great Britain, in dated marine sediment cores from Queensland/Australia and in man-made lake sediment cores from Mississippi, USA. Typical for all samples is the almost total absence of PCDFs and the nearly identical congener/isomer distribution throughout all geographies.

Almost all possible 210 congeners are released from anthropogenic sources and, due to chemical, physical and biological stability and long-range transport, are ubiquitous and have been detected in all environmental compartments. Due to the persistence of the 2,3,7,8-substituted congeners and the lipophilicity of these compounds, PCDDs/PCDFs accumulate in fatty tissues and in carbon-rich matrices such as soils and sediments.

National release inventories

With this mandate to facilitate a convention on reduction and elimination of releases of persistent organic pollutants (POPs), UNEP Chemicals will '. . . assist countries in the identification of national sources of dioxin/furan releases by promoting access to the information on available sources of dioxins/furans . . .'. Table 7.1 summarizes initial findings obtained from national inventories of releases of dioxins and furans, which have been compiled by the United Nations Environment Programme (UNEP) in 1999 and have been updated since then. The updated UNEP report for a reference year around 1995 would estimate annual releases to air of approximately 13,000 g I-TEQ $year^{-1}$ from about 20 countries. This amount is based on best estimates from most countries and the lower bound emission for the rest of the countries. The upper estimate would be around 30,000 g I-TEQ $year^{-1}$ and would also include another 2400 g I-TEQ in preliminary estimates from US sources, which have been addressed only recently (US-EPA, 2000a). The PCDD/PCDF releases into air per year and country are shown in Table 7.1. It should also be noted that, for example, Japan updates its inventory on an annual basis and for its last reporting year estimated much lower emissions, namely 2260–2440 g TEQ $year^{-1}$ for 1999 coming down from 6301–6370 g TEQ $year^{-1}$ in 1997 (Environment Agency Japan, 2000).

Most data are available for industrialized countries from Western Europe and North America. From Asia, there is only an inventory for Japan and an additional estimate of 22 g I-TEQ $year^{-1}$ for emissions from

Table 7.1. National dioxin and furan inventories: PCDD/PCDF emissions to air (UNEP, 1999, updated).

Country	Emission (g TEQ year^{-1})	Reference year	Source[a]
Austria	29	1994	1
Australia	150–2,300	1998	1
Belgium	661	1995	1
Canada	164	1999	2
Croatia	95.5	~1997	
Denmark	19–170	1998/99	3
Finland	98.3–198	~1997	1
France	873–2,737	1998	1
Germany	327	1994	1
Hungary	112–8,436	1995	1
Japan	6,301–6,370 (2,260–2,440)	1997 (1999)	4
The Netherlands	486	1991	1
New Zealand	14–51		5
Norway	9.15	~1997	1
Slovak Republic	42	1994	1
Sweden	22–88	1993	1
Switzerland	181	1995	1
UK	569–1,099	1995	1
USA	2,501	1995	6
Global flux	12,655–25,945		

[a]Source: 1 = Fiedler (1999); 2 = Environment Canada (2001); 3 = Hansen (2001); 4 = Environment Agency Japan (2000); 5 = Buckland *et al.* (2000); 6 = US-EPA (2000a).

waste incinerators in the Republic of Korea. From the southern hemisphere, only Australia and New Zealand have estimated annual emissions. From Africa, Central and South America and the rest of Asia there are no data at all. At present, the geographical coverage is not sufficient to estimate global emissions of PCDDs/PCDFs. Further, the present inventories do not cover all known sources of dioxins and furans. There are several efforts underway to identify dioxin sources in parts of the world where, so far, there is no information available. Existing inventories will be updated, as it is obvious that countries have initiated measures to reduce emissions of dioxins and furans.

Environmental concentrations, fate and transport

Many data are available for PCDD/PCDF concentrations in soils, sediments and air. Biomonitors, such as vegetation or cows' milk, have been applied successfully to identify or monitor ambient air concentrations in the neighbourhood of potential point sources, although a linear correlation between PCDD/PCDF concentrations in vegetation and air samples cannot be established. Due to public concern regarding dioxins and furans, many studies have been aimed at identifying potential 'hotspots' of contamination. As a result, the overall presentation of data is often biased towards contaminated samples and higher concentrations, rather than baseline information.

When evaluating concentrations of PCDDs/PCDFs in the environment, it should be taken into account that some matrices are sensitive to short-term inputs, e.g. ambient air or short-lived vegetation, whereas other matrices, such as sediments and soils, are relatively insensitive to temporal variation. Further important factors for the interpretation of results are season (e.g. in winter PCDD/PCDF concentrations in air may be higher by a factor of ten on a toxic equivalent (TEQ) basis than in summer), length of the sampling or exposure (e.g. a few hours vs. weeks), location (e.g. urban vs. rural), the

sampling method (e.g. high volume sampling vs. particulate deposition), sampling depth (e.g. surface vs. core), etc.

Soils are natural sinks for persistent and lipophilic compounds such as PCDDs/PCDFs, which adsorb to the organic carbon of the soil and, once adsorbed, remain relatively immobile. Soil is a typical accumulating matrix with a long memory; in other words, dioxin inputs received in the past will remain and, due to the very long half-lives of PCDDs/PCDFs in soils, there is hardly any clearance. Soils can receive inputs of environmental pollutants via different pathways, of which the most important are: atmospheric deposition, application of sewage sludge or composts, spills, and erosion from nearby contaminated areas. Sediments are the ultimate sink for PCDDs/PCDFs (and other persistent and lipophilic organic substances). As with soils, sediment samples are accumulating matrices for lipophilic substances and can receive inputs via different pathways: atmospheric deposition, industrial and domestic effluents, stormwater, spills, etc. Today, PCDDs/PCDFs can be detected ubiquitously and have been measured in the Arctic, where almost no dioxin sources are present. It became clear that the lipophilic pollutants, such as PCDDs/PCDFs, at the North and the South Poles originated from lower (warmer) latitudes. Emission of most PCDDs/PCDFs from combustion sources into the atmosphere occurs in the moderate climate zones; PCDDs/PCDFs then undergo long-range transport towards the North Pole, condensing in the cooler zones when the temperatures drop. This process of alternating re-volatilization and condensing, also named the 'grasshopper effect', can carry pollutants thousands of kilometres in a few days. Thus, the air is an important transport medium for PCDDs/PCDFs. An indirect method of determining ambient air concentrations is the use of biomonitors, such as vegetation. The outer waxy surfaces of pine needles, kale or grass absorb atmospheric lipophilic pollutants and serve as an excellent monitoring system for PCDDs/PCDFs (Buckley-Golder *et al*., 1999, Task 2).

In most countries, a broad range of PCDD/PCDF concentrations has been detected in all media. Table 7.2 presents the range of reported background concentrations and maximum concentrations measured in contaminated locations from European countries. As illustrated in Table 7.2, the lowest concentrations for all matrices are below 1 ng I-TEQ kg^{-1} dry matter (DM) and the highest background values are around 100 ng I-TEQ kg^{-1} DM. At contaminated locations, measured concentrations in soils range from several hundred to around 100,000 ng I-TEQ kg^{-1} DM (Finland, sites contaminated with wood preservatives; and The Netherlands, close to a scrap car and scrap wire incinerator) and in sediments up to 80,000 ng I-TEQ kg^{-1} DM (Finland, downstream from a wood preservative-producing site). The extremely high concentration of 14,800 fg I-TEQ^{-3} was measured in 1992/93 at the Pontyfelin House site, in the Panteg area of Pontypool in South Wales, which is very close (~150 m) to an industrial waste incinerator (Buckley-Golder *et al*., 1999, Task 2).

Fish and shellfish frequently have been used as biomonitors for the aquatic environment as they are highly bioaccumulative

Table 7.2. Concentrations of PCDDs/PCDFs measured in EU Member States.

Environmental matrix	Measured range background	Maximum concentration at contaminated sites	Units
Soil	< 1–100	100s–100,000	ng I-TEQ kg^{-1} DM
Sediment	< 1–200	100s–80,000	ng I-TEQ kg^{-1} DM
Air (ambient)	< 1–100s	14,800	fg I-TEQ m^{-3}
(deposition)	< 1–100s		pg I-TEQ m^{-2} day^{-1}
Sewage sludge	< 1–200 (average 10–40)	1,200	ng I-TEQ kg^{-1} DM
Spruce/pine needles (biomonitors)	0.3–1.9	50–100	ng I-TEQ kg^{-1} DM

for PCDDs/PCDFs, and concentrations of several hundred pg TEQ g^{-1} fat have been detected. These concentrations are much higher than those found in terrestrial animals, such as cattle, pigs or chickens. Top-predators, such as sea eagles or guillemots, also showed high concentrations of PCDDs/PCDFs: as an example, 830–66,000 pg TEQ g^{-1} fat were found in Finnish white-tailed sea eagles (Buckley-Golder *et al*., 1999, Task 2).

Understanding of the environmental fate of PCDDs/PCDFs is fundamental to evaluating human exposure. Although the TEQ approach was developed and proven as a helpful tool for risk assessment, input data for models and exposure assessment have to be congener specific.

Knowledge of the numerical values of certain parameters characterizing the properties of individual PCDDs/PCDFs is necessary in order to predict the behaviour of the mixtures found in the environment. The physical and chemical properties, which are measures of or control the behaviour of dioxins are:

- their low vapour pressure (ranging from 4.0×10^{-8} mmHg for 2,3,7,8-Cl_4DF to 8.2×10^{-13} mmHg for Cl_8DD);
- their extremely low solubility in water (ranging from 419 ng l^{-1} for 2,3,7,8-Cl_4DF, 7.9 and 19.3 ng l^{-1} for 2,3,7,8-Cl_4DD to 0.074 ng l^{-1});
- their solubility in organic/fatty matrices (log K_{ow} range from 5.6 for Cl_4DF and 6.1/7.1 for Cl_4DD to 8.2 for Cl_8DD);
- their preference for binding to organic matter in soil and sediments (log K_{oc} values for 2,3,7,8-Cl_4DD were between 6.4 and 7.6).

The processes by which PCDDs/PCDFs move through the environment are reasonably well known. PCDDs/PCDFs are multimedia pollutants and, once released to the environment, become distributed between environmental compartments (Buckley-Golder *et al*., 1999, Task 3).

PCDDs/PCDDFs are semi-volatile compounds and, in the atmosphere, can exist in both the gaseous phase and bound to particles, depending upon the congener and the environmental conditions. Especially during the warmer (in the northern hemisphere, summer) months, the lower chlorinated PCDD/PCDF congeners tend to be found predominantly in the vapour phase. PCDD/PCDF in the vapour phase can undergo photochemical transformation, with a dechlorination process leading to more toxic congeners if octa- and heptachlorinated congeners degrade to tetra- and pentachlorinated and finally to non-toxic compounds with only three or fewer chlorine atoms. PCDDs/PCDFs attached to particulate matter seem to be resistant to degradation.

In the terrestrial food chain (air → grass → cattle → milk/meat → man), PCDDs/PCDFs can be deposited on plant surfaces via wet deposition, via dry deposition of chemicals bound to atmospheric particles or via diffusive transport of gaseous chemicals in the air to the plant surfaces. Each of these processes is governed by a different set of plant properties, environmental parameters and atmospheric concentrations. Investigations with native grassland cultures showed that dry gaseous deposition played the dominant role for the accumulation of the lower chlorinated PCDDs/PCDFs, whereas dry particle-bound deposition played an important role in the uptake of the PCDDs/PCDFs with six and more chlorine atoms. There was also some evidence indicating an input of the higher chlorinated PCDD/PCDF from wet deposition (Welsch-Pausch *et al*., 1995). Levels in, for example, grass reflect recent exposure to PCDDs/PCDFs, as vegetation is only exposed for a relatively short time, with new growth replacing old and crops being harvested. For agricultural leaf crops, the main source of contamination is direct deposition from the atmosphere and soil splash. Root uptake and translocation of dioxin contamination into the crop has been confirmed for courgette and cucumber only. Grazing animals are exposed to dioxins by ingesting contaminated pasture crops, and PCDDs/PCDFs are found to accumulate primarily in the fatty tissues and milk.

For agricultural soils, an additional source of PCDD/PCDF can be the application of sewage sludge. Small amounts of PCDDs/PCDFs deposited on to soil can be returned to the atmosphere by the resuspension of previously deposited material or revolatilization

of the less chlorinated congeners. Because of their chemical characteristics and very low solubility, PCDDs/PCDFs accumulate in most soil types, with very little water leaching and negligible degradation of the 2,3,7,8-substituted PCDD/PCDF congeners.

PCDDs/PCDFs partition quickly to organic matter and so accumulate in sediments. They accumulate in aquatic fauna as a result of the ingestion of contaminated organic matter. The concentration of PCDDs/PCDFs in fish tissue is found to increase up the food web (biomagnification) as a result of the progressive ingestion of contaminated prey.

Carry-over rates: from environment to food

The transfer of dioxins from grass into cattle has been studied, and carry-over rates have been determined. In general, carry-over rates decrease with increasing degree of chlorination of the chemical, indicating that absorption through the gut also decreases. This decrease in absorption is attributed to the greater hydrophobicity of the higher chlorinated PCDDs/PCDFs, which inhibits their transport across aqueous films in the digestive tract of the cow.

In studies conducted at background concentrations, the highest transfer was determined for two lower chlorinated dibenzo-*p*-dioxins and one dibenzofuran, namely 2,3,7,8-Cl_4DD (2,3,7,8- tetrachlorodibenzo-*p*-dioxin), 1,2,3,7,8-Cl_5DD (1,2,3,7,8-pentachlorodibenzo-*p*-dioxin), and 2,3,4,7,8-Cl_5DF (2,3,4,7,8-pentachlorodibenzofuran). For these three congeners about 30–40% are transferred from feed to cow's milk. About 20% are transferred for the 2,3,7,8-substituted Cl_6DD (hexachlorodibenzo-*p*-dioxin) and Cl_6DF (hexachlorodibenzofuran) homologues. For the hepta- and octachlorinated PCDDs and PCDFs, not more than 4% of the ingested congeners find their way into the milk. Although highly dependent on the characteristics of each congener, the overall transfer on a TEQ basis is about 30%; in other words: about 30% of the most toxic PCDD/PCDF congeners which are ingested by the cow are excreted via the milk (Welsch-Pausch and McLachlan, 1998).

Distribution in Foods

The largest database on dioxin concentrations in food exists for some European countries, and the major findings are discussed in this following section. From North America, especially from the USA, the database on dioxin concentrations in food is small compared with the European database (US-EPA, 2000b). The Organochlorine Programme in New Zealand found very low concentrations of PCDDs/PCDFs in the foodstuffs (NZ, 1998). Concentrations of PCDD/PCDF ranged from 0.072 to 0.57 pg I-TEQ g^{-1} fat for meats and meat products; 0.056–0.26 pg I-TEQ g^{-1} fat for dairy products, 0.41–1.82 pg I-TEQ g^{-1} fat for fish, and 0.12 and 0.29 pg I-TEQ g^{-1} fat for eggs and poultry, respectively. Cereal products and bread were between 0.19 and 0.66 pg I-TEQ g^{-1} fat (all numbers include half of the detection limit for non-quantifiable congeners when calculating the TEQ)

In 2000, a database with information on concentrations of PCDDs, PCDFs and/or dioxin-like PCBs (polychlorinated biphenyls) in food products and human milk was established and evaluated. The samples originated from rural and industrial sites in ten EU Member States and were collected between 1982 and 1999. Due to the high demands on dioxin and furan analyses, broad field surveys based on a large number of samples are rare. Nevertheless, the current database can be considered relatively complete for PCDDs and PCDFs, but rather incomplete for dioxin-like PCBs.

With respect to dioxin contamination, highest relevance is for foods of animal origin, where in principle only 2,3,7,8-substituted congeners are found. These are the most toxic and most persistent. Foods of plant origin normally have lower concentrations of dioxins and furans but, for example, grass plays an important role as feedstuff for cattle, sheep, etc., and the contamination in the grass translocates into the animal and its products, e.g. meat, milk. Humans and breast-fed infants are the last steps in the food chain and thus have the highest concentrations.

A survey of European food data can be summarized as follows:

- The national average concentration of PCDDs/PCDFs in eggs, fats, oils, meat (and its products) and milk (and its products) is generally less than 1 pg I-TEQ g^{-1} fat, with an upper limit of 2–3 pg I-TEQ g^{-1} fat.
- PCDDs/PCDFs in fish ranged from 0.25 pg I-TEQ g^{-1} fresh weight (FW) up to 10–20 pg I-TEQ g^{-1} FW.
- Concentrations in fruits, vegetables and cereals were generally close to the limits of quantification.
- Concentrations in meat and meat products and fish and fish products seem to vary with the organ analysed, e.g. higher concentrations on a fat basis in liver than in adipose tissue. Further, there is a difference between animal species, e.g. lower concentrations on a fat basis in pork than in beef, poultry or mutton.
- Decreasing trends in the concentration of PCDDs and PCDFs in foods, especially in consumer milk and some types of meat, have been determined in a few countries. However, the available information is insufficient and too incomplete to draw a general conclusion on temporal trends for other types of foods.
- Although the data on concentrations of dioxin-like PCBs in foods are scarce, the available information indicates that these PCB congeners may add one to two times of the PCDD/PCDF TEQ. In particular, PCB congeners 126 and 118 may contribute much more strongly to the total TEQ content of foods than do the PCDDs and PCDFs.
- The largest database exists for PCDDs and PCDFs in human milk, and for some countries strong downward trends have been observed. Since 1995, the national average concentrations have ranged between 8 and 16 pg I-TEQ g^{-1} fat. Although the database is incomplete, results from the years 1990–1994 indicate that, on a TEQ basis, PCBs can account for the same to up to three times the concentration of the PCDDs and PCDFs (7–29 pg TEQ g^{-1} fat).

Milk and milk products

Analysis of dioxins in cow's milk has been performed since 1986. As dairy products are the main contributor to the human dioxin burden and cow's milk also serves as a biomonitor, the database for milk samples is large. When comparing dioxin concentrations in cow's milk, seasonal variations of up to 25% can occur due to changes in animal feeding stuffs. The differences between certain regions can be even higher. In the late 1980s, contamination of cow's milk with dioxins by chlorine-bleached cardboard containers was established. After elimination of elemental chlorine in the bleaching process, the PCDD/PCDF levels in cow's milk were no longer influenced by cardboard containers. At the end of 1997, increasing levels of dioxins in cow's milk were detected in Baden-Württemberg (Germany). Finally, contaminated citrus pulp, a component of feedingstuff imported from Brazil, was found to be the cause of elevated dioxin concentrations (Malisch, 1998a,b). This contamination was found in other federal Länder of Germany and later other European countries as well.

The most recent surveys show national average concentrations in the range of 0.3–2.1 pg I-TEQ g^{-1} for PCDDs/PCDFs and 0.2–1.8 pg PCB TEQ g^{-1} fat for dioxin-like PCBs. To explain the concentrations of PCDDs/PCDFs/PCBs in milk and milk products, several factors have to be considered: obviously, deposition of dioxins and related compounds emitted from either point or diffuse sources on pasture as well as contaminations present in animal feedstuffs are important routes of exposure for cattle. Due to stringent enforcement of limit values, the national average concentrations of dioxins in dairy products have decreased over the last decade in many European countries.

Meat and meat products

PCDD/PCDF concentrations in foodstuffs of animal origin depend on the animal. Thus, distinctions have to be made between

different types of meat. The lowest levels were found in pork. As sausages mainly contain pork, their dioxin concentrations are similar but tend to be slightly higher than those of pure pork due to the addition of beef and liver and occasionally through the smoking process.

In general, beef, veal, poultry and mixed meat have quite similar concentrations of PCDDs/PCDFs, in the range of 0.5–0.7 pg I-TEQ g^{-1} fat. The mean for pork is lower and around 0.3 pg I-TEQ g^{-1} fat. Game meat and liver had significantly higher dioxin concentrations than the other meat subgroups (SCF, 2000).

Eggs

The most recent surveys on concentrations of PCDDs/PCDFs in eggs gave mean concentrations between 0.5 and 2.7 pg I-TEQ g^{-1} fat with an overall mean around 1 pg I-TEQ g^{-1} fat (SCF, 2000). Older studies tend to give a similar picture, suggesting that concentrations have not changed substantially. German studies have shown that the PCDD/PCDF levels depend on the type of the chicken's housing (cage, ground or field). Higher concentrations were found for eggs from hens which can take up dioxins from soil. Lower levels were detected in eggs from hens housed in elevated wire cages. Recent results indicate that these dependencies are decreasing. From the limited information on PCBs, it can be assumed that the contribution of the PCBs to a total TEQ is in the same range as that of PCDDs/PCDFs (SCF, 2000).

Fish and fish products

PCDD/PCDF concentrations in fish are highly variable. It is problematic to generate representative data on dioxin levels in fish, as a lot of fish species and fishing grounds exist. In this context, a recent representative study including 184 samples of fish and fish products is of great importance. Sampling was based on the real intake according to the share of different species and fishing grounds. Because of large differences in the fat content of fish, levels on a whole weight basis were preferred.

Wild fish and farmed freshwater fish had mean concentrations of around 10 pg I-TEQ g^{-1} fat for PCDDs/PCDFs and 30 pg PCB-TEQ g^{-1} fat for co-planar and mono-*ortho* PCBs. The threefold higher mean PCB concentration reflects the fact that PCB levels are constantly higher than the combined PCDD/PCDF levels. It should be noted that PCDD/PCDF concentrations range over three orders of magnitude and PCB concentrations over two orders of magnitude (SCF, 2000).

Fruit and vegetables

There are no recent data for dioxin concentrations in foods of plant origin. Products of vegetable origin, such as cereals with less than 2% fat, fruit and vegetables, had very similar contamination levels, with a mean concentration of around 0.02–0.3 pg I-TEQ g^{-1} on a whole food basis (SCF, 2000).

Human milk

A large database exists for dioxin concentrations in human milk; the national average concentrations of PCDDs, PCDFs and dioxin-like PCBs, expressed in I-TEQ and PCB-TEQ, respectively, are presented in Table 7.3. For the period 1995–1999, the current database shows national average concentrations between 8 and 16 pg I-TEQ g^{-1} fat. For the period before 1995, the national averages ranged between 10 and 34 pg I-TEQ g^{-1} fat. Generally, the data demonstrate a downward trend for human milk concentrations of PCDDs/PCDFs. Most complete time trends can be established for Germany based on more than 1732 individual samples collected in various German 'Bundesländer' during 1985–1998. The German database shows a 60% decline in the average as well as in the highest PCDD/PCDF levels found in human milk between the late 1980s and 1998. The database is too incomplete to draw

Table 7.3. National average concentrations of dioxins and related PCBs (in pg TEQ g^{-1} fat) in representative human milk samples.

	$TEQ_{PCDD/PCDF}$			TEQ_{PCB}		
Country	< 1990	1990–1994	1995–1999	< 1990	1990–1994	1995–1999
Belgium		24.8			6.63	
Germany	30.7	20.6	13.8			
Denmark	18.1	16.7			18.0	
Finland	20.0	13.2		25.3	12.0	
France			16.4			
Italy	25.0					
The Netherlands	34.2	23.5			20.9	
Norway		10.4			29.1	
		13.3	7.90		19.4	
Range of means	18–34	10–25	8–16	25	7–29	No data

conclusions about the TEQ contribution of dioxin-like PCBs.

Food consumption data

The consumption data from the participating countries are generally produced from studies performed rather recently. The survey methods differ, including consumption record studies (2–28 days) as well as 24 h recall, household budget and food frequency questionnaire studies. The study populations were generally adults (from teenagers to the elderly), but the UK and Germany have also studied separate groups of consumers, including breast-fed infants, toddlers, schoolchildren and adults. The food consumption data reveal variations between countries in consumption of different food groups, a mirror of the country-specific food traditions and habits.

Dietary intakes

Based on the data collected on PCDD, PCDDF and PCB concentrations in food, mean dietary intakes can be calculated by multiplying the average concentrations by average consumption of major food groups. In EU Member States, and for the period after 1995, the average dietary intakes of PCDDs and PCDFs ranged between 29 and 97 pg I-TEQ day^{-1}, which on a body weight (BW) basis corresponds to 0.4–1.5 pg I-TEQ kg^{-1} BW day^{-1}. Surveys of chemical analyses of foods collected in the 1970s and 1980s gave much higher estimates, ranging from 127 to 314 pg I-TEQ day^{-1}, corresponding to 1.7–5.2 pg I-TEQ kg^{-1} BW day^{-1}. The 95 percentile (or 97.5 percentile) intake, based on data from The Netherlands and the UK, was two to three times the mean intake.

The intake of co-planar and mono-*ortho* PCBs would add another 48–110 pg PCB-TEQ day^{-1} (= 0.8–1.8 pg PCB-TEQ kg^{-1} BW day^{-1}). Whereas the contribution of PCBs to the total TEQ equals the intake of PCDDs and PCDFs in countries such as Finland, The Netherlands, Sweden and the UK, studies in Norway showed that the contribution from dioxin-like PCBs is up to four times the TEQ contribution of the PCDDs and PCDFs. Thus, average human daily intake of PCDDs, PCDFs and dioxin-like PCBs in European countries has been estimated to be 1.2–3.0 pg WHO-TEQ kg^{-1} BW day^{-1}. More than 90% of the human exposure derives from food. Foodstuffs of animal origin normally contribute to more than 80% of the overall exposure (SCF, 2000).

In European countries, milk and dairy products are the main contributors to the average daily intake of PCDDs and PCDFs on an I-TEQ basis with 16–39%, meat and meat products are second (6–32%) and fish and fish products are third (2–63%). Other products, mainly of plant origin, such as vegetables and cereals, contributed 6–45% in those countries

for which data were available. The total intake of I-TEQ differed from country to country. Reasons for these differences may result from different food consumption habits but also from applied sampling strategy and the large variations in concentrations of dioxin-related substances in some of the food groups (e.g. vegetables and fruits, eggs and fish).

It is well known that during the breast-feeding period, on a body weight basis, the intake of PCDDs and PCDFs is 1–2 orders of magnitude higher than the average adult intake. A few countries (i.e. Finland, Germany, The Netherlands, Sweden and the UK) reported clear downward trends for the exposure of the general population to dioxins and furans and, for Germany (see Table 7.4), Finland, The Netherlands and Sweden, this decline is also noted for concentrations in human milk.

Although different dietary habits make direct comparison of results from different countries difficult, the daily intakes of PCDDs and PCDFs by males living in New Zealand are consistently lower than those of other countries. The intakes are also below the WHO-recommended tolerable daily intake (TDI) of 1–4 pg TEQ kg^{-1} BW day^{-1}. The dietary intake estimated for an 80 kg adult male consuming a median energy (10.8 MJ day^{-1}) diet was 14.5 pg I-TEQ day^{-1} (equivalent to 0.18 pg I-TEQ kg^{-1} BW day^{-1}) and an additional 12.2 pg TEQ day^{-1} (= 0.15 pg TEQ kg^{-1} BW day^{-1}) for dioxin-like PCBs. Dietary intakes estimated for a 70 kg adolescent male consuming a high energy (21.5 MJ day^{-1}) diet were 30.6 pg I-TEQ day^{-1} (= 0.44 pg I-TEQ kg^{-1} BW day^{-1}) and 22.7 pg PCB-TEQ day^{-1} (= 0.32 pg TEQ kg^{-1} BW day^{-1}) (NZ, 1998).

Food and feedingstuff-related accidents

In the past, high exposures occurred through accidents. Well-known examples are the contamination of edible rice oils, such as the Yusho in Japan in 1968 and the Yu-Cheng in Taiwan in 1978. In these cases, PCBs from hydraulic oils leaked into edible oils, which were sold and consumed by thousands of people. Severe toxic effects were detected in both populations due to high levels of PCDFs and PCBs (Needham, 1993; Guo *et al*., 1994; Masuda, 1994).

Each year from 1997 to 1999, cases of dioxin (and PCB) contamination of animal feeds and foods occurred. Among these are the dioxin contamination of citrus pulp pellets (an ingredient for feeding stuffs) from Brazil in the years 1997–1998, the contamination of animal feeds with PCBs and dioxins in Belgium in spring of 1999, and the dioxin contamination of kaolinitic clay (a feed additive) from some mines in the USA and Germany. In each of these cases, preventive measures were taken to avoid a further distribution of contaminated products and to protect the consumer against foods with elevated levels.

Table 7.4. Dietary intake of PCDDs/PCDFs of breast-fed infants in Germany.

Age (months)	Year	Mean intake (pg TEQ day^{-1})	Mean intake (pg TEQ BW day^{-1})	Comments
1	1998	291	70	Fully breast-fed
2	1998	338	68	Fully breast-fed
3	1998	360	62	Fully breast-fed
4	1998	370	57	Fully breast-fed
6	1998	369	48	Fully breast-fed
5	1998	271	38	Partly breast-fed
6	1998	180	24	Partly breast-fed
7–9	1998	108	13	Partly breast-fed
4	1986–1990	879	135	Fully breast-fed
4	1992	604	93	Fully breast-fed
4	1994	502	77	Fully breast-fed
4	1996	402	62	Fully breast-fed

The citrus pulp pellet contamination

From mid-1997 until March 1998, on average, twice the concentrations of PCDDs/PCDFs in cow's milk were detected by German Food Control laboratories: starting from a level of about 0.6 pg I-TEQ g^{-1} fat in summer 1997, the average concentration increased to 1.41 pg I-TEQ g^{-1} fat in different regions of Germany in February 1998. The highest value was 7.86 pg I-TEQ g^{-1} fat and thus exceeded the concentration of 5 pg I-TEQ g^{-1} fat, the maximum permissible concentration to place milk products on the German market. Although this observation was made in Germany first, later the same observation was found in the 12 Member States of the EU. Whereas feedingstuff samples typically had concentrations in the range from 100 to 300 pg I-TEQ kg^{-1}, a compound feed for milk production, which had been found at two different dairy farms, had about 1800 pg I-TEQ kg^{-1}. It affected the level of dioxins and furans in cow's milk, beef and veal (Malisch, 1998a,b).

The contamination was traced back to citrus pulp pellets imported from Brazil and used in compound feed for ruminants all over Europe. The Brazilian citrus pellet production had been contaminated by dioxin-containing lime, which was a by-product from a chemical factory. The lime apparently was used for feed production against the advice of the supplier, who believed it was for construction. As mentioned earlier, this contamination may have had an impact on the general level of dioxin exposure of the European population and a slight increase in the dioxin content in breast milk and tissue.

The Belgian chicken accident

In March 1999, serious animal health problems in poultry production were discovered in Belgium. There was a marked reduction in egg hatchability and an increased mortality of chickens. At the end of May, analysis of feedingstuff samples, hens and breeding eggs showed high levels of dioxins and furans. The first analyses showed dioxin concentrations 1000 times above background level; the contamination dropped by more than 100 times from February to March 1999 (Table 7.5). The contamination seems to have been caused by the discharge of about 25 l of PCB transformer oil into a waste collection unit for animal fats recycled into animals feed contaminating 107 t of fat. From this, about 90 t of fat was used for production of feedstuff for poultry, and the remaining fat was used for production of milk and meat. At the beginning of October 1999, the number of affected or suspected farms was 505 poultry farms, 1625 pig farms and 411 cattle farms. The estimated costs for Belgium in connection with the dioxin food contamination is about US$1 billion; indirect costs are estimated to be three times higher. A correct waste disposal of the 25 l of transformer oil would have cost about US$1000. Though the Belgian dioxin contamination had a major effect on the Belgian food production economy, it gave only a short-term peak exposure to dioxins and furans for humans, which cannot be detected in the general population.

Table 7.5. Belgian dioxin accident – PCDD/PCDF concentrations in feedingstuff and food.

	Concentration (pg WHO-TEQ kg^{-1})	
Poultry feed	811,000	
Poultry fat	775	1,009
Egg fat	266	713

The kaolinitic (ball) clay case

In 1999, a dioxin contamination of poultry and mink was traced back to the use of kaolinitic clay as an anti-caking agent in poultry feed and in mineral feed for mink. The origin of the contamination was traced back to a ball clay mine in Germany. Similar examples were found in the USA, where catfish and beef had been impacted through the use of ball clay in animal feedingstuff production.

The Brandenburg case

Repeated detection of elevated dioxin levels in eggs produced in the German state of Brandenburg was identified in 1999 when, in an open system, grass meal (for feedingstuff

production) was dried by burning wood as the fuel. All types of wood were burned, including waste wood with chemical contamination from former painting or use of wood preservatives.

The choline chloride case

In the year 2000, a dioxin contamination in choline chloride pre-mixtures for feedstuff was detected in Germany. The original choline chloride (= vitamin K) from a Belgian producer was not contaminated but the Spanish feedstuff pre-mix producer who sold the pre-mix to Germany had added pine sawdust to the product as a carrier. This pine sawdust was heavily contaminated with dioxin-containing pentachlorophenol.

Uptake and Human Exposure, Maternal Transmission

For humans (and animals), the major uptake of dioxins and furans is via ingestion. The 2,3,7,8-substituted congeners have long half-lives, generally of the order of years, and this causes these compounds to bioaccumulate. Metabolism is almost negligible and, to calculate body burdens, intake is the parameter for countermeasures. Protection of the fetus is of particular concern when precautionary actions are to be taken.

For the general population, the major pathway of exposure to PCDDs, PCDFs and PCBs is through food. More than 90% of human exposure occurs via the diet, with foods of animal origin usually being the predominant sources. Contamination of food is caused primarily by deposition of emissions from combustion sources such as waste incineration, the metal industry, energy production or household heating, and subsequent accumulation in the food chain. Due to their lipophilicity, PCDDs, PCDFs and PCBs are associated with fat. Contamination of food may also occur through contaminated feed, improper application of sewage sludge, flooding of pastures, waste effluents and certain types of food processing or packaging (SCF, 2000).

Some subpopulations may have higher exposure to dioxins, furans and PCBs as a result of particular consumption habits, e.g. nursing infants and subsistence fishermen living close to contaminated waters.

Toxicity and Clinical Effects

Toxic effects in laboratory animals

The extraordinary potency of 2,3,7,8-TCDD (tetrachlorodibenzo-*p*-dioxin) and related 2,3,7,8-substituted PCDDs and PCDFs has been demonstrated in many animal species. They elicit a broad spectrum of responses in experimental animals such as: liver damage (hepatoxicity); suppression of the immune system (immunotoxicity); formation and development of cancers (carcinogenesis); abnormalities in fetal development (teratogenicity); developmental and reproductive toxicity; skin defects (dermal toxicity); diverse effects on hormones and growth factors; and induction of metabolizing enzyme activities (which increases the risk of metabolizing precursor chemicals to produce others which are more biologically active).

It is generally believed that 2,3,7,8-substituted PCDDs and PCDFs exhibit the same pattern of toxicity. The toxic responses are initiated at the cellular level, by the binding of PCDDs/PCDFs to a specific protein in the cytoplasm of the body cells, the aryl hydrocarbon receptor (AhR). The 2,3,7,8-substituted PCDDs/PCDFs bind to the AhR and induce *CYP1A1* (cytochrome P_{450} 1A1) and *CYP1A2* (cytochrome P_{450} 1A2) gene expression. The binding to the AhR constitutes a first and necessary step to initiate the toxic and biochemical effects of dioxins, although it is not sufficient alone to explain the full toxic effects. This mechanism of action of 2,3,7,8- Cl_4DD parallels in many ways that of the steroid hormones, which have a broad spectrum of effects throughout the body and where the effects are caused primarily by the parent compound. However, TCDDs and steroid hormone receptors (e.g. oestrogen, androgen, glucocorticoid, thyroid hormone, vitamin D_3 and retinoic acid receptors) do

not belong to the same family. AhR-binding affinities of 2,3,7,8-Cl_4DF, 1,2,3,7,8-Cl_5DF and 2,3,4,7,8-Cl_5DF are of the same order of magnitude as observed for 2,3,7,8-TCDD. With increasing chlorination, receptor-binding affinity decreases. The induction of the cytochrome P_{450} 1A1 enzyme is frequently used as a convenient biomarker for PCDDs/ PCDFs and other dioxin-like compounds.

Cancer promotion

2,3,7,8-TCDD is a multisite carcinogen in animals as well as in humans. TCDD causes liver tumours in animals at lower concentrations than any other man-made chemical. Dioxins are not genotoxic (i.e. do not initiate cancer development), but 2,3,7,8-TCDD and other dioxins and furans are strong promoters of tumour development. TCDD interferes with several functions that probably influence the tumour promotion process, such as growth factors, hormone systems, oxidative damage, intercellular communication, cell proliferation (division and growth), apoptosis (cell death), immune surveillance and cytotoxicity (cellular toxicity).

In all mammalian species tested so far, lethal doses of 2,3,7,8-TCDD result in delayed death preceded by excessive body weight loss ('wasting'). Other signs of 2,3,7,8-TCDD intoxication include thymic atrophy, hypertrophy/hyperplasia of hepatic, gastrointestinal, urogenital and cutaneous epithelia, atrophy of the gonads, subcutaneous oedema and systemic haemorrhage. The lethal dose of 2,3,7,8-TCDD varies more than 5000-fold between the guinea-pig ($LD_{50} = 1\ \mu g\ kg^{-1}$ BW), the most sensitive, and the hamster, the least sensitive species.

In tissue culture, 2,3,7,8-TCDD affects growth and differentiation of keratinocytes, hepatocytes and cells derived from other target organs. Toxicity of 2,3,7,8-TCDD segregates with the AhR, and relative toxicity of other PCDD congeners is associated with their ability to bind to this receptor. PCDDs cause suppression of both cell-mediated and humoral immunity in several species at low doses. PCDDs have the potential to suppress resistance to bacterial, viral and parasitic challenges in mice.

Kinetics

In most vertebrate species, the 2,3,7,8-substituted PCDD and PCDF congeners are predominantly retained; in other words, if chlorine atoms are present on all 2,3,7,8 positions, the biotransformation rate of PCDDs/ PCDFs is strongly reduced, resulting in significant bioaccumulation. In most species the liver and adipose tissue are the major storage sites. Although the parent PCDD/ PCDF congeners cause the biological effects, biotransformation to more polar metabolites should be considered to be a detoxification process. Oxidation by cytochrome P_{450} primarily occurs at the 4 and 6 positions in the molecule, and the presence of chlorine atoms at these positions reduces metabolism more than substitution at the 1 and 9 positions. The half-lives of especially the PCDFs in humans are much longer than those in experimental animals.

2,3,7,8-TCDD is both a developmental and a reproductive toxicant in experimental animals. The developing embryo/fetus appears to display enhanced sensitivity to the adverse effects of PCDDs. Perturbations of the reproductive system in adult animals require overtly toxic doses. In contrast, effects on the developing organism occur at doses more than 100 times lower that those required in the mother. Sensitive targets include the developing reproductive, nervous and immune systems. Perturbation of multiple hormonal systems and their metabolism due to PCDD exposure may play a role in these events.

One effect that has been observed recently is the altered sex ratio (increased females) seen in the 6 years after the accident in Seveso, Italy. Particularly intriguing in this latest evaluation is the observation that exposure before and during puberty is linked to this sex ratio effect. Other sites have been examined for the effect of TCDD exposure on sex ratio with mixed results, but with smaller numbers of offspring (US-EPA, 2000c).

Toxic effects in humans

In humans, effects associated with exposure to dioxins are observed mainly in accidental

and occupational exposure situations. A number of cancer locations, as well as total cancer, have been associated with exposure to dioxins (mostly TCDD). In addition, an increased prevalence of diabetes and increased mortality due to diabetes and cardiovascular diseases have been reported. In children exposed to dioxins and/or PCBs in the womb, effects on neurodevelopment and neurobehaviour (object learning) and effects on thyroid hormone status have been observed at exposures at or near background levels. At higher exposures, children exposed transplacentally to PCBs and PCDFs show skin defects, developmental delays, low birth weight, behaviour disorders, a decrease in penile length at puberty, reduced height among girls at puberty and hearing loss. It is not totally clear to what extent dioxin-like compounds are responsible for these effects, when considering the complex chemical mixtures to which human individuals are exposed. However, it has been recognized that subtle effects might already be occurring in the general population in developed countries, at current background levels of exposure to dioxins and dioxin-like compounds and, due to the high levels of persistence of the dioxin-like compounds, the concentrations in the environment, as well as in food, will only decrease slowly.

There are a number of cohorts with high exposure to PCDDs/PCDFs (and PCBs), e.g. NIOSH (National Institute of Occupational Safety and Health, USA) and Boehringer occupational studies, veterans of Operation Ranch Hand in Vietnam, residents of Seveso, etc. The NIOSH population who were highly exposed for more than 1 year, and with a 20 year latency period, had an increase of all cancers; the Ranch Hand population showed an increase in diabetes with increasing dioxin levels (no other effects seen); Seveso residents had high levels of dioxin and, although the number of births was relatively low for 7 years post-exposure, there were significantly more girls born than boys (change in normal sex ratio). From these results obtained in high-exposure groups, it seems unlikely that clinically observable health effects will be found in the general adult population (Büchert *et al*., 2001).

The PCDD/PCDF pattern in humans may yield information as to different sources. Also, people from certain geographical regions may have specific patterns because of predominant exposures from different sources, e.g. Europeans have higher 2,3,4,7,8-Cl_5DF concentrations compared with US residents (Büchert *et al*., 2001).

For humans, chronic effects are of greater concern than acute toxicity. Amongst the most sensitive end points are reproductive, developmental, immunotoxic and neurotoxic effects. One of the 17 so-called toxic congeners, 2,3,7,8-TCDD, is the most toxic synthetic chemical (LD_{50} for guinea-pigs $= 1\ \mu g\ kg^{-1}$ BW day^{-1}).

Human exposure to 2,3,7,8-TCDD or other PCDD congeners due to industrial or accidental exposure has been associated with chloracne and alterations in liver enzyme levels in both children and adults. Changes in the immune system and glucose metabolism have also been observed in adults. Infants exposed to PCDDs and PCDFs through breast milk exhibit alterations in thyroid hormone levels and possible neurobehavioural and neurological deficits.

Carcinogenicity

Four epidemiological studies of high-exposure industrial cohorts in Germany, The Netherlands and the USA found an increase in overall cancer mortality. Overall, the strongest evidence for the carcinogenicity of 2,3,7,8-TCDD is for all cancers combined, rather than for any specific site. The relative risk for all cancers combined in the most highly exposed and longer latency subcohorts is 1.4.

In these cohorts, the blood lipid 2,3,7,8-TCDD levels estimated to the last time of exposure were 2000 ng kg^{-1} (mean) (up to 32,000 ng kg^{-1}) in the US cohort, 1434 ng kg^{-1} geometric mean (range, 301–3683 ng kg^{-1}) among accident workers in the Dutch cohort, 1008 ng kg^{-1} geometric mean in the group

of workers with severe chloracne in the BASF accident cohort in Germany, and up to 2252 kg^{-1} in the Boehringer cohort in Germany. These calculated blood 2,3,7,8-TCDD levels in workers at time of exposure were in the same range as the estimated blood levels in a 2-year rat carcinogenicity study. In rats exposed to 100 ng kg^{-1} BW 2,3,7,8-TCDD day^{-1}, hepatocellular carcinomas and squamous cell carcinomas of the lung were observed. Estimated blood levels were 5000–10,000 ng kg^{-1} 2,3,7,8-TCDD. In the same study, in rats exposed to 10 ng kg^{-1} BW 2,3,7,8-TCDD day^{-1}, hepatocellular nodules and focal alveolar hyperplasia were observed. Estimated blood levels were 1500–2000 ng kg^{-1} 2,3,7,8-TCDD. These results indicate parallel tumorigenic responses to high exposure to 2,3,7,8-TCDD in both humans and rats.

In view of the results mentioned above, it should be noted that the present background levels of 2,3,7,8-TCDD in human populations (2–3 ng kg^{-1}) are 100–1000 times lower than those observed in this rat carcinogenicity study. Evaluation of the relationship between the magnitude of the exposure in experimental systems and the magnitude of the response (i.e. dose–response relationships) does not permit conclusions to be drawn on the human health risks from background exposures to 2,3,7,8-TCDD (IARC, 1997).

A Working Group for IARC (International Agency for Research on Cancer, Lyon, France) classified 2,3,7,8-TCDD as being carcinogenic to humans (IARC, 1997). In making this overall evaluation, the working group took into consideration the following supporting evidence:

1. 2,3,7,8-TCDD is a multisite carcinogen in experimental animals that has been shown by several lines of evidence to act through a mechanism involving the AhR.
2. This receptor is highly conserved in an evolutionary sense and functions the same way in humans as in experimental animals.
3. Tissue concentrations are similar both in heavily exposed human populations in which an increased overall cancer risk was observed and in rats exposed to carcinogenic dosage regimens in bioassays.

Other PCDDs and non-chlorinated dibenzo-*p*-dioxin are not classifiable as to their carcinogenicity in humans.

The IARC concluded that there is inadequate evidence in humans for the carcinogenicity of PCDFs. There is inadequate evidence in experimental animals for the carcinogenicity of 2,3,7,8-Cl_4DF. There is limited evidence in experimental animals for the carcinogenicity of 2,3,4,7,8-Cl_5DF and 1,2,3,4,7,8-Cl_6DF. The overall evaluation states that PCDFs are not classifiable as to their carcinogenicity in humans (group 3).

In its recent dioxin reassessment, the US-EPA basically follows the IARC classifications (US-EPA, 2000c) and concludes that 'under EPA's current approach, TCDD is best characterized as a "human carcinogen"'. This means that, based on the weight of all of the evidence (human, animal, mode of action), TCDD meets the stringent criteria that allow EPA and the scientific community to accept a causal relationship between TCDD exposure and cancer hazard. The guidance suggests that 'human carcinogen' is an appropriate descriptor of carcinogenic potential when there is an absence of conclusive epidemiological evidence to clearly establish a cause and effect relationship between human exposure and cancer, but there are compelling carcinogenicity data in animals and mechanistic information in animals and humans demonstrating similar modes of carcinogenic action. The 'human carcinogen' descriptor is suggested for TCDD because all of the following conditions are met. Occupational epidemiological studies show an association between TCDD exposure and increases in cancer at all sites, in lung cancer, and perhaps at other sites, but the data are insufficient on their own to demonstrate a causal association. There is extensive carcinogenicity in both sexes of multiple species of animals at multiple sites (IARC, 1997).

Risk Assessment

First risk assessments only focused on the most toxic congener, 2,3,7,8-TCDD. Soon it

was recognized, though, that all PCDDs/PCDFs substituted at least in positions 2, 3, 7 or 8 are highly toxic and thus major contributors to the overall toxicity of the dioxin mixture. In addition, despite the complex composition of many PCDD/PCDF-containing 'sources', only congeners with substitutions in the lateral positions of the aromatic ring, namely the carbon atoms 2, 3, 7 and 8, persist in the environment and accumulate in food chains.

For regulatory purposes so-called toxicity equivalency factors (TEFs) have been developed for risk assessment of complex mixtures of PCDDs/PCDFs (NATO/CCMS, 1988). The TEFs are based on acute toxicity values from *in vivo* and *in vitro* studies. This approach is based on the evidence that there is a common, receptor-mediated mechanism of action for these compounds. Although the scientific basis cannot be considered as solid, the TEF approach has been adopted as an administrative tool by many agencies and allows conversion of quantitative analytical data for individual PCDD/PCDF congeners into a single TEQ. As TEFs are interim values and administrative tools, they are based on the present state of knowledge and should be revised as new data become available. Today's most commonly applied TEFs were established by a NATO/CCMS Working Group on Dioxins and Related Compounds as international toxicity equivalency factors (I-TEFs) (NATO/CCMS, 1988). However, in 1997, a WHO/IPCS (World Health Organization/Intergovernmental Programme on Chemical Safety) working group re-evaluated the I-TEFs and established a scheme, which besides human and mammalian TEFs, also established TEFs for birds and fish (Table 7.6). The same expert group also assessed the dioxin-like toxicity of PCB and assigned TEF values for 12 co-planar and mono-*ortho*-substituted PCB congeners (see Table 7.7) (WHO, 1997).

It should be noted that most existing legislation and most assessments still use the I-TEF scheme. However, the recently agreed Stockholm Convention on POPs (for reference see UNEP, 2001) refers to the combined WHO-TEFs as the starting point as a reference.

Different international expert groups have performed health risk assessment of

Table 7.6. International toxicity equivalency factors (I-TEFs) for PCDDs/PCDFs (NATO/CCMS, 1988) and WHO-TEFs for PCDDs/PCDFs (WHO, 1997).

		WHO-TEF		
Congener	I-TEF	Humans/mammals	Fish	Birds
2,3,7,8-Cl_4DD	1	1	1	1
1,2,3,7,8-Cl_5DD	0.5	1	1	1
1,2,3,4,7,8-Cl_6DD	0.1	0.1	0.5	0.05
1,2,3,7,8,9-Cl_6DD	0.1	0.1	0.01	0.01
1,2,3,6,7,8-Cl_6DD	0.1	0.1	0.01	0.1
1,2,3,4,6,7,8-Cl_7DD	0.01	0.01	0.001	< 0.001
Cl_8DD	0.001	0.0001	—	—
2,3,7,8-Cl_4DF	0.1	0.1	0.05	1
1,2,3,7,8-Cl_5DF	0.05	0.05	0.05	0.1
2,3,4,7,8-Cl_5DF	0.5	0.5	0.5	1
1,2,3,4,7,8-Cl_6DF	0.1	0.1	0.1	0.1
1,2,3,7,8,9-Cl_6DF	0.1	0.1	0.1	0.1
1,2,3,6,7,8-Cl_6DF	0.1	0.1	0.1	0.1
2,3,4,6,7,8-Cl_6DF	0.1	0.1	0.1	0.1
1,2,3,4,6,7,8-Cl_7DF	0.01	0.01	0.01	0.01
1,2,3,4,7,8,9-Cl_7DF	0.01	0.01	0.01	0.01
Cl_8DF	0.001	0.0001	0.0001	0.0001

For all non-2,3,7,8-substituted congeners, no TEF has been assigned.

Table 7.7. TEFs for PCBs (WHO, 1997).

Congener	Humans/mammals	Fish	Birds
3,4,4′,5-TCB (81)	0.0001	0.0005	0.1
3,3′,4,4′-TCB (77)	0.0001	0.0001	0.05
3,3′,4,4′,5-PeCB (126)	0.1	0.005	0.1
3,3′,4,4′,5,5′-HxCB (169)	0.01	0.00005	0.001
2,3,3′,4,4′-PeCB (105)	0.0001	< 0.000005	0.0001
2,3,4,4′,5-PeCB (114)	0.0005	< 0.000005	0.0001
2,3′,4,4′,5-PeCB (118)	0.0001	< 0.000005	0.00001
2′,3,4,4′,5-PeCB (123)	0.0001	< 0.000005	0.00001
2,3,3′,4,4′,5-HxCB (156)	0.0005	< 0.000005	0.0001
2,3,3′,4,4′,5′-HxCB (157)	0.0005	< 0.000005	0.0001
2,3′,4,4′,5,5′-HxCB (167)	0.00001	< 0.000005	0.00001
2,3,3′,4,4′,5,5′-HpCB (189)	0.0001	< 0.000005	0.00001

dioxins and related compounds. A Nordic expert group (for Scandinavian countries) proposed a TDI for 2,3,7,8-TCDD and structurally similar chlorinated PCDDs and PCDFs of 5 pg kg^{-1} BW, based on experimental studies on cancer, reproduction and immunotoxicity. A first WHO meeting in 1990 established a TDI of 10 pg kg^{-1} BW for 2,3,7,8-TCDD, based on liver toxicity, reproductive effects and immunotoxicity, and making use of kinetic data in humans and experimental animals. Since then, new epidemiological and toxicological data have emerged, in particular with respect to neurodevelopmental and endocrinological effects. In May 1998, a joint WHO–European Centre for Environment and Health (ECEH) and IPCS expert group re-evaluated the old TDI and came up with a new TDI (which is a range) of 1–4 pg TEQ kg^{-1} BW, which includes all 2,3,7,8-substituted PCDDs and PCDFs as well as dioxin-like PCBs (for reference, see the 12 PCBs in Table 7.7). The TDI is based on the most sensitive adverse effects, especially hormonal, reproductive and developmental effects, which occur at low doses in animal studies, e.g. in rats and monkeys at body burdens in the range of 10–50 ng kg^{-1} BW. Human daily intakes corresponding to body burdens similar to those associated with adverse effects in animals were estimated to be in the range of 10–40 pg kg^{-1} BW day^{-1}. The 1998 WHO-TDI does not apply an uncertainty factor to account for interspecies differences in toxicokinetics since body burdens have been used to scale doses across species. However, the estimated human intake was based on lowest observed adverse effect levels (LOAELs) and not on no observed adverse effect levels (NOAELs). For many end points, humans might be less sensitive than animals; uncertainty still remains regarding animal to human extrapolations. Further, differences between animals and humans exist in the half-lives for the different PCDD/PCDF congeners. To account for all these uncertainties, a composite uncertainty factor of 10 was recommended. As subtle effects might already be occurring in the general population in developed countries at current background levels of exposure to dioxins and related compounds, the WHO expert group recommended that every effort should be made to reduce exposure to below 1 pg TEQ kg^{-1} BW day^{-1} (WHO, 1998).

In November 2000, the Scientific Committee on Food (SCF) for the European Commission recommended a temporary tolerable weekly intake (t-TWI) of 7 pg 2,3,7,8-TCDD kg^{-1} BW using the body weight approach. It was also concluded that the TEQ approach should be applied to include all 2,3,7,8-substituted PCDDs/PCDFs and dioxin-like PCBs. Thus, the t-TWI of 7 pg TEQ kg^{-1} BW day^{-1} is applicable for these compounds (seven PCDDs, ten PCDFs and 12 PCBs). The t-TWI is based on the most sensitive end points from animal studies, e.g. developmental and reproductive effects in rats and monkeys and endometriosis in monkeys (SCF, 2000).

When compared with adults, breast-fed infants are exposed to higher intakes of PCDDs, PCDFs and PCBs on a body weight basis, although for a limited time only. Despite the higher exposure to contaminants, the WHO, like other agencies noted the beneficial effects associated with breast feeding and therefore promote and support breast feeding. Further, the subtle effects detected in infants were associated with transplacental rather than lactational exposure (WHO, 1998).

Risk Management

As PCDDs and PCDFs have never been produced intentionally, their production and use cannot be regulated by chemical legislation and a prohibition of production. Indirect measures have to be taken by, for example, banning production and use of chemicals that are known to be contaminated with PCDDs/PCDFs and taking measures to reduce emissions into the environment from known sources of dioxins and furans (see next section).

The SCF concluded that, although dioxin source reduction has been accomplished successfully in many European countries, a considerable proportion of the European population still exceeds the t-TWI. Therefore, further measures are needed to limit environmental releases of PCDDs/PCDFs and dioxin-like compounds (SCF, 2000).

The recent incidents of food and feed contamination have shown that present regulation is non-existent or inadequate, and a root cause analysis is required to develop appropriate monitoring, prevention and management. Setting feed and food limits alone will not prevent further accidents and there is no way to exclude the possibility of similar incidents occuring in the future unless specific measures are taken. However, regulatory levels would build the legal basis at least to eliminate products with extraordinary contamination levels from the market.

Monitoring of the animal feed production chain could mitigate impacts and identify causes. In contrast to former dioxin cases, which mainly originated from high emissions of individual sources, recent incidents have been caused by entry of contaminants more directly into the human food chain. Dealing with these accidents, there are mainly three distinct objectives to address. These require different approaches for assessment, prevention, monitoring and regulatory response (Büchert *et al*., 2001):

- identification and response to an emergency situation of an acute contamination (e.g. the Belgian case);
- identification and seizure of products with exceptionally high levels (e.g. the citrus pellet, choline chloride and Brandenburg cases) which can even affect the general population if used to a large extent in the feed and food chains;
- measures aiming to reduce exposure of the general population by ceasing use of feed ingredients that are more highly contaminated than comparable components (e.g. fish meal and fish oil from the northern hemisphere).

Each case should be addressed carefully and it should be recognized that solutions for one case will not necessarily prove effective for others.

Legislation/Regulatory Issues

Several countries have taken action to reduce exposure to dioxins and furans and, in many places, especially in industrialized countries of the northern hemisphere, environmental concentrations of PCDDs/PCDFs are decreasing. Legislation includes establishment of limit values for stack emissions, e.g. for waste incinerators and other industrial plants, limit values for pulp mill effluents, limit values for sewage sludge spread on agricultural land or guidelines, for example, for soil uses. In Europe, emissions limits for incineration processes are usually set on the basis of stack gas converted to normal temperature and pressure (273 K,

101.3 kPa), dry gas, and expressed at 11% oxygen. In the USA, the convention often uses a reference oxygen level of 7% and temperature of 298 K. These differences can be very important, e.g. the emission limit for a European incinerator of 0.1 ng I-TEQ Nm^{-3} (per normal cubic metre) dry gas at 11% oxygen is equivalent to approximately 0.13 ng I-TEQ $dscm^{-1}$ (per dry standard cubic metre) at 7% oxygen as specified under the US regulation.

Within the food and feedingstuff regulations, the only recommended limit values exist for dairy products. To keep the agricultural food chain free of dioxins and furans, an EU Directive sets a maximum tolerance level of 500 pg WHO-TEQ kg^{-1} for citrus pellets and for lime used as additive in animal feed production. The same limit also applies to the maximum limit for dioxin content of 500 pg WHO-TEQ kg^{-1} for most additives belonging to the group 'binders, anti-caking agents and coagulants'. Lastly, as a secondary measure, the use of 'wood, sawdust and other materials derived from wood treated with wood protection products' is prohibited in compound feedingstuffs (see Table 7.8).

Many countries have established guideline values for various foods or food categories. Table 7.9 shows present regulations for PCDDs/PCDFs and, for comparison and completeness, for PCBs in foods for European countries.

Conclusions

Many actions taken since the late 1980s have resulted in a reduction of the daily intake of dioxins and furans for many European countries. However, recent accidents have shown the vulnerability of the food chain to contamination with these compounds. There are strong indications that the citrus pellet contamination has reversed the former downward trend in body burden on a broad basis all over Europe. Special attention has to be paid to the high intake of dioxins and furans for infants, which is still in a range that poses risk to the developing organism.

Exposure issues relating to dioxins (and other contaminants) should not be considered in isolation. As shown in Scandinavia, the Finnish population is eating more fish, which has contributed to an important improvement in cardiovascular disease prevention, although this may seem inadvisable from a PCDD/PCDF/PCB exposure point of view as these fish can be highly contaminated with dioxins and other lipophilic contaminants. The relative risk is important and needs to be considered, and knee-jerk reactions should be avoided. Also, reduction of dioxin emissions should be seen in context and must occur together with the controls of other pollutants and contaminants, whether chemical, residues or pathological. Lessons learned in this field must be used to improve understanding in other fields, and vice versa.

Table 7.8. EU directives addressing PCDD/PCDF in feedingstuffs.

EU Directive	Description
98/60/EC Citrus pulp pellets as feedingstuffs (Amendment to 74/63/EEC)	Sets an upper-bound detection limit of 500 pg I-TEQ kg^{-1}; in force since 1 July 1998
2439/1999/EC and 739/2000/EC	Maximum limit for dioxin content of 500 pg WHO-TEQ kg^{-1} for most additives belonging to the group 'binders, anti-caking agents and coagulants' (applies from 1 March 2000; to be re-examined before October 2000)
91/516/EC	The use of 'wood, sawdust and other materials derived from wood treated with wood protection products'[a] is prohibited in compound feedingstuffs

[a]Wood preservatives may contain high concentrations of PCDDs/PCDFs, e.g. PCPs, other chlorophenols, chlorobenzenes.

Table 7.9. Guidelines and maximum levels for concentrations of PCDDs, PCDFs and PCBs in foods in European countries.

	Foodstuffs of animal origin	
Country	PCDDs and PCDFs	PCBs
Austria	Provisional limits WHO-TEQ (PCDD/PCDF) g^{-1} fat: pork 2, milk 3, poultry and eggs 5 and beef 6 pg	
Belgium	Milk, bovine, poultry, animal fats and oils, eggs and derived products, if > 2% fat: 5 pg WHO-TEQ (PCDD/PCDF) g^{-1} fat Pork and derived products, if > 2% fat: 3 pg WHO-TEQ (PCDD/PCDF) g^{-1} fat	For the sum of PCBs 28, 52, 101, 118, 138, 153 and 180 Milk and derived products, if > 2% fat: 100 ng g^{-1} fat Bovine, pork, poultry, animal fats and oils, eggs and derived products, if > 2% fat: 200 ng g^{-1} fat
Denmark	No national limits	No national limits
Finland	No national limits	No national limits
France	Milk and dairy products: 5 pg g^{-1} fat	No national limits
Germany	Recommendations for milk and dairy products in pg I-TEQ g^{-1} milk fat: • < 0.9 (desirable target) • 3.0 (identification of sources; measures to reduce input; recommendations for land use; recommendation to stop direct supply of milk products to consumers) • > 5.0 (ban on trade of contaminated milk products)	Congener-specific limits for PCBs 28, 52, 101, 138, 153 and 180 in foods of animal origin: 0.008–0.6 mg kg^{-1} fat or whole weight basis
Greece	No national limits	No national limits
Ireland	International norms	International norms
Italy	No national limits	Action level for the sum of tri- to octachlorobiphenyls in various foods of animal origin (excluding freshwater and marine fish and derived products): 100 ng g^{-1} fat
Luxemburg	Recommended: pork 2, beef 6, poultry 5, milk 3 and eggs 5 pg g^{-1} fat	
Norway	No national limits	No national limits
Portugal	No national limits	No national limits
Spain	Levels > 5 pg g^{-1} fat are considered as non-acceptable in dairy products	No national limits
Sweden	No national limits	PCB 153: meat products > 10% fat: 0.1, milk and milk products > 2% fat: 0.02, and eggs: 0.1 mg kg^{-1} fat Meat products < 10% fat: 0.01, milk and milk products < 2% fat: 0.001, and fish: 0.1 mg kg^{-1} wet weight
The Netherlands	Dairy products and foods with milk or dairy product as ingredients: 6 pg TEQ g^{-1} fat	Congener-specific limits for PCBs 28, 52, 101, 118, 138, 153 and 180 in foods of animal origin: 0.02–2 mg kg^{-1} fat (for fish mg kg^{-1} wet weight)
UK	Guideline for cows' milk: 0.66 ng WHO-TEQ kg^{-1} whole milk (16.6 ng WHO-TEQ kg^{-1} fat)	

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8 Polycyclic Aromatic Hydrocarbons in Diverse Foods

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants which represent a very important group of carcinogens or co-carcinogens. They are found in coal, asphaltic rocks and petroleum, and are also formed by the incomplete combustion of organic matter, that is to say by the incomplete combustion of some of the above materials, as well as that of proteins, lipids and carbohydrates. In addition, it has been suggested that these compounds could be synthesized during the metabolic processes of plants, seaweed and bacteria.

Due to industrial and engine combustion emissions and other processes, these compounds contaminate air, water and soil, and so are passed on to foods; they can also be generated during incorrect food processing and cooking. As a result, they are present in both unprocessed and processed foods.

The relationship between exposure to combustion emissions and carcinogenicity in humans has been known for a long time. It was noticed by Pott in 1775 with regard to skin cancer in chimney sweeps. Afterwards, both the observation of a higher frequency of cancer in human groups whose diets were rich in smoked foods and studies showing the carcinogenicity of some PAHs in animals were the starting point for many studies of these compounds, some aspects of which have been reviewed (Howard and Fazio, 1980; Guillén, 1994; Shaw and Connell, 1994; Guillén *et al*., 1997).

Nature of Polycyclic Aromatic Hydrocarbons

PAHs are a very numerous group of compounds formed by fused aromatic rings made up of carbon and hydrogen atoms, the most simple of which is naphthalene. The number of PAHs is very large and, furthermore, these compounds can either be partially hydrogenated or have alkyl substituents. There are other compounds with fused aromatic rings in the molecule, which also include heteroatoms and other functional groups, such as amine, phenol or nitro groups; these latter, together with PAHs, constitute a wider group named polycyclic aromatic compounds.

PAHs have been classified in two classes: *peri*- and *cata*-condensed. *Peri*-condensed PAHs can be defined as those systems whose

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graphs, or lines which connect the ring centres, form cycles; these can be subdivided further into two classes: alternants, which are formed exclusively by six-membered rings, and non-alternants, which include some five-membered rings. *Cata*-condensed PAHs can be defined as those systems whose graphs do not form cycles, and can be classified further as branched or not branched, the former being thermodynamically more stable and chemically less reactive than non-branched systems of the same size. *Cata*-condensed PAHs are always alternant systems. Figure 8.1 shows some examples.

In PAH topology, some regions and carbon atom positions have been related to biological activity: the K region defined as the external corner of a phenanthrenic moiety; the L region consisting of a pair of opposed open anthracenic point atoms; the 'bay' region defined as an open inner corner of a phenanthrenic moiety; the distal bay region also known as the M region; and the *peri* position, which corresponds to the carbon atom opposite the bay region and adjacent to the angular ring. Figure 8.2 shows these regions and the *peri* position in the benz(*a*)anthracene molecule.

Table 8.1 names and gives formulae, structures, molecular weights, boiling points and some other properties such as water solubility and octanol/water partition coefficient of some of the PAHs most frequently studied in foods. All these compounds are solid at room temperature; their boiling points are high and their volatility is low. They are lipophilic, so their water solubility is low and their octanol/water partition coefficients are fairly high; these two latter properties have been related to their biological activity.

Compound	Formula	Graph	Characteristics
	Peri-condensed		
Pyrene			cycle alternant
Fluoranthene			cycle non-alternant
	Cata-condensed		
Chrysene			linear non-branched alternant
Triphenylene			linear branched alternant

Fig. 8.1. Different types of structures in PAH.

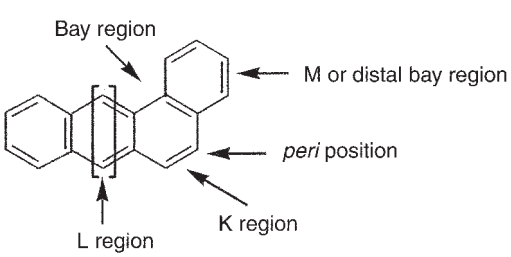
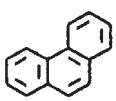
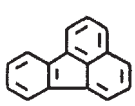
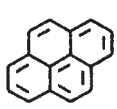
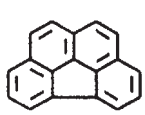
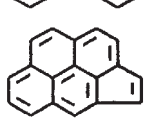
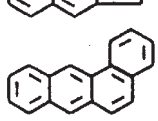
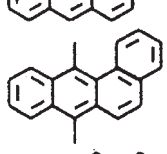
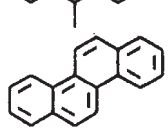
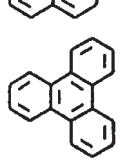
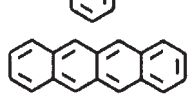


Fig. 8.2. Regions related to biological activity.

Table 8.1. Some of the PAHs most frequently studied in foods: structure, molecular weight and other properties.

Nomenclature	Formula	Structure	MW[a]	BP[b] (°C)	WS[c] (μg l^{-1})	PCow[d]
Naphthalene	$C_{10}H_8$		128	218	31,700.0	3.37
Acenaphthylene	$C_{12}H_8$		152	270	16,100.0	3.92
Phenanthrene	$C_{14}H_{10}$		178	338	1,290.0	3.24
Anthracene	$C_{14}H_{10}$		178	340	73.0	4.54
Fluoranthene	$C_{16}H_{10}$		202	383	260.0	5.22
Pyrene	$C_{16}H_{10}$		202	393	135.0	5.18
Benzo(*ghi*)fluoranthene	$C_{18}H_{10}$		226	432		
Cyclopenta(*cd*)pyrene	$C_{18}H_{10}$		226	439		
Benz(*a*)anthracene	$C_{18}H_{12}$		228	435	14.0	5.91
7,12-Dimethylbenz(*a*)anthracene	$C_{20}H_{16}$		256		61.0	6.00
Chrysene	$C_{18}H_{12}$		228	441	2.0	5.61
Triphenylene	$C_{18}H_{12}$		228	439	43.0	5.49
Naphthacene	$C_{18}H_{12}$		228	450	0.6	5.76

continued

Table 8.1. *Continued.*

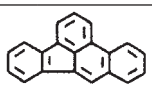
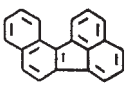
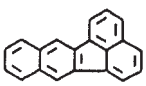
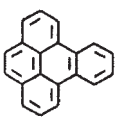
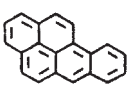
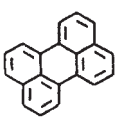
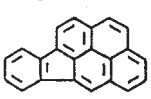
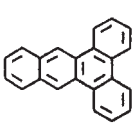
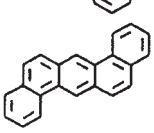
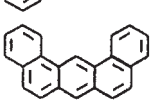
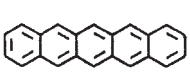
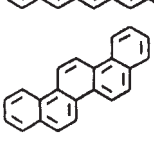
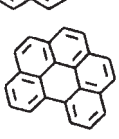
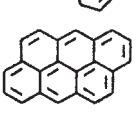
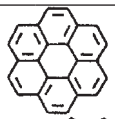
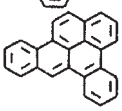
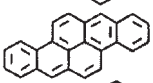
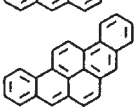
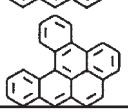
Nomenclature	Formula	Structure	MW[a]	BP[b] (°C)	WS[c] (μg l^{-1})	PCow[d]
Benzo(*b*)fluoranthene	$C_{20}H_{12}$		252	481	1.5	5.8–6.1
Benzo(*j*)fluoranthene	$C_{20}H_{12}$		252	480	2.5	6.12
Benzo(*k*)fluoranthene	$C_{20}H_{12}$		252	481	0.8	6.0–6.8
Benzo(*e*)pyrene	$C_{20}H_{12}$		252	493	4.0	
Benzo(*a*)pyrene	$C_{20}H_{12}$		252	496	4.0	6.04
Perylene	$C_{20}H_{12}$		252	495	0.4	
Indeno(1,2,3-*cd*)pyrene	$C_{22}H_{12}$		276	536		6.58
Dibenz(*a*,*c*)anthracene	$C_{22}H_{14}$		278		1.6	
Dibenz(*a*,*h*)anthracene	$C_{22}H_{14}$		278	524	0.6	6.50
Dibenz(*a*,*j*)anthracene	$C_{22}H_{14}$		278		12.0	
Pentacene	$C_{22}H_{14}$		278			
Picene	$C_{22}H_{14}$		278	519		
Benzo(*ghi*)perylene	$C_{22}H_{12}$		276		0.3	6.50
Anthanthrene	$C_{22}H_{12}$		276	547		

Table 8.1. *Continued.*

Nomenclature	Formula	Structure	MW[a]	BP[b] (°C)	WS[c] (μg l^{-1})	PCow[d]
Coronene	$C_{24}H_{12}$		300	525	0.1	6.75
Dibenzo(*a*,*e*)pyrene	$C_{24}H_{14}$		302			
Dibenzo(*a*,*h*)pyrene	$C_{24}H_{14}$		302			
Dibenzo(*a*,*i*)pyrene	$C_{24}H_{14}$		302			
Dibenzo(*a*,*l*)pyrene	$C_{24}H_{14}$		302			

[a]Molecular weight; [b]boiling point; [c]water solubility; [d]octanol/water partition coefficient.
Data are taken from Mackay and Shiu (1977), Bjorseth (1983) and Dabestani and Ivanov (1999).

Distribution of PAHs in Foods

Before beginning a description of the results obtained by several authors concerning distribution of PAHs in foods, some aspects should be considered. First, PAHs are generated as complex mixtures, so the presence of only one PAH in contaminated foods is not common. However, in some studies of the ocurrence of PAHs in foods, only the concentration of benzo(*a*)pyrene has been determined, because this compound is considered an indicator of other PAHs. Others study only those PAHs whose analysis is recommended by international organizations such as WHO (World Health Organization) or EPA (the US Environmental Protection Agency); other studies concern all those PAHs found in the food sample.

Secondly, it should be pointed out that the methodology used for the extraction, clean-up, separation, identification and quantification of PAHs in food samples decisively influences the results obtained. For this reason, all data and comments on distribution of PAHs in foods should be seen in the context of each study.

The ocurrence of PAHs in foods of vegetable origin is due basically to environmental contamination, especially of air and soil. In fruits and vegetables, the concentrations of PAHs detected vary considerably depending on the food surface/weight ratio, time of exposure, proximity to contamination source and level of contamination in the air. Fruits and vegetables growing in regions free of contamination are also free of PAHs. Furthermore, grilled vegetables show higher concentrations than raw vegetables (Tateno *et al.*, 1990). Table 8.2 gives the concentrations of PAHs found in lettuce growing at different distances from a highway, as well as in raw and grilled vegetables.

Detected PAH levels are not high in raw cereals and beans, and contamination is due basically to aerial deposition (Jones *et al.*, 1989); this is in agreement with the ocurrence of PAHs in higher concentrations in bran than in flour (Dennis *et al.*, 1991). Drying techniques used in some countries for cereal preservation, such as direct combustion gas heating, can increase their PAH concentrations. Likewise, cereal and bean smoking or toasting also contribute to the level of PAHs (Klein *et al.*, 1993). Table 8.2 gives concentrations found in raw wheat grains, white flour and bran, as well as in raw and toasted coffee bean samples.

Raw sugarcane does not contain PAHs but, in some countries, sugarcane plantations

Table 8.2. PAH concentrations (μg kg^{-1} dry weight) in several foods of vegetable origin.[a]

Compound	L_{50S}	L_{12S}	WG_{UK}	WF_{UK}	B_{UK}	RC_G	TC_G	RV_J	TV_J
Phenanthrene	5.0	7.5	–	–	–	–	–	2.2	3.9
Anthracene	0.2	0.3	–	–	–	–	–	0.1	0.1
1-Methylphenanthrene	0.6	1.6	–	–	–	–	–	0.0	n.d.[b]
2-Methylphenanthrene	0.7	1.6	–	–	–	–	–	–	–
9-Methylanthracene	–	–	–	–	–	–	–	n.d.	0.0
Fluoranthene	5.3	9.1	0.6	0.2	0.7	8.0	14.3	1.3	0.8
Pyrene	5.8	10.4	0.4	0.5	0.1	8.1	16.3	0.3	0.6
Benzo(*ghi*)fluoranthene	–	–	0.0	–	–	–	–	–	–
Cyclopenta(*cd*)pyrene	–	–	0.1	–	–	–	–	–	–
Benz(*a*)anthracene	0.9	4.6	0.2	0.1	0.7	0.3	1.2	0.0	0.4
Chrysene	3.3[c]	7.1[c]	0.8[c]	0.1	0.8	1.8[c]	2.6[c]	–	–
Benzo(*b*)fluoranthene	0.6	7.3	0.6[d]	0.0	0.3	1.9[d]	1.4[d]	–	–
Benzo(*k*)fluoranthene	0.4[e]	6.1[e]		0.1	0.5			0.0	0.0
Benzo(*e*)pyrene	0.8	6.7	0.3	0.2	0.4	0.8	0.6	0.2	0.2
Benzo(*a*)pyrene	0.5	6.2	0.3	0.1	0.4	0.9	0.8	0.1	0.7
Perylene	0.0	1.7	–	n.d.	–	0.3	0.2	0.0	1.3
Indeno(1,2,3-*cd*)pyrene	0.6	8.3	0.3	0.1	1.1	0.5	0.4	–	–
Dibenz(*a*,*c*)anthracene	–	–	–	–	–	0.1[f]	0.0[f]	n.d.	1.6
Dibenz(*a*,*h*)anthracene	–	–	0.0	0.0	0.1			n.d.	0.1
Benzo(*ghi*)perylene	0.5	10.8	0.3	0.1	0.5	0.6	0.6	–	–
Coronene	–	–	0.1	–	–	–	–	0.2	5.0

[a]L_{50S}, lettuce grown at 50 m from a Swedish highway (μg kg^{-1} fresh weight) (Larsson and Sahlberg, 1981); L_{12S}, lettuce grown at 12 m from a Swedish highway (μg kg^{-1} fresh weight) (Larsson and Sahlberg, 1981); WG_{UK}, wheat grain from Broadbalk (UK) (Jones *et al.*, 1989); WF_{UK}, wheat flour (Dennis *et al.*, 1991); B_{UK}, bran (Dennis *et al.*, 1991); RC_G, raw coffee (Klein *et al.*, 1993); TC_G, toasted coffee (Klein *et al.*, 1993); RV_J, raw vegetables (Tateno *et al.*, 1990); TV_J, toasted vegetables (Tateno *et al.*, 1990).

[b]n.d., not detected

[c]Concentration of chrysene + triphenylene.

[d]Concentration of benzo(*b*)fluoranthene + benzo(*j*)fluoranthene + benzo(*k*)fluoranthene.

[e]Concentration of benzo(*j*)fluoranthene + benzo(*k*)fluoranthene.

[f]Concentration of dibenz(*a*,*c*)anthracene + dibenz(*a*,*h*)anthracene.

are usually set alight before harvesting, contaminating the sugarcane, then the unrefined sugar, and so the sugarcane spirits (Serra *et al.*, 1995). Sugar refining may contribute to avoiding this contamination. The contamination level in nuts, roots and tubers is low (Dennis *et al.*, 1991).

Although there are hardly any studies of PAH contamination in oilseeds and olives (Dennis *et al.*, 1991), oils from different vegetable sources such as virgin and refined olive oil, sunflower, soybean, maize, coconut, rapeseed, cotton, groundnut, grapeseed, rice, palm and palm kernel oils, cocoa butter, as well as other commodities derived from vegetable oil such as margarines, cream substitutes and some infant formulae powders have been widely studied. The presence of these contaminants in oils, discarding the biosynthetic route, can be attributed both to environmental contamination, basically from the air, and to contamination during processing. Oilseeds are sometimes dried directly by combustion gases, which causes contamination; this is the case with copra and grapeseeds. In addition, the possibility of contamination by PAHs contained in the organic solvent used in the oil extraction process has been commented on. The way to reduce the PAH level in these foods is by means of the refining process, especially if activated charcoal is used in the bleaching step. Table 8.3 gives the results of several oil studies, and great variations in PAH content in the different samples

Table 8.3. PAH concentration (μg kg^{-1}) in several vegetable oil and fat samples.[a]

Compound	VO	O	S	So	G	CC	RC	Mc
Acenaphthylene	–	–	4.4	0.9	–	–	–	–
Phenanthrene	15.3	4.7	2.3	2.2	–	970.0	2.8	6.0
Anthracene	0.9	–	0.0	2.1	–	200.0	0.3	0.9
1-Methylphenanthrene	–	0.9	–	–	–	120.0	2.5	1.8
2-Methylphenanthrene	–	1.0	–	–	–	140.0	1.7	1.3
2-Methylanthracene	–	–	–	–	–	60.0	0.6	0.3
4,5-Methylphenanthrene	–	–	–	–	–	59.0	1.5	–
Fluoranthene	4.2	0.4	6.7	8.9	17.1	520.0	18.0	9.0
Pyrene	5.0	2.1	5.0	2.6	7.2	440.0	20.0	15.0
1-Methylpyrene	–	–	–	–	–	38.0	3.6	2.9
Benz(*a*)anthracene	0.2	2.8[b]	3.1	21.9	78.5	76.0	1.3	21.0[b]
Chrysene	0.5		1.7[c]	17.3[c]	63.3[c]	120.0[c]	4.1[c]	
Benzo(*b*)fluoranthene	0.1	–	2.2	24.8	85.3	55.0	0.7	4.5[d]
Benzo(*k*)fluoranthene	0.1	–	2.0[e]	27.6[e]	98.8[e]	–	–	
Benzo(*e*)pyrene	–	–	4.1	25.2	87.6	20.0	0.4	1.8
Benzo(*a*)pyrene	0.0	–	1.5	28.4	105.7	22.0	0.2	2.2
Perylene	–		0.6	10.0	36.2	6.3	< 0.1	0.6
Indeno(1,2,3-*cd*)pyrene	0.2		1.3	22.8	80.6	9.8	< 0.1	0.7
Dibenz(*a*,*c*)anthracene	–	0.0[f]	0.0[f]	4.7[f]	12.9[f]	–	–	0.2[f]
Benzo(*ghi*)perylene	0.0	0.6	1.7	16.9	65.7	9.6	< 0.1	0.7
Coronene	–	–	0.3	2.1	7.4	–	–	0.2

[a]VO, virgin olive oil (Moret *et al.*, 1997); O, olive oil (Hopia *et al.*, 1986); S, refined sunflower oil (Kolarovic and Traitler, 1982); So, refined soybean oil (Kolarovic and Traitler, 1982); G, refined groundnut oil (Kolarovic and Traitler, 1982); CC, crude coconut oil (Larsson *et al.*, 1987); RC, refined coconut oil (Larsson *et al.*, 1987); Mc, cooking margarine (Hopia *et al.*, 1986).
[b]Concentration of benz(*a*)anthracene + chrysene + triphenylene.
[c]Concentration of chrysene + triphenylene.
[d]Concentration of benzo(*b*)fluoranthene + benzo(*j*)fluoranthene + benzo(*k*)fluoranthene.
[e]Concentration of benzo(*j*)fluoranthene + benzo(*k*)fluoranthene.
[f]Concentration of dibenz(*a*,*c*)anthracene + dibenz(*a*,*h*)anthracene.

have been observed. The high PAH concentrations in some oil samples is a cause for concern because vegetable oils and fats are ingredients in a great number of manufactured foods.

Another group of foods of concern comes from the aquatic environment. Studies on the ocurrence of PAHs in oysters, mussels, fish, shellfish and other marine organisms such as seals and sea lions have been made, and great variations have also been found, due both to the level of contamination where these organisms grow and to their ability to metabolize PAHs. Molluscs and fish accumulate light PAHs to a similar degree; however, heavy PAHs seem to accumulate more in molluscs than in fish. These facts can be observed in Table 8.4, which gives PAH content data for oyster (Sanders, 1995) and fish (Vassilaros *et al.*, 1982) samples, coming from two very differently contaminated places, as well as of mussels and fish, coming from the same place (Baumard *et al.*, 1998). The known ability of some seafoods to accumulate PAHs is why the concentration of PAHs in these organisms has been considered as an indicator of the contamination of their habitat. However, these same organisms can release, in a short period of time, their accumulated PAHs if they are transferred to clean water; this fact should be taken into account for lowering their PAH levels. In addition, processing and cooking techniques such as smoking and grilling can contribute to increasing the PAH levels of these commodities. Table 8.4 also gives PAH concentrations of fresh and smoked fish samples.

Table 8.4. PAH concentrations (μg kg^{-1} dry weight) in oyster, mussel, and fresh and smoked fish samples.[a]

Compound	O_{MP}	O_{OC2}	CF_{BR}	CF_{BL}	M_{FB}	F_{FB}	FF	SF
Methylnaphthalene	–	–	6.0	5.0	–	–	–	–
Dimethylnaphthalene	–	–	100.0	17.0	–	–	–	–
Acenaphthylene	–	–	270.0	n.d.[b]	–	–	–	–
Phenanthrene	76.0	18.0	2700.0	2.0	10.4	19.2	n.d.	61.8
Anthracene	30.0	6.0	–	–	1.1	0.8	–	–
Fluoranthene	680.0	32.0	1800.0	4.0	5.7	25.8	18.8	20.7
Pyrene	407.0	n.d.	1500.0	4.0	4.7	35.1	83.0	117.0
Benz(*a*)anthracene	228.0	n.d.	22.0	n.d.	1.8	0.5	0.4	4.5
Chrysene	261.0	n.d.	–	6.0	5.4[c]	2.1[c]	175.0	290.0
Triphenylene	–	–	–	–			–	–
Benzo(*b*)fluoranthene	53.0	n.d.	–	–	2.8[d]	1.7[d]	11.5	30.0
Benzo(*k*)fluoranthene	161.0	n.d.	–	–			0.3	1.0
Benzo(*j*)fluoranthene	–	–	–	–			–	–
Benzo(*e*)pyrene	–	–	14.0	n.d.	2.2	0.8	–	–
Benzo(*a*)pyrene	31.0	n.d.	7.0	n.d.	0.7	1.3	44.0	48.0
Perylene	–	–	8.0	n.d.	1.0	0.0	–	–
Indeno(1,2,3-*cd*)pyrene	–	–	–	–	1.2	0.0	–	–
Dibenzanthracene	–	–	–	–	0.1[e]	0.0[e]	4.9	8.0
Benzo(*ghi*)perylene	41.0	n.d.	–	–	1.8	0.0	149.0	201.0

[a]O_{MP}, oyster, *Crassostrea virginica*, from MP-Murrells Inlet (South Carolina) (Sanders, 1995); O_{OC2}, oyster, *Crassostrea virginica*, from OC2-Murrells Inlet (South Carolina) (Sanders, 1995); CF_{BR}, catfish, *Ictalurus nebulosus*, from Black River (Ohio) (Vassilaros *et al*., 1982); CF_{BL}, catfish, *I. nebulosus*, from Buckeye Lake (Vassilaros *et al*., 1982); M_{FB}, mussel, *Mytills galloprovincialis*, from Fort Brescou (France) (Baumard *et al*., 1998); F_{FB}, fish, *Serranus scriba*, from Fort Brescou (France) (Baumard *et al*., 1998); FF, fresh fish, *Pseudotolithus elongatus*, from Lagos (Nigeria) (Akpan *et al*., 1994); SF, smoked fish, *Pseudotolithus elongatus*, from Lagos (Nigeria) (Akpan *et al*., 1994).
[b]n.d. = not detected.
[c]Concentration of chrysene + triphenylene.
[d]Concentration of benzo(*b*)fluoranthene + benzo(*j*)fluoranthene + benzo(*k*) fluoranthene.
[e]Concentration of dibenz(*a*,*c*)anthracene + dibenz(*a*,*h*)anthracene.

Drinking water may also be contaminated. Atmospheric pollution contaminates the surface of open-air supplies and the runoff from waste deposits may contaminate ground water; in addition, the use of tar-coated water pipes increases PAH levels in water. Levels of benzo(*a*)pyrene up to 1 μg kg^{-1} have been detected in tap water (IARC, 1983).

Foods of animal origin such as meat, fat and lard, milk, butter, cheese and eggs generally do not contain high levels of PAHs. However, recent studies of animal products from contaminated zones show significant levels (Husain *et al*., 1997); this has been detected especially in egg and milk samples, as can be observed in Table 8.5. In addition, some smoking processes, some cooking procedures and some types of heat sources used for cooking contribute to PAH levels in foods. Examples of PAH levels in raw and barbecued beef (Lodovici *et al*., 1995) and in frankfurters grilled on a log fire or fried in a pan (Larsson *et al*., 1983) are given in Table 8.5.

Finally, manufactured foods made up of several ingredients have a PAH content which is a function of the PAH content of each ingredient as well as of the processes involved in their manufacture.

Estimations of PAH intake from food, carried out in several countries such as Austria, Germany, Italy, The Netherlands, the UK, Sweden and the USA, range from 0.1 to 1.6 μg of benzo(*a*)pyrene per person per day. The PAH intake and the foods that mainly contribute to this depend on the eating habits of the country, on the environmental contamination of the region in which the foods are produced, on the techniques used for food preserving

Table 8.5. PAH concentrations (μg kg^{-1} dry weight) in several foods of animal origin.[a]

Compound	E_1	E_2	M_1	M_2	B_1	B_2	F_{LF}	F_{FP}
Phenanthrene	–	18.5	–	3.0	–	–	168.0	4.5
Anthracene	–	29.7	–	0.5	–	–	35.4	0.7
2-Methylphenanthrene	–	–	–	–	–	–	15.2	1.1
2-Methylanthracene	–	–	–	–	–	–	7.3	0.1
1-Methylphenanthrene	–	–	–	–	–	–	14.4	0.7
9-Methylanthracene	–	–	–	–	–	–	2.1	n.d.[b]
Fluoranthene	0.1	1.2	0.1	3.4	1.0	10.8	119.0	1.9
Pyrene	n.d.	5.5	0.0	35.5	0.0	1.3	127.0	1.8
1-Methylpyrene	–	–	–	–	–	–	16.2	n.d.
Benz(*a*)anthracene	0.0	4.5	0.0	2.4	2.2	0.5	44.5	0.3
Chrysene	n.d.	5.6	n.d.	8.6	n.d.	24.7	44.1[c]	0.6[c]
Triphenylene	–	–	–	–	–	–		
Benzo(*b*)fluoranthene	0.4	3.5	0.0	3.1	0.6	1.2	29.8	n.d.
Benzo(*k*)fluoranthene	0.0	4.5	0.0	n.d.	0.1	0.6	41.9[d]	n.d.[d]
Benzo(*j*)fluoranthene	–	–	–	–	–	–		
Benzo(*e*)pyrene	–	–	n.d.	n.d.	–	–	21.8	n.d.
Benzo(*a*)pyrene	0.0	7.5	0.0	1.5	0.6	1.4	54.2	0.1
Perylene	–	–	–	–	–	–	7.9	n.d.
Indeno(1,2,3-*cd*)pyrene	–	8.7	n.d.	n.d.	–	–	41.4	n.d.
Dibenz(*a*,*h*)anthracene	n.d.	4.7	n.d.	n.d.	1.0	1.5	3.5[e]	n.d.[e]
Benzo(*ghi*)perylene	n.d.	1.2	0.0	n.d.	0.0	0.0	35.5	n.d.
Anthanthrene	–	–	–	–	–	–	14.9	n.d.

[a] E_1, egg samples studied by Lodovici *et al.* (1995); E_2, egg samples studied by Husain *et al.* (1997) (data in μg kg^{-1} wet weight); M_1, milk sample studied by Dennis *et al.* (1983); M_2, cow milk sample studied by Husain *et al.* (1997) (data in μg kg^{-1} wet weight); B_1, beef meat (Lodovici *et al.*, 1995); B_2 barbecued beef meat (Lodovici *et al.*, 1995); F_{LF}, frankfurters grilled on a log fire (Larsson *et al.*, 1983); F_{FP}, frankfurters grilled in frying pan (Larsson *et al.*, 1983).

[b] n.d. = not detected.

[c] Concentration of chrysene + triphenylene.

[d] Concentration of benzo(*j*)fluoranthene + benzo(*k*)fluoranthene.

[e] Concentration of dibenz(*a*,*c*)anthracene + dibenz(*a*,*h*)anthracene.

and processing, and finally on the cooking methods. In spite of the differences found, there is general agreement that food is an important source of PAH exposure in humans.

Uptake and Metabolism

Once PAHs have entered the body orally, they reach the intestine, where they can be absorbed and distributed to other organs through enterohepatic circulation. Food components may alter the uptake of PAHs, either enhancing or reducing their absorption and, as a result, potentiating or inhibiting their toxic effects. According to Stavric and Klassen (1994), the adsorption of PAHs to some components of diet, such as the carbon present in certain processed foods, can reduce their availability for absorption. These authors also observed that food polyphenols such as quercetin and chlorogenic acid produce a reduction in the absorption of benzo(*a*)pyrene and its metabolites, although to a lesser extent than carbon. The formation of complexes with some food components can also lead to a reduction in the bioavailability of some PAHs ingested with food. The solubility of PAHs in food ingested also plays an important role in their absorption. Water, in which benzo(*a*)pyrene and other PAHs are not soluble, may reduce the transfer to the intestinal mucosa, whereas 'oily' foods, in which PAHs are soluble,

facilitate this transfer (Stavric and Klassen, 1994). It is clear from these findings that the uptake of PAHs from diet is influenced markedly by the composition of foods with which they are ingested. This could help explain the difficulty in establishing a correlation between the presence of PAHs in the diet and the development of cancer, since there could be food components exerting some protective effect.

It is known that PAHs undergo metabolic transformation in the organism, which can result either in the formation of active metabolites that can finally form covalent adducts with DNA, or in the formation of products which will be excreted further. Given that adduct formation is considered the initial event in chemical carcinogenesis, the formation of active metabolites is considered to be closely related to the carcinogenicity of PAHs. As an example of the metabolic transformations undergone by PAHs, a model of the metabolic path of benzo(*a*)pyrene, including both activation and detoxification routes, is shown in Fig. 8.3. One of the most widely accepted approaches to explain the PAH biotransformation process begins with a cytochrome P_{450}-mediated epoxidation of the molecule (see Fig. 8.3). This epoxidation is catalysed by an enzyme complex called mixed-function oxidase (MFO), which is located in the endoplasmic reticulum or microsomal fraction. The second step involves a hydroxylation process with the formation of diols, and is catalysed by a hydrase, the so-called epoxidohydrase (EH), which is closely linked to the MFO enzyme complex. The enzyme complex including the hydrase is often referred to as an aryl-hydrocarbon hydroxylase (AHH). The diols formed can be converted further into dihydrodiol epoxides. From a chemical and biological point of view, the dihydrodiol epoxides (especially those formed in the bay region) are very reactive because they can attack critical nucleophilic sites in DNA, either directly in an S_N2 reaction or after forming a carbocation in an S_N1 reaction (Guillén *et al.*, 1997). Nevertheless, the intermediate diols can also undergo a detoxification process by conjugating with glucuronic acid or glutathione, leading to conjugated metabolites, which can be excreted by renal or biliary channels. It is worth noting that some authors (Jacob *et al.*, 1995) make a distinction between phase I metabolism, which includes the steps leading to the formation of *trans*-dihydrodiols (diols), and phase II metabolism, which refers to the further reactions of the phase I metabolites. Metabolites of PAHs with two and three rings are excreted preferentially in the urine, while higher molecular metabolites are released in the faeces.

Ingested PAHs potentially can be metabolized by the gut microflora, by the intestinal wall and by the liver. The intestinal epithelium contains all the enzymes which have been identified as being involved in activation and detoxification of PAHs, although these activities are generally much lower than in the liver (Benford and Bridges, 1985). Moreover, the low levels of inducible P_{450} isozymes in the intestinal tract could influence the occasional development of tumours in the small and large intestine as a consequence of the ingestion of PAH-containing food (Stavric and Klassen, 1994). Nevertheless, the resulting biological activity of ingested PAHs is determined not only by their degree of absorption and metabolization, but also by the presence of compounds which can act as inducers, promoters or inhibitors of the PAH metabolism by acting on enzymatic factors. Thus, the activity of intestinal enzymes that metabolize PAHs into ultimate carcinogens may be induced by drugs, certain vegetables, environmental pollutants such as polychlorinated biphenyls, and gastric hormones (Benford and Bridges, 1985). The activity of the cytochrome P_{450}-dependent-mono-oxygenases can also be induced by the PAHs themselves. Among PAHs, benzo(*a*)pyrene and 3-methylcholanthrene are the most studied inducer agents. There are other PAHs which, despite their inability as inducer agents, can play a role in carcinogenesis as promoters of the process initiated by other compounds (Jacob, 1996). There are also some dietary factors such as certain flavones present in vegetables which can promote or activate certain cytochrome P_{450}-dependent reactions, including benzo(*a*)pyrene hydroxylation, in both liver and intestine. On the contrary, food components such as antioxidants, certain flavones

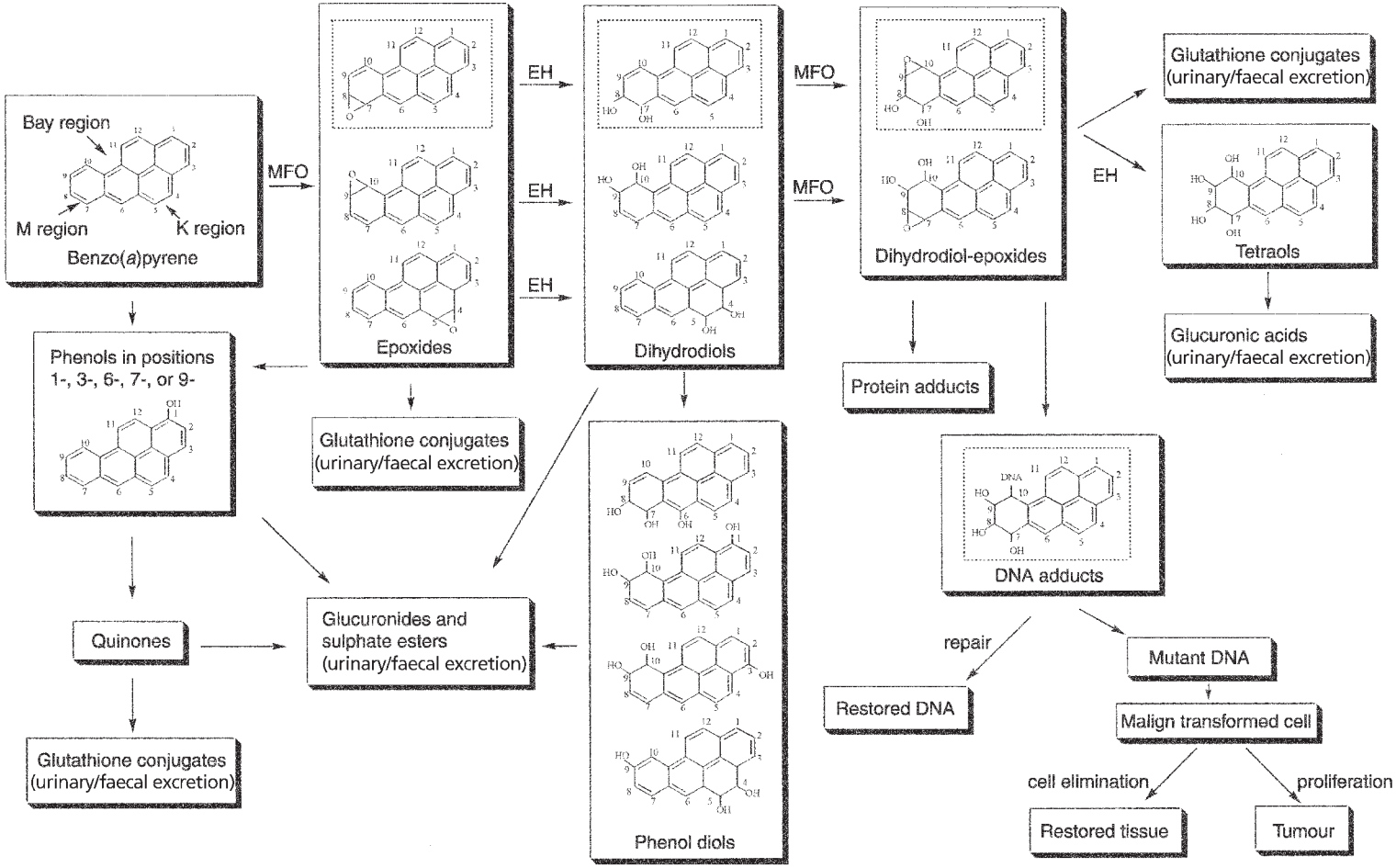


Fig. 8.3. Metabolic path of benzo(*a*)pyrene and possible effects.

and vitamins A, C and E may inhibit PAH metabolism (Benford and Bridges, 1985). It should be noted that some substances have been considered to act as both inducers and inhibitors.

It must be taken into account that most of this information comes from experiments with animals and with a limited number of PAHs. Therefore, the extrapolation of the results to different species, including humans, or from one compound to another, can sometimes lead to erroneous conclusions and predictions. Jacob *et al*. (1995), in an experiment with embryonic epithelial lung cells from rats, hamsters and humans, found qualitative and even quantitative similarities in the pattern of primary (or phase I) metabolites in the cases of pyrene, benzo(*a*)pyrene, chrysene and anthanthrene, but significant differences in the phase II metabolism. In another study, the same author (Jacob, 1996) obtained significant differences in the phase I metabolism of benz(*a*)anthracene with liver microsomes from human, rats, dogs, mice and rabbits. Considerable interindividual variations exist in the metabolism and excretion of PAHs, regardless of the administration route considered. Although the mechanism for such a variable response is unclear, it may be due to interindividual differences in constitutive or induced physiological mechanisms such as digestion, absorption, metabolism or excretion. Consequently, each subject exhibits an individual and invariant PAH metabolite profile, with a certain ratio of carcinogenic and non-carcinogenic PAH metabolites, which may indicate an individual equipment of PAH-metabolizing enzymes and a potential predisposition for cancer risk.

Toxicity and Carcinogenicity

PAH exposure is associated with many adverse effects in experimental animals, including reproductive toxicity, cardiovascular toxicity, bone marrow toxicity, immune system suppression and liver toxicity (Collins *et al*., 1998). In addition, teratogenic, mutagenic and carcinogenic properties have been reported for many PAHs (IARC, 1973, 1983). Some general considerations must be taken into account when evaluating the toxicity and biological effects of PAHs: (i) studies have been carried out on animals, so care must be taken before extrapolation to humans; (ii) most of the experiments involve administration of PAHs by routes which are not oral ingestion, considered the best way to predict the possible effects of dietary PAHs; and (iii) there are studies of the biological effects of PAHs whose results are ambiguous or inconclusive, so further investigations would be necessary to reach definitive conclusions.

Non-carcinogenic effects

There are few studies regarding the effects of oral exposure to PAHs. The results from Nousiainen *et al*. (1984) do not reveal acute toxic effects in rats given 50 or 150 mg benzo(*a*)pyrene kg^{-1} day^{-1} by gavage for 4 consecutive days, except for alterations of the enzyme activity of the gastrointestinal mucosa and induction of hepatic carboxylesterase activity, which cannot be considered adverse effects *per se*.

Reproductive toxicity of PAHs has been reported. In general, the reproductive effects of benzo(*a*)pyrene, the best documented PAH, include resorptions, malformations, stillbirths and decreased fertility in the progeny. This is because active metabolites can cross the placenta and reach the fetuses of orally exposed animals. The inducibility of the cytochrome P_{450} system of the animals has been shown to be related directly to the embryotoxicity of PAHs. The dose orally administered also seems to have an effect on the embryotoxic effects observed. Thus, in a group of pregnant CD-1 mice treated with benzo(*a*)pyrene during gestation, a marked reduction in the viability of litters from individuals exposed to the highest dose was found (Mackenzie and Angevine, 1981). An embryotoxic effect in rats has also been observed for dibenz(*a*,*h*)-anthracene when given in high doses (IARC, 1983). More recently, an ovotoxic effect has been reported for 9,10-dimethylbenz(*a*)-anthracene, 3-methylcholanthrene and benzo-

(*a*)pyrene. These compounds administered intraperitoneally produced the destruction of primordial follicles in mice and rats when given in repeated low doses, so they might be related to the early menopause seen in women exposed to cigarette smoke (Borman *et al*., 2000).

Immunotoxic effects have also been found in rats after oral administration of benzo(*a*)pyrene, affecting bone marrow, thymus, spleen and lymph nodes. Davila *et al*. (1996) examined the toxic effects of nine different PAHs on human peripheral blood T-cell mitogenesis. They found that benzo(*a*)pyrene, 3-methylcholanthrene and 7,12-dimethylbenz-(*a*)anthracene were highly immunotoxic in the human system, while dibenz(*a*,*c*)anthracene and dibenz(*a*,*h*)anthracene were of intermediate toxicity, 9,10-dimethylanthracene, benzo-(*e*)pyrene and benz(*a*)anthracene were mildly immunotoxic, and anthracene had no measurable toxicity at the concentrations tested.

Genotoxicity

Many PAHs show mutagenic activity to *Salmonella typhimurium* and even to mammalian cells *in vitro* in the presence of an exogenous metabolic system (IARC, 1973, 1983), although this activity is not always related to the production of tumours. However, it must be noticed that some PAHs which have been found to be mutagenic are also active as initiators in the mouse skin initiation–promotion assay, so their influence cannot be ruled out when evaluating the biological effects of mixtures of PAHs. Fluoranthene or coronene can be cited as examples.

In addition to mutagenic activity, some PAHs can also induce unscheduled DNA synthesis, sister chromatid exchange, morphological transformation or chromosomal aberrations in mammalian cells either in culture or *in vivo* (IARC, 1973, 1983).

Carcinogenicity

In spite of the variety of the toxic effects related to PAHs, the one of most concern is cancer. Many PAHs have been shown to be carcinogenic to experimental animals by different administration routes but, as mentioned previously, there are not many studies concerning oral administration. Most of these latter have been carried out with benz(*a*)-anthracene, dibenz(*a*,*h*)anthracene and benzo--(*a*)pyrene, resulting in hepatomas, lung adenomas, squamous papillomas, forestomach papillomas and carcinomas, and stomach tumours. There are several factors which have an influence on the effects observed. These factors can be external, such as the dose of the PAHs administered, the administration route, the vehicle supporting the PAHs, the presence of several PAHs and the frequency of exposure, or individual, such as age, sex, genetic factors and nutritional status. The dose administered can determine the extent of the carcinogenic effects observed. Goldstein *et al*. (1998), in a 2-year feeding experiment with mice, observed that animals given 17.5 µg benzo(*a*)pyrene day^{-1} did not develop tumours, whereas doses of 350 µg benzo(*a*)pyrene day^{-1} produced forestomach, oesophagus and tongue tumours. The administration route can also lead to differences in the carcinogenic response of experimental animals. The response of rats or hamsters to benzo(*a*)pyrene administered orally is small, even though they rapidly develop skin tumours after skin application. It has also been observed that when benzo(*a*)pyrene is administered to rats by gavage in a specific solution, a higher tumorigenic response is observed than when benzo(*a*)pyrene is given with the diet. This could be a result of the protective effect exerted by some components of the diet which can interact with the absorption and metabolism of PAHs. It must be noticed that there are some PAHs which, in spite of their inability to produce tumours *per se*, contribute to increasing the incidence of some types of tumours produced by complete carcinogenes such as benzo-(*a*)pyrene when administered with them. These compounds include benzo(*ghi*)-perylene, fluoranthene and pyrene (IARC, 1973, 1983). These two latter deserve special attention for the purposes of risk assessment, because of their wide distribution in the environment and in foods. Some studies have

revealed that the frequency of administration also has an influence on the toxic effect of a certain PAH. Qing *et al*. (1997) split a 1 mg dosage of dimethylbenz(*a*)anthracene given once a week for 6 weeks into five daily doses of 200 µg given intragastrically to female SENCAR mice each week for 6 weeks, and found that the toxicity was higher. It must also be said that oral administration of PAHs to young individuals can result in a higher incidence of tumours than treatment at other ages, showing the greater sensitivity of infant animals to carcinogens compared with adults of the same species (Lijinski, 1991).

To get an overall view of the carcinogenicity of PAHs, it can be said that, in general, the hydrocarbons with fewer than four fused rings are non-carcinogenic, although there are some methyl derivatives such as 9,10-dimethylanthracene and 1,2,3,4-tetramethylphenanthrene which are carcinogens of moderate potency. The compounds containing four fused rings are non-carcinogenic (triphenylene, naphthacene and pyrene) or weakly carcinogenic (benz(*a*)anthracene and chrysene). However, as with the three-ring compounds, methyl substitution in some of these compounds gives rise to hydrocarbons of very great carcinogenic potency, although all monomethyl compounds are not equal and the carcinogenicity depends to a large extent on the position of substitution in the molecule, leading to products as lacking in carcinogenic activity as the parent hydrocarbon or to compounds of great carcinogenic potency. As far as the five-ring compounds are concerned, these exhibit a varying carcinogenic potency, ranging from the non-carcinogenic picene, pentacene, perylene and benzo(*e*)pyrene to the potent benzo(*a*)pyrene and dibenz(*a*,*h*)-anthracene, including also weak carcinogens, such as dibenz(*a*,*c*)anthracene. Most of the six-ring hydrocarbons examined are carcinogenic, although there are some of them, such as benzo(*ghi*)perylene, which are non-carcinogenic. Coronene, a seven-ring hydrocarbon, is also non-carcinogenic (Lijinsky, 1991). The great differences observed among PAHs in relation to their carcinogenic activity can be explained because some characteristics are necessary both in the parent PAHs and in their metabolites to form adducts with DNA, which, as has been mentioned, represents the initial event in chemical carcinogenesis.

One of the first mechanisms proposed for the formation of active intermediates involved the formation of simple K region epoxides (see Fig. 8.3). However, it was later recognized that nucleic acid adducts formed with K region epoxides were not identified in those formed in tissues treated with the parent PAHs (Shaw and Connell, 1994). Furthermore, Jacob (1996) pointed out that the metabolic activation at the K region mainly results in non-toxic and non-carcinogenic metabolites and hence plays a role in detoxification. It is now admitted that PAHs are activated mainly by the formation of vicinal diol-epoxides and that, in most cases, the diol-epoxides are formed adjacent to a bay region (see Fig. 8.3). The existence of a bay region in the molecule has been considered for many authors as a prerequisite for carcinogenic activity, because the bay region-derived dihydrodiolepoxides exhibit the most pronounced tendency to form carbonium ions and turn out to be the most reactive. (The bay region activation mechanism of benzo(*a*)-pyrene is marked in Fig. 8.3 by means of dotted lines.) However, this characteristic alone does not predict the carcinogenicity of a PAH. It has been found that methyl substitution at the *peri* position reduces carcinogenic activity by blocking distal bay region diol formation (Loew *et al*., 1985). The other main region of activity in PAH molecules which can also play a role in the carcinogenicity of some PAHs is the L region, which features localization of π electrons across *para* positions, e.g. the 7,12 positions of benz(*a*)anthracene. The presence of an L region in a PAH was recognized as being responsible for the absence of carcinogenic activity in certain PAHs. However, substitution at this region can enhance the carcinogenicity of the unsubstituted PAH, such as in the case of 7,12-dimethylbenz-(*a*)anthracene, which is a potent carcinogen compared with benz(*a*)anthracene (Shaw and Connell, 1994). Nevertheless, there are many other factors such as metabolic, stereochemical and conformational factors, as well as the biological reactivity of the metabolites, which contribute to the marked differences in tumorigenicity of various PAHs. As an

example, in the case of picene, quantum mechanical calculations predicted its carcinogenicity, but none has been detected in several animal studies. This absence could result from the inability of microsomal enzymes to transform its M region dihydrodiol to dihydrodiol bay region epoxides in sufficient amounts to initiate carcinogenesis (Platt *et al.*, 1988).

In spite of the role of PAH–DNA adducts in carcinogenesis, it must be pointed out that the formation of PAH–DNA adducts does not necessarily imply the development of tumours, since, as shown in Fig. 8.3, the damaged template can be repaired before cell replication has occurred (Shaw and Connell, 1994).

Despite the fact that the bay region theory explains the carcinogenicity of many PAHs, there are others which, although tumorigenic, do not have bay regions or it has been shown that they are not activated via a bay region epoxide. Thus, Cavalieri and Rogan (1985) suggested an alternative hypothesis to explain activation of PAHs, based on a one-electron oxidation that yields a reactive radical cation intermediate which acts as an ultimate carcinogen. Flesher and Myers (1991) also developed some rules of molecular geometry for predicting the carcinogenic activity of unsubstituted PAHs. Other approaches have correlated carcinogenicity with a superdelocalizability index, which represents the potential reactivity at the bond adjacent to the bay region in the dihydrodiol intermediate (Berger *et al.*, 1978).

The degree of carcinogenic activity of PAHs has been expressed by different codes or indexes. The IARC (International Agency for Research on Cancer) has defined categories which refer only to the strength of the evidence that an exposure is carcinogenic, and not to the extent of its carcinogenic activity (potency) nor to the mechanism involved. Therefore, this is a classification which may change as new information becomes available. The evidence relevant to carcinogenicity is classified into four categories: sufficient evidence of carcinogenicity (SE), limited evidence of carcinogenicity (LE), inadequate evidence of carcinogenicity (IE) and evidence suggesting lack of carcinogenicity (LC). Each category is defined in a different way depending on whether the studies are carried out in humans or experimental animals. Taking into account not only the strength of the evidence derived from studies in humans, but also studies in experimental animals and other relevant data, the IARC categorize carcinogens in five groups, as indicated in Table 8.6: 1 (carcinogenic to humans), 2A (probably carcinogenic to humans), 2B (possibly carcinogenic to humans), 3 (unclassifiable as to carcinogenicity to humans) and 4 (probably not carcinogenic to humans). Group 1 is used when there is SE in humans, or when there is less than SE in humans but SE in experimental animals, with strong evidence in exposed humans that the agent acts through a relevant mechanism of carcinogenicity. Group 2A is used when there is LE in humans and SE in experimental animals, or when there is IE in humans and SE in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Group 2B is used when there is LE in humans and less than SE in experimental animals, or when there is IE in humans but SE in experimental animals. Group 3 is used most commonly when there is IE in humans and IE or LE in experimental animals, or when there is IE in humans but SE in experimental animals, with strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans. Finally, group 4 is used when there is LC in humans and in experimental animals. It is worth pointing out that there is no PAH included in group 1.

As well as the classification of the carcinogenic activity of PAHs given by the IARC, there are other ways to express the degree of carcinogenicity of the different PAHs, such as the Badger index, in which carcinogenicity ranges from (–) to (++++), the carcinogenic scale proposed by Cavalieri and co-workers, who characterize carcinogenicity from (–) to (+++++) (Cavalieri *et al.*, 1983), or the less commonly reported Iball index I (Braga *et al.*, 1999). This latter is proportional to the fraction of subject animals that show a carcinogenic response divided by the mean latent period. Carcinogenicity data according to the scale of Cavalieri and co-workers and to the Iball index for a group

Table 8.6. Carcinogenicity and other parameters related to carcinogenicity of PAHs.

Compound	Carcinogenicity				RPs	TEFs	TEFs	PEFs
Naphthalene	n.d.[a,b]	n.d.[c]	–[d]	n.d.[e]	n.d.[f]	0.001[g]	n.d.[h]	n.d.[i]
Phenanthrene	IE	3	–	00	n.d.	0.001	n.d.	n.d.
Anthracene	LC	3	–	n.d.	n.d.	0.01	n.d.	n.d.
Fluoranthene	NE	3	n.d.	n.d.	n.d.	0.001	n.d.	n.d.
Pyrene	NE	3	–	n.d.	0.081	0.001	n.d.	n.d.
Benz(*a*)anthracene	SE	2A	±	07	0.145	0.1	0.014	0.1
Chrysene	LE	3	±	05	0.0044	0.01	0.026	0.01
Triphenylene	IE	3	–	00	n.d.	n.d.	n.d.	n.d.
1-Methylchrysene	IE	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Methylchrysene	LE	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-Methylchrysene	LE	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-Methylchrysene	LE	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-Methylchrysene	SE	2B	+++	n.d.	n.d.	n.d.	n.d.	1.0
6-Methylchrysene	LE	3	±	n.d.	n.d.	n.d.	n.d.	n.d.
7,12 Dimethylbenz(*a)* anthracene	n.d.	n.d.	+++++	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo(*b*)fluoranthene	SE	2B	n.d.	n.d.	0.141	0.1	0.11	0.1
Benzo(*j*)fluoranthene	SE	2B	n.d.	n.d.	0.061	n.d.	n.d.	0.1
Benzo(*k*)fluoranthene	SE	2B	n.d.	n.d.	0.066	0.1	0.037	0.1
Benzo(*a*)pyrene	SE	2A	++++	72	1.0	1.0	1.0	1.0
Benzo(*e*)pyrene	IE	3	–	02	0.004	n.d.	n.d.	n.d.
Perylene	IE	3	–	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenz(*a*,*c*)anthracene	LE	3	+	03	n.d.	n.d.	n.d.	n.d.
Dibenz(*a*,*h*)anthracene	SE	2A	+++	26	1.11	5.0	0.89	n.d.
Dibenz(*a*,*j*)anthracene	LE	3	+	04	n.d.	n.d.	n.d.	n.d.
Indeno(1,2,3-*cd*)pyrene	SE	2B	n.d.	n.d.	0.232	0.1	0.067	0.1
Benzo(*ghi*)perylene	IE	3	–	n.d.	0.022	0.01	0.012	n.d.
Anthanthrene	LE	3	±	n.d.	0.320	n.d.	n.d.	n.d.
Cyclopenta(*cd*)pyrene	LE	3	–	n.d.	0.023	n.d.	n.d.	n.d.
Coronene	IE	3	–	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo(*a*,*e*)pyrene	SE	2B	+++	50	n.d.	n.d.	n.d.	1.0
Dibenzo(*a*,*h*)pyrene	SE	2B	++++	68	n.d.	n.d.	n.d.	10
Dibenzo(*a*,*i*)pyrene	SE	2B	++++	74	n.d.	n.d.	n.d.	10
Dibenzo(*a*,*l*)pyrene	SE	2B	+++++	33	n.d.	n.d.	n.d.	10

[a]n.d. no data.
[b]IARC (1973, 1983), evidence of carcinogenicity in experimental animals (SE: sufficient evidence of carcinogenicity; LE: limited evidence; IE: inadequate evidence; LC: evidence suggesting lack of carcinogenicity; NE: no evidence that it is carcinogenic *per se* to experimental animals).
[c]IARC, overall evaluation (1: carcinogenic to humans; 2A: probably carcinogenic to humans; 2B: possibly carcinogenic to humans; 3: unclassifiable as to carcinogenicity to humans; 4: probably not carcinogenic to humans).
[d]Data from Cavalieri *et al.* (1983): extremely active, +++++ ; very active, ++++ ; active, +++; moderately active, ++ ; weakly active, +; very weakly active, ±; inactive, –.
[e]Iball index.
[f]RP, relative carcinogenic potencies. Adapted from Krewski *et al.* (1989) (Collins *et al.*, 1991).
[g]From Nisbet and LaGoy (1992) (Collins *et al.*, 1998).
[h]From Muller *et al.* (1997) (Thomson and Muller, 1998).
[i] From the Office of Environmental Health Hazard Assessment (OEHHA) (Collins *et al.*, 1998).

of PAHs are presented in Table 8.6. In addition, it is worth mentioning some parameters related to the carcinogenicity of PAHs which express carcinogenic potencies relative to that of benzo(*a*)pyrene; these are relative potencies (RPs) and toxicity equivalency factors (TEFs)

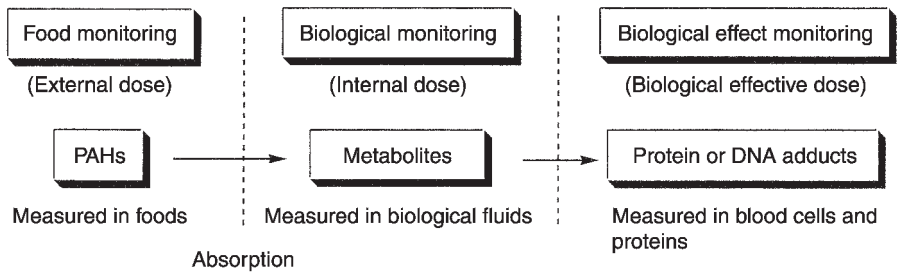


Fig. 8.4. Different levels of monitoring for risk assessment.

or potency equivalency factors (PEFs). These parameters have been established to assign a carcinogenic activity to PAHs with unknown cancer potency values, and they are used to estimate quantitatively the cancer risk associated with exposure to PAHs. Some authors (Collins *et al*., 1998) suggest the use of PEFs instead of TEFs, since nearly all PEFs for PAHs are based on cancer bioassay information and they do not take into account other studies such as acute toxicity determinations, structure–activity relationships or short-term tests such as AHH induction. TEFs or PEFs are usually indexed at increments of a factor of 10. Data relative to the RP, TEFs and PEFs of a group of PAHs can be seen in Table 8.6.

Risk Assessment

According to the EPA, risk assessment is the process that scientists and government officials use to estimate the increased risk of health problems in people who are exposed to different amounts of toxic substances. The assessment of human health effects associated with exposure to chemical carcinogens is normally performed in four stages: (i) hazard identification (what health problems are caused by the toxic substance); (ii) exposure assessment (how much enters the body); (iii) dose–response assessment (the health problems at different exposures); and (iv) risk characterization (the extra risk to health). Combining the results of the exposure assessment and the dose–response assessment gives an estimate of the increased lifetime risk of cancer for an individual exposed to the maximum predicted long-term concentration.

Figure 8.4 shows the main monitoring levels in the risk assessment of PAHs. The first approach to evaluating the risk associated with dietary exposure to PAHs is based on the measurement of PAH levels in foodstuffs, since high concentrations of PAHs in ingested food will probably result in adverse effects for human health. However, this analysis of external exposure, although very useful, does not take into account either the absorption, metabolism, distribution or excretion mechanisms of PAHs or interindividual differences relative to these mechanisms, which clearly determine the final effects of PAHs. Biomarkers can be used as a means of obtaining information on an individual's internal exposure to a xenobiotic or on the actual or potential effects of that exposure. Biomarkers are parameters that can be evaluated quantitatively, semi-quantitatively or qualitatively in body fluids, cells or tissues. In general, biomarkers can be classified as: biomarkers of exposure, which reflect dose of toxic agents; biomarkers of effect, which indicate biological response to exposure with potential toxicological implications; and biomarkers of susceptibility, which provide information about the intrinsic sensitivity of an individual to the toxic agent. As examples of the different types of biomarkers, PAH metabolites in urine, DNA and protein adducts, mutations or chromosomal aberrations can be cited, and they provide information about different stages of the process comprised between exposure to PAHs and development of malignant effects.

Biomarkers for PAHs

The most widely used biomarkers for dietary exposure to PAHs are measurement of PAH–DNA adducts, generally in nucleated white blood cells, and PAH metabolites excreted in urine, but protein adducts also appear as a promising tool to assess PAH exposure. Table 8.7 summarizes the main features of the biomarkers most used for PAHs.

Among all the PAH metabolites studied, 1-hydroxypyrene (1-OHP), which can be determined by high-performance liquid chromatography (HPLC) with fluorescence detection (FL) or by gas chromatography–mass spectrometry (GC–MS), has been considered as the preferred biomarker for routine assessment of exposure to PAHs. It must be noticed that, although 1-OHP is used preferentially as an indicator for PAH exposure at workplaces, it can also be used for exposure to dietary PAHs. Thus, increased levels of urinary excreted 1-OHP have been observed in humans after consumption of grilled meat (Van Maanen *et al*., 1994). Moreover, a significant correlation between urinary 1-OHP and peripheral blood PAH–DNA adducts has been found in a study of dietary exposure to PAHs (Kang *et al*., 1995). Neverthelss, in spite of the sensitivity and ease of measurement of 1-OHP, a potential disadvantage of urine biomarkers is that, in general, they only reflect recent exposure. Besides, effective biological monitoring based on the determination of 1-OHP requires an understanding of its excretion kinetics and it must be used for exposure assessment of homogeneous groups, since the relative proportion of pyrene in complex mixtures of PAHs can vary among different sources.

PAH–DNA adducts provide information on the molecular or biologically effective dose of PAHs reaching a critical target, which can be considered as an integration of internal exposure and metabolism. Many investigations have been focused on the study of the relationship between PAH exposure and formation of DNA adducts, but contradictory results have been obtained. Thus, the results from studies in which dietary exposure to PAHs has been examined, reveal that the concentration of PAH–DNA adducts in blood cells after controlled consumption of chargrilled beef increased only in some subjects (Kang *et al*., 1995). On the contrary, a dose-dependent response to PAH ingestion was observed in a feeding study carried out by Van Maanen *et al*. (1994). The discrepancy between results could be due to the great variability observed in the response of different individuals, to some difficulties in DNA adduct measurement by agent-specific immunoassays or by non-agent specific ^{32}P-post-labelling assay, and to the kinetics of formation and elimination of PAH–DNA adducts. The level of DNA adducts correlates directly with the concentration of the carcinogen in the diet when the rate of adduct formation is compensated by the rate of adduct removal (steady state), whereas this correlation fails if adduct measurement is made after this period. Finally, it must be noticed that the

Table 8.7. Main characteristics of the most-used biomarkers for PAHs.

Biomarker	Characteristics	Determination
1-Hydroxypyrene	Effective as biomarker of PAH recent exposure	HPLC-FL
	Sensitive and easy to determine	GC–MS
	Certain correlation with DNA adducts	
	Valid for homogeneous groups of exposure	
	No clear relationship with cancer risk	
DNA adducts	Effective as biomarker of internal exposure	^{32}P-post-labelling
	Limited usefulness as biomarker of effect	Immunochemical methods
	No clear relationship with tumour induction	
	Affected by a great variability	
Protein adducts	Effective to assess longer exposure	GC–MS
	Great accessibility and stability	Immunoassays
	No clear relationship with cancer risk	HPLC-UV/FL

persistence of biomarkers measured in blood cells, such as PAH–DNA adducts, is determined by the lifetime of the cell types chosen for their analysis.

It can be said that, currently, the presence of a DNA adduct in human tissue indicates that exposure has occurred and, in some cases, how much exposure there has been. However, an association between adduct formation and cancer risk has not yet been shown for PAHs. An attempt to correlate tumour induction and adduct formation was made by Goldstein *et al*. (1998), who directly compared both parameters in mice given benzo(*a*)pyrene and coal tars, either orally or intraperitoneally. DNA adducts were found in both tumours and tumour-free tissue, but neither quantitation of total DNA adducts nor quantitation of the DNA adducts formed by benzo(*a*)pyrene could predict the development of tumours. Moreover, the data indicated significant differences for tumour induction by benzo(*a*)-pyrene compared with coal tars.

At present, no clear and accepted models of risk assessment on the basis of DNA adduct levels are available. However, it must be said that the relationship between DNA adduct formation and human cancer has been elucidated in the case of tobacco smoking and lung cancer (Poirier *et al*., 2000).

The formation of adducts of PAHs with proteins, generally haemoglobin and serum albumin, is considered to be a valuable surrogate for DNA adduct formation, since many chemical carcinogens bind to both DNA and protein in blood with similar dose–response kinetics (Poirier *et al*., 2000). The main reasons for the use of protein adducts in biochemical effect monitoring are their relative easy accessibility of target tissues and their relative stability in comparison with DNA adducts, which constantly undergo repair. Because of this stability, protein adducts tend to represent exposure over the life of the tissue monitored (Shaw and Connell, 1994). The level of protein adducts, which can be determined by GC–MS, immunoassays or HPLC with UV or FL detection, has been found to be directly proportional to the daily carcinogen dose at steady state and is typically linear over a large dose range (Poirier *et al*., 2000).

Protein adducts have been employed as biomarkers for many human exposures including tobacco-related, workplace and medicinal (psoriasis) PAHs (Poirier *et al*., 2000). However, there are few studies on the correlation between protein adducts and cancer risk and, besides, protein adducts are less well accepted than DNA adducts as indicators of carcinogenic potential.

It seems that, in the future, approaches to cancer risk assessment will take into account not only a single biomarker, but the results of a battery of biomarker tests, including DNA adduct and protein adduct analyses.

Quantitative Risk Assessment

There are very few studies in which a quantitative risk assessment from exposure to dietary PAHs has been achieved, and they are based on an extrapolation of the cancer potency of a single PAH, benzo(*a*)pyrene, from animal studies to humans. This can be explained by the lack of information relative to the carcinogenicity of PAHs in humans. The wide range and the high variability of the data used in the risk assessment of PAHs from dietary sources result in different cancer potency estimates, which makes it difficult to make an accurate estimation of cancer risk. The largest data set currently available is that obtained by Neal and Rigdon (1967) in a dietary study with mice. However, although these data have been used together with those of Thyssen *et al*. (1981) by the EPA for risk assessment, their use for quantitative low-dose extrapolation results in some uncertainty in the determination of the cancer potency factor, which makes it difficult to obtain a reliable quantitative risk assessment (Collins *et al*., 1991). More recently, human cancer potency figures for oral exposure to PAHs have been derived from inhalation potencies of coke oven emissions (Thomson and Muller, 1998), assuming that the relative potency of PAHs by oral and inhalation routes in humans and rodents is similar.

One proposed method for establishing quantitative risk estimates for PAH mixtures is based on the use of the TEFs or PEFs

mentioned above. In a TEF approach for PAHs, overall potency of the mixture is expressed relative to benzo(*a*)pyrene, and contributions of individual carcinogenic PAHs are taken to be additive (Goldstein *et al*., 1998). No risk assessments for oral exposure to PAHs have been reported using this methodology, except for that of Thomson and Muller (1998). These authors tried to estimate the cancer risk from dietary sources of PAHs by using four different approaches. Two methods were based on the sum of risk from individual PAHs, using cancer potency values from rodent studies or from human inhalation data. The other two used benzo(*a*)pyrene as a surrogate, either representing a proportion of the risk from the total mixture, or being assigned a potency representative of the PAH mixture as a whole. The differences among the results may be due to the assumptions that each approach involves, to the number of PAHs included or to the data used to estimate cancer potencies. These authors also suggest that an assessment based on benzo(*a*)pyrene as a surrogate for the potency of the PAH fraction of an orally administered mixture is more realistic than an assessment based on summing the risk from a limited group of identified PAHs. However, there are other authors (Goldstein *et al*., 1998) who have questioned both the accuracy of risk assessment based on benzo(*a*)pyrene and the use of TEFs.

Risk Management

Risk management takes the information generated in the risk assessment and translates it into a policy decision. It must be pointed out that, to the best of our knowledge, no risk management of the ingestion of PAH-containing foods exists. Instead, considering the adverse effects associated with exposure to these compounds, some measures have been suggested to reduce the intake of PAHs. The first goal in reducing the levels of ingested PAHs would be the reduction of environmental PAH pollution, which is responsible for the contamination of many food sources such as vegetables or marine organisms. Efforts should be made to avoid PAH contamination of foods during their processing and cooking. To this end, some measures can be implemented, involving some of the most contaminated foodstuffs. The contamination of vegetable oils can be reduced by avoiding the contamination of seeds during processes such as drying, and by removing PAHs during refinement. Deodorizing removes some of the light PAHs, whereas the use of activated carbon during the bleaching step can have a significant effect on heavy PAH reduction. Contamination of oils and fats can also be avoided by purifying extraction solvents by filtration through silica gel, or by using non-hydrocarbon solvents. With regard to smoked foods, a rigorous control of the smoking process and the use of liquid smokes could give rise to lower PAH concentrations in the products. To reduce the PAH content of grilled or broiled meat and fish, the use of foods with lower proportions of fat and the control of the cooking temperature are recommended. The contamination of grilled food with PAHs can also be minimized by using charcoal as fuel, by avoiding open flames and by special grill constructions that prevent the fat from dripping on to the heat source. In the case of vegetables, washing them in water before an adequate processing can help reduce the levels of environmental PAHs, especially those of the heavy PAHs (Larsson and Sahlberg, 1981). However, despite all the measures suggested, it is clear that, at the moment, the environmental PAH load constitutes an important source of contamination for some raw materials used in the food industry. Therefore, efforts should be made to avoid ingredients from very polluted areas.

Legislation

The most outstanding feature concerning the regulation of PAHs in foods is the lack of measures to limit or avoid the presence of these compounds, shown to be detrimental for human health. In fact, few countries have established a limit for PAHs in foodstuffs. Germany has limited the benzo(*a*)pyrene

content of smoked meat to 1 μg kg^{-1}. This limit was adopted subsequently in Austria and Poland. In 1988, the European Union established a maximum limit of 0.03 μg kg^{-1} of benzo(*a*)pyrene in food as a result of the use of smoke flavourings. In Finland, smoking additives must have a benzo(*a*)-pyrene concentration lower than 30 μg kg^{-1}, and their incorporation into foods is limited to 0.5 g kg^{-1}. The use of liquid smokes in foods traditionally subjected to smoking has been authorized by the FAO/WHO Joint Committee on Food Additives, provided that the benzo(*a*)pyrene content does not exceed 10 μg kg^{-1}. In Germany, the German Society for Fat Science (DGF) has proposed a value of 5 μg kg^{-1} as the limit value for heavy PAHs and a value of 25 μg kg^{-1} for the sum of both light and heavy PAHs in refined fats and oils. The 'Czech guidelines for additives and contaminants in foods' proposal from the Czech Republic includes limiting values of 2 μg kg^{-1} for each PAH enumerated (no light PAHs are involved) and 20 μg kg^{-1} for total PAHs in oils, fats and oil products. Recently, the Spanish government has established limiting values of 2 μg kg^{-1} for each heavy PAH enumerated and 5 μg kg^{-1} for total heavy PAHs in olive pomace oil. However, no legislation exists regarding either benzo(*a*)pyrene or other PAH levels in other types of food.

Conclusions

PAHs constitute a group of environmental contaminants including compounds with different degrees of carcinogenicity. They are also widespread in foods as a result of both environmental pollution and inadequate processing and cooking. Since toxic and carcinogenic effects after oral administration of some of these compounds have been demonstrated in experimental animals, their presence in foods should be avoided and controlled. Consequently, the use of reliable methods that allow the accurate determination of PAHs in foodstuffs as a first estimate of human exposure is encouraged. Moreover, it would be valuable to develop screening methods usable in routine analysis which could help estimate the levels of PAHs in different foodstuffs. The measurement of certain parameters, known as biomarkers, in body fluids, cells or tissues could also constitute a very useful tool for assessing exposure to PAHs or even for identifying early biological effects which can lead further to the development of cancer; however, much work remains to be done before establishing clear correlations between biomarkers and cancer risk. Although some attempts have been made to estimate the risk derived from ingestion of PAHs, the difficulty in evaluating dietary PAH intake and the lack of accurate data regarding carcinogenic potency of PAHs by oral exposure make it difficult to estimate reliably the cancer risk of ingested PAHs. Finally, even though epidemiological studies point to the contribution of PAHs to human cancer, legal dispositions concerning the regulation of PAHs in foods are very scarce. Besides, they refer only to very concrete groups of foods and to a single PAH, benzo(*a*)pyrene, although there are other PAHs with equal or greater carcinogenic potential. Consequently, considerable efforts must be made to avoid PAH contamination of foods, to ensure that regulatory directives are adhered to and human health effects are minimized.

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9 Heavy Metals

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Introduction

Definition

Heavy metal: a metal or alloy with a density higher than 4.5–5.0 kg dm^{-3}.

Heavy metals include metals that are essential as well as toxic. In the public mind, however, heavy metals usually cover all metals that have been connected to negative properties in some way, even including aluminium (2.7 kg dm^{-3}). Arsenic is qualified by its density (5.7 kg dm^{-3}), but is by definition not a metal.

Nature of the compounds

In Table 9.1 are described some of the more commonly used physical properties that are important in the categorization of metals and other elements.

The metals that usually first come to mind when heavy metals are mentioned are lead, cadmium and mercury, all well known due to their documented toxic effects. Two other commonly encountered heavy metals are chromium and nickel, which are not toxic in the concentrations normally found in food but are used in vast quantities, not least in equipment coming into contact with food.

Another concept that needs to be mentioned is trace metals, which many people use as a synonym for heavy metals. Trace has been defined in the analytical nomenclature as the range 10^{-4}–10^{-2} parts per million (ppm = mg kg^{-1}). In up-to-date nomenclature, the term trace is not mentioned, but popular expressions die hard. There is thus some confusion in terms, since heavy metals mostly occur at trace, or microtrace, concentrations in foodstuffs.

Analytical Quality Assurance

'It is easy to make an analysis, but difficult to get the right result!'

A tremendous amount of data on heavy metals in foodstuffs have been published since the 1950s. Only quite recently has the importance of analytical quality assurance (AQA) procedures in analytical chemistry been realized. The sad implication of this is that most of the data for heavy metals in foods at trace or ultra-trace levels published prior to the early 1980s are highly unreliable. This is not to say that everything published later is reliable (or, for that matter, that everything published earlier is unreliable). However, the probability for reliability has increased. This is, of course, very fine, but

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Table 9.1. Physical properties of the five heavy metals described in this chapter.

Metal	Atomic number	Density (kg dm^{-3})	Melting point (°C)	Boiling point (°C)
Cadmium (Cd)	48	8.65	321	765
Chromium (Cr)	24	7.2	1857	2672
Mercury (Hg, hydrargyrum)	80	13.55	– 39	357
Nickel (Ni)	28	8.9	1453	2732
Lead (Pb, plumbum)	82	11.35	327.5	1740

how can I, 'the consumer' of results, elucidate what is reliable or not? It is not always possible, but there are several means available to help evaluate the reliability of an analytical survey.

When it comes to assessing food safety from the point of view of heavy metal content, we must keep in mind that the analysis of heavy metals at the concentration levels commonly found in foods is a fairly difficult analysis, with several traps on the way. All current results are relative, a result of a comparison, which may go wrong. Although researchers are working on absolute methods, it will probably be a long while before any practical results are available.

'How certain is uncertainty?'

A result, as it comes out of an analytical instrument, may have a virtually infinite number of decimals, which to the unsuspecting eye may give the impression of absolute reliability, but this is an illusion. Assume that you for some reason decide to reanalyse a sample and you get a result quite different from the first. How do you know which is right? Or are both wrong? Maybe both are correct! In order to determine this, we need to know the measurement uncertainty (MU) for the results. The MU is the sum of all uncertainties introduced during the analytical process, and can be rather large at low concentrations. A result of, for example, Pb of 0.025 mg kg^{-1} may have an MU of ±0.010 mg kg^{-1}, which means that the 'true' value can be anywhere between 0.015 and 0.035 mg kg^{-1}. In light of this, a large number of decimals does not make much sense.

'Analytical error'

Two of the main reasons for analytical unreliability in heavy metal analysis are the neglected importance of contamination and analytical interferences, which are not to be confused with MU.

Very few foods are totally free from heavy metals, although they may not be detectable. They are usually occurring either naturally or through contamination. Analytical blanks, carried out appropriately, would indicate the level of contamination during the analytical steps prior to the determination. Interferences can often be corrected for if they are known to occur. In light of this, it is quite easy to understand that past results are not always what they seem to be. A third reason is over-reliance on recovery tests. Recoveries are mostly useful in analyses involving an extraction step, or other procedures in which you might expect to lose analyte prior to determination. In the analysis of heavy metals, full recovery (allowing for measurement uncertainty) is, generally, expected. A fourth reason is simply analytical carelessness or incompetence.

The two main pillars of quality assurance

Certified reference materials

Certified reference materials (CRMs) are materials that provide you with the possibility to check your performance against a sample with a 'known' quantity of the analyte of interest. When it comes to heavy metals, the 'known' level is, of course, only the best estimate available with all its shortcomings.

CRMs are used to evaluate the performance over time, as well as the result of a specific determination. Many aspects of the use of CRMs are discussed in a book by Stoeppler *et al*. (2001).

Proficiency testing

Proficiency testing (PT) is probably the only objective way to assess the analysis (method) as carried out in a specific laboratory at a specific time. The result of the participation in a PT programme is invariably worse than the result of a CRM, probably because the 'assigned' value is unknown at the time of analysis. In combination, PT and CRMs are the backbone of quality assurance (QA) in heavy metal, or trace element, analysis.

'Analysts are only human'

It is natural that an analyst who authors a paper focuses on his assets and suppresses his shortcomings. It is consequently important that the 'consumer' of published data is to some degree able to 'read between the lines'. If what you are looking for, in terms of AQA, in the report is not mentioned, it has probably not been done. If a specific item is given more room than seems justified, it might be hiding something else.

Effects of QA

The general effects of QA procedures may not always be visible. When it comes to heavy metals at trace levels, however, it may show up when publications with different degrees of QA are compared (Engman and Jorhem, 1998) and can be summarized as follows:

1. Papers with satisfactory QA, describing the content of, for example, Cd or Pb at trace levels in uncontaminated foods, usually find the results within a limited range.
2. Papers with unsatisfactory QA, describing the content of, for example, Cd or Pb in uncontaminated foods, often find the results spreading over two to three orders of magnitude.

ISO-Guide 17025 (ISO/IEC, 1999) provides a good picture of what is required for a satisfactory QA procedure.

Cadmium

A soft silvery metal that can easily be cut with a knife. It was discovered in 1817 in Germany by F. Stromeyer.

The most well known event of toxic effects of Cd on man is probably the 'Itai-itai' disease ('ouch-ouch' disease). In a district of Japan, after the Second World War and up to the early 1970s, it was found that many people suffered from a disease that, under much pain and suffering, resulted in severe bone deformation and, in many cases, death. It was found to be the result of river water being polluted by Cd-containing waste from mining activities. The river water was used for irrigation of rice fields, which resulted in Cd-contaminated rice, often with Cd levels between 0.5 and 1 mg kg^{-1}. The consumers, women in particular, then suffered from decalcification of the skeleton (osteomalacia), which led to skeletal deformation and frequent bone fractures. Even the slightest exertion, such as coughing, could result in, for example, broken ribs (Friberg *et al*., 1974).

Uses

One of its main uses is in Ni–Cd batteries. Another common use has been for surface plating. Red–yellow Cd pigments are used in paints and on ceramics. Cd has also been used as a stabilizer in plastics.

Distribution in foods

The Cd content can vary drastically between different food products, from less than 0.001 to 100 mg kg^{-1}. Most of the more commonly consumed products contain low levels of Cd. Muscle tissues from most animals,

including fish, contain levels below 0.01 mg kg^{-1}. A notable exception is horse meat, which generally contains levels in excess of 0.1 mg kg^{-1}. Kidney from older domestic animals is the tissue in which the highest levels are found. Levels approaching 100 mg kg^{-1} have been detected in crab hepatopancreas. In liver and kidney of game animals and horses, the Cd level is often so high that their consumption should be severely restricted.

In vegetable foods the Cd level normally does not vary that much, and seldom exceeds 0.05 mg kg^{-1}. However, there are some notable exceptions. Certain seeds, for example sunflower and flax, often have Cd levels approaching 0.5 mg kg^{-1}. Very high levels have been found in mushrooms from the *Agaricus* genus (e.g. *Agaricus augustus*), in which levels of 10–20 mg kg^{-1} are not uncommon.

The levels in wheat, rice and potatoes are of particular interest. The Cd levels are usually not very high (≤ 0.05 mg kg^{-1}). However, being basic foodstuffs, consumed in large quantities, their influence on the total intake is considerable. Table 9.2 indicates the normal levels for most types of food. Higher levels than those indicated may, however, be found occasionally.

Normal intake levels

As evident from the different levels encountered in foodstuffs, the intake of Cd may vary considerably depending on eating habits. Apparently, most people, regardless of nationality, have eating habits that seldom include items with abnormal Cd levels, as can be seen in Table 9.3. The two most common methods used to establish the actual intake

Table 9.2. Examples of Cd levels in uncontaminated foodstuffs (based on results from Jorhem and Sundström, 1993, 1995; Marro, 1996; Hardy, 1998; Ysart *et al.*, 1999, 2000).

Foodstuff	mg kg^{-1} fresh weight
Meat (excluding horse), including most fishes, dairy products	≤ 0.005
Most vegetables, certain fish (e.g. herring), rye flour, oats, eggs	≤ 0.025
Wheat flour, potatoes, certain vegetables (e.g. spinach, carrots)	≤ 0.05
Wild mushrooms, horse meat, wheat bran	≤ 0.2
Liver and kidney from domestic animals, sunflower and flax seeds	≤ 0.5
Mussels and oysters	≤ 1
Certain mushrooms (e.g. *Agaricus augustus*), horse kidney, crab hepatopancreas	≥ 10

Table 9.3. Average daily intake of Cd (mg) through the diet in certain countries.

	Adults	Adult females	Adult males	Reference
Australia 1994 (MB)[a]		0.013	0.019	Marro (1996)
Australia 1996 (MB)		0.021	0.027	Hardy (1998)
Belgium 1992 (DD)[b]	0.023			Cauwenbergh *et al.* (2000)
Germany 1991 (MB)		0.011	0.014	Müller *et al.* (1998)
Germany 1996 (DD)		0.007	0.009	Seifert and Anke (1999a)
Japan 1992 (DD)		0.027		Tsuda *et al.* (1995)
Spain (MB)	0.024			Cuadrado *et al.* (1995)
Sweden 1987 (MB)			0.012	Becker and Kumpulainen (1991)
Sweden 1996 (DD)		0.010		Vahter *et al.* (1996)
UK 1994 (MB)	0.014			Ysart *et al.* (1999)
UK 1997 (MB)	0.011			Ysart *et al.* (2000)

[a]MB, market basket study.
[b]DD, duplicate diet study.

of food components are duplicate diet (DD) studies, in which duplicate portions of everything ingested are collected, and market basket (MB) studies, in which food items are collected, e.g. in relation to national consumption statistics.

When the measured levels are below the limit of detection (or quantification), this gives rise to problems in estimating the intake. Depending on the method used, the intake may be under- or overestimated. It is thus rather difficult to compare results from reports and publications, since they are not always that explicit on the background to their figures.

In the UK, the Cd intake has been monitored through MB surveys since 1976 (Ysart *et al.*, 2000). They show a slight tendency to decrease, but more data are probably needed to verify this (Table 9.4).

Uptake and metabolism in humans

The Cd uptake by adults is in the order of 5%, and is stored primarily in the kidneys. Several studies have shown that factors such as the dose, the composition of the food and the individual's nutritional status may influence the uptake rate of ingested Cd (Fox, 1988; Sandström, 1988; Andersen *et al.*, 1992). Iron deficiency is one factor which enhances Cd uptake. There are also indications that a sudden exposure to a foodstuff with a high concentration may result in a higher uptake of Cd than a lower level of exposure over time (Lind *et al.*, 1997).

Toxicity and clinical effects

Cd has no known function in the human metabolism and, since its damaging effects are well documented, it is desirable to keep the intake as low as possible. Examples of acute effects are vomiting and diarrhoea. One of its chronic effects is slight kidney damage, which results in low molecular proteins in the urine (proteinuria). This may be followed by severe kidney damage (uraemia, possibly lethal), osteomalacia and osteoporosis.

Risk assessment

Better diagnostic procedures have led to the conclusion that kidney damage may occur at much lower intake levels than was earlier thought. A consequence of this is that the difference between normal intake levels and intake where negative effects may start to occur is without a safety margin. A Belgian survey (Buchet *et al.*, 1990) has shown that as much as 10% of the general, non-smoking, population has an internal dose of Cd sufficient to cause slight renal dysfunction.

An international expert group (WHO, 1993a) has agreed on a provisional tolerable weekly intake (PTWI) for Cd of 0.007 mg kg^{-1} body weight (BW). For an adult weighing 70 kg, this means a PTWI of 0.490 mg of Cd per week.

Risk management

The rationale for the maximum limits (MLs) laid down by the EU working group (Table 9.5) states that

> Cadmium may accumulate in the human body and may induce kidney dysfunction, skeletal damage and reproductive deficiencies. It cannot be excluded that it acts as a human carcinogen. The SCF (Scientific Committee for Food) recommended in its opinion of 2 June 1995 greater efforts to reduce dietary exposure of cadmium since

Table 9.4. Comparison of population dietary exposure results for cadmium from the UK Total Diet Studies 1976–1997 (Ysart *et al.*, 2000). Average daily intake in mg.

Year of study	1976	1978	1980	1982	1984	1986	1988	1991	1994	1997
Intake of Cd	0.02	0.02	0.026	0.018	0.019	0.017	0.019	0.018	0.014	0.012

foodstuffs are the main source of human intake of cadmium. Therefore, maximum levels should be set as low as reasonably achievable (EC, 2001).

Legislation/intake recommendations

The European Union has reached an agreement to set MLs for Cd in certain foods. These are binding for the Member States after 22 April 2002 and will replace existing nationally set limits. The MLs are summarized in Table 9.5.

Conclusions

Cd constitutes a serious risk for, primarily, kidney damage, even at today's level of exposure. The intake via food must thus be kept as low as possible, and hopefully be lowered even further. It is therefore important to reduce the distribution of Cd into the environment, from where it may find its way into the food chain.

Chromium

Bluish-white, hard, brittle, lustrous and resistant to corrosion, it was discovered in 1797 in France (Paris) by Nicolas-Louis Vauquelin.

Uses

Its main uses are as a plating metal and as an alloy in stainless steel. It is also often used in tanning of hides.

Distribution in foods

The Cr content of foods varies considerably, with most major foods at the low end of the spectrum (Table 9.6). It is probable that a substantial part of Cr present in foods is due to contamination during the various steps of production. Cr is a major component of stainless steel, which, in the form of, for example, knives, benches, tanks and

Table 9.5. Maximum levels of Cd in certain foodstuffs (EC, 2001). All references in the official list are omitted.

Product	Maximum level (mg kg^{-1} wet weight)
Meat of bovine animals, sheep, pig and poultry. Muscle meat of most fish.	0.05
Vegetables and fruits, excluding leafy vegetables, fresh herbs, all fungi, stem vegetables, root vegetables and potatoes	
Cereals, excluding bran, germ, wheat grain and rice. Muscle meat of wedge sole, eel, European anchovy, louvar or luvar, horse mackerel or scad, grey mullet, common two-banded seabream, European pilchard or sardine	0.1
Meat of horse. Bran, germ, wheat grain and rice. Soybeans. Leafy vegetables, fresh herbs, celeriac and all cultivated fungi	0.2
Liver of cattle, sheep, pig and poultry. Crustaceans, excluding brown meat of crab	0.5
Kidney of cattle, sheep, pig and poultry. Bivalve molluscs. Cephalopods (without viscera)	1.0

Table 9.6. Examples of Cr levels in uncontaminated foodstuffs (based on results from, for example, Anderson *et al.* 1992; Jorhem and Sundström, 1993; Hardy, 1998; Ysart *et al.*, 1999, 2000).

Foodstuff	mg kg^{-1} fresh weight
Meat, fish, milk and milk products, vegetables, fruits and berries, cereals, cattle liver and kidney	≤ 0.02
Beans, lentils, seeds, blue poppy seeds, pig liver and kidney, wild mushrooms	≤ 0.1
Dark chocolate – cocoa, white poppy seeds, buckwheat, sugar	0.1–5

machinery, frequently comes into contact with food during processing. Another source for Cr is the stainless steel utensils used for cooking in the household, especially in cooking of acidic foods. In older papers, meat is often cited as a good source for Cr, but this is probably due to contamination, or other types of interferences during the analysis. Table 9.6 exemplifies the Cr level normally found in food in newer studies.

A seldom recognized source for Cr in the diet is food preserved in tin cans. The tin plate undergoes a passivation treatment using, for example, sodium dichromate in order to improve resistance to oxidation and lacquer adherence. A survey of Cr in canned fruit and vegetables showed a median Cr level of 0.06 mg kg^{-1} in products from unlacquered cans, whereas the median level in corresponding products in cans with a lacquered inside, as well as fresh products, was 0.01 mg kg^{-1} (Jorhem and Slorach, 1987).

Normal intake levels

There are several studies, both MB and DD, available on the daily intake of Cr (Table 9.7). With the exception of the UK studies (Ysart *et al.*, 1999, 2000), the levels are quite consistent at ≤ 0.05 mg day^{-1}. The reason for the higher levels in the UK studies probably stems from the fact that those foods are prepared for consumption, which means that the foods have been exposed to the kind of contamination normally encountered during preparation. Thus, they probably give a truer picture of the actual intake than the other studies. For presumably the same reason, some of the UK foods in Table 9.6 exceed the exemplified levels.

Uptake and metabolism in humans

Cr in foods is present mostly in its trivalent form (Cr^{3+}), which plays an important role in the metabolism of sugar, and functions through insulin in maintaining normal glucose tolerance. Less insulin is required in the presence of optimal amounts of biologically active Cr (Anderson, 1992). Body uptake of Cr^{3+} is estimated to be in the order of 0.5%. The hexavalent form (Cr^{6+}) is more toxic and may give rise to, for example, allergy and contact eczema, and may also be carcinogenic. This form is normally not found in foods, but may be present in water. Most analytical methods for Cr in foods do not distinguish between the two forms; only the total is therefore usually known.

Toxicity and clinical effects

Negative effects are due mainly to occupational exposure, and not via food. Drinking water contaminated with Cr^{6+}, however, has been known to produce gastrointestinal symptoms (e.g. abdominal pain, vomiting and diarrhoea). Hexavalent Cr is also allergenic, i.e. allergenic contact eczema may result from contact with Cr-containing products. There is today no information available indicating that intake of Cr via the diet

Table 9.7. Average daily intake of Cr (mg) through the diet in certain countries.

	Adults	Adult females	Adult males	Reference
USA (MB)[a]		0.012[b]	0.019[b]	Anderson *et al.* (1992)
Austria (DD)[c]		0.031	0.038	Wilplinger *et al.* (1996)
Belgium 1992 (DD)	0.053			Cauwenbergh *et al.* (1996)
Sweden 1988 (DD)		0.020		Jorhem *et al.* (1998)
UK 1994 (MB)	0.30			Ysart *et al.* (1999)
UK 1997 (MB)	0.10			Ysart *et al.* (2000)

[a]MB, market basket study.
[b]Mean level 1000 $kcal^{-1}$.
[c]DD, duplicate diet study.

should cause, or have a negative effect on people having, Cr allergy (NAS, 2001).

Risk assessment

There seems to be little risk of overexposure to Cr through the diet, including drinking water. Risks are related mainly to contact with products containing Cr, e.g. phosphate detergents and tanned leather. Metallic Cr, e.g. Cr-plated surfaces and stainless steel, is not known to give rise to contact eczema.

The US National Academy of Science has concluded that an acceptable intake (AI) of Cr for women 19–50 years of age is 25 µg day^{-1}, and for men of the same age 35 µg day^{-1}. A tolerable upper intake level was not established, since few serious adverse effects have been associated with excess intake from food (NAS, 2001).

Risk management

Intake recommendations

As Cr is not known to give any toxic effects at the concentrations found in normal foodstuffs, no maximum levels are laid down. As it is, to some degree, essential, there are some requirements on intake. In the UK, the Committee on Medical Aspects of Food Policy has recommended that chromium intakes should be above 25 µg day^{-1} for adults and between 0.1 and 1.0 µg kg^{-1} BW day^{-1} for children and adolescents (COMA, 1991).

Conclusions

Cr is occurring at low concentrations in most foods, although the concentration may vary considerably. It plays a part in the metabolism of glucose. The intake via (unprocessed) food is in some countries so low that intake recommendations are barely met. Low level contamination of food during processing and cooking is probably a considerable source for Cr in the diet.

Mercury

Silver-white; liquid at room temperature; stable in air, water and alkali. Mercury was one of the earliest metals known to man. It has been used in medicine and in cosmetics for millennia.

During the 20th century there were several major catastrophes of Hg poisoning through contaminated food, of which 'Minamata' probably is one of the more well known. This was caused by consumption of fish and shellfish contaminated by waste water containing Hg from chemical plants in the Minamata bay area in Japan. Methyl mercury was formed from inorganic Hg and accumulated in fish, which in turn poisoned the consumers. A large number of people died of the effects (Fujiki, 1972). In Iraq in 1972, over 6000 people were poisoned after eating bread made from wheat treated with methyl mercury. More than 400 people died (Bakir *et al*., 1973).

Uses

Although debated, it is still used in dental amalgam as well as in batteries. A large but decreasing use is as a catalyst in industrial processes. Another decreasing use is in electric switches in instruments and in thermometers.

Distribution in foods

Hg is not widely distributed in uncontaminated foodstuffs. The main source is fish, of which the predatory species, e.g. pike and swordfish, who are at the top of the marine food chain, have the highest levels, whereas herring, for example, usually have levels at the lower end (Table 9.8). Hg occurs predominantly (in the order of 50–80%) as methyl mercury in fish.

Normal intake levels

The great difference between the three fish surveys in Table 9.8 is probably due to

differences in fish species. As can be seen from Table 9.9, the daily intake of Hg via the diet is rather similar in all studies, independent of country. This would indicate that large predatory fish are not a staple food even in Japan, where consumption of marine products is high.

Uptake and metabolism in humans

Inorganic Hg in food is absorbed up to approximately 10%, whereas methyl mercury is absorbed efficiently to nearly 100%. As methyl mercury is both stable and lipophilic, it can penetrate cell membranes as well as the blood–brain barrier and be absorbed in the brain, where it can cause severe damage. It can also pass the placenta and be taken up by the fetus and affect the development of the nervous system. Children exposed to methyl mercury prior to birth may thus experience negative effects on their mental development (EHC, 1990).

Toxicity and clinical effects

Hg is a toxic metal with no known function in human metabolism. Inorganic Hg gives rise to a number of both acute and chronic symptoms. Some acute symptoms are: thirst; metallic taste; inflammation of the mouth, the lining of the stomach and the lining of the colon; nausea; abdominal pain; tenesmus (a continual inclination to evacuate the bowels or bladder, accompanied by a painful straining); and kidney degeneration. Some chronic symptoms are: excessive salivation; loosened teeth and inflammation of the gums; nervousness and irritability; tremors; and slurred speech. Symptoms from inorganic Hg, however, are not likely to occur through intake via food.

As previously described, methyl mercury is a highly toxic substance that has caused a lot of injury. Some of its early symptoms are fatigue, paraesthesia (sensation of prickling, burning, etc. on the skin) in, for example, the tongue and extremities, and headache. Later symptoms are insomnia, depression,

Table 9.8. Examples of Hg levels in uncontaminated foodstuffs (based on results from, for example, Ohlin, 1993; Cuadrado *et al.*, 1995; Ysart *et al.*, 1999, 2000).

Foodstuff		mg kg^{-1} fresh weight
Fruit, vegetables, dairy products, beverages		≤ 0.001
Meat and meat products, offal, eggs, cereals		≤ 0.01
Fish	Spain: Cuadrado *et al.* (1995)	0.13–0.19[a]
	Sweden: Ohlin (1993)	0.02–1.5
	UK: Ysart *et al.* (1999, 2000)	0.054 and 0.043

[a]Recalculated from dry weight.

Table 9.9. Average daily intake of Cr (mg) through the diet in certain countries.

	Adults	Adult females	Adult males	Reference
Sweden 1987 (MB)[a]	0.0018			Becker and Kumpulainen (1991)
Japan 1992 (DD)[b]		0.0099		Tsuda *et al.* (1995)
Japan 1992 (MB)		0.0035		Tsuda *et al.* (1995)
Spain (MB)	0.006			Cuadrado *et al.* (1995)
Australia 1994 (MB)		0.013	0.016	Marro (1996)
Australia 1996 (MB)		0.014	0.018	Hardy (1998)
UK 1994 (MB)	0.005			Ysart *et al.* (1999)
UK 1997 (MB)	0.003			Ysart *et al.* (2000)

[a]MB, market basket study.
[b]DD, duplicate diet study.

spasticity, constricted visual field, blurred speech, paralysis and fetal neurodevelopmental effects.

Risk assessment

An international expert group (WHO, 1993a) have decided on a PTWI for total Hg of 0.005 mg kg^{-1} BW, which is equal to a maximum intake of 0.350 mg of Hg $week^{-1}$ for a person weighing 70 kg. In addition, only 0.0033 mg kg^{-1} BW may be present as methyl mercury (= 0.23 mg^{-1} week for a 70 kg person). It was noted furthermore that pregnant women and nursing mothers were likely to be at greater risk for the negative effects of methyl mercury (WHO, 2000).

Risk management

Foodstuffs other than fish are generally very low in Hg and pose no threat to the health of the general population. The EC working group that established the MLs in Table 9.9 has given a good assessment of the risk.

> Methyl mercury may induce alterations in the normal development of the brain of infants and at higher levels may induce neurological changes in adults. Mercury contaminates mostly fish and fishery products. To protect public health, maximum levels of mercury in fishery products are laid down by Commission Decision 93/351/EEC. The levels should be as low as reasonably achievable, taking into account that for physiological reasons certain species concentrate mercury more easily in their tissues than others (EC, 2001).

Legislation/intake recommendations

The EC has reached an agreement on MLs for Hg in fish and fishery products, which will be binding for the Member States after 22 April 2002 and will replace existing national limits. These MLs are summarized in Table 9.10.

Conclusions

Food in general contains very low levels of Hg and it is no threat to human health. Certain predatory fish may contain very high levels and could constitute a health risk. For these species, an ML of 1.0 mg kg^{-1} has been established. Women should limit their consumption of fish during pregnancy and lactation.

Nickel

A silver-white, lustrous, ductile, corrosion-resistant metal. It was discovered in 1751 in Sweden by Axel Fredrik Cronstedt.

Uses

Its main use is as an alloy in stainless steel and coins. It is also used for metal plating and in batteries. Another important area of use is as a catalyst in chemical processes.

Distribution in foods

Ni can be found in virtually every foodstuff. The lowest levels are usually found

Table 9.10. Maximum levels of Hg in foodstuffs (EC, 2001). All references in the official list are omitted.

Product	Maximum level (mg kg^{-1} wet weight)
Fishery products, except those listed below	0.5
Examples of species excepted from above: Anglerfish, Atlantic catfish, bass, eel, halibut, marlin, pike, rays, shark (all species), sturgeon, swordfish, tuna	1.0

in animal products and milk. Cereals, fruit and berries have intermediate levels, and high levels are found, for example, in cocoa. More information is found in Table 9.11. The number of reliable surveys of Ni in foods and diets is, however, rather limited.

Normal intake levels

Much of the Ni content in diets probably stems from contamination during processing and preparation. It is therefore rather surprising to see the very high degree of agreement between the different types of study as well as within and between countries (Table 9.12).

Electric water heaters may constitute a considerable source of Ni in the diet, a source more or less unlikely to be included in intake studies. Studies in Denmark and Sweden have shown that heaters with Ni-plated or stainless steel elements can give hot water with up to 1 mg Ni l^{-1}, although the variation is considerable. (Pedersén and Petersén, 1995; Jorhem *et al*., 1997).

Uptake and metabolism in humans

Absorption of Ni from food is estimated to be in the order of 0.7%, if ingested together with food, whereas Ni in beverages is absorbed more efficiently, especially if ingested on an empty stomach. Ni has been shown to be essential in animal studies, but not in the human metabolism (NAS, 2001).

Toxicity and clinical effects

Toxic effects are due mainly to occupational or accidental exposure and not via food. Accidental ingestion of Ni salts has resulted in nausea, diarrhoea and vomiting. The most pronounced negative effect of Ni is its strong allergenic properties. It is estimated that approximately 10% of all women and 1% of men in Denmark and Sweden develop allergic reactions to Ni-containing items such as jewellery, coins and metal buttons (Jorhem *et al*., 1996). Eczema usually develops on skin that is directly exposed to Ni-containing objects. There are, however, people who

Table 9.11. Examples of Ni in uncontaminated foodstuffs (based on results from, for example Jorhem and Sundström, 1993; Ysart *et al*., 1999, 2000).

Foodstuff	mg kg^{-1} fresh weight
Meat, fish, milk, liver and kidney	≤ 0.02
Fruit and vegetables, cereals	≤ 0.1
Milk chocolate, berries, wild mushrooms	≤ 1
Cocoa – dark chocolate, buckwheat, lentils, seeds, beans, nuts	1–5

Table 9.12. Average daily intake of Ni (mg) through the diet in certain countries.

	Adults	Adult females	Adult males	Reference
Sweden 1987 (MB)[a]	0.082			Becker and Kumpulainen (1991)
Sweden 1988 (DD)[b]		0.11		Jorhem *et al*. (1998)
Germany 1992 (DD)		0.14	0.17	Seifert and Anke (1999b)
Germany 1996 (DD)		0.090	0.097	Seifert and Anke (1999b)
UK 1994 (MB)	0.13			Ysart *et al*. (1999)
UK 1997 (MB)	0.12			Ysart *et al*. (2000)

[a]MB, market basket study.
[b]DD, duplicate diet study.

develop eczema or blisters on non-exposed skin. For this group of people, it has been suggested that intake of Ni via food can have an enhancing effect (Veien and Menné, 1990).

Risk assessment

The risk of consuming foods with toxic levels of Ni seems highly improbable. A major source of Ni for some people could be water heaters. Beverages from such heaters can have high levels of Ni. As such beverages often are consumed on an empty stomach, which promotes Ni absorption, they could constitute a risk for enhanced problems for people with grave Ni allergy (Jorhem *et al*., 1996).

As nickel is not known to be toxic at the concentrations found in normal foodstuffs, no maximum levels are laid down. The WHO has set a tolerable daily intake (TDI) of 0.005 mg kg^{-1} BW, which corresponds to 0.35 mg day^{-1} for a person weighing 70 kg (WHO, 1993b). The US National Academy of Science (NAS, 2001) has, for certain population groups, established an upper intake level (UL) which is 'the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects for almost all individuals'. For adolescents and during pregnancy and lactation, the UL is set at 1.0 mg day^{-1} (of soluble Ni salts).

Conclusions

The Ni level in food constitutes no health risk to the general population. Groups with severe Ni allergy may be helped by selecting food with low Ni levels. The major source of Ni in the diet is probably contamination during processing and, for some people, through the use of water heaters. People with serious Ni allergy may benefit from avoiding foods with high Ni levels.

Lead

Soft, malleable, dark greyish metal, which has been known by man for more than 6000 years. Pb and Pb compounds have found wide use over the millennia. Pots and pans of Pb and pewter were popular for cooking in Roman times. Grape syrup boiled down in a lead pot acquired a nice sweet taste (lead acetate), which made it useful for sweetening of sour wines. It was also an excellent preservative. It has been used in medicine since ancient times, e.g. Pb acetate against diarrhoea and even as tooth fillings. Different Pb compounds have been used as cosmetics, e.g. galena (lead sulphate) as eye make-up. Pb poisonings of epidemic proportions have been reported repeatedly during the last millennium, e.g. in 1738 in Devon through Pb-contaminated cider. In the USA, vast numbers of inner city children were identified as lead poisoned into the 1980s. One source was identified as the ingestion of Pb-containing paint flakes. Ceramics with poorly applied Pb glazing have always been a 'reliable' source for Pb intake (Gilfillan, 1965; Lin-Fu, 1980; Nriagu, 1983; Wooley, 1984). The introduction of tetramethyl- and tetraethyl lead as an anti-knocking agent in petrol during the 1920s assisted in spreading enormous quantities of Pb into the environment and to man, through contaminated food and (urban) air.

Pb compounds have also been used for outright adulteration of foodstuffs: curry by addition of lead chromate (yellow), butter and sugar with 'white lead', and paprika powder with 'red lead'.

Uses

Pb is used in car batteries, pewter, as a stabilizer in plastics, colour pigments, porcelain glazes and for radiation protection.

Distribution in foods

Pb can be detected in most foods, but there are only a few foods that naturally contain high levels (Table 9.13). Many of the most consumed foods, such as meat, potatoes and milk, have levels below, or even far below, 0.01 mg kg^{-1}. Wine

usually has Pb levels below 0.05 mg kg^{-1}, but can reach higher levels, mostly in wine from 'old' wine countries or in vintage wine.

During the last two decades two parallel events have led to a considerable decrease in the intake of Pb via food.

1. Welding of the side seam of tin cans has gradually replaced the Pb-soldered side seams during the last two decades. The impact of the introduction of this new production technique cannot be overemphasized. The earlier type of tin can with Pb-soldered side seams resulted in foodstuffs often containing more than 0.1 mg of Pb kg^{-1}, not seldom exceeding 0.5 mg kg^{-1}. This lead source is now, fortunately, history. In cans with welded side seams, the lead content is not very different from that in the corresponding fresh food. Foodstuffs in cans with an unlacquered inner surface may, however, still have a slight increase in Pb due to contamination from the exposed tin layer (Jorhem and Slorach, 1987).

2. The reduction/elimination of petrol with added tetraethyl or tetramethyl has resulted in a much reduced level of Pb contamination of, in particular, vegetables. The effect of the reduced exposure to Pb via food is clearly visible in Table 9.14, which is based on the results from the UK Total Diet Studies 1976–1997 (Ysart *et al*., 2000).

Normal intake levels

The present-day intake levels in the UK are very similar to what has been found in several other countries from the late 1980s and onwards (Table 9.15).

Uptake and metabolism in humans

The uptake of Pb from food by adults is in the order of 10%, whereas children may have an uptake of up to 50%. Most of the Pb is accumulated in the skeleton. Pb can pass the placenta barrier and the blood–brain barrier in children.

Toxicity and clinical effects

Pb has no known function in human metabolism. Some of its negative effects have been known for millennia. Some acute effects are headache, irritability and colic (gripes). Pb displays several chronic effects, such as colic, constipation, anaemia, pallor, palsy, disturbed reproduction, fetal neurodevelopmental effects and reduced learning capacity in children. Experiments on mice have shown that Pb poisoning may have a negative effect on female reproduction for three generations (Wide, 1985).

Table 9.13. Examples of Pb levels in uncontaminated foodstuffs (based on results from, for example, Jorhem and Sundström, 1993; Marro, 1996; Hardy, 1998; Ysart *et al*., 1999, 2000).

Foodstuff	mg kg^{-1} fresh weight
Meat including most fish, milk, eggs, potatoes	≤ 0.01
Most vegetables, wheat and rye flour, oats, wine	≤ 0.05
Leafy vegetables, liver, kidney from domestic animals	≤ 0.2
Most wild mushrooms, mussels	≤ 0.5
Certain wild mushrooms, liver from game animals	≤ 1

Table 9.14. Comparison of population dietary exposure results for lead from the UK Total Diet Studies 1976–1997 (Ysart *et al*., 2000). Average daily intake in mg.

Year of study	1976	1978	1980	1982	1984	1986	1988	1991	1994	1997
Intake level	0.11	0.11	0.12	0.069	0.065	0.06	0.06	0.028	0.024	0.026

Risk assessment

Intake of Pb via food should be kept as low as possible. A PTWI for Pb (0.025 mg kg^{-1} BW) has been decided by an international expert group. This is equal to 1.75 mg of Pb $week^{-1}$ for a person weighing 70 kg (WHO, 1993a).

Risk management

The banning of Pb additives in petrol and the phasing out of tin cans with Pb-soldered side seams have, as mentioned, radically reduced the Pb burden in man as well as the environment. Although several sources for Pb have been drastically reduced/eliminated over the last decades, risks still remain. This has been nicely described by the EC working group who established the MLs in Table 9.16:

> Lead absorption may constitute a serious risk to public health. Lead may induce reduced cognitive development and intellectual performance in children and increased blood pressure and cardiovascular diseases in adults. Over the past decade the levels in food decreased significantly due to the awareness of lead being a health problem, source-related efforts to reduce the emission of lead and improvements in quality assurance of chemical analysis. The SCF concluded in its opinion of 19 June 1992 that the mean

Table 9.15. Average daily intake of Pb (in mg) through the diet in certain countries.

	Adults	Adult females	Adult males	Reference
Australia 1994 (MB)[a]		0.021	0.027	Marro (1996)
Australia 1996 (MB)		0.030	0.038	Hardy (1998)
Germany 1996 (DD)[b]		0.019	0.019	Seifert and Anke (2000)
Spain (MB)	0.18			Cuadrado *et al.* (1995)
Sweden 1987 (MB)			0.017	Becker and Kumpulainen (1991)
Sweden 1988 (DD)		0.026		Vahter *et al.* (1990)
UK 1994 (MB)	0.023			Ysart *et al.* (1999)
UK 1997 (MB)	0.024			Ysart *et al.* (2000)

[a]MB, market basket study.
[b]DD, duplicate diet study.

Table 9.16. Maximum levels of Pb in foodstuffs (EC, 2001). All references in the official list are omitted.

Product	Maximum level (mg kg^{-1} wet weight)
Cow's milk, infant formulae	0.02
Fruit juices, concentrated fruit juices (for direct consumption) and fruit nectars	0.05
Meat of bovine animals, sheep, pig and poultry; vegetables, excluding brassica, leafy vegetables, fresh herbs and all fungi. In the case of potatoes, the maximum level applies to peeled potatoes. Fruits, excluding berries and small fruits, fats and oils	0.1
Muscle meat of most fish, berries and small fruits, cereals, legumes and pulses, wines	0.2
Brassica, leafy vegetables and all cultivated fungi	0.3
Muscle meat of wedge sole, eel, spotted seabass, horse mackerel or scad, grey mullet, common two-banded seabream, grunt, European pilchard or sardine	0.4
Edible offal of cattle, sheep, pig and poultry; crustaceans, excluding brown meat of crab	0.5
Bivalve molluscs, cephalopods (without viscera)	1.0

> level of lead in foodstuffs does not seem to be a cause of alarm, however, longer term action should follow with the objective of further lowering the mean levels of lead in foodstuffs. Therefore, the maximum levels should be as low as reasonably achievable (EC, 2001).

Another amelioration, perhaps small but with a great symbolic value, is the banning of Pb seals on wine bottles, which came into effect in the early 1990s.

Legislation/intake recommendations

The EU has reached an agreement on MLs for Pb in certain foods. These will be binding for the Member States after 22 April 2002 and will replace existing national limits (Table 9.16).

Conclusions

The contamination of food with Pb has been drastically reduced over the last decades through source-related actions, such as the phasing out of organic Pb compounds in petrol, introduction of Pb-free tin cans and prohibiting Pb seals on wine bottles. This has, in turn, led to a radically decreased intake of Pb via food. Although no immediate danger can be seen, there are continuous on-going efforts to reduce further the intake of Pb via food.

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10 Dietary Nitrates, Nitrites and *N*-nitroso Compounds and Cancer Risk with Special Emphasis on the Epidemiological Evidence

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Introduction

The present chapter is an update of our review article published in 1998 (Eichholzer and Gutzwiller, 1998) on the epidemiological evidence relating estimated dietary intake of *N*-nitroso compounds (NOCs), nitrates and nitrites and the human risk of cancer of various sites. Thus, this chapter is not intended to assess health risks other than cancer, such as nitrite-induced methaemoglobinaemia in infants. Nor does it intend to be a comprehensive survey on subjects such as distribution in foods, uptake and metabolism, and animal toxicity of nitrates, nitrites and *N*-nitroso compounds. Rather, its main purpose is to highlight information which may be of relevance of human cancer risk assessment of these components in the diet. No attempt is made to consider non-dietary routes of exposure (e.g. inhalation).

Nature of Nitrate, Nitrite and *N*-nitroso Compounds

Nitrate is usually referred to as the NO_3^- ion or as sodium nitrate ($NaNO_3$); similarly, nitrite is expressed as the NO_2^- ion or sodium nitrite ($NaNO_2$). *N*-nitroso compounds can be divided into two categories: the class of nitrosamines (e.g. *N*-nitrosodimethylamine (NDMA)) and the class of nitrosamide-type compounds including *N*-nitrosoureas, *N*-nitrosocarbamates and *N*-nitrosoguanidines. Compounds of both groups differ considerably in chemical formation and biological effectiveness (Council of Europe, 1995).

Distribution in Foods

Vegetables usually contribute 75–80% of the total daily intake of nitrate, with high levels in lettuce, spinach, celery, beetroot, turnip greens, etc. The nitrate concentration of drinking water, another contributor to the total exposure to nitrate, varies widely depending on the source (high concentrations in private water supplies), season and proximity to arable land. Nitrates and nitrites are widely used in the production of cured meat products and added as preservatives to fish in some countries. Nitrites are also found naturally in some grains and vegetables.

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Many NOCs have been detected in foods, but only NDMA is well studied; thus, more is known, for example about the sources of human exposure to nitrosamines than to nitrosamides. NDMA has been found in various processed meats (salted, cured or smoked, such as bacon) and fish, and in beer, etc. Overall, nitrate, nitrite and NOC concentrations in food products can vary widely for the same food or for drinking water from different sites. Furthermore, nitrosation may occur, before food intake, between nitrite which has been formed from nitrate by contaminating bacteria and amines and amides present in the same food. In assessing the health risk to man from dietary exposure to nitrate, nitrite and NOCs it is therefore important to recognize that the analysis of the exposure is particularly complex. In addition, endogenous formation of NOCs and their precursors, may be an important source of exposure (see below) (Ikins *et al*., 1986; Walker, 1990; Gangolli *et al*., 1994; Council of Europe, 1995).

Uptake and Metabolism in Humans/Animal Models and Maternal Transmission

Dietary nitrate is absorbed from the proximal small intestine into the plasma. About 25% of the ingested nitrate is secreted in the saliva and, of this, approximately 20% is converted to nitrite in the mouth by nitrate-reducing bacteria. Nitrate and nitrite can also be formed endogenously in enzymatic reactions mediated by bacteria, macrophages and neutrophils. It has been postulated that in the stomach nitrite might nitrosate, for example, secondary amines ingested in food to form nitrosamines. Such endogenous formation of NOCs has been demonstrated in experimental animals and in humans; it occurs by nitrosation of amines or amides via their acid- or bacterial (gastric hypochlorhydria) catalysed reaction with nitrite, or by reaction with products of nitric oxide generated during inflammation and infection. Reducing substances, such as ascorbic acid, actively secreted by the gastric mucosa and present in vegetables, etc., on the other hand prevent nitrosation. Thus humans are exposed to pre-formed NOCs and to NOCs produced *in vivo*, but it is very difficult to estimate the daily amount formed endogenously. In relation to maternal transmission, it has, for example, been observed that ethyl urea and nitrite fed to pregnant rats caused formation of ethyl nitrosourea brain tumours in all their offspring (Preston-Martin *et al*., 1982; Bartsch *et al*., 1988, 1992; Bartsch, 1991; Gangolli *et al*., 1994; Mirvish, 1995; Hecht, 1997; Lee *et al*., 1997; Hill, 1999; McKnight *et al*., 1999).

Toxicity and Clinical Effects

The carcinogenicity of nitrate, nitrite and NOCs has been reviewed extensively and updated continually in the light of new data emerging from ongoing research on these important chemicals, and this chapter is not intended to be a comprehensive survey on the carcinogenic effects of these compounds. Nitrate in itself is not carcinogenic. There is also no (or controversial) evidence of direct nitrite (other than through nitrosamine formation) carcinogenicity in animals. On the contrary, various NOCs have been found to be carcinogenic to multiple organs in at least 40 animal species, including higher primates (Bogovski and Bogovski, 1981). The cellular and molecular changes induced by some NOCs in animals have been shown to be very similar to those in human tissues (Bartsch, 1991). Distinct organ specificity is an important characteristic of NOCs (Gangolli *et al*., 1994; Council of Europe, 1995; McKnight *et al*., 1999).

Risk Assessment in Epidemiology

Ecological studies are typically a starting point for more detailed and better controlled epidemiological research, i.e. analytical epidemiological studies such as case–control, cohort and intervention studies. Evidence from ecological studies cannot, in isolation, amount to more than a possible causal relationship. Greater reliance can be placed on aggregate evidence from individually based

case–control and cohort studies. Although case–control studies are of shorter duration and less expensive than cohort studies, risk estimates may be distorted by selection and recall bias. Cohort studies are less susceptible to such bias, as information is collated before a disease develops. Both types of studies are, however, prone to confounding, in that the variables analysed are merely surrogates for the actual (but unmeasured) active agent. In randomized, placebo-controlled, intervention trials, subjects are allocated at random to either active treatment or placebo, and this type of study design avoids known and unknown confounding. When the results of case–control and cohort studies are repeatedly consistent, this strengthens the case for causal links. Large, well-designed controlled trials may produce strong and consistent conclusions, but they are not feasible in the present context. Data from experimental studies in animal models, on the contrary, may reinforce human evidence, but, in isolation, are of limited evidence (Hennekens and Buring, 1987; World Cancer Research Fund and American Institute for Cancer Research, 1997). Despite extensive information regarding carcinogenicity of NOCs to animals, there have been few analytical epidemiological studies investigating the risk in humans, and what is available is mainly limited to ecological and case–control studies. The present chapter is an update of our review (Eichholzer and Gutzwiller, 1998) on the epidemiological evidence (excluding ecological studies) relating estimated dietary intake of NOCs, nitrates and nitrites (and some examples of individual foods rich in these substances when no data on NOCs, nitrate and nitrite are available) and the risk of brain, stomach, oesophageal and nasopharyngeal cancers. The main emphasis is given to brain tumours, as most of the newer data are related to this cancer site. For a number of additional cancer sites such as leukaemia, non-Hodgkin's lymphoma, renal cell and testicular cancers and cancer of the bladder and colon, single studies exist showing associations with NOCs and precursors (Foster *et al*., 1997; Moller, 1997; Yuan *et al*., 1998; Law *et al*., 1999; Mohsen *et al*., 1999; Roberts-Thomson *et al*., 1999). These cancer sites as well as cancer of the oesophagus will not be discussed in the present chapter, the latter due to the fact that not enough new data have been published since we reviewed the evidence in 1998.

Brain Tumours

Incidence rates and pathogenesis

Astrocytoma, medulloblastoma, ependymoma, glioblastoma and meningioma are the most common types of brain tumours. The age curve of these tumours shows a peak during the first decade of life followed by peaks in adults, except for medulloblastoma, which is rarely observed in adults, and meningioma, which is less prevalent in children than in adults. Brain tumours account for about one in five childhood cancers. The highest rates of tumours of the central nervous system are observed in Israeli females and in the female population of Iceland. Intermediate rates are seen in most western countries. Rates in Asian populations are lowest. In children, the highest rates are observed in Nordic countries. Very little is known about the aetiology of brain tumours. Inherited syndromes that predispose to brain tumour development such as neurofibromatosis are present in fewer than 5% of patients. Ionizing radiation, the only established environmental cause, similarly accounts for no more than a few per cent of cases (Higginson *et al*., 1992; Preston-Martin *et al*., 1996). One postulated risk factor that has been the subject of investigation is exposure to NOCs and precursor nitrates and nitrites, some of which are nervous system carcinogens in animals, especially when exposure occurs transplacentally (see above). The hypothesis has been expanded recently to include adults as well as childhood brain cancer.

Epidemiological evidence

Under the auspices of the SEARCH programme of the International Agency for Research on Cancer (IARC), a series of multi-

centred international coordinated case–control studies was initiated to evaluate, *inter alia*, the roles of NOCs, their precursors and modulators of their metabolism in the occurrence of childhood and adult brain tumours. Common methods of exposure and specificity of diagnosis will allow pooling of the data of all studies. This increases statistical power, and analysis by histological subtypes will be possible (Giles *et al*., 1994; McCredie *et al*., 1994).

Diet during pregnancy and risk of childhood brain tumours

The observation that various NOCs are potent nervous system carcinogens, particularly when animals are exposed transplacentally, prompted Preston-Martin *et al*. (1982) to propose that pre-natal and early exposures might be related to childhood brain tumours in humans. Since 1982, 11 case–control studies of childhood brain tumours and maternal diet during pregnancy have focused on aspects of diet related to the hypothesis that transplacental exposure to NOCs increases the risk of brain tumours in childhood. Three studies also considered dietary intake of children (Howe *et al*., 1989; Sarasua and Savitz, 1994; Lubin *et al*., 2000). The methods and the results of these studies are described in Table 10.1. Most studies investigated all childhood brain tumours combined, despite the fact that different brain tumours may have different aetiologies. Furthermore, in most studies, consumption of cured meat was used as a crude indicator of NOCs and nitrite exposure; in five studies, intake of nitrates, nitrites and/or NOCs was estimated. In Los Angeles County, Preston-Martin and co-workers (1982) questioned mothers of 209 young brain tumour patients and mothers of 209 population-based controls about experiences of possible aetiological relevance which they had during pregnancy, including frequency of consumption of cured meats. Results suggested an aetiological role for cured meats (odds ratios (ORs) = 1.2 for moderate, 2.3 for high vs. low intake; P trend = 0.008) and other NOC-containing substances in childhood brain tumours. In a small Canadian case–control study (Howe *et al*., 1989) comparing the children's intake of cured meats prior to diagnosis, no significant association was observed. Beer consumption during pregnancy, on the other hand, increased the risk of childhood brain tumour significantly. Two other rather small studies, i.e. surveys with a low power to detect existing associations, generally observed no associations with cured meat consumption in pregnancy (Cordier *et al*., 1994; Sarasua and Savitz, 1994), but in the study by Sarasua and Savitz (1994) an increased risk for consumption of hot dogs during pregnancy (OR = 2.3 (95% confidence interval (CI) = 1.0–5.4)) and childhood (OR = 2.1 (95% CI = 0.7–6.1)) was found. A slightly increased risk was also observed with consumption of ham, sausage and bacon by the child. Non-significant positive and negative associations, respectively, were observed by Cordier *et al*. (1994) with intake of nitrate and nitrite. Despite the small number of cases, in the Australian case–control study by McCredie *et al*. (1994), the risk of childhood brain tumours rose significantly with reported increasing consumption, during pregnancy, of cured meats. The same was true for the much larger studies of Preston-Martin *et al*. (1996) and Schymura *et al*. (1996). In the former study, nitrite from cured meat, but neither total nitrite nor nitrite from vegetables, was related to brain cancer risk. A recently published survey carried out in Israel (Lubin *et al*., 2000) observed no association between intake of nitrate and nitrite during pregnancy or childhood and risk of brain tumour.

Few studies have concentrated on a single type of brain tumour in children. Gestational and familial risk factors were investigated for their association with astrocytoma in a case–control study of 163 pairs performed in Pennsylvania, New Jersey and Delaware (Kuijten *et al*., 1990). A significant trend showing more frequent consumption of cured meats in mothers of astrocytoma cases compared with control mothers was observed. However, the association was only significant among more highly educated mothers (OR = 6.8 (95% CI = 1.8–26.3)). Conversely, a study by Bunin and co-workers (1993) showed no elevated risk with frequent maternal consumption of cured meats,

Table 10.1. Case–control studies on dietary intake of nitrates, nitrites and *N*-nitroso compounds (or the corresponding foods) during pregnancy and the risk of brain tumours in children.

Reference and number of cases	Brain tumour	Dietary variable (intake during pregnancy)	Comparison	Association	OR (95% CI)	Adjusted/ matched for	Population
Preston-Martin *et al.* (1982) *n* = 209	'Brain tumours'	Cured meats	High vs. lower	↑	2.3; *P* trend 0.008	A–D	Los Angeles County, USA, children < 25 years
Howe *et al.* (1989) *n* = 74	'Brain tumours'	Cured meats (child)	> 1 × week^{-1} vs. ≤ 1 × week^{-1}	NS	1.13 (0.55–2.31)	A, B, E, F	Southern Ontario, Canada, children ≤ 19 years
		Beer (pregnancy)	Ever vs. never	↑	3.53 (1.16–10.8)		
Kuijten *et al.* (1990) *n* = 163	Astrocytoma	Cured meat	Yes vs. no	NS	1.9 (0.9–4.2); *P* trend 0.04	A, C, E, R[a]	Children < 15 years old in New Jersey, Delaware and Pennsylvania, USA
		Mothers	Frequency				
		highly educated	high	↑	6.8 (1.8–26.3)		
		less educated	high	NS	1.2 (0.4–3.8)		
Bunin *et al.* (1993) *n* = 166	Primitive neuroectodermal tumour	Nitrate	Quartile 4 vs. quartile 1	NS	0.54 (–)	A, C, R, S	USA and Canada, children < 6 years
		Nitrate		NS	1.06 (–)		
		Nitrosamines		NS	1.55 (–)		
		Cured meats		NS	1.10 (0.60–2.03)		
Bunin *et al.* (1994) *n* = 155	Astrocytic glioma	Cured meats	Quartile 4 vs. quartile 1	NS	1.7 (0.8–3.4)	A, C, D, R	USA and Canada, children < 6 years
		Nitrite		NS	1.3 (0.7–2.6)		
		Nitrate		NS	0.7 (0.3–1.4)		
		Dimethylnitrosamine		NS	0.8 (0.4–1.8)		
Cordier *et al.* (1994) *n* = 75	'Brain tumours'	All cured meats	≥ 1 × week^{-1} vs. < 1 × week^{-1}	NS	0.7 (0.2–3.0)	A, B, E, G, H	Ile de France, children ≤ 15 years
		ham		NS	0.9 (0.2–3.4)		
		other		NS	0.7 (0.3–1.6)		
		Nitrite	Quartile 4 vs. quartile 1	NS	0.4 (0.1–1.4)		
		Nitrate	Quartile 4 vs. quartile 1	NS	1.5 (0.5–4.6)		
McCredie *et al.* (1994) *n* = 82	Tumour of brain or cranial nerves	Cured meats	Quartile 4 vs. quartile 1	↑	2.5 (1.1–5.7)	B, E, H, K, J, L	New South Wales, Australia, children ≤ 14 years

continued

Table 10.1. *Continued.*

Reference and number of cases	Brain tumour	Dietary variable (intake during pregnancy)	Comparison	Association	OR (95% CI)	Adjusted/ matched for	Population
Sarasua and Savitz (1994)	'Brain tumour'	During pregnancy	≥ 1 week^{-1} vs. < 1 × week^{-1}	NS	1.0 (0.5–2.1)	B, D, E, F, I	Denver, Colorado, USA, children < 14 years
n = 45		ham, bacon, sausage, hot dogs, lunch meats	> 0 week^{-1} vs. 0 week^{-1}	(↑)	2.3 (1.0–5.4)		
			≥ 1 week^{-1} vs. < 1 × week^{-1}	↓	0.4 (0.2–0.8)		
		Child up to diagnosis					
		ham, bacon, sausage, hot dogs, lunch meats	≥ 1 week^{-1} vs. < 1 × week^{-1}	NS	1.4 (0.6–3.1)		
			≥ 1 week^{-1} vs. < 1 × week^{-1}	NS	2.1 (0.7–6.1)		
			≥ 1 week^{-1} vs. < 1 × week^{-1}	NS	0.6 (0.3–1.4)		
Preston-Martin *et al.* (1996)	'Brain tumour'	Cured meats	> Daily vs. never	↑	2.1 (1.3–3.2)	A–F	19 counties, US West Coast, children < 20 years
n = 540		Nitrite total	Quartile 4 vs. quartile 1	NS	1.1 (0.79–1.50)		
		from cured meat	Quartile 4 vs. quartile 1	↑	1.9 (1.3–2.6)		
		from vegetables	Quartile 4 vs. quartile 1	NS	0.98 (0.71–1.3)		
Schymura *et al.* (1996)	'Brain tumour'	Hot dogs	Once week^{-1} vs. less	(↑)	1.33 (1.00–1.76)	A–C, M	New York, USA, children
n = 338		'Other' cured meat	2–3 × week^{-1} vs. less	↑	2.01 (1.10–3.63)		
			Once week^{-1} vs. less	↑	6.04 (1.89–19.31)		
Lubin *et al.* (2000)	'Brain tumour'	During pregnancy				A, B, N–Q	Israel, children < 18 years
n = 300		nitrate	Intermediate vs. low	NS	1.10 (0.83–1.46)		
		nitrite		NS	0.97 (0.74–1.27)		
		Child life					
		nitrate		NS	1.07 (0.81–1.40)		
		nitrite		NS	0.91 (0.66–1.25)		

CI, confidence intervals; NS, not statistically significant; ↑, statistically significant direct association; (↑), lower 95% CI = 1; ↓, statistically significant inverse association; A, birth year; B, sex; C, race; D, socio-economic status; E, age at diagnosis; F, residence; G, maternal age; H, maternal education; I, other types of meat (charcoal grilled foods, hamburgers, lunch meats and each other); J, mother's body mass index just before pregnancy; K, vegetables; L, fruit; M, potential confounders; N, country of birth; O, energy intake; P, vitamin C; Q, each other; R, telephone exchange; S, food components and supplements.

[a]'Controls were pair matched to cases for telephone exchange, i.e. a series of telephone numbers was formed by retaining the area code exchange, and next two digits of the phone number and randomly generating the final two digits' (Kuitjen *et al.*, 1990).

nitrates (OR = 0.54) or nitrites and primitive neuroectodermal tumour in children, but a non-significant increased risk of 1.5 for high intake of nitrosamines. A parallel study of astrocytic glioma in children (155 case–control pairs) was conducted by the same investigators and interviewers using the identical questionnaire (Bunin *et al*., 1994). Non-significant elevated risks between cured meat and nitrite consumption during pregnancy and risk of astrocytic glioma were shown, but high vs. low intake of dimethylnitrosamines was associated with an OR of 0.8 (95% CI = 0.4–1.6).

Adult brain tumours

More recently, the NOCs hypothesis has been expanded to include adult as well as childhood brain cancer. The methods and the results of these studies are described in Table 10.2. Burch *et al*. (1987) studied 215 adult males (25–80 years of age) and an equal number of hospital-based controls. The study included many dead cases. Thus, the quality of dietary data was poor because of the large number of proxy respondents. The investigators observed elevated risks for reported use of spring water (OR = 4.33 (95% CI = 1.24–15.2)) and wine consumption (OR = 2.14 (95% CI = 1.28–3.60)) (ever vs. never) for brain tumours in general. Although wine and spring water consumption is consistent with a role for NOCs in the aetiology of brain tumours, for several other factors related to this hypothesis (e.g. consumption of processed meat and fish products), no significant association was found. Preston-Martin *et al*. (1989) studied employment histories and other potential risk factors of 272 men aged 25–69 years with a primary brain tumour first diagnosed during 1980–1984 in Los Angeles County. Separate analyses were carried our for 202 glioma pairs and 70 meningioma pairs. No significant direct association between NOC-rich beer, wine and hard liquor consumption, and risk of gliomas or meningiomas in males was observed, but there was a significant inverse association of glioma with beer consumption and a non- significant increased risk with hard liquor. A small Swedish case–control study (Ahlbom *et al*., 1986) found a marginally significant OR of 2.1 (95% CI = 1.0–4.4) for the consumption of bacon or smoked ham, and non-significant elevated ORs for the consumption of smoked sausage or fish when astrocytoma cases were compared with community controls. Deleting proxy data from the analysis of this study did not affect the ORs. A German case–control study (Boeing *et al*., 1993) revealed an increased glioma risk associated with the consumption of various NOCs (NDMA, *N*-nitrosopyrrolidine and *N*-nitrosopiperidine), but no association with endogenous *N*-nitrosation, i.e. consumption of nitrate or nitrite, was observed. In an Australian study, risk of glioma or meningioma in adults was decreased with consumption of beer, wine or spirits; for wine, these inverse associations were statistically significant (Ryan *et al*., 1992). In a population-based case–control study in Melbourne (Giles *et al*., 1994) comprising 416 gliomas, a significantly elevated OR in men and a non-significant OR in women were associated with the intake of NDMA. In women but not in men, the intake of nitrate was significantly inversely associated with gliomas. In men but not in women, the intake of nitrite showed a non-significant increased risk. A case–control study in Los Angeles County (Blowers *et al*., 1997) of 94 women with gliomas found that risk increased with increasing consumption of cured meats and fish (OR = 1.7 (95% CI = 0.8–3.8)), most notably of bacon (OR = 6.6 (95% CI = 1.9–22.5)), and estimated nitrite intake from cured meats (OR = 2.1 (95% CI = 1.0–4.6)) but not significantly with nitrite intake from all foods (OR = 1.4 (95% CI = 0.6–3.5)). In a case–control study in Israel, intake of NOCs was associated with an increased risk of meningiomas (OR = 1.98 (95% CI = 0.97–4.05)) but not gliomas (OR = 0.79 (95% CI = 0.32–1.96)) in adults aged 18–75 years (Kaplan *et al*., 1997). In the case–control study by Lee *et al*. (1997), for both men and women, glioma cases were more likely than controls to be categorized as having a high risk diet (high consumption of cured foods and low consumption of fruits and vegetables rich in vitamin C, or high consumption of nitrite

Table 10.2. Case–control studies on dietary intake of nitrates, nitrites and *N*-nitroso compounds (or the corresponding foods) and the risk of brain tumours in adults.

Reference and number of cases	Brain tumour	Dietary variable	Comparison	Association	OR (95% CI)	Adjusted/ matched for	Population
Ahlbom *et al.* (1986)	Astrocytomas	Bacon, smoked ham,	≥ 1 × week^{-1} vs. < 1 ×	(↑)	2.1 (1.0–4.4)	A–C	Stockholm,
n = 78		smoked sausage,	week^{-1}	NS	1.7 (0.9–3.1)		Sweden, adults
(population control)		smoked fish		NS	1.5 (0.4–5.6)		20–75 years
Burch *et al.* (1987)	'Brain tumour'	Spring water	Ever vs. never	↑	4.33 (1.24–15.2)	A–E	Southern
n = 215		Wine	Ever vs. never	↑	2.14 (1.28–3.60)		Ontario, Canada, adults 25–80 years
Preston-Martin *et al.* (1989)	Gliomas (G) Meningiomas	Beer	> 1 × month^{-1} vs. less	NS ↓	G: 0.7 (0.5–1.2) M: 0.4 (0.1–0.9)	A, C, F	Los Angeles County, USA,
n = 272	(M)	Wine		NS	G: 0.7 (0.5–1.1)		men 25–69
				NS	M: 0.7 (0.3–1.4)		years
		Hard liquor		NS	G: 1.3 (0.8–1.9)		
				NS	M: 0.7 (0.3–1.4)		
Ryan *et al.* (1992)	Gliomas (G)	Gliomas	Yes vs. no		0.77 (0.47–1.27)	A–C	Adelaide,
n = 170	Meningiomas	Beer		NS	0.58 (0.38–0.91)		Australia, adults
	(M)	Wine		↓	0.78 (0.49–1.24)		25–74 years
		Spirits		NS			
		Meningiomas			0.51 (0.25–1.06)		
		Beer		NS	0.54 (0.30–0.97)		
		Wine		↓	0.66 (0.35–1.27)		
		Spirits		NS			
Boeing *et al.* (1993)	Gliomas	Nitrate	Tertile 3 vs. tertile 1	NS	0.9 (0.5–1.5)	A–C, G, H	Germany, adults
n = 115		Nitrite		NS	1.1 (0.6–2.0)		25–75 years
		NDMA		↑	2.8 (1.5–5.3)		
		NPYR		↑	3.4 (1.8–6.4)		
		NPIP		↑	2.7 (1.4–5.2)		

Giles *et al*. (1994)	Gliomas	Males	Tertile 3 vs. tertile 1			A–C, G, H	Melbourne,
n = 416		Nitrate		NS	1.13 (0.68–1.86)		Australia, adults
		Nitrite		NS	1.58 (0.96–2.58)		20–70 years
		NDMA		↑	1.78 (1.12–2.84)		
		Females					
		Nitrate		↓	0.53 (0.28–0.96)		
		Nitrite		NS	0.98 (0.55–1.72)		
		NDMA		NS	1.45 (0.78–2.68)		
Blowers *et al*. (1997)	Gliomas	Nitrite	Quartile 4 vs. quartile 1		1.4 (0.6–3.5)	A, C, F, I, J	Los Angeles
n = 94		All foods		NS	2.1 (1.0–4.6)		County,
		Cured meats		(↑)	0.7 (0.2–1.8)		California, USA,
		Nitrate		NS			women 25–74 years
Kaplan *et al*. (1997)	Gliomas	NOCs	≥ 1.06 vs. ≤ 0.75 μg	NS	0.79 (0.32–1.96)	A, B, F, K	Israel, adults
n = 139	Meningiomas		1000 calories^{-1}	NS	1.98 (0.97–4.05)		18–75 years
Lee *et al*. (1997)	Gliomas	Cured foods plus	High and low vs. low and			A, B, F, L, M	San Francisco
n = 434		vitamin C-rich fruit	high intake				Bay Area,
		and vegetables	men	↑	2.0 (1.2–3.5)		California, USA,
			women	NS	1.5 (0.8–2.7)		adults ≥ 20 years
		Nitrite and vitamin C	High and low vs. low and high intake				
			men	↑	2.1 (1.1–3.8)		
			women	NS	1.5 (0.7–3.1)		

CI, confidence intervals; NS, not statistically significant; ↑, statistically significant direct association; (↑), lower 95% CI = 1; ↓ statistically significant inverse association; A, birth year; B, sex; C, residence; D, marital status; E, date of diagnosis or death; F, race; G, alcohol consumption; H, smoking; I, body mass index; J, total grams of food; K, total energy intake; L, income; M, education; NMDA, *N*-nitrosodimethylamine; NPYR, *N*–nitrosopyrrolidine; NPIP, *N*–nitrosopiperidine.

and low vitamin C intake), although the associations were stronger and statistically significant only in men.

Conclusions

In summary, the general impression from 11 case–control studies on childhood brain tumours and maternal diet during pregnancy is that mothers of cases were more likely than controls to consume cured meats. There is also limited evidence that consumption of cured meat in childhood increased risk of brain tumours in children, and all three studies on adult brain tumours showed an increased risk with intake of some sort of cured meats. Intravenous administration of NOCs induced gliomas in experimental animals. Pregnant rats fed nitrites plus amides produced offspring at increased risk of brain tumours, with the effect being suppressed by vitamins C and E, which interfered with *in vivo* nitrosation reactions. It has been hypothesized that nitrite levels of cured meats in the stomach may be highly concentrated in the relative absence of vitamins. Conversely, nitrite formed in the saliva from nitrate in vegetables may be considerably more diluted, and nitrosation inhibitors are present. This hypothesis would explain why, for nitrate and nitrite intake overall, no clear risk pattern emerged in the discussed studies and why in two studies nitrate/nitrite from cured meat, but not total nitrate/nitrite, was related to brain tumour risk. A causal association between cured meat and (childhood) brain tumours cannot be concluded on the basis of the available data. Alternative explanations, such as chance findings by multiple statistical comparisons and effects of bias and confounding, are possible. The potential for recall bias is a particular concern, given the widespread perception that at least some cured meats are unhealthy. Parents of children who developed brain cancer may over-report (or report more accurately than controls) their consumption of foods that are believed to be undesirable. The causes of brain cancers are not well understood, so few known risk factors could be considered confounders. Nevertheless, it is possible that additional (dietary) factors, such as vegetable and fruit intake or some component of cured meats other than pre-formed NOCs and NOC precursors (e.g. heterocyclic amines), could at least in part be responsible for the observed positive association between cured meat and brain tumours. The present data, on the other hand, do not allow us to rule out the possibility that cured meat consumption may increase risk of (childhood) brain cancer. Cohort studies, which limit recall bias and consider potential confounders in the analyses, are needed to evaluate the effect of total as well as specific cured meat intake on brain tumour risk (Hennekens and Buring, 1987; Preston-Martin *et al*., 1996; Blowers *et al*., 1997; Lee *et al*., 1997; Bunin, 1998; Blot *et al*., 1999).

Stomach Cancer

Incidence rates and pathogenesis

Stomach cancer is the second most common incident cancer and cause of cancer mortality throughout the world, with a distinct geographical pattern. The highest incidence rates are found in Japan, South America and eastern Asia; intermediate rates are found, for example, in Switzerland and France; and low rates are found in North America, Canada and Greece. The decline of stomach cancer rates over the past decades, most of all in developed countries, and the results of migrant studies suggest a predominant aetiological role for external environmental factors generally believed to be dietary. Diets high in vegetables and fruits and low in salt and the use of refrigeration are considered to be the most effective means of preventing stomach cancer. An important non-dietary risk factor for stomach cancer is infection with the *Helicobacter pylori* bacterium. Other potential risk factors such as high consumption of grilled and barbecued meat and fish and cured meats are discussed. The stomach is an established site for NOC carcinogenesis in animals (Higginson *et al*., 1992; World Cancer Research Fund and American Institute for Cancer Research, 1997).

Epidemiological evidence

Dietary nitrate and risk of stomach cancer

Of six case–control studies that estimated dietary intake of nitrate and its association with stomach cancer risk (for references, see Eichholzer and Gutzwiller, 1998), all showed a decreased risk with high vs. low consumption, one of them when adjusted for age, gender, vitamin C, β-carotene and α-tocopherol moving close to unity (Hansson *et al*., 1994). Three of the inverse associations were statistically significant (Risch *et al*., 1985; González *et al*., 1994; La Vecchia *et al*., 1994). These findings might result from the fact that vegetables, the main source of nitrate, might themselves or some of their constituents protect against gastric cancer (Block *et al*., 1992). Risch *et al*. (1985) adjusted their analyses only for total food consumption (in grams) and ethnicity. González *et al*. (1994) and La Vecchia *et al*. (1994) adjusted only for total energy. The two existing cohort studies do not support a positive association between dietary intake of nitrate and stomach cancer incidence. In the study by van Loon *et al*. (1998), the relative risk of the highest versus the lowest quintile of intake from food was 0.80 (95% CI = 0.47–1.37) (adjusted for age, sex, smoking, education, coffee consumption, intake of vitamin C and β-carotene, family history of stomach cancer, prevalence of stomach disorders, and use of refrigerator and freezer). Similarly, in a Finnish cohort study, a non-significant inverse trend ($P = 0.09$) between dietary nitrate intake and risk of stomach cancer was observed (relative risk (RR) highest vs. lowest quartile = 0.56 (95% CI = 0.27–1.18); adjusted for age, sex, municipality, smoking and energy intake) (Knekt *et al*., 1999). Despite the fact that, in contrast to vegetables, drinking water does not contain protective substances, in the study by van Loon *et al*. (1998) nitrate intake from drinking water was associated with a slightly reduced RR of 0.88 (95% CI = 0.59–1.32) (adjusted for the variables mentioned above). Similarly, Rademacher *et al*. (1992) found no association between nitrate levels in water (central or private water sources) and cancer risk in a case–control study of Wisconsin residents. This may be due to the fact that the place of residence listed on the death certificate (hospitals or nursing homes excluded) was assumed to be the source of the subjects' nitrate exposure via drinking water for at least 20 years prior to death. Conversely, Boeing *et al*. (1991) did report in a German case–control study a significantly elevated risk for users of well water compared with users of central water supplies at some time during a subject's life (OR = 2.26 (95% CI = 1.19–4.28)). These results were adjusted only for smoking of meat at home, years of refrigerator use, age, sex and hospital. Nitrate content of drinking water was not measured, but analyses from other countries have shown that private water sources can contain considerable amounts of nitrate. In a newer case–control study from Taiwan, nitrate content of drinking water was significantly associated with an increased risk of stomach cancer mortality (OR = 1.14 (95% CI = 1.04–1.25)) for those with nitrate levels higher than 0.45 mg NO_3-N l^{-1}, when the results were adjusted for urbanization level of residence, sex, year of birth, year of death and calcium and magnesium levels in drinking water (Yang *et al*., 1998).

Dietary nitrite and risk of stomach cancer

Of seven case–control studies that estimated the intake of nitrite (de Stefani *et al*., 1998; Eichholzer and Gutzwiller, 1998), five showed an increased risk of stomach cancer. In the above-mentioned study of Risch *et al*. (1985), the positive association was statistically significant (OR = 2.61 (95% CI = 1.61–4.22); adjusted for dietary fibre, chocolate, carbohydrates, no refrigerator, total food consumption and ethnicity). The same held true for a case–control study conducted in the Greater Milan area (La Vecchia *et al*., 1997) when the interaction between methionine and nitrites was considered. Compared with subjects with low methionine and low nitrite intake, the OR was 2.45 (95% CI = 1.9–3.2) in the high methionine and high nitrite stratum. Conversely, in a case–control study in Uruguay (de Stefani *et al*., 1998), the

highest quartile of nitrite consumption was associated with a significantly decreased risk of stomach cancer when compared with the lowest quartile (OR = 0.55 (95% CI = 0.48–0.62); adjusted for age, sex, residence, urban/rural status, smoking, alcohol and 'mate' consumption). In one of the two existing cohort studies, multivariate analysis (including the variables mentioned above) revealed an RR of the highest versus the lowest quintile of nitrite intake of 1.44 (95% CI = 0.95–2.18) (van Loon *et al*., 1998). In the mentioned Finnish cohort study by Knekt *et al*. (1999), a slightly decreased risk of stomach cancer was observed in those with intake of nitrite in the highest quartile compared with those in the lowest (RR = 0.71 (95% CI = 0.28–1.78); adjusted for age, sex, municipality, smoking and energy intake).

Dietary NOCs and risk of stomach cancer

Of five case–control studies that estimated NOC intake, four showed a statistically increased risk with high intake of NDMA (Eichholzer and Gutzwiller, 1998). In the French case–control study conducted by Pobel *et al*. (1995), the OR for the third versus the first tertile of intake was 7.00 (95% CI = 1.85–26.46; adjusted for age, sex, occupation and total energy intake). Only dietary exposure to NDMA was assessed, although it may not be representative of the whole group of pre-formed nitrosamines in food. In the study by González *et al*. (1994), it was suggested that high consumption of a protective factor, such as vitamin C, neutralizes the increased risk related to the consumption of pre-formed nitrosamines (OR = 2.09 in the highest quartile, adjusted for total energy). In the study by La Vecchia *et al*. (1995) the multivariate OR for the highest NDMA intake tertile was 1.37 (95% CI = 1.1–1.7) including age, sex, education, family history of gastric cancer, combined food score index, and intake of β-carotene, vitamin C and total energy, nitrite and nitrate. No information on *H. pylori* in cases and controls was available, although *H. pylori* antibody prevalence has not been shown to correspond to high risk areas of gastric cancer in Italy. In a more recent case–control study in Uruguay (de Stefani *et al*., 1998), NDMA intake was associated with an increased risk of gastric cancer, with an OR = 3.6 (95% CI = 2.4–5.5) for the highest category of exposure. The dose–response pattern was highly significant. Joint exposure to NDMA and heterocyclic amines (2-amino-1-methyl-6-phenylimidazo(4,5-*b*)-pyridine (PhIP)) displayed independent effects by both chemicals, and their interaction followed a multiplicative model with an elevated OR of 12.7 (95% CI = 7.7–21.2). When nutrients and related chemicals (methionine, nitrite, NDMA, PhIP, vitamin C and β-carotene) were in the same model simultaneously, NDMA and PhIP were both associated with significantly elevated ORs. The only existing cohort study (Knekt *et al*., 1999) revealed a non-significant decreased risk of stomach cancer in those with a dietary intake of NDMA in the highest quartile (RR = 0.75 (95% CI = 0.37–1.51)).

Conclusions

In summary, the toxicological data unequivocally show that pre-formed NOCs cause carcinoma in animals. Four of five case–control studies that estimated NOC intake in humans showed a statistically significant increased risk of stomach cancer with high intake of NDMA. The only cohort study found a slightly decreased risk with high NDMA intake. Humans are exposed to pre-formed NOCs and to NOCs produced *in vivo*. Dietary nitrites and nitrates have been suggested to be precursors of endogenous synthesis of NOCs, and by this to increase human cancer risk. In five of seven case–control studies on nitrite and gastric cancer risk, a positive association was reported. In one of these studies, the association was statistically significant; conversely, another case–control study showed a significant decreased risk with high nitrite intake. One of two cohort studies found a non-significant increased, the other a slightly reduced stomach cancer risk with high intake of nitrite. In six case–control and two cohort studies, inverse associations were reported between nitrate intake and gastric cancer risk; in three studies, these results were statistically significant. These findings might result from the fact that vegetables –

the main source of nitrate – also contain protective factors such as vitamin C. Based on results from ecological studies, the hypothesis of an increased risk of stomach cancer with high intake of nitrate from drinking water (does not contain protective factors?) was postulated. So far, only three case–control and one cohort study have evaluated this hypothesis. Two of the case–control studies showed significant increased risks. Misclassification or low levels of exposure (van Loon *et al*., 1998) could have influenced the negative findings of the other studies. In addition, Yang *et al*. (1998) observed in their case–control study inverse associations between calcium and magnesium concentrations in drinking water and stomach cancer, i.e. drinking water could also contain protective factors. Overall, the association between nitrate in drinking water and stomach cancer risk should be evaluated in additional case–control and cohort studies with special emphasis on accurate estimation of exposure.

Nasopharyngeal Cancer

Incidence rates and pathogenesis

Nasopharyngeal carcinoma (NPC) is rare in most countries, including the USA and Western Europe. NPC occurs in an endemic form in Chinese people in South-east Asia, Arabs in North Africa and in Alaskan Inuits of mongoloid origin. Known and suspected causes are genetic factors, Epstein–Barr virus (EBV), inhaled substances, smoking and diet, especially Cantonese salted fish during childhood (Higginson *et al*., 1992; Vokes *et al*., 1997; World Cancer Research Fund and American Institute for Cancer Research, 1997).

Epidemiological evidence

Several case–control studies (for references see Eichholzer and Gutzwiller, 1998) in southern China, Malaysia, Hong Kong and Thailand demonstrated an association between the consumption of salted fish, especially during weaning, and the risk of NPC. Ning *et al*. (1990) observed in a case–control study performed in a low risk region for NCP that exposure to salted fish (ever vs. never) was significantly associated with an increased risk of NPC (OR = 2.2 (95% CI = 1.3–3.7)). Controls were matched to cases by age, sex and race. The following four characteristics of exposure to salted fish independently contributed to the increased risk: earlier age at first exposure, increasing duration and frequency of consumption and steaming fish rather than frying, grilling or boiling it. Results were not adjusted for other risk factors. In a separate analysis, a significant increased risk was observed for the consumption of salted shrimp paste and salted fish when adjusted for each other and for carrot consumption, but not for infection with EBV and other factors. The case–control study of Zheng *et al*. (1994) (88 NPC cases, 176 age-, sex- and neighbourhood-matched controls) was conducted in Zangwu County, Guangxi, China. The multivariate analysis (including use of wood fuel, consumption of herbal tea and a socio-demographic score) found a significantly increased risk (OR = 3.8 (95% CI = 1.5–9.8)) for the consumption of salted fish in rice porridge before the age of 2 years. These results may be affected by recall bias, as subjects provided data on their diet from almost 30 years previously. Additionally, Sriamporn *et al*. (1992) conducted a case–control study with data from 120 NPC cases and the same number of hospital-, age- and sex-matched controls in North-east Thailand, a region which shows an intermediate risk for this neoplasm. The consumption of sea-salted fish at least once a week versus never in adult life was a significant risk factor for nasopharyngeal cancer (OR = 2.5 (95% CI = 1.2–5.2); adjusted for alcohol, cigarette consumption, occupation, education and area of residence). Again, EBV infection as a potential confounder was not assessed. Similarly, in a recent case–control study in Shanghai, a region with intermediate risk for NPC (Yuan *et al*., 2000), adults who ate salted fish at least once a week had an 82% increase in risk of NPC, relative to those who ate salted fish less than once a month ($P = 0.07$).

As already mentioned, rates of NPC comparable with those in South-east Asia have been reported in Inuit populations in Canada, Alaska and Greenland and in the Arabs of northern Africa. Cantonese Chinese, Maghrebian Arabs and Inuits were compared in anthropological studies by Hubert *et al*. (1993). It should be noted that, for example, the diet of Maghrebian Arabs is very different from that of the Chinese, and does not include salted fish. The conclusion of Hubert's study was that traditional preserved food preparations could be the common factors linking these groups. Laboratory analyses of food samples of South China, Macao, Tunisia and Greenland revealed, among other things, the presence of volatile nitrosamines (Poirier *et al*., 1987). In a third step of the study by Hubert *et al*. (1993), case–control studies in Tunisia and China tested the hypotheses based on these data. The results suggested that the consumption in early youth of salted and preserved foods other than salted fish, for example, fermented fish sauce, salted shrimp paste, mouldy bean curd and two kinds of preserved plums, was also associated with an increased risk of NPC (Eichholzer and Gutzwiller, 1998). Correspondingly, a more recent case–control study in Nagaland, India, revealed a direct association of NPC with consumption of smoked meat (adjusted OR = 10.8 (95% CI = 3.0–39.0)) (Chelleng *et al*., 2000). Similarly, in the case–control study by Yuan *et al*. (2000), in addition to salted fish (see above), subjects in the highest quartile of intake of protein-containing preserved foods compared with those with low intake (first quartile) also experienced a statistically significant 78% increase in risk of NPC (OR = 1.78 (95% CI = 1.37–2.31)). When the joint effect of preserved food and oranges/tangerines on risk of NPC was examined, subjects in the highest tertile of preserved food and the lowest tertile of orange/tangerine intake had a threefold increase in risk (95% CI = 2.08–4.91) compared with those in the lowest tertile of preserved food and the highest tertile of orange/tangerine intake. In a case–control study in Malaysian Chinese (Armstrong *et al*., 1998), consumption of four salted preserved foods (fish, leafy vegetables, egg and root), fresh pork/beef organ meats, and beer and liquor 5 years prior to diagnosis exhibited significant positive associations with NPC risk. The associations were less strong for dietary intake at age 10 years. In addition, in a case–control study in the USA, where the annual incidence of the disease is low, risk of non-keratinizing and undifferentiated tumours of the nasopharynx was increased in frequent consumers of preserved meats (including bacon, hot dogs and sausage), which contain high levels of added nitrites (RR: highest vs. lowest quartile 4.59 (95% CI = 0.78– 27.01); *P* trend 0.04). For squamous cell carcinoma, the corresponding RR was 1.15 (95% CI = 0.46–2.87; *P* trend 0.58). The results indicate that future studies should consider the effects of dietary risk factors on the risk of specific histological subsets of NPC, and not assume that the disease is aetiologically homogeneous (Farrow *et al*., 1998). Overall, studies have not estimated exposure to NOCs directly. In a recent case–control study in Taiwan (Ward *et al*., 2000), intake of nitrosamines and nitrite (based on 66 foods) as an adult was not associated with risk of NPC. High intake of nitrosamines and nitrites (from foods other than soy products, which contain inhibitors of nitrosation) during childhood and weaning were associated with significantly increased risks of NPC.

Conclusions

In high risk areas such as China, studies on NPC found elevated risks with higher consumption of salted fish, particularly during childhood. Correspondingly, in 1997, the World Cancer Research Fund and the American Institute of Cancer Research considered the overall evidence that diets high in Cantonese-style salted fish increase the risk of NPC as convincing. Salted fish has a high level of secondary amines. These amines are believed to interact with nitrite salts used as preservatives, leading to the formation of NOCs, which are possibly organotrophic for the nasopharynx (World Cancer Research Fund and American Institute of Cancer Research, 1997). This has been demonstrated *in vivo* by Yu *et al*. (1989), who induced malignant nasal cavity tumours in rats fed salted fish. In areas with food habits very different

from those of Chinese people, such as those of Arabs in North Africa, or of the low risk US population, other (traditionally) preserved foods (meats, etc.) with high content of NOCs may be of importance in the aetiology of NPC. As already discussed for brain tumours, for the observed association between preserved foods and NPC, alternative explanations, such as confounding, are possible. For example, the preserved meats most commonly consumed in the US diet, including bacon, hot dogs and sausage, are often grilled or pan-fried, processes that result in the formation of heterocyclic amines; thus, the increased risk of NPC associated with these foods may result not from their nitrite or nitrosamine content, but from the methods used for cooking them. So far, with few exceptions, studies have not estimated exposure to NOCs from the whole diet directly. This should be done in future studies by simultaneously adjusting for dietary nitrosation inhibitors in the analyses.

Summary and Overall Conclusions

NOCs are potent carcinogens in animal studies. Many cancer sites are suspected to be related to NOCs in humans, but for most cancer locations only a few epidemiological studies exist. So far, high consumption of cured meats and salted fish was associated with increased risk of brain tumours and NPC. Exogenous and endogenous exposure to NOCs is suspected to be the causal link, but dietary intake of NOCs and precursor nitrates and nitrites has not yet been studied adequately for these cancer sites. For stomach cancer, four of six epidemiological studies showed a significant increased risk with high intake of NDMA. For nitrite, the positive association was weaker and, for nitrate from vegetables (the main contributor to total daily intake), a rather consistent inverse association was observed. Overall, a causal relationship between dietary NOC, nitrite and nitrate cannot be concluded or excluded on the basis of the available data. Alternative explanations, such as effects of bias and confounding, are possible. The present studies are mainly of case–control design, thus particularly prone to recall and misclassification bias. Furthermore, it is possible that other (dietary) factors such as intake of vegetables, fruit and nitrosation inhibitors, or some component of cured meat and salted fish other than pre-formed NOCs and NOC precursors, for example, heterocyclic amines, could at least in part be responsible for the observed associations. Cohort studies, which limit recall bias and consider potential confounders in the analyses, are needed to evaluate properly the effect of dietary NOC, nitrite and nitrate on human cancer risk. Meanwhile, present legal measures to limit overall dietary NOC exposure, and exposure to nitrites and nitrates as food additives, are reasonable. As high intake of vegetables and fruit is promoted for cancer prevention (World Cancer Research Fund and American Institute for Cancer Research, 1997), restrictive legal nitrate levels in vegetables, on the other hand, may be counteractive. Drinking water, another dietary contributor of nitrate, does not contain nitrosation inhibitors; thus its cancer risk should be evaluated separately.

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11 Adverse Reactions to Food Additives

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Introduction

Food additives are different from other compounds shown in other chapters in view of the fact that food additives are added intentionally with some purpose, whereas other compounds described in other chapters such as polychlorinated biphenyls, dioxins and polycyclicaromatic hydrocarbons occur as contaminants. The Codex Alimentarius by the Joint FAO/WHO Codex Alimentarius Commission (1991) defined food additives as follows.

> 'Food additive' means any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly) in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods.

The term does not include 'contaminants' or substances added to food for maintaining or improving nutritional qualities. The definitions of food additives, however, differ with countries. Some countries allow nutrients as food additives. Postharvest pesticides are allowed as food additives in some countries, but some other countries categorize them as residual pesticides. A food act in each country takes precedence over the Codex Alimentarius.

Estimates are that 2000–20,000 agents are added to the food that we consume (Collins-Williams, 1983). These include preservatives, stabilizers, conditioners, thickeners, colourings, flavourings, sweeteners and antioxidants (Box 11.1). Despite such enormous exposure to these agents, only a small number have been associated with hypersensitivity reactions. In a questionnaire study of US households (Altman, 1996), self-reported adverse reactions to food additives in family members ranged from 1.2% for food dyes and colourings to 2.7% for monosodium glutamate (MSG). Because the perceived adverse reactions to food additives were not verified by appropriate diagnostic challenge procedures, the true frequency of food additive reactions in the general population remains largely unknown. In a Dutch study that started with a survey of 1483 Dutch adults and proceeded through clinical challenge trials, only three individuals were identified with food additive sensitivities (Niestijl Jansen *et al*., 1993), which amounts to 0.2% of the population. In a large study of food additive-induced sensitivities that started with a survey of 4274 Danish schoolchildren

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and proceeded through clinical trials, an intolerance to food additives confirmed by double-blind challenge occurred in 2% of the children selected from the survey on the basis of atopic history but only in 0.13% of the entire surveyed population (Fuglsang *et al*., 1993, 1994). Young *et al*. (1987) evaluated the prevalence of sensitivities to food additives among a British population using a combination of a survey questionnaire given to 18,582 individuals and a series of mixed additive challenges conducted at home with self-reporting of symptoms. They estimated the prevalence of adverse reactions to food additives as 0.01–0.23% (Young *et al*., 1987). Thus, food additive-induced sensitivities seem to occur rarely in the overall population.

For years, some investigators have suggested that a significant number of patients with asthma or chronic urticaria and angio-oedema have symptoms caused by the ingestion of food additives. Despite many studies that have attempted to establish the prevalence of reactions to additives in patients, the true incidence of reactions remains unknown. This is due primarily to the lack of properly controlled studies. Although many anecdotal reports exist, rigorously controlled studies are rarely found in this area of clinical investigation.

Box 11.2 lists the additives that have been reported to be most commonly associated with adverse reactions. Figure 11.1 illustrates the chemical structure of selected additives. A common chemical structure does not link these compounds together into a single molecular configuration. These agents will be

Box 11.1. Common food additives.

- Antioxidants
  - Butylated hydroxyanisole (BHA)
  - Butylated hydroxytoluene (BHT)
- Extraction solvents
  - Dichloromethane (methylene chloride)
  - Trichloroethylene (TCE)
- Flavouring agents
  - *Trans*-anethole
  - Benzyl acetate
  - (+)-Carvone and (–)-carvone
  - Ethylmethylphenolglycidate
- Food colour: FD&C dyes
  - Tartrazine (FD&C yellow no. 5)
  - Erythrosine (FD&C red no. 3)
  - Indigotin (FD&C blue no. 2)
- Preservatives
  - Benzoates
  - Sulphites
- Sweetening agents
  - Aspartame
  - Hydrogenated glucose syrup
  - Saccharin
- Thickening agents
  - Ethyl cellulose
  - Karaya gum
  - Tragacanth gum
- Miscellaneous food additives
  - Ammonium phosphate, monobasic (monoammonium orthophosphate)
  - Insoluble polyvinylpyrrolidone or polyvinyl polypyrrolidone (PVPP)
  - Polyvinylpyrrolidone (PVP) (Polyvidone)
  - Potassium bromate
  - L-(+) Tartaric acid, ammonium, calcium and magnesium salts

Box 11.2. Additives most commonly associated with adverse reactions.

- FD&C dyes
  - Azo dyes
    - Tartrazine (FD&C yellow no. 5)
    - Ponceau (FD&C red no. 4)
    - Sunset yellow (FD&C yellow no. 6)
    - Amaranth (FD&C red no. 5)
  - Non-azo dyes
    - Brilliant blue (FD&C blue no. 1)
    - Erythrosine (FD&C red no. 3)
    - Indigotin (FD&C blue no. 2)
- Parabens/benzoates
  - Parahydroxy benzoic acid
    - Methyl paraben
    - Ethyl paraben
    - Butyl paraben
  - Sodium benzoate
  - Hydroxy benzoic acid
- Butylated hydroxyanisole (BHA)
- Butylated hydroxytoluene (BHT)
- Nitrates
- Nitrites
- Monosodium glutamate (MSG)
- Sulphites
  - Sulphur dioxide
  - Sodium sulphite, potassium sulphite, bisulphite, metabisulphite

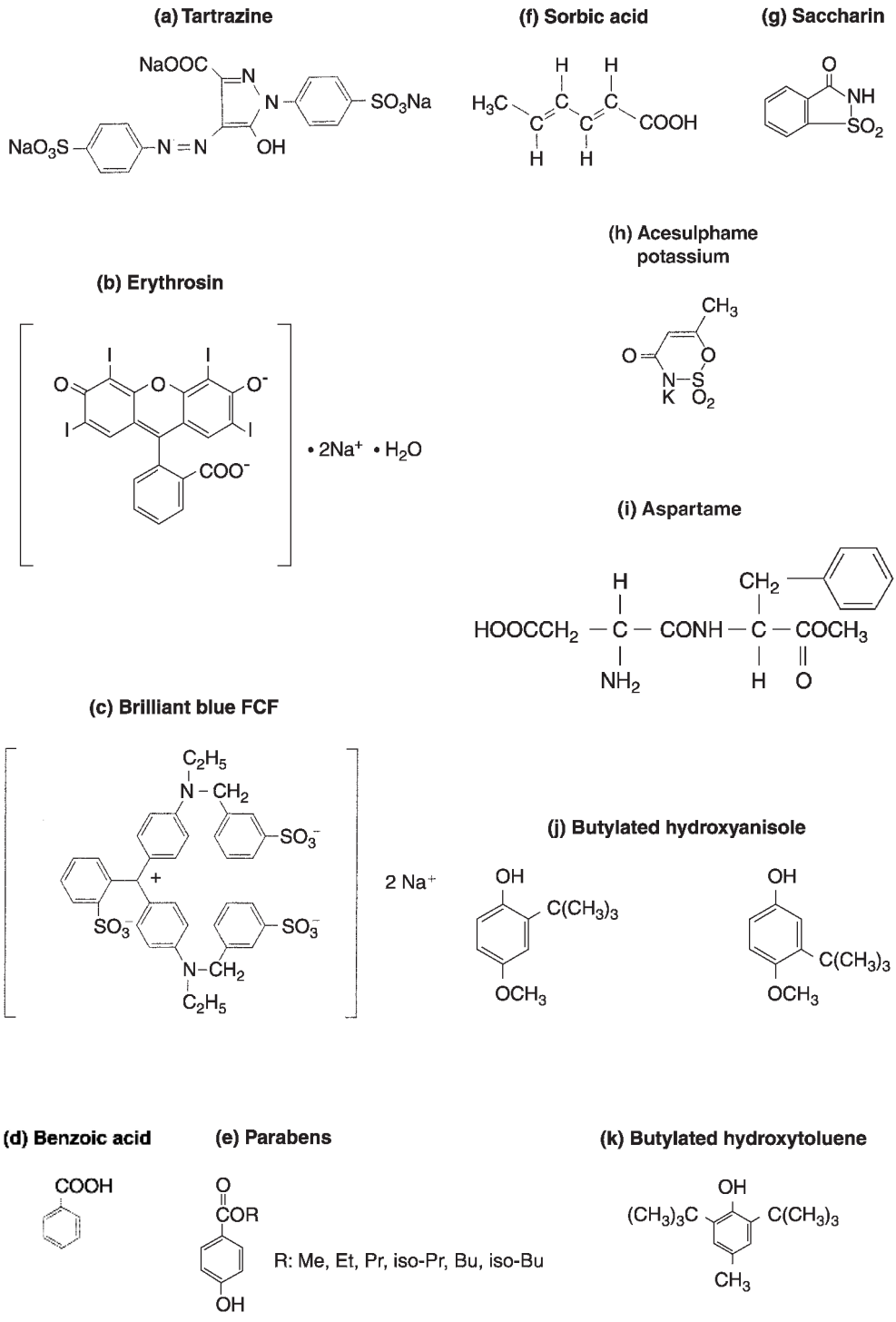

Fig. 11.1. Chemical structures of selected additives.

discussed individually as they relate to urticaria and angio-oedema, anaphylaxis or anaphylactoid reactions, and asthma.

General Considerations and Descriptions of General Food and Drug Additives

Food colours

Both synthetic and natural colours are used for food colouring. Synthetic food colours, coal-tar colours, need a certification by the official chemical examination. Many countries allow 10–15 synthetic food colours, but allowable colours differ in every country. Some food colours are prepared as aluminium lakes. Caramel, carrot carotene, turmeric, annatto extract, etc. are natural colours. Food colour changes easily by heating or oxidation, and thus food colours are used to compensate for colours of food. Food colours are also used to sharpen the consumer's appetite.

Dyes

Dyes approved by the Food Dye and Coloring Act (FD&C) are coal tar derivatives, the best known of which is tartrazine (FD&C yellow no. 5). All dyes contain aromatic rings. In addition to tartrazine, the azo dyes (containing *N*:*N*-linkages) include ponceau (FD&C red no. 4) and sunset yellow (FD&C yellow no. 6). Amaranth (FD&C red no. 5) was banned from use in the USA in 1975 because of claims of carcinogenicity. Non-azo dyes include brilliant blue (FD&C blue no. 1), erythrosine (FD&C red no. 3) and indigotin (FD&C blue no. 2).

Tartrazine (CI food yellow 4, FD&C yellow no. 5, EEC no. E102)

The structural formula is given in Fig. 11.1(a). Molecular weight: 534.37 Da. Description: light orange powder or granules. The λ maximum of 10 mg l^{-1} in 0.15% ammonium acetate solution is 426–430 nm. Functional use: food colour, a mono azo colour. Natural occurrence: not known. Use: one of most widely used food colours in the world. Used alone or with other food colours in soft drinks, sweets, salted vegetables, jam, confections and a variety of processed foods. Acceptable daily intake (ADI) 0–7.5 mg kg^{-1} body weight (BW).

Erythrosin (CI food red 14, FD&C red no. 3, EEC no. E127)

The structural formula is given in Fig. 11.1(b). Molecular weight: 897.88 Da. Description: red powder or granules. The λ maximum of 3 mg l^{-1} in 0.15% ammonium acetate solution is 524–528 nm. Functional use: food colour, a xanthene colour. Natural occurrence: not known. Use: used alone or with other food colours in canned fruit cocktail, canned cherry, ice cream, sherbets and confections. This colour precipitates in acidic conditions and thus is not used in acidic beverages or drops. ADI: 0–0.1 mg kg^{-1} BW.

Brilliant blue FCF (CI food blue 2, FD&C blue no. 1, EEC no. E133)

The structural formula is given in Fig. 11.1(c). Molecular weight: 792.86 Da. Description: blue powder or granules. The λ maximum of 5 mg l^{-1} in 0.15% ammonium acetate solution is 628–632 nm. Functional use: food colour, a triphenylmethane colour. Natural occurrence: not known. Use: used alone or with other food colours in confections, soft drinks, sweets and bakery products. Stable to light, heat, salts and acids. Usable in acidic beverages or drops. ADI: 0–12.5 mg kg^{-1} BW.

Sulphites/sulphur dioxide

In AD 79, the respiratory death of Pliny the Elder was attributed to the sulphur dioxide (SO_2)-rich gases emanating from the eruption of Mount Vesuvius. Sulphur oxides, including SO_2 and particulate sulphuric acid (H_2SO_4), are generated from the combustion

of sulphur-containing fossil fuels and may be significant aeropollutants for asthmatic persons (Boushey, 1982a; Sheppard, 1988). Plumes of exhaust fumes in the vicinity of power plants and smelters reach concentration peaks of 0.5–1 ppm SO_2 for 5–10 min intervals and yet do not exceed the National Ambient Air Quality standard of 0.14 ppm average for 24 h (Balmes *et al*., 1987).

Sulphur dioxide gas inhalation challenges in asthmatic subjects induce bronchoconstriction dependent on the concentration of SO_2 (Shepphard *et al*., 1980; Fine *et al*., 1987), the minute ventilation at which it is inhaled (Linn *et al*., 1987) and the severity of asthma as measured by non-specific bronchial hyper-reactivity to histamine or methacholine (Sher and Schwartz, 1985). The response occurs more readily with oral than with nasal breathing (Bethel *et al*., 1983a) and is mitigated during breathing at high temperature, high humidity as compared with low temperature, low humidity conditions (Linn *et al*., 1985). The maximal bronchoconstrictor response occurs over 5–10 min and, if not sufficient to produce symptoms necessitating bronchodilator treatment, it does not progress over ensuing hours of continued exposure. Eucapnic hyperventilation or moderate exercise while breathing SO_2 at 0.5–1 ppm has produced clinically significant bronchoconstriction in most asthmatics studied (Bethel *et al*., 1983a,b; Linn *et al*., 1983, 1985; Balmes *et al*., 1987; Fine *et al*., 1987); at 0.25 ppm, the effect is small (Bethel *et al*., 1985).

Likewise, sulphuric acid aerosols at a concentration of 1000 μg m^{-3}, the threshold limit value of the Environmental Protection Agency for occupational exposure, produce significant declines of forced expiratory volume in 1 s (FEV_1) in asthmatic subjects (Utell *et al*., 1983), whereas aerosols at 100 μg m^{-3}, the 'worst case' for ambient exposure, do so only in asthmatic subjects during exercise (Koenig *et al*., 1983; Utell *et al*., 1991; Hanley *et al*., 1992). The prevalence of persistent cough and phlegm is significantly higher among adults living in a Utah community near a smelter, with exposure at the 100 μg m^{-3} mean annual level, than among subjects in communities with exposure at one-third of that level or less (Chapman *et al*., 1985).

The effects, if any, on the induction or maintenance of bronchial hyper-reactivity of lesser concentrations of SO_2 and sulphuric acid particles – for example, the annual averages in urban southern California are in the range of 0.001–0.01 ppm SO_2 – singly or in combination with other aeropollutants or other factors associated with asthma, such as atopic state and viral respiratory infection, have not yet been clearly elucidated.

The term *sulphiting agents* is used to describe SO_2 and several inorganic sulphites that may be added to foods, beverages and pharmaceuticals. Until recently, SO_2 and five sulphite salts (sodium sulphite SO_3, sodium or potassium bisulphite HSO_3, or metabisulphite S_2O_5) have been listed in the Code of Federal Regulations (1984) as 'generally recognized as safe' (GRAS) with the provision that they are not be used on foods considered to be a source of thiamine (vitamin B_1).

SO_2 is a non-flammable, colourless gas that readily dissolves in water and undergoes hydration to form sulphurous acid, which then dissociates to bisulphite and sulphite (Schroeter, 1966). Under physiological conditions at pH 7.4, sulphite is the predominant chemical species. However, in acid solution (e.g. the gastric lumen with pH near 1), sulphite undergoes proton association to form bisulphite and sulphurous acid. The latter dehydrates once again to form SO_2 (Schroeter, 1966). In addition to acid pH, the generation of SO_2 is also enhanced by heat.

Because of this interchangeability, sulphite concentrations in food may be expressed as parts per million (ppm) of SO_2 or, vice versa, SO_2 content can be expressed as SO_2 equivalent (SDE) or milligrams of sulphite. The conversion is ppm of SO_2 = mg of sulphite per kg of food.

Sulphites are highly reactive and combine with a number of biological compounds including carbohydrates and pyridinonucleotides (Schroeter, 1966). Sulphites also react with disulphide bonds present in proteins (Cecil, 1963). Therefore, when identifying sulphite (or SO_2) concentrations in foods,

results will be expressed as free or bound SO_2. The bound SO_2 is usually reported as total SO_2, although, even with the harshest of chemical extractions, all potential SO_2 forms probably cannot be measured. The importance of bound sulphites as causing sensitivity reactions is difficult to evaluate.

Use of sulphites in foods and beverages

Sulphiting agents are still widely used in the food and beverage industry (Box 11.3). Sulphites can inhibit a number of enzymatic reactions, for example polyphenoloxidase, ascorbate oxidase, lipoxygenase and peroxidase (Cecil, 1963). Although the mechanism of action is unknown, sulphite inhibition of polyphenoloxidase is important in the control of enzymatic browning. This was the primary reason for adding sulphites to items in salad bars, such as lettuce, avocados and guacamole, as well as to cut potatoes and apples, fresh mushrooms and table grapes (Komanowsky *et al*., 1970; Ponting *et al*., 1971; Nelson, 1983; Taylor and Bush, 1983). The enzyme tyrosinase, a type of

Box 11.3. Sulphite-containing foods and drugs.

Foods

High content	*Low content (< 10 ppm)*[a]
Dried fruit (excluding dark raisins and prunes)	Corn starch
Lemon juice (non-frozen)	Dry maize
Lime juice (non-frozen)	Frozen potatoes
Wine	Maple syrup
Molasses	Imported jams and jellies
Sauerkraut juice	Fresh mushrooms
Grape juice (white, white sparkling, pink sparkling, red sparkling)	Malt vinegar
	Dried cod
	Canned potatoes
	Beer
	Dry soup mix
Moderate content	Soft drinks
Dried potatoes	Instant tea
Wine vinegar	Pizza dough (frozen)
Gravies, sauces	Pie dough
Fruit topping	Sugar (especially beet sugar)
Maraschino cherries	Gelatin
Pectin	Coconut
Shrimp (fresh)	Fresh fruit salad
Sauerkraut	Domestic jams and jellies
Pickled peppers	Crackers
Pickled cocktail onions	Cookies
Pickles/relishes	Grapes
	High fructose corn syrup

Drugs

Sulphite-preserved inhalants	0.5–1.5	High rare[b]
Sulphite-preserved subcutaneous injectants	0.3–10	Very low
Sulphite-preserved intravenous injectants	0.3–10	Low

[a]Foods with low sulphite content have not been implicated in inducing reactions in sulphite-sensitive individuals.
[b]Rare bronchoconstriction but (?) no bronchodilation.

polyphenoloxidase, catalyses tyrosine oxidation, which leads to black spot formation on shrimp. This oxidative reaction (not infection) can be prevented by sulphites.

Sulphites can also be used as inhibitors of non-enzymatic browning in wines, dried fruits, dehydrated vegetables (especially potatoes), vinegar, white grape juice, coconut, and pectin (Taylor and Bush, 1983). The chemistry of these reactions is complex and beyond the scope of this chapter, but has been reviewed by McWheeney *et al*. (1974).

The antimicrobial actions of sulphites are useful as sanitizing agents for food containers and fermentation equipment because sulphites reduce or prevent microbial spoilage of food (e.g. table grapes), and act as selective inhibitors of undesirable organisms during fermentation.

The antioxidative effects of sulphites serve a major function in the brewing process, where oxidative changes impede development of the beer's flavour (Roberts and McWheeney, 1972). The ability of sulphites to break disulphide bonds in the gluten reaction of dough accounts for their widespread use (although in minimal residual quantities) as dough conditioners for biscuits, cookies, crackers, frozen pizza dough and pie crusts (McWheeney *et al*., 1974). In the production of maraschino cherries, sulphites are used to bleach the fruit before injecting red dye.

Regulator restrictions

After the discovery of sulphite-sensitive asthmatic persons (Stevenson and Simon, 1981), the Food and Drug Administration (FDA), the Bureau of Alcohol, Tobacco and Firearms (BATF) and the Environmental Protection Agency (EPA) moved to regulate the uses of sulphites in 1986. The FDA required the declaration of sulphites on the food labels when sulphite residues exceeded 10 ppm. The BATF followed suit with wines. The FDA banned the use of sulphites from fresh fruits and vegetables other than potatoes. This ban affected the practice of sulphiting lettuce, cut fruits, guacamole, mushrooms and many other foods, and in particular the once common practice of sulphiting fresh fruits and vegetables in salad bars. The FDA also moved to ban sulphites from fresh, prepeeled potatoes. The EPA required that imported table grapes be detained at their port of entry until sulphite residues can no longer be detected. The FDA has also enacted a regulation specifying the allowable residue levels for sulphites in shrimp. Foods that currently contain sulphites are listed in Box 11.3. Foods with low levels of sulphites contain ≤ 10 ppm SO_2 and have not been associated with producing reactions in sulphite-sensitive subjects.

Mechanisms of sulphite sensitivity

The mechanisms of sensitivity reactions to sulphiting agents are unknown. Depending on the route of exposure, a number of mechanisms have been postulated. It is known that asthmatic subjects, upon inhalation of less than 1.0 ppm of SO_2, develop bronchoconstriction (Boushey, 1982b). Fine *et al*. (1987) demonstrated that bronchoconstriction developed in asthmatic subjects who inhaled SO_2 and bisulphite (HSO_3^-) but not sulphite (SO_3^-). Alteration of airway pH was not a cause of bronchoconstriction. Thus, depending upon pH and the ionic species, asthmatics develop bronchoconstriction after exposure to certain forms of sulphite. It is also recognized that some asthmatic individuals respond to either oral or inhalation challenge with sulphite, but that inhalation is more apt to induce bronchoconstriction (Schwartz and Chester, 1984). Variability in the response to sulphites in capsule and acidic solutions, administered via the oral route, has also been observed (Lee *et al*., 1986). The same individuals may not always develop bronchoconstriction when challenged on repeated occasions with sulphites. The following represent further attempts to understand more fully the variables and reasons for this inconsistent response.

INHALATION DURING SWALLOWING Delohery *et al*. (1984) studied ten sulphite-sensitive asthmatic subjects. All subjects reacted to a challenge with acidic metabisulphite solution when it was administered as a mouthwash or swallowed, but not when it was instilled through a nasogastric tube. Furthermore,

these same individuals did not respond with changes in pulmonary function when they held their breath while swallowing the solution. Ten non-sulphite-sensitive asthmatic subjects did not react to sulphites when administered as a mouthwash or swallowing challenge. Researchers therefore hypothesized that some individuals respond to sulphites during oral challenges because of inhalation of SO_2 during the swallowing process.

LINKAGE OF SULPHITE SENSITIVITY WITH AIRWAY HYPER-REACTIVITY Asthmatic persons are known to respond to various stimuli (airway irritants) at concentrations lower than normal individuals (i.e. to have airway hyperresponsiveness); therefore, attempts have been made to link sulphite sensitivity with airway responsiveness as measured during histamine and methacholine inhalation challenges. Australian investigators were unable to demonstrate a relationship between the degree of airway responsiveness to inhaled histamine and the presence of sulphite sensitivity (Delohery *et al*., 1984). Taylor *et al*. (1997) attempted to induce sulphite sensitivity in a group of 16 asthmatic subjects. They first established the provocative dose of methacholine producing a 20% decrease in FEV_1 (PD20). Then the researchers used a sulphite bronchial/oral challenge using an acidic sulphite solution to determine the presence of sulphite sensitivity, and three of the 16 subjects reacted to the sulphiting agent with a 20% decrease in FEV_1. One week later, the patients underwent bronchial challenge with an antigen to which they were known to be sensitive. They returned 24 h later for a repeat methacholine challenge. This was followed 24 h later by a second sulphite challenge. After antigen challenge, only one additional subject showed a response to sulphiting agent that had not been present before the antigen challenge, and there was no significant increase in airway response to methacholine. Therefore, these investigators were unable to induce sulphite sensitivity by exacerbating airway hyper-reactivity.

CHOLINERGIC REFLEX Because SO_2 may produce bronchoconstriction through cholinergic reflex mechanisms, the effect of atropine and other anticholinergic agents has been studied (Simon *et al*., 1984a; Simon and Stevenson, 1991; Taylor *et al*., 1997). Pre-inhalation of atropine blocked the airway response to oral challenge with sulphiting agents in three of five subjects and partially inhibited the response in two others. Doxepin, which possesses anticholinergic as well as antihistaminic properties, was protective in three of five individuals undergoing oral challenge with sulphites.

POSSIBLE IgE-MEDIATED REACTIONS Some investigators have attempted to identify an immunological basis for these reactions. Positive patch tests with sulphites suggested a delayed hypersensitivity mechanism in patients with contact dermatitis (Epstein, 1970). The presence of precipitating antibodies to sulphites (Prenner and Stevens, 1976) or alterations in complement activity (Twarog and Leung, 1982) have not been detected. A more likely explanation would be the presence of an IgE-mediated response in selected subjects. Prenner and Stevens (1976) observed a positive skin scratch test to an aqueous solution of sodium bisulphite at 10 mg ml^{-1} in their patient who experienced laryngeal oedema after sulphite challenge. This patient also exhibited a dramatic response with intradermal testing at the same concentration. Three non-sensitive control subjects had negative skin tests to sulphites. Of the five asthmatic subjects studied by Stevenson and Simon (1981), none showed positive skin tests to sulphites. However, the patient of Twarog and Leung (1982), who experienced anaphylaxis after sulphite exposure, showed a positive intradermal skin test response to an aqueous solution of bisulphite at 0.1 mg ml^{-1}. Control subjects were found to have negative skin tests with 1.0 mg ml^{-1} of this solution. Meggs *et al*. (1985) reported that a patient developed wheezing when skin-tested with sodium bisulphite at 100 μg ml^{-1}. Yang *et al*. (1986) identified two asthmatic subjects with either positive prick or intradermal skin tests to sulphites, as well as one subject with urticaria and one with anaphylaxis, who also was found to have positive intradermal tests to sulphites. Boxer *et al*. (1988) reported two

additional cases with positive skin tests and oral challenges to sulphiting agents that induced bronchoconstriction. Selner *et al*. (1987) reported positive intradermal and skin puncture tests with 0.1 and 10 mg ml^{-1} potassium metabisulphite solutions, respectively, in a sulphite-sensitive asthmatic subject. Two non-sensitive control subjects had negative skins tests. Simon and Wasserman (1986) also reported two sulphite-sensitive asthmatics with positive intradermal skin tests to bisulphites at a concentration of 10 mg ml^{-1}, a concentration that did not produce wheal-and-flare cutaneous responses in controls. These observations are consistent with an IgE-mediated mechanism, with sulphites acting as chemical haptens.

Further evidence for an IgE-mediated mechanism is supported by passive transfer experiments (Prausnitz–Küstner transfer). Several investigators have successfully transferred skin-test reactivity to non-sensitive subjects with sera from sulphite-sensitive individuals (Prenner and Stevens, 1976; Simon and Wasserman, 1986; Yang *et al*., 1986). Skin sensitizing activity was abolished by heating the sera to 56°C for 30 min (Simon and Wasserman, 1986). Others have not been successful in repeating these experiments (Epstein, 1970). These data suggest the presence of a serum factor, presumably IgE, but, to date, specific IgE antibodies to sulphiting agents or sulphiting agents conjugated to human serum albumin have not been demonstrated successfully (Meggs *et al*., 1985; Boxer *et al*., 1988). That sulphiting agents could by themselves stimulate direct mediator release from mast cells or basophils in the absence of IgE has also been considered. Histamine release from mixed peripheral blood leucocytes could not be demonstrated in the five subjects studied by Stevenson and Simon (1981), but none of the five had positive cutaneous wheal-and-flare responses to sulphites. Simon and Wasserman (1986) also found inconsistencies in leucocyte histamine release from peripheral blood leucocytes from a patient whose skin tests to sulphites were positive. In contrast, Twarog and Leung (1982) found that 20% of the total basophil histamine was released in the patient they studied using concentrations of 10^{-3}–10^{-7} M sodium bisulphite. Cells from control subjects did not release histamine. Moreover, the histamine release was enhanced by pre-incubating the patient's serum with sodium bisulphite.

Similarly, inconsistencies in the measurement of mast cell or basophil mediators in the peripheral blood of challenged patients have been reported. No rise in plasma histamine levels were observed in patients experiencing hypotension and gastrointestinal response during sulphite challenges (Delohery *et al*., 1984). Likewise, Altman *et al*. (1985) failed to observe changes in serum neutrophil chemotactic activity in sulphite-sensitive individuals during sulphite challenges. In contrast, Meggs *et al*. (1985) observed a significant rise in plasma histamine levels in two of seven subjects with systemic mastocytosis undergoing sulphite challenges (Stevenson and Simon, 1981). However, no clinical response was observed. In an asthmatic subject, whose skin tests to sulphites were positive, the plasma histamine level tripled during the time of the asthmatic response to sulphite challenge. Four subjects with asthma or rhinitis, attributed to sulphite exposure, when challenged intranasally with 5 mg of potassium metabisulphite in distilled water, demonstrated increased histamine levels in nasal lavage fluid 7.5 min after challenge (Ortolani *et al*., 1987). In control subjects with chronic rhinitis, similar results were also obtained, although the level of histamine in nasal secretions was generally less than in the patients with sulphite sensitivity.

Indirect evidence for mast cell mediators playing a role in the production of bronchoconstriction resulting from sulphiting agents has been reported. Freedman (1980) mentions that inhaled sodium cromolyn prevented the asthmatic response to acidic solutions of sulphite. Simon and Stevenson (1997) found that inhaled cromolyn inhibited sulphite-induced asthma in four of six subjects and partially inhibited the response in two other subjects undergoing oral challenge with sulphites. Schwartz (1986) reported that oral cromolyn at a dose of 200 mg blocked an asthmatic response to an oral sulphite challenge in one subject.

SULPHITE OXIDASE DEFICIENCY It has been proposed that a deficiency in the enzyme that metabolizes sulphite to sulphate (sulphite oxidase) may be responsible for some adverse reactions to sulphites. (Simon, 1986; Simon and Stevenson, 1997). Six subjects, found to be sulphite-sensitive by oral provocative challenge exhibited less sulphite oxidase activity in skin fibroblasts when compared with normal control subjects. The major source of sulphite oxidase activity in humans, however, is in the liver.

Preservatives

Preservatives are used for protection from food poisoning by the prevention of putrefaction and deterioration with microorganisms, and for the improvement of the shelf-life of processed foods. Some organic acids and their salts or esters, plant extracts and some proteins are used as preservatives. The use of preservatives is limited by regulations in most cases. Compounds to adjust pH or water activity are not called preservatives.

Benzoic acid

The structural formula is given in Fig. 11.1(d). C_6H_5-COOH. Molecular weight: 122.12 Da. Description: white laminar crystals or needles. It is odourless or has a slight odour of benzaldehyde. Solubility of benzoic acid in water is 0.29% at 20°C. Calcium, potassium and sodium salts are also used as preservatives. Functional use: an acid-type antimicrobial preservative, a growth inhibitor for mould, yeast and bacteria. The minimum inhibition concentrations on the growth of *Aspergillus orizae* are 1/8000 at pH 3.0, 1/2000 at pH 4.5 and 1/500 or less at pH 6.0 (Suzuki *et al*., 1999). The same tendency is observed in other moulds, bacteria and yeast. Natural occurrence: raspberries, plums, anise, tea, etc. (Juhlin, 1977; Williams, 1978). Use: margarine, baked goods, beverages, soy sauce, etc., at the concentration 0.6–2.5 g kg^{-1}. The allowable concentration depends on foods. ADI: 0–5 mg kg^{-1} BW.

Parabens (parahydroxy benzoic acid esters)

The structural formula is HO-(C_6H_4)-COO-R, where R: alkyl group (Fig. 11.1(e)). Methyl, ethyl, propyl or butyl esters are cited in the Compendium of Food Additive Specifications (Joint FAO/WHO Expert Committee on Food Additives (JECFA), 1992). In addition to the above esters, isopropyl, isobutyl and heptyl eaters are also used as preservatives. Molecular weight: 152.15 Da for the methyl ester, 166.18 Da for the ethyl ester, 180.21 Da for the propyl ester and 194.23 Da for the butyl ester. Description: almost odourless, small, colourless crystals or a white, crystalline powder. Functional use: neutral-type antimicrobial preservative for moulds and yeast. The mixture of esters is used for adjusting the solubility. The solubility of methyl, ethyl, propyl and butyl esters in 100 ml of water at 25°C is 0.25, 0.17, 0.05 and 0.02 g, respectively. Inhibition concentrations on the growth of moulds are 100–160 mg l^{-1} (Suzuki *et al*., 1999). Natural occurrence: methylparaben is known as a pheromone produced by the queen honey bee (Barbier *et al*., 1960), and is present in royal jelly at a concentration of 15–30 mg kg^{-1} (Ishiwata and Yamada, 2000). Use: beverages (0.001–0.003%), baked goods (0.003–0.01%), sweets (0.003–0.01%), jams and preserves (0.1%). Parabens are also used in cosmetics and medicines as preservatives. ADI: 0–10 mg kg^{-1} BW. Benzoic acid, sodium benzoate, methylparaben, propylparaben and heptylparaben are approved as food and drug additives by the US FDA and have been assigned a GRAS status (Jacobsen, 1997).

Sorbic acid

Structural formula: H_3C-CH=CH-CH=CH-COOH (Fig. 11.1(f)). Molecular weight: 112.12 Da. Description: colourless needles or white free-flowing powders, having a slight characteristic odour. Solubility in water is 0.25% at 30°C, and that of potassium sorbate is 58.2% at 20°C. Functional use: an acid-type antimicrobial preservative having a wide spectrum for microorganisms, a fungistatic agent and a growth inhibitor for moulds, yeast and aerobic bacteria. Inhibition

concentrations on the growth of moulds and yeast are 1/4000–1/16,000 at pH 3.0, 1/2000–1/4000 at pH 4.5 and 1/500–1/2000 at pH 5.5 (Suzuki *et al*., 1999). Natural occurrence: may be obtained from berries of the mountain ash (Budavari *et al*., 1996), but no other natural occurrence is known. Use: sorbic acid and its calcium, magnesium, potassium or sodium salt is used for many kinds of foods such as beverages, cheese, baked goods, cakes, fish and meat products. Concentrations in foods range from 0.03 to 3 g kg^{-1}. ADI: 0–25 mg kg^{-1} BW.

Monosodium glutamate

Glutamic acid is a non-essential, dicarboxylic amino acid that constitutes 20% of dietary protein. Glutamate appears naturally in some foods in significant amounts, e.g. 100 g of Camembert cheese contains as much as 1 g of MSG; however, the greatest exposure to MSG occurs after it is added to foods as a flavour enhancer. The sodium salt of glutamic acid (MSG) is added to a wide variety of foods by manufacturers, restaurant chefs and individuals. A Japanese chemist established 90 years ago that MSG was responsible for the flavour-enhancing properties of the seaweed *Laminaria japonica* (traditionally used in Japanese cooking) (Marshall, 1948). MSG is added routinely to Chinese, Japanese and other South-east Asian foods and soups. Up to 6 g of MSG may be ingested in a highly seasoned Chinese meal. A single bowl of wonton soup can contain 2.5 g of MSG. MSG is also one of Kentucky Fried Chicken's secret herbs and spices for its fried chicken. MSG is found in most manufactured meat and chicken products, particularly soup stocks and the increasingly popular diet foods (lean/light/low-calorie/low-fat/low-cholesterol).

MSG currently remains among the additives listed by the FDA as GRAS. The fact that MSG is added to a particular food is usually displayed on the label. However, the amount of MSG added is seldom revealed. MSG may also appear on a label as 'hydrolysed vegetable protein'. The flavour-enhancing properties of MSG stem from its excitatory (depolarizing) action on sensory taste receptors. Current research suggests that adverse reactions are linked to the ingestion of large amounts of MSG, which are rapidly absorbed, particularly when ingested in solution and on an empty stomach (Allen, 1991).

Chinese restaurant syndrome

Chinese restaurant syndrome (CRS) was first described in 1968 by a Chinese physician, Dr Robert Homan Kwok, in the *New England Journal of Medicine* (Kwok, 1968). This syndrome, occurring within hours of a Chinese restaurant meal, is characterized by headache, a burning sensation along the back of the neck, chest tightness, nausea and sweating. In 1969, Schaumburg *et al*. reported the first formal study of the effects of MSG in humans. These investigators suggested that MSG elicits three categories of symptoms: a burning sensation, facial pressure and chest pain. Headache was considered to be a consistent complaint in only a minority of individuals. Symptoms appeared in susceptible individuals only if the meal contained free MSG and was ingested on an empty stomach. Such individuals responded to 3 g or less of free MSG, an amount found to be present in a 200 ml serving of wonton soup in one New York restaurant.

Schaumburg *et al*. (1969) determined that the systemic reactions in their subjects were caused by L-glutamate. The intensity and duration of symptoms were related to the dosage of MSG. The onset of symptoms was usually 15–25 min after ingestion. After intravenous administration, the first symptoms appeared in 17–20 s, with a threshold concentration for inducing minimum symptoms ranging from 25 to 125 mg. In two subjects, intravenous injection of MSG into the forearm, while the circulation was occluded by an axillary cuff, produced a burning sensation over the entire arm. The burning sensation was felt over the chest and neck 17 s after the cuff was removed. Schaumburg *et al*. concluded that this burning sensation was a peripheral neuroexcitatory phenomenon and was not due to central nervous system stimulation. Ghadimi *et al*. (1971) reported similar studies and showed that onset and severity of

symptoms are both dose related. Subsequent studies of the CRS by Kenny and Tidball (1972) and Reif-Lehrer (1977) suggest that the prevalence of the CRS in the population eating in Chinese restaurants is about 30%.

Sweeteners

Both synthetic and natural sweeteners are allowed as food additives. Sugar alcohols such as xylitol and D-sorbitol are naturally occurring sweeteners. Artificial sweeteners such as saccharin, acesulphame potassium, and sucralose are non-calorie sweeteners.

Saccharin

The structural formula is given in Fig. 11.1(g). Molecular weight: 183.19 Da. Description: white crystals or a white, crystalline powder, odourless or with a faint aromatic odour having a sweet taste even in very dilute solutions with about 500 times the sweetness of sugar. Slightly soluble in water. Sodium saccharin is freely soluble in water. Functional use: non-calorie sweetening agent. Natural occurrence: not known. Use: used alone or with other sweeteners. The sodium salt is one of the widely used sweeteners added in dentifrices and lipsticks. Free saccharin is effective in keeping the taste in chewing gum (not more than 0.05 g kg^{-1} in Japan) because of its insolubility in water. The allowable limit of sodium saccharin in Japan is: vinegar pickles 2.0 g kg^{-1}, soft drinks 0.30 g kg^{-1}, jam 0.20 g kg^{-1} and confections and sweets 0.10 g kg^{-1}. ADI: 0–2.5 mg kg^{-1} BW (tentative).

Acesulphame potassium

The structural formula is given in Fig. 11.1(h). Molecular weight: 201.14 Da. Description: odourless, white crystalline powder having an intensely sweet taste, with about 200 times the sweetness of sugar. Functional use: non-calorie sweetening agent. Natural occurrence: not known. Use: confections, chewing gum, jam, wines, soft drinks, fermented milk, tabletop sweetener. ADI: 0–15 mg kg^{-1} BW.

Aspartame (L-α-aspartyl-L-phenylalanine methyl ester)

The structural formula is given in Fig. 11.1(i). Molecular weight: 294.31 Da. Description: white, odourless, crystalline powder, having a strong sweet taste, with about 200 times the sweetness of sugar. A dipeptide. Functional use: sweetening agent. Natural occurrence: not known. Use: tabletop sweetener, sweets, soft drinks, chewing gum, salted vegetables, etc. Decomposition rate at 80°C for 2 h is 3% at pH 3.0 and 4.0, but increases to 92.5% at PH 6.5, The major decomposition product is diketopiperidine. ADI: 0–40 mg kg^{-1} BW.

Aspartame was discovered serendipitously in 1965 by a chemist seeking to find an inhibitor of gastrin which might function as an antiulcer agent (Mazur, 1984). In 1973, G.D. Searle petitioned the FDA for approval to market aspartame as a sweetener (United States General Accounting Office/HRD, 1987). In 1974, aspartame was approved for use in dry foods. In December 1975, the FDA held the approval for marketing aspartame because of concern over problems noted in studies by Searl Laboratories (Chicago, Illinois) and because of allegations that aspartame was unsafe and could cause mental retardation and endocrine dysfunction. In July 1981, the FDA Commissioner reapproved aspartame as a food additive, and marketing was initiated that same year. In July 1983, aspartame was approved for use in carbonated beverages (Garriga and Metcalfe, 1988).

Antioxidants

These are substances to protect foods from deterioration by the oxidation of fats, oils and some other food components. Antioxidants such as BHA, BHT and TBHQ (tertiary butylhydroquinone) inhibit the formation of peroxides of fats and oils causing food poisoning, and inhibit the browning of cut vegetables and fruits by the oxidation of polyphenols. Ascorbic acid (vitamin C, water soluble) and tocopherol (vitamin E, oil soluble) are known as natural antioxidants.

Butylated hydroxyanisole (BHA)

The structural formula is given in Fig. 11.1(j). Molecular weight: 180.25 Da. Description: white or slightly yellow crystals or waxy solid, with a faint characteristic odour. Insoluble in water. Functional use: antioxidant. Natural occurrence: not known. Use: fat, oil and butter, dried fish, salted fish, mashed potato, frozen marine products, cereals, dry yeast, dried vegetables, processed meat, etc., at the concentration of 0.001–0.02%. BHA is sometimes used in combination with BHT. The limit is the total amount with BHT. The antioxidative activity of 3-isomer is 1.5–2 times higher than that of the 2-isomer. Recent BHA consists of the 3-isomer. ADI: 0–0.5 mg kg^{-1} BW.

Butylated hydroxytoluene (BHT)

The structural formula is given in Fig. 11.1(k). Molecular weight: 220.36 Da. Description: white, crystalline or flaked solid, odourless or having a characteristic faint aromatic odour. Functional use: antioxidant. Natural occurrence: not known. Use: same as BHA, or used in a wider variety of foods. Used with other antioxidants such as vitamin C or citric acid. BHT is also used as an antioxidant for plastics and petroleum products. ADI: 0–0.3 mg kg^{-1} BW.

BHA and BHT are commonly used in cereal and other grain products. They were developed originally as antioxidants for petroleum and rubber products, but were discovered to be effective in preventing oxidation of animal fatty acids in the mid-1950s (Babich, 1982). BHT has been promoted by some as an anticancer, antiageing substance and as a treatment for genital herpes (Pearson and Shaw, 1984). In addition to such claims being unsubstantiated, legitimate toxic side effects, including severe gastrointestinal and neurological toxicities, have been reported after ingestion of standard and suggested doses (Grogan, 1986; Sklian and Goldstone, 1986).

Colour fixatives

Nitrates and nitrites are used as colour fixatives. The sodium and potassium salts are common for colour fixatives. Nitrate is reduced to nitrite by bacteria in foods, and reacts with myoglobin to form nitrosomyoglobin, which has a stable pink colour. Nitrite inhibits the growth of bacteria, and therefore is also used as a preservative. Nitrates and nitrites are widely used as preservatives. However, their popularity as additives stems from their ability to add flavouring and colouring. These agents are added to processed meats (e.g. frankfurters, salamis).

Sodium nitrate

Structural formula: $NaNO_3$. Molecular weight: 84.99 Da. Description: clear, colourless, odourless, transparent crystals, or white granules or powder, and deliquescent in moist air. Functional use: colour fixative, antimicrobial agent, preservative. Natural occurrence: in vegetables (Walker, 1990) at concentrations of more than 1000 mg kg^{-1} as a natural component of plants. Nitrate is reduced to nitrite by microorganisms or chemically, and the nitrite formed acts as a colour fixative. Use: cured meats, meat products, dried fish, cheese. ADI: 0–3.7 mg kg^{-1} BW as nitrate.

Sodium nitrite

Structural formula: $NaNO_2$. Molecular weight: 69.00 Da. Description: white or slightly yellow, hygroscopic and deliquescent granules, powder, or opaque, fused masses of sticks. Functional use: colour fixative, antimicrobial agent, preservative, flavour enhancer. Natural occurrence: vegetables (0–6 mg kg^{-1}) (Walker, 1990); some salted vegetables contain up to 50 mg kg^{-1} as nitrite (Suzuki *et al.*, 1999). About 5% of ingested nitrate is reduced to nitrite in human saliva (Walker, 1990). Use: cured meats, meat products, dried fish, cheese. Effective to inhibit the growth of *Clostridium botulinum*. ADI: 0–0.06 mg kg^{-1} BW as nitrite.

Acidulants

All acidulants used as food additives are known as natural components in plants or

animal bodies. Acidulants are used for some other purposes such as flavour enhancement, controlling the pH of food, preventing growth of microorganisms and antioxidation by trapping metals, in addition to their major role. The following compounds are used as acidulants: acetic acid, adipic acid, citric acid, fumaric acid, lactic acid, malic acid, phosphoric acid, succinic acid, tartaric acid, etc. The sodium, potassium and calcium salts are also used for the above purposes.

Acetic acid

Structural formula: CH_3COOH. Molecular weight: 60.05 Da. Description: clear, colourless liquid having a pungent characteristic odour, miscible with water and alcohol. Functional use: acidifier, flavouring agent, pH control agent. Natural occurrence: vinegar, fermented foods, fruits. Use: acetic acid is the principal component of vinegar. Diluted acetic acid (4–5%) is used as vinegar mixing with sugar, sweetener and amino acids. Acetic acid (vinegar) is also employed in preparing salad dressings, sauce, mayonnaise, pickles, ketchups, syrups and cheese. ADI: not limited.

Citric acid

Structural formula: $CH_2COOH\text{-}HO\text{-}C\text{-}COOH\text{-}CH_2COOH$. Molecular weight: 192.13 Da. Description: white or colourless, odourless crystalline solid, having a strongly acid taste. The anhydrous and monohydrate forms are listed. Functional use: acidifier, antioxidant, synergist, sequestrant, flavouring agent. Natural occurrence: citrus fruits. Use: soft drinks (0.1–0.3%), juice, jelly, jam, sweets (1%). ADI: not limited.

Lactic acid

Structural formula: $CH_3CH(OH)COOH$. Molecular weight: 90.08 Da. Description: colourless or yellowish, nearly odourless, syrupy liquid with an acid taste, consisting of a mixture of lactic acid and lactic acid lactate. It is obtained by the lactic fermentation of sugars or is prepared synthetically. Common products of commerce are 50–90% solutions. Functional use: acidifier. Natural occurrence: lactic fermented milk, muscle. Use: 0.05–0.2% in soft drinks, sweets, jam, sherbet, etc. as an acidifier, with expecting preservative effect. ADI: not limited.

Distribution in foods and daily intake

ADIs of food additives have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (1996) to ensure that consumers can always confidently choose healthy and enjoyable diets from a safe and varied food supply. The use of many food additives is regulated by food acts or food sanitation law. Some food additives are allowed for use only in limited foods, and some food additives are limited by the concentrations that may be used. The determination of food additives in foods and the estimation of the daily intake, especially in comparison with the ADI, are very important to ensure public health.

Food additives are not always used in allowable foods or up to the allowable limits. Some food additives decompose gradually prior to consumption. The allowable limit differs depending on the type of foods. The concentrations of some food additives in foods in Japan were determined in 1995 (Ishiwata *et al*., 1995). Preservatives, benzoic acid, dehydroacetic acid, *p*-hydroxybenzoic acid esters and sorbic acid, were detected in 16,660 (14.9%) of the total of 112,131 allowable and non-permissible food samples. The average concentration of sorbic acid in foods was 14.1% of the allowable limits.

The JECFA (1999) assessed (first draft) the daily intake of benzoic acid, BHA, BHT, sulphites and TBHQ, and concluded that the estimates of national mean intake by consumers of these food additives were unlikely to exceed the ADI. In general, the daily intake of food additives is estimated by the poundage method (production or used amount), market basket method (total diet study), individual food analysis method, etc. Every method includes both overestimation and underestimation factors. For example, in the case of poundage method, it is possible to estimate

overall intake of food additives, and the labour and cost for analysis of food additives are relatively low compared with other methods. However, natural sources such as organic acid or inorganic compounds are not included in the estimated intakes. A market basket method is suitable to estimate the total intake of some food additives in foods, both intentionally added and naturally contained, but there are labour and analysis costs involved. The individual food analysis method utilizes analytical results in published papers or official inspection for the estimation of the daily intake. Therefore, daily intakes are estimated based on a huge number of analytical results, but food additives for which the daily intake can be estimated are limited. Estimated daily intakes of some food additives are shown in Table 11.1 together with the ADI.

No daily intake of food additives except that of nitrate exceeded the ADI. Nitrate intake is close to or above the ADI. The major functional use of nitrate is as a preservative and a colour fixative for meat products. The daily intake of nitrate was 0.73 mg per person (Ito, 2000) from the food category of meat and fish products or 0.62 mg per person from meat products (Ishiwata *et al*., 2000). The daily intake of nitrate in Japan estimated by a market basket method was 232 mg per person in 1996 (Ito, 2000) and 189 mg per person in 1999 (Yamada, 2000), and most (~90% or more) nitrate came from unprocessed foods such as fruits and vegetables (Ito, 2000). When the body weight of adults was assumed to be 50 kg, the daily intake was at or above the ADI. The JECFA (1995) evaluated the intake of nitrate from vegetables as 'the Committee considered it inappropriate to compare exposure to nitrate from vegetables directly with the ADI and hence to derive limits for nitrate in vegetables directly from it'.

Urticaria, Angio-oedema, Anaphylaxis and Additives

In 1959, Lockey first reported three patients with a history of urticaria after the ingestion of tablets containing tartrazine. One patient was in the middle of treatment for a skin

Table 11.1. Estimated daily intake of some food additives and comparison with ADI.

		Daily intake (mg per person)				
		Poundage		Based on the inspection results		Total diet (market basket)
Food additive	ADI (mg kg^{-1} body weight)	UK[a] 1984–1986	Japan[b] 1995	Japan[c] 1996	Finland[d] 1980	Japan[e] 1994–1995
Benzoic acid	0–5	48.9	4.05	11	40	2.4
p-Hydroxy benzoic acid ester	0–10[f]	0.1	0.38	1.1	0.18	0.124
Sorbic acid	0–25	29.4	33.9	26	37	27.5
Nitrate	0–3.7	1.3	0.19	1.4	6.4	232
Sulphur dioxide	0–0.7[g]	18.4	8.46	1.5	4.0	0.088
BHA	0–0.5	0.4	0.009	0.11	0.17	0.002
BHT	0–0.3	0.2	0.26	0.22	6.6	0.066
Sodium saccharin	0–5.0[h]	2.8	2.27	7.6		0.416

[a]Ministry of Agriculture, Fisheries and Food, UK (1993).
[b]Fujii (1996).
[c]Ishiwata *et al*. (2000); Yamada *et al*. (2000).
[d]Penttilä *et al*. (1988).
[e]Ito (2000).
[f]Group ADI of methyl, ethyl and propyl esters.
[g]Group ADI of sulphur dioxide and sulphites.
[h]Group ADI of saccharin and its salts.

eruption caused by another agent. The other two subjects were challenged with tartrazine sublingually in an open manner. One 'reacted' (no further description offered). The other had only mild complaints localized to the mouth.

The incidence of reactions to any additives, including tartrazine, in patients with chronic urticaria and angio-oedema is unknown. This is not because of inadequate numbers of studies, but rather because of the lack of properly and vigorously controlled studies and inherent problems in challenging patients with chronic urticaria.

Food additive challenge studies in urticaria patients

Design considerations

PATIENT SELECTION Selection of patients for study may include: (i) all available patients with chronic urticaria (or only those with chronic idiopathic urticaria); (ii) only those with histories suggestive of food additive-provoked urticaria; or (iii) those whose urticaria improved after a diet free of commonly implicated additives. Depending upon the group selected and the challenge process, different percentages of so-called positive reactors have been reported. These variables, often omitted or poorly stated in reports and studies, add more confusion to the already difficult task of comparing results between different studies.

ACTIVITY OF URTICARIA AT THE TIME OF STUDY The relative degree of activity or inactivity of urticaria or angio-oedema at the time of challenge appears to determine the ability of the skin to respond with cutaneous reactions during subsequent additive challenges. Patients with active urticaria are more likely to develop further urticarial activity, while challenges performed upon patients whose urticaria is in remission are more likely to yield negative results. In the study of Lumry *et al*. (1982), only one of 15 patients whose urticaria was in remission experienced a reaction to acetylsalicylic acid (ASA), whereas seven of ten patients whose urticaria was active at the time of challenge reacted to ASA. These challenges were conducted using objective reaction criteria, and reactions were compared with a baseline observation period in the same patient.

MEDICATIONS In several studies, reference is not made as to whether medications, particularly antihistamines, are continued or withheld during challenges. However, there are important caveats to bear in mind when interpreting challenge studies that mention details of withdrawing medications: (i) discontinuation of antihistamines immediately before or within 24 h of challenge is likely to induce false-positive reactions; (ii) continuation of antihistamines during challenges may block some of the milder additive-induced cutaneous responses and therefore promote false-negative results; and (iii) subjects are also more likely to experience breakthrough urticaria the longer the interval from the last antihistamine dose to the test substance associated with a 'positive challenge'. This phenomenon is accentuated if placebo controls are given first, before additive challenges, and in closest proximity to the protective effect of the last antihistamine tablet.

REACTION CRITERIA In most studies, a period of baseline observation for comparison with reaction data was never made. Most challenge studies reported loosely defined and subjective criteria for identifying urticarial response. The reaction criteria simply consisted of 'clear signs of urticaria developing within 24 h'. The studies by Stevenson *et al*. (1986) and Lumry *et al*. (1982) represent the only reported challenge studies which utilized an objective system of scoring urticarial responses.

PLACEBOS The importance of placebo-controlled studies in additive challenges cannot be overemphasized. Studies that did not utilize placebo controls are useless in specifically linking urticarial responses to a challenge substance. There are a surprising number of published studies of additive challenges that never employed placebo controls. Even in most placebo-controlled studies, the placebo was usually the first challenge substance, followed by ASA and then an

additive. Thus, a spontaneous flare of urticaria was least likely to coincide with the first placebo challenge, particularly if antihistamine was last ingested just before beginning challenges. The use of multiple placebos, including randomization of placebos, enhances the design of placebo-controlled challenges and eliminates the bias of first challenge placebo alone.

CONTROLS Among the most important features of any food additive challenge protocol is a double-blind challenge. Because exacerbations of urticaria may be stress provoked, it is necessary to blind the study subjects. Furthermore, nurses and physicians can transmit unspoken signals of concern and apprehension when the 'real' test substance is administered. In addition, it is important to eliminate observer bias whenever possible because positive responses always consist of the appearance of hives or hives in greater numbers than were observed at baseline.

DOSES Box 11.4 lists maximum doses for common additives implicated in adverse reactions. Starting doses should be individualized depending upon the estimated amount ingested at the time of the reported reaction/severity of reaction/level of sensitivity. Doses usually double between initial and maximum doses.

Multiple additive challenges in chronic urticaria

EXAMPLES OF STUDIES WITH LESS STRINGENT DESIGN CRITERIA One of the earliest open additive challenge studies in chronic urticaria patients was reported by Doeglas (1975). He found that seven of 23 patients (30.4%) reacted to tartrazine and 'four or five' (17.4 or 22.7%) reacted to sodium benzoate. Placebo-controlled challenges were not performed. Thune and Granholt (1975) reported that 20 of 96 patients reacted to tartrazine, 13 of 86 reacted to sunset yellow, five of seven reacted to parabens, and six of 47 reacted to BHA/BHT. Furthermore, in their total group of 100 patients with chronic idiopathic urticaria, 62 reacted to at least one of the 22 different additives used in challenges. Because none of the challenges were placebo controlled, conclusions about the specificity of reactions, linked to a particular additive, are difficult to support.

In a study of 330 patients with recurrent urticaria, Juhlin (1981) performed single-blind challenges using multiple additives and only a single placebo, which was always given first, preceding the additive challenges. He found one or more positive reactions in 31% of patients challenged. Reaction criteria were subjective. Reactions were judged to be 'uncertain' in 33% of patients because, as the author stated, 'judging whether a reaction is positive or negative is not always easy'. Furthermore, if patients 'reacted' to the lactose placebo, a wheat starch placebo was then used in re-testing, presumably because the author assumed that the original burst of urticaria was due to the placebo. Questionable reactors were re-tested and, if the repeat test was positive, it was assumed that the first test was positive; the same logic applied for re-testing with negative response.

Supramaniam and Warner (1986) reported that 24 out of 43 children reacted to one or more additives in their double-blind challenge study. However, a baseline observation period was not used, and only one placebo was interspersed among the nine different additives used as challenge substances. Furthermore, whether antihistamines were withheld prior to or during challenges was not mentioned.

In 1985, Genton *et al.* performed single-blind additive challenges on 17 patients with chronic urticaria and/or angio-oedema of unknown type. All medications were also discontinued at the beginning of the diet, and patients were subjected to a 14-day

Box 11.4. Suggested maximum doses for additives used in challenge protocols.

Tartrazine	50 mg
Sulphites	200 mg
MSG	5 g
Aspartame	150 mg
Parabens/benzoates	100 mg
BHA/BHT	100 mg

elimination diet (free of food additives) before any challenges. Of the 17 patients, 15 developed urticaria after at least one of the six additives used for challenge in this study.

EXAMPLES OF STUDIES WITH BETTER DESIGN AND REACTION CRITERIA Ortolani *et al*. (1988) studied 396 patients with recurrent chronic urticaria and angio-oedema as a follow-up to a study performed in 1984 (Ortolani *et al*., 1984). Double-blind, placebo-controlled, oral food provocation challenges were performed on patients who had significant remissions in their urticaria while following an elimination diet. Medications were discontinued during challenges, but the timing of discontinuation of medications was not mentioned. Based on history alone, 179 patients were considered for, but only 135 patients participated in, an elimination diet for suspected food or food additive intolerance. Only eight out of 87 patients that reported significant improvement on the 2-week elimination diet had positive challenges to foods. Of the 79 patients with negative food challenge, 72 underwent double-blind, placebo-controlled, oral food additive provocation challenges. Twelve patients had positive challenges to one or more additives; many of these patients were reported to have reacted to two or three of the test additives. Five of 16 patients with positive ASA challenges had positive additive challenges, four of these to sodium salicylate. The similarity of chemical structures between ASA and sodium salicylate supports the concept of cross-reactivity between ASA and sodium salicylate; however, the doses used (> 400 mg) in the sodium salicylate challenge far exceeded that encountered in conventional diets and therefore had little to do with native dietary exposure. Considering that the proposed mechanisms for reactions to additives such as tartrazine, sodium benzoate and sulphites are so different, the meaning of 'positive challenges' in this study is unclear. Furthermore, although a patient's history is important to the consideration of food sensitivity, it is usually a poor indicator of a possible additive hypersensitivity, since patients are usually not aware of all the additives they consume daily and are always reporting urticarial flare-ups in relation to external events and ingestions. Elimination of more than 50% of the original study population may have been proper for food sensitivity determinations, but it was not justified for selection of patients for additive challenges.

Hannuksela and Lahti (1986) challenged 44 chronic urticaria patients with several food additives, including sodium metabisulphite, BHA/BHT, β-carotene and benzoic acid, in a prospective, double-blind, placebo-controlled study. Only one of the 44 patients had a positive challenge (to benzoic acid). However, one of 44 patients also reacted to a placebo challenge. All medications were discontinued 72 h before the first challenge and during the study. Patients were not following an additive-free diet before the challenges. The challenge dose of metabisulphite was quite low (only 9 mg). Similarly, Kellett *et al*. (1984) noted that approximately 10% of 44 chronic idiopathic urticaria patients reacted to benzoates and/or tartrazine, but 10% of the same subjects also reacted to placebos.

STUDIES OF ELIMINATION DIETS An alternative way to investigate urticaria which is presumed to be secondary to food additives is to eliminate all additives from the diet and observe a decrease in hives. Unfortunately, blind or placebo-controlled studies of this type have not been reported in the literature. In uncontrolled studies, Ros *et al*. (1976) reported an additive-free diet to be 'completely helpful' in 24% of patients with chronic urticaria. Another 57% of patients were 'much improved', and 19% were 'slightly better' or experienced 'no change' in their urticaria. Rudzki *et al*. (1980) reported that 50 of 158 patients responded to a diet free of salicylates, benzoates and azo dyes. These studies did not address the question as to which, if any, additives had been inducing urticaria.

In another study, Gibson and Clancy (1980) found that 54 of 76 patients who underwent a 2-week additive-free diet 'responded'. Using the same study population, they then challenged the responders with individual

additives. Although the challenges were placebo controlled, the placebo was always given first. Furthermore, no mention was made as to whether the challenges were blinded. A diet free of the offending additive was then continued for 6–18 months, followed by a repeat challenge. All three patients who initially experienced a positive challenge after tartrazine did not develop urticaria upon rechallenge with tartrazine 6–18 months later. One of the four patients with initially positive benzoate challenges also experienced a negative challenge upon re-exposure 6–18 months later. Therefore, despite a dietary elimination approach, the incidence of additive-induced urticaria continues to be elusive.

Reports of additive sensitivity using single or limited challenges

TARTRAZINE (FD&C YELLOW NO. 5) Even the incidence of reactions to tartrazine, the most commonly implicated additive causing reactions in patients with urticaria, is not known. In a double-blind, placebo-controlled study, three of 38 patients with chronic urticaria (8%) reacted to tartrazine (Gibson and Clancy, 1980). All three patients were probably sensitive to aspirin; however, the details of the challenge protocols were not presented and the challenge dose of tartrazine was only 0.22 mg. The choice of challenge dose was based on the quantity of tartrazine added to pharmaceutical tablets. Much greater amounts of tartrazine are found in foods and drinks (25–50 mg).

Settipane and Pudupakkam (1975) also report tartrazine sensitivity in some patients with urticaria who were also sensitive to aspirin. However, in a single-blind study of the incidence of aspirin sensitivity in chronic idiopathic urticaria at Scripps Clinic, we administered 25 and 50 mg doses of tartrazine (up to a total dose of 75 mg in most patients during one challenge day), and only one of 24 patients reacted with urticaria. This single suspected tartrazine reactor was then rechallenged using a double-blind, placebo-controlled tartrazine challenge and again developed urticaria after 25 mg of tartrazine (Stevenson *et al*., 1986). This patient did not experience any reaction to aspirin (receiving a total of 975 mg, with 650 mg as a final single dose) and gave an excellent history of reported urticaria after exposure to tartrazine in her diet previously.

SUNSET YELLOW (FD&C YELLOW NO. 6) A single case report described a 43-year-old physician with acute episodes of severe abdominal pain and hives believed to be secondary to yellow dye no. 6. Despite ongoing ingestion of this dye, the subject experienced only four isolated episodes of hives in 2 years. Two challenges, one single and the other double-blind, provoked 'reactions'. The single-blind challenge was associated with both abdominal pain and urticaria. However, the double-blind challenge was only associated with pain and not with urticaria (Gross *et al*., 1989).

SULPHITES In 1976, Prenner and Stevens reported the occurrence of an anaphylactic reaction after the ingestion of food sprayed with sodium bisulphite. The patient, a 50-year-old male, experienced generalized urticaria and pruritis, swelling of the tongue, difficulty with swallowing and tightness in his chest within minutes after eating lunch at a restaurant. He responded promptly to treatment with subcutaneous adrenaline. Subsequently, a prick test to sulphite as well as an intradermal test were significantly positive (with negative controls). The authors were able to demonstrate passive transfer, via Prausnitz–Küstner (P–K) testing, to a non-atopic human recipient.

In 1980, Clayton and Busse described a non-atopic female who developed generalized urticaria that progressed to life-threatening anaphylaxis within 15 min of drinking wine. Her symptoms were not reproduced by ingestion of other alcoholic beverages. In retrospect, this may have been a case of sulphite-provoked urticaria and anaphylaxis.

Habenicht *et al*. (1983) described two patients with several episodes of urticaria and angio-oedema following restaurant meals. Only one of these patients underwent a single-blind oral challenge with potassium metabisulphite; generalized urticarial lesions developed in this patient within 15 min of the 25 mg challenge dose. However, a placebo

challenge was not performed. Avoidance of potential sulphite sources apparently has led to resolution of this patient's recurrent symptoms.

Schwartz reported two patients with restaurant-related symptoms who underwent oral metabisulphite challenges (Schwartz, 1983). Both patients had symptoms including weakness, a feeling of dissociation from body, dizziness, nausea, chest tightness and possible hives temporally related to ingestion of salads. Upon challenge, both patients experienced abdominal distress, dizziness, borderline hypotension and bradycardia. These signs and symptoms were more consistent with vasovagal reactions than anaphylaxis. In a 1985 report, Schwartz and Sher (1985b) described a patient who received less than 2 ml of procaine (Novocaine®) with adrenaline subcutaneously. Within several minutes, she developed a sense of flushing, warmth and pruritis followed by scattered urticaria, dyspnoea and a sense of anxiety. Skin tests, using various local anaesthetics and sulphite, were negative. She developed 'a sense of fullness in her head, nasal congestion and a pruritic erythematous blotchy eruption' 30 min after a single-blind oral dose of 10 mg of sodium bisulphite. Respiratory symptoms did not develop and pulmonary function test results remained normal. This patient went on to tolerate the same local anaesthetics without adrenaline. It is critical to note that this patient did not describe a history of food-related symptoms. Furthermore, the usual dose of aqueous adrenaline contains only 0.3 mg of sulphite, and local anaesthetics contain only up to 2 mg ml^{-1} of sulphite. Therefore, usual doses of such anaesthetics, even in the most sensitive individuals, would not be expected to provoke reactions. The mechanism of this patient's 'reaction' cannot be linked confidently to sulphite and was probably a vasomotor response secondary to anxiety and/or to the effects of adrenaline.

There are now two publications demonstrating the inability of investigators to provoke reactions to sulphites in patients with idiopathic anaphylaxis, some with histories of restaurant-associated symptoms (Sonin and Patterson, 1985; Kulczycki, 1986). In a study describing food skin testing in 102 patients with idiopathic anaphylaxis, only one patient was found to have cutaneous sensitivity to metabisulphite (Stricker *et al*., 1986). In addition, we have performed sulphite ingestion challenges in 25 patients with chronic idiopathic urticaria and angio-oedema without encountering any reactions. Therefore, except for the reports by Prenner and Stevens (1976) and Yang *et al*. (1986), no other studies using properly controlled challenges confirm sulphite-induced urticaria, angio-oedema and/or anaphylaxis. Yang *et al*. (1986) described one patient with a history of sulphite-provoked anaphylaxis. A borderline intradermal skin test was demonstrated, as was a positive single-blind oral provocation challenge to 5 mg of potassium metabisulphite. This patient's cutaneous reactivity was also transferred passively via the P–K reaction. However, these investigators were unable to elicit positive challenges in nine patients with histories of hives after eating restaurant food. In conclusion, IgE-mediated immediate hypersensitivity reactions to sulphites (possibly via a hapten recognition) appear to be extraordinarily rare, if they exist at all, in inducing urticaria and anaphylaxis. In the overwhelming majority of cases, the mechanism of sulphite-provoked urticaria, angio-oedema and anaphylaxis (or anaphylactoid reactions) remains an enigma.

BENZOATES AND PARABENS In the literature, we are aware of a total of two reports (three cases) of apparent IgE-mediated, paraben-induced urticaria and angio-oedema (Aldrete and Johnson, 1969; Nagel *et al*., 1977). Parabens in pharmaceutical preservatives were the presumed source of these additives. All three patients had positive skin tests to parabens, but not to the drugs themselves, which were free of paraben preservatives. These patients were able to tolerate oral benzoates in their diet without reaction. Michels *et al*. (1991) reported the case of a teenager who had experienced several food-associated reactions in which sodium benzoate seemed to be the common factor. One of these episodes involved flush, angio-oedema, dyspnoea and severe hypotension. An oral

challenge with 20 mg of sodium benzoate produced itching and urticaria.

MONOSODIUM GLUTAMATE A 1987 letter in the *Lancet* (Squire, 1987) described a 50-year-old man with recurrent angio-oedema of the face and extremities which was related by history to ingestion of a soup which contained MSG. A single-blind, placebo-controlled challenge with the soup base resulted in 'a sensation of imminent swelling' within a few hours, but visible angio-oedema appeared 24 h post-challenge. In a graded challenge, angio-oedema occurred 16 h after challenge with 250 mg of MSG alone. Avoidance of MSG led to an extended remission. Details of the challenge were not reported, nor was there any mention of whether medications were withheld during challenges.

ASPARTAME Two cases of aspartame-provoked urticaria and angio-oedema have been reported (Kulczycki, 1986). In both individuals, hives began after aspartame was approved as a sweetener in carbonated beverages in 1983. Both patients reported the onset of urticaria within 1 h of ingesting aspartame-sweetened soft drinks. Double-blind, placebo-controlled challenges reproduced urticaria with doses of aspartame (25–75 mg) below the amount contained in a typical 12-ounce can (100–150 mg). Despite the widespread use of aspartame in diet drinks and elsewhere, other reports have not followed these initial findings. Even an attempt to recruit patients believing themselves to be sensitive to aspartame did not yield additional subjects for challenge studies (Nagel *et al*., 1977). In this study, 12 subjects with urticaria, but without a history of aspartame-associated urticaria, were challenged with aspartame and none experienced a reproducible adverse reaction.

BHA/BHT Roed-Petersen and Hjorth (1976) found four patients with eczematous dermatitis who had positive patch tests to BHA and BHT. Dietary avoidance of the antioxidants resulted in remissions in two patients. When challenged with ingestion of 10–40 mg BHA or BHT, both patients experienced exacerbations of their dermatitis. Osmundsen (1980) reported a case of contact urticaria, apparently due to BHT contained in plastic folders; the patient had positive wheal-and-flare responses to 1% BHA and BHT in ethanol. A case of acute urticarial vasculitis related to BHT in chewing gum has also been reported (Moneret-Vautrin *et al*., 1986).

Two patients with chronic idiopathic urticaria, in whom remissions were achieved while following dye and preservative elimination diets, had exacerbations of their urticaria when challenged under double-blind, placebo-controlled conditions with BHA and BHT (Goodman *et al*., 1990). After elimination of BHA and BHT from their diets, the patients were observed to have marked abatement of the frequency, severity and duration of their urticaria.

Natural food colourants

Many natural colourants are allowed for use in foods, including annatto, carmine, carotene, turmeric, paprika, beet extract and grape skin extract. These types of colourants are not used to any extent in pharmaceutical applications. Several studies have reported positive reactions after challenges with natural colours (Mikkelsen *et al*., 1978; Juhlin, 1981; Fuglsang *et al*., 1994) or mixtures of natural and synthetic colours (Veien *et al*., 1987). The natural colourants involved in these challenges were annatto, betanin, curcumin, turmeric, β-carotene, canthaxanthin and beet extract. The adverse reactions were asthma, urticaria, atopic dermatitis, colic and vomiting. Of course, no one colour can be identified as the causative factor when challenges are conducted with mixtures.

Annatto

Annatto is obtained as an extract from the seeds of the fruit of the Central and South American tree, *Bixa orellana*. Bixin, the principal pigment in annatto, is a carotenoid. The extracts are red in colour, but annatto is often used to impart an orange or deep-yellow colour to the finished food.

Nish *et al*. (1991) reported a case of a possible IgE-mediated allergic reaction to annatto extract. The patient experienced angio-oedema, urticaria and severe hypotension within 20 min of ingesting a breakfast cereal containing annatto. The patient had a strongly positive skin test to annatto extract, and an IgE-binding protein was identified through sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with immunoblotting. Because annatto extract is derived from a seed, the presence of proteins in the extracts is likely. IgE-mediated allergies to annatto proteins are possible, although this is the only reported case in the medical literature. Young *et al*. (1987) estimated the prevalence of annatto sensitivity at 0.01–0.07%.

Carmine

Carmine and cochineal extract are derived from dried female insects of the species *Dactylopius coccus*, which lives as a parasite on the prickly pear cactus. An aqueous alcoholic extract of the dried insects is made and concentrated, by removal of the alcohol, to obtain the colour additive, cochineal extract. The colouring principle of cochineal extract is carminic acid. Carmine is the aluminium or calcium–aluminium lake of the colouring principles, primarily carminic acid, obtained by aqueous extraction of cochineal. Carmine and cochineal extract have a red colour.

Carmine is widely used in cosmetics, but only a few cases of dermatological reactions have been attributed to it (Sarkany *et al*., 1961; Kagi *et al*., 1994). Park (1981) reported a case of severe anaphylactic shock possibly linked to the cutaneous use of carmine. A soldier involved in a casualty simulation drill was smeared with a make-up stick to simulate burns. An immediate anaphylactic response ensued, characterized by severe hypotension and tachycardia. Unfortunately, no follow-up was done on this patient to confirm the role of carmine in this case.

Two individuals with carmine-associated occupational asthma also reacted to oral challenges with carmine solution (Burge *et al*., 1979). One responded with asthma and gastrointestinal upset after challenge with 1 ml of cochineal extract diluted in 100 ml of water. The other experienced asthma after drinking Campari, a beverage that contains carmine. Kagi *et al*. (1994) described an individual with anaphylaxis characterized by rhinitis, asthma, urticaria and multiple gastrointestinal complaints after ingestion of Campari-orange. This individual had positive skin prick tests to the Campari beverage, carmine and carmine-containing cosmetics, indicating a possible IgE-mediated reaction. Another case of probable IgE-mediated allergy to carmine has been described in an individual who reacted with urticaria, angio-oedema and asthma after ingestion of a carmine-containing yoghurt (Beaudouin *et al*., 1995). A histamine-release assay using the patient's basophils was also positive, another indication of an IgE-medicated reaction (Beaudouin *et al*., 1995). Because carmine is obtained from an extract of insect bodies, it might contain proteins and could elicit IgE-mediated reactions in rare cases such as these.

Summary

Tartrazine and other dyes, benzoates and parabens occasionally may aggravate but have not been reported as the cause of chronic urticaria. Parabens have been shown to provoke (rarely) anaphylaxis when given parenterally. Sulphites, although not implicated in chronic urticaria, occasionally have been reported to provoke anaphylaxis. MSG has been reported to provoke angio-oedema in a single case report. Aspartame, BHA and BHT have been shown to be the cause of isolated cases of chronic idiopathic urticaria. Nitrates and nitrites have not been associated with urticaria, angio-oedema or anaphylaxis.

Asthma and Additives

Design considerations for challenge studies

General considerations

A screening challenge should be conducted in a single-blind, open fashion. Because so

few patients are sensitive to additives, single-blind challenges simplify the procedure. More importantly, such a challenge fulfils the important safety requirement of individualizing doses. At each successive challenge step in the protocol, the doses increase two-fold. If a patient has a 10–15% drop in FEV_1 after a particular dose, one may wish to add a dose at half the usual increment of increase. This would not be possible with a double-blind challenge protocol.

Other safety factors include performing the challenge when the patient's asthma is stable (FEV_1 value of at least 1.5 l and 70% of predicted or prior best values). At times, especially in patients most likely to be sulphite-sensitive (e.g. chronic asthmatic patients are corticosteroid dependent), these patients may require a burst of corticosteroids to stabilize their asthma activity (as in aspirin-sensitive subjects). Challenges routinely begin in the morning and can take place in the physician's office if one is prepared to treat rapidly moderate to severe asthma with inhaled bronchodilators. Using the recommended protocol and individualized doses, we have not encountered severe reactions during hundreds of sulphite challenges, and all patients' bronchoconstriction could be reversed rapidly with albuterol or metaproterenol administered with a hand-held nebulizer.

On the day of challenge, patients should withhold their inhaled and oral β_2 agonists and inhaled anticholinergics, and antihistamines and cromolyn should be withheld for 24 h before the challenge. We continue theophylline at therapeutic levels and continue (even increase) inhaled and oral steroids. In our experience, theophylline and steroids do not interfere with sulphite (or aspirin) challenges (Stevenson and Simon, 1981; Pleskow *et al.*, 1983; Simon and Stevenson, 1987). Although a recent study suggests that corticosteroids may increase the threshold reaction dose (Nizankowska and Szczeklik, 1989), withholding theophylline and steroids may lead to a false-positive challenge in an unstable asthmatic patient. To control this variable, and to establish stability of their bronchial airways, we recommend that patients undergo a placebo challenge for a length of time equal to that of the sulphite challenge.

Pulmonary function is measured before challenge and before the next dose or sooner if symptoms occur. A 20% drop in FEV_1 value from the baseline is considered a positive challenge.

Sulphite challenge

Specific considerations

Since the FDA regulation on sulphite usage in the late 1980s, the indications for challenges with SO_2, sulphite and sulphurous acid particles have become limited to scientific investigation and very occasional clinical purposes.

Challenges for investigation of the environmental and occupational hazards of SO_2 and sulphuric acid exposures include epidemiological studies and studies of asthmatic populations aimed at better drawing the lines for regulating emissions of these chemicals (Boushey, 1982a). Likewise, scientific investigations aimed at better understanding the bronchial hyper-reactivity associated with asthma may employ SO_2 or sulphite challenges akin to histamine, methacholine, exercise, water, osmolar, allergen, mediator and pharmacological challenges outlined in other chapters in this volume.

For clinical purposes, when an asthmatic patient recognizes reactivity to SO_2/sulphite, as is most likely to occur with exposure-ingestion of dried fruits or wine, he or she can avoid these sulphite sources and is protected further by regulations of the FDA requiring labelling of processed food containing more than 10 ppm SO_2 equivalents. If there is suspicion, particularly if there is uncertainty regarding reaction to dried fruits, wine, processed potatoes or shrimp, or when an asthmatic patient must be reassured that he or she is not relevantly sensitive to sulphites, oral challenge with the suspected food or beverage (Taylor *et al.*, 1988) or capsule doses of sulphite under single-blind and, if the result is apparently positive, then double-blind conditions are appropriate.

At Scripps Clinic, we use the following protocol for sulphite capsule challenges: patients are challenged at a time when their asthma is in remission as documented by FEV_1 greater than 70% of the predicted or best previously observed value and at least 1.5 l absolute. Inhaled cromolyn (Koenig *et al*., 1988) and anticholinergic (Simon *et al*., 1984b), antihistamine (Simon *et al*., 1982) and adrenergic bronchodilator medications are withheld on the day of challenge; other medications are continued. Open oral challenges with capsule doses of 5, 25, 50, 100 and 200 mg of potassium metabisulphite at 30-min intervals are administered, and spirometric measurements are performed before each dose. If there is a fall of FEV_1 of 20% or more, the challenge is suspended, and obstruction is reversed with inhalations of adrenergic bronchodilator. Apparent reactions need to be verified with double-blind, placebo-controlled challenges.

Before concluding that a patient is sulphite sensitive, one should repeat the challenge in a double-blind, placebo-controlled manner, starting with the patient's previously established provoking dose and using at least two other placebo challenges. For suggested challenge doses for common food additives, see Box 11.4.

Specific additives and asthma

Tartrazine

Data on the incidence of reactivity to additives in patients with asthma are, on the whole, only slightly better than those for patients with urticaria. The additive most frequently implicated in provoking asthmatic reactions has been tartrazine. Critical review of the medical literature, however, suggests that sensitivity to tartrazine in patients with asthma is distinctly unusual, if it exists at all (Simon, 1984; Stevenson, 1991). In 1958, Speer stated that agents used in artificial colouring were the cause of asthma in sick children; however, the author presented no details about how this conclusion was reached. In 1967, Chafee and Settipane reported a patient with severe asthma who experienced angio-oedema after aspirin ingestion and severe attacks of asthma shortly after ingesting a number of unrelated drugs. After approximately 2 years of such reactions and a great deal of investigative activity, the attacks disappeared when benzoates and tartrazine were eliminated from this patient's food and medication. During eight double-blind, placebo-controlled challenges with various dyes, significant symptoms (tickling of throat, tight cough and wheeze) occurred only after receiving tartrazine. Unfortunately, no pulmonary function studies were conducted and the double-blind challenge for tartrazine was not repeated.

In their classic monograph on aspirin intolerance, Samter and Beers (1968) discussed the fact that benzoates and tartrazine were commonly used in the food ingested by their aspirin-sensitive individuals. In their first report, 80 patients with asthma were challenged with unknown doses of tartrazine and three 'reacted' (Samter and Beers, 1967). However, essential information concerning withholding or continuing medications, use of placebo controls, criteria for positive reaction, etc. were not provided. Juhlin *et al*. (1972) reported that seven of eight patients with asthma who were sensitive to aspirin also reacted to tartrazine. However, the investigator's criteria for a positive reaction were subjective, and details of the placebo challenges were not discussed. As the studies of Stenius and Lemola (1976) point out, such details are important. Their protocol called for withholding bronchodilators on the day of challenge, then giving a placebo first, followed by aspirin and finally tartrazine. All these challenges took place on the same day; therefore, any patient requiring bronchodilators would be least likely to 'react' to placebo and more likely to 'react' to aspirin. Finally, as the day wore on and any possible bronchodilator effects wore off, patients' bronchial trees became most likely to constrict, in the absence of bronchodilator, or 'react' to tartrazine. It is also unclear from these studies what happened to the patients who 'reacted' to aspirin. That is, when exactly was the tartrazine challenge performed in the sequence of

challenges? Was it performed after treatment for the aspirin reaction, after the aspirin reaction spontaneously resolved, in the middle of an unresolved aspirin reaction or on another day? In view of any of these uncertainties, how would one interpret a 'reaction' to tartrazine? Finally, the criteria for a positive reaction was a 20% fall in peak expiratory flow. Their data were also reported inexactly: '. . . about 25% of 140 asthmatics were aspirin-sensitive and 20% tartrazine-sensitive.'

In another study without placebo controls, Freedman (1977) challenged 14 of 30 patients with asthma who gave a history of asthma after ingestion of orange-coloured drinks. Only one patient experienced a 'reaction' to tartrazine; her maximal fall in FEV_1 was only 14% after ingestion of a 20 mg dose (apparently the criterion for a reaction was a 14% decline in FEV_1 values). In a more recent study by Rosenhall (1982), 2.3% of 542 patients with asthma had a 'definitely' positive response to tartrazine, and another 6% had a 'probably' positive response. Some problems with this study included single-blind challenges and the fact that placebo studies were conducted only if challenges to other substances were 'difficult to interpret'. Furthermore, non-respiratory tract changes, including cutaneous responses such as urticaria and gastrointestinal complaints such as vomiting or diarrhoea, were included as criteria for a positive response in these asthmatic subjects.

A decade earlier, one of the few double-blind, placebo-controlled challenges with tartrazine was performed in 38 patients with a history of aspirin-provoked asthma (Settipane and Pudupakkam, 1975). Although only 0.44 mg tartrazine was used as the highest provoking dose, three of 38 patients were found to be responsive to tartrazine (experiencing a > 20% fall in vital capacity, FEV_1 and expiratory flow rates).

Spector *et al*. (1979) performed placebo-controlled aspirin and tartrazine challenges in more than 200 patients. Of 230 patients, 44 had positive reactions to aspirin (an incidence of almost 20%). Of 277 patients, 11 reacted to tartrazine (FEV_1 falls of > 20%), an incidence of less than 4% in the population studied; however, five of these 11 patients did not undergo placebo challenges. All 11 patients who were reported to have reacted to tartrazine also had a reaction to aspirin during another challenge. In other words, tartrazine sensitivity was not observed in patients with asthma who were not sensitive to aspirin. One could extrapolate from these data that 15–25% of ASA-sensitive asthmatic patients are also sensitive to tartrazine. Yet, in double-blind, placebo-controlled challenges of 45 patients who had a history of moderately severe asthma (one-half of whom also had nasal polyps and up to 45% of whom were sensitive to aspirin), Weber *et al*. (1979) did not find any who were sensitive to tartrazine in doses up to 20 mg. Along these lines, Vedanthan *et al*. (1977) conducted tartrazine challenges in 54 children (aged 10–17 years) with asthma and found none who were sensitive to tartrazine. Five of the 54 children were sensitive to aspirin during challenges conducted at another time.

Tarlo and Broder (1982) performed double-blind ingestion challenges with tartrazine, benzoate and aspirin. Of the 28 subjects, only one responded to tartrazine (15 mg producing a 20.4% drop in FEV_1) and one to benzoate (25 mg provoking a 29% drop). Neither of these patients was found to be aspirin sensitive during challenges with this drug, and neither responded to dietary elimination of the two suspected additives.

In 1985, Genton *et al*. reported challenge results with additives, including tartrazine, in 17 asthmatic subjects. Attempts were not made to mask the flavour or colour of the agents tested. β Agonists were withheld and only a single placebo was administered, versus multiple doses of other substances. A positive challenge was defined as a 20% drop in peak flow rates up to 8 h after a challenge. Even with this protocol, only one subject 'reacted' to tartrazine.

For more than 20 years, investigators at Scripps Clinic have been studying aspirin-sensitive asthma. One should note that tartrazine is not a cyclo-oxygenase inhibitor (Gerber *et al*., 1979) and therefore would not be expected to cross-react with aspirin, as do non-steroidal anti-inflammatory drugs, in such patients. In any case, we performed

tartrazine challenges before aspirin challenges as a routine procedure in more than 150 single-blind, placebo-controlled challenges (with 25 and 50 mg of tartrazine) in our aspirin-sensitive asthmatic population. In this single-blind screening study, we identified six patients whose FEV_1 declined after tartrazine (Stevenson, 1991). In five of six patients, rechallenge with tartrazine using double-blind, placebo-controlled challenge sequences was negative. One patient who experienced a decline in FEV_1 values during single-blind tartrazine challenges moved out of town, and we have been unable to rechallenge this patient in a double-blind, placebo-controlled fashion. We remain sceptical that tartrazine-induced asthma attacks even exist. Certainly, evidence linking tartrazine to aspirin sensitivity has not been forthcoming.

Other dyes

Reactions to non-azo dyes and azo dyes other than tartrazine are reported far less commonly than those to tartrazine, even in those studies that reported tartrazine sensitivity. Therefore, these agents will not be discussed further.

Sulphites

In 1973, Kochen described a child who developed acute bronchospasm after opening cellophane packages and ingesting dried fruits treated with SO_2. Although the term sulphite was not mentioned, this report may in fact be the first example of sulphite inhalation-induced asthma. The first case report actually specifying sulphite was in 1976 by Prenner and Stevens. They described a non-asthmatic but atopic individual with a history of hay fever. After a sulphite-containing restaurant meal, the subject developed generalized urticaria, angio-oedema and possibly laryngeal oedema. During an unlinked oral challenge with a 10 mg sulphite capsule, the patient developed itching, cough and tightness in the throat. The challenge was considered positive and was discontinued without producing urticaria, angio-oedema or laryngeal oedema. Pulmonary function tests to determine whether there was an asthmatic response were not performed.

In 1977, Freedman, in the UK, noted that many asthmatic patients gave histories of reacting to citrus drinks. These drinks contain tartrazine (FD&C yellow no. 5) and benzoate as well as SO_2. Freedman's 'sulphur dioxide challenges' were performed by subjects ingesting solutions into which sulphites had been dissolved. Some of the asthmatic subjects did have wheezing after SO_2 challenges. However, the challenges were not placebo controlled and the amount of SO_2 inhaled is not clear. The author considered a fall in FEV_1 of as little as 12% to be a positive reaction.

In 1981, Stevenson and Simon first reported five adult asthmatic patients with a history of severe restaurant-provoked asthma and even anaphylaxis who underwent single-blind, placebo-controlled capsule challenges. A 20% or greater fall in FEV_1 10–20 min after ingesting capsules containing 5–50 mg of potassium metabisulphite ($K_2S_2O_5$) was reproduced in all five patients during single-blind oral challenges (Stevenson and Simon, 1981). Simultaneously, Baker *et al*. (1981) reported asthmatic reactions to sulphites contained in pharmaceutical products. In 1982, Twarog and Leung observed an 18-year-old asthmatic subject with a history of recurring asthma attacks after restaurant meals. In addition, on two separate occasions while hospitalized for non-asthma-related problems, the patient received Bronkosol® and experienced severe attacks of asthma resulting in respiratory arrest. This patient also developed an equally severe reaction after receiving intravenous metoclopramide (Reglan®). Comparison of the constituents of these two agents revealed bisulphite as the only common substance. A similar patient with a paradoxical bronchospastic reaction following Bronkosol® inhalation was reported by Koepke *et al*. (1984). Genton *et al*. (1985) found four of 17 adult asthmatics who reacted to high concentrations of acidic solutions of sulphites. Also in 1985, in a letter, a patient was described who experienced episodes of bronchoconstriction after application

of an eye solution containing sulphite preservatives (Schwartz and Sher, 1985a).

Clinical characteristics of sulphite-sensitive asthmatic individuals

Patient profile

The typical clinical features of sulphite-sensitive asthmatic individuals were first described in 1981 (Stevenson and Simon, 1981). All have chronic asthma, usually are corticosteroid dependent and are provoked by multiple other factors (e.g. upper respiratory infections, irritants and exercise). The irritant effects of smog, presumably on afferent receptors in the trachea, are particularly troublesome for these individuals. Characteristically, the most severe corticosteroid-dependent, sulphite-sensitive subjects did not have asthma until their first isolated sulphite reactions. Within months, they progressed from asymptomatic to chronic asthma with corticosteroid dependency. This indicates that any member of the population is at risk for sulphite sensitivity. The typical sulphite-sensitive asthmatic is usually non-atopic with chronic vasomotor-type rhinosinusitis. These individuals are differentiated from aspirin-sensitive patients because they lack nasal polyps and eosinophilia. Sinus X-rays are abnormal in a high percentage of both aspirin-sensitive and sulphite-sensitive asthmatics.

Cross-sensitivity

Studies by Stevenson and Simon in 15 sulphite-sensitive asthmatics had shown that none reacted to aspirin during oral challenges and, vice versa, none of a group of 15 aspirin-sensitive asthmatic subjects had positive oral sulphite challenges (Simon and Stevenson, 1987). Moreover, careful review of the medical literature does not confirm that aspirin and sulphite sensitivity co-exist in the same individual. One report described dual positive challenges in some patients with chronic asthma (Kochen, 1973). However, the details of the challenges and the clinical characteristics of the patients were not available in the report. One sulphite-sensitive asthmatic patient originally described as aspirin sensitive (Koepke *et al*., 1984) was subsequently rechallenged with both aspirin and sulphite at Scripps Clinic and found to have only sulphite sensitivity (Simon, 1985). None of our sulphite-sensitive asthmatic patients have experienced positive challenges to tartrazine (50 mg) or MSG (2500 mg), nor have they manifested IgE-mediated sensitivity with food antigens by skin testing.

Although Baker *et al*. (1981) described sulphite-sensitive patients who also had sensitivity to aspirin and, in some cases, other additives, such as MSG and benzoates, these data were largely historical and not confirmed by challenges. In addition, the dose of sulphite used during their challenges was 500 mg. We now recognize that this dosage is excessive. Finally, one report described an individual with a history of MSG sensitivity who reacted to both MSG and sulphite (50 mg in solution) in a double-blind, placebo-controlled challenge (Koepke and Selner, 1986).

Prevalence

The prevalence of adverse reactions to sulphiting agents is not known despite attempts to establish the prevalence of sulphite sensitivity in asthmatic subjects. Because of the nature of the populations studied and the challenge methods employed, the incidence can only be estimated. Simon *et al*. (1982), in a preliminary study, examined the prevalence of sensitivity to ingested metabisulphite in a group of 61 adult asthmatic subjects. None gave a history of sulphite sensitivity. Challenges were conducted with potassium metabisulphite capsules and solutions. Positive responses were confirmed by a placebo-controlled challenge. Of 61 patients, five (8.2%) had a 25% or greater decline in FEV_1 values during sulphite challenge. Koepke and Selner (1982) conducted open challenges with sodium metabisulphite in 15 adults with a history of asthma after ingestion of sulphited foods and beverages. One of 15 (7%) had a 28% decline in FEV_1. A confirmatory challenge was not conducted. In a larger study by Buckley *et al*.

(1985), 134 patients underwent single-blind challenges with potassium metabisulphite capsules. Of these, 4.6% were reported to have sulphite sensitivity. In these three preliminary studies, the patient populations consisted of asthmatics with corticosteroid dependency who were being evaluated at an allergy referral centre. Thus, the prevalence, based on these observations, is probably not applicable to the asthma population as a whole, because corticosteroid dependency is one of the clinical characteristics of sulphite-sensitive asthmatics.

In the largest study reported to date, Bush *et al*. (1986) conducted capsule and neutral solution sulphite challenges in 203 adult asthmatic subjects. Patients were not selected for a history of sulphite sensitivity. Of these patients, 120 were not receiving corticosteroids, while 83 were corticosteroid dependent. Of the non-corticosteroid-dependent group, only one experienced a 20% or greater decline in FEV_1 values after single-blind and confirmatory double-blind challenges. The response rate in the corticosteroid-dependent asthmatic group was higher (8.4%). The prevalence in the asthmatic population as a whole was less than 3.9%, and corticosteroid-dependent asthmatic patients appeared to be most at risk for sulphite sensitivity.

Although corticosteroid-dependent asthmatic individuals have the highest incidence of positive sulphite challenge results, they are not the only group at risk. Notable in the history of sulphite-sensitive asthmatic patients is that originally they were not corticosteroid dependent or even asthmatic while undergoing their initial sulphite reactions. In fact, most did not have asthma when they began having severe restaurant-provoked bronchospastic reactions. Only later did they develop chronic asthma that became corticosteroid dependent. Therefore, the population at risk can be an early asthmatic or pre-asthmatic individual, indistinguishable from the general population. Fortunately, based upon studies and reports, the number of these patients is small (Simon and Stevenson, 1997).

The incidence of sulphite sensitivity in the paediatric population is also unknown. As noted earlier, the first case of sulphite-induced bronchospasm was in a child (Kochen, 1973). Since then, there have been two other isolated case reports of alleged sulphite-sensitive children (Sher and Schwartz, 1985; Wolf and Nicklas, 1985). However, these reports did not include properly designed and blinded challenges. Whether any of these three patients are, in fact, sulphite sensitive remains in question. In one study of chronic asthmatic children, almost two-thirds were reported to be sulphite sensitive after open ingestion challenge (Towns and Mellis, 1984). Challenges did not include controls, and the children reacted only to large doses of sulphite solutions. These children may have reacted to the higher levels of SO_2 generated in solution which are being swallowed. Whatever the prevalence, investigators generally agree that sulphite reactions have decreased markedly since 1986 when the FDA banned the use of sulphites in fresh food and required labelling for other sources of sulphites.

Benzoates and parabens

The next most commonly reported additives that might cause bronchospasm in patients with asthma are the parabens. In Freedman's (1977) study, four of 14 patients with a history of sensitivity to orange drinks had positive bronchospastic reactions to sodium benzoate in uncontrolled challenges. Maximum decreases in FEV_1 values ranged from 23 to 33% between 10 and 30 min after 20–100 mg sodium benzoate. Samter and Beers (1968), as noted earlier for tartrazine, reported that sodium benzoate was a commonly used preservative in the foods which their aspirin-sensitive patients reacted to by history. In Rosenhall's (1982) study, despite poorly designed challenges, only one of 504 patients reacted to a dose of sodium benzoate (< 100 mg). Weber *et al*. (1979) found only one of 43 patients with a positive reaction to 250 mg of sodium benzoate or hydroxybenzoic acid in double-blind studies. Furthermore, when this patient was rechallenged 2 years later, he did not react to the same provoking dose of sodium benzoate.

The study of Genton *et al*. (1985) also examined asthmatic reactions to sodium benzoate and only found one of 17 subjects who was reported to have 'reacted'.

The only authors to report a double-blind, placebo-controlled, benzoate-induced reaction were Tarlo and Broder (1982). Once again, their patient was described as being aspirin insensitive. Additionally, no improvement was noted in this patient's asthma when benzoate was removed from the diet.

Monosodium glutamate

The initial report of MSG-provoked asthma described two patients with delayed asthmatic reactions (12 h post-ingestion) (Allen and Baker, 1981b); however, a subsequent report described four other patients with a history of CRS and asthma in the fourth patient experiencing a respiratory arrest within 3 h of the Chinese meal (Allen and Baker, 1981a). Challenges performed open or single-blind with changes in peak respiratory flow rates were used to confirm positive reactions. In addition, three of 12 asthmatics without a history of Chinese restaurant-provoked asthma had positive challenges to MSG (all late). With single-blind, placebo-controlled screening challenges, 100 subjects with asthma (30 subjects with a history of Chinese restaurant asthma attacks; 70 patients with a negative history) were challenged with 2.5 g of MSG. No patient had a significant fall in FEV_1 value or the development of asthma symptoms during the MSG challenge. The mean change in FEV_1 with MSG challenge was no different from that of placebo challenge. A case describing MSG-provoked asthma has been reported. This individual had positive double-blind, placebo-controlled MSG and sulphite challenges (Koepke and Selner, 1986). Not surprisingly, there are two reports involving small numbers of mild asthmatics, without a suggestive history, who were not found to react to MSG during oral challenges (Schwartzstein *et al*., 1987; Germand *et al*., 1991). We have not seen a positive early or late reaction to MSG in our Scripps Clinic asthma population (Woessner *et al*., 1999).

BHA/BHT

In 1973, Fisherman and Cohen reported seven patients with either asthma or rhinitis who were said to be intolerant to BHA and BHT. These patients were identified by a doubling of their earlobe bleeding times. Clinical details, or the reason why BHA or BHT was ever suspected to cause difficulty, were not given. Rationale for the reported effect on the bleeding time was not given. The next year, performing a similar study, Cloninger and Novey (1974) refuted these findings (Fisherman and Cohen, 1976).

Other chemicals

Benzalkonium chloride

Paradoxical responses to nebulized ipatropium bromide (Beasley *et al*., 1987) and beclomethasone dipropionate (Clark, 1986) led to the discovery that the antibacterial preservative benzalkonium chloride causes bronchoconstriction in about 60% of asthmatic subjects; the characteristics of the responses – rapid onset with slow recovery over 60 min, and inhibition by cromolyn but not ipatropium – suggest a mechanism of action via release of mediators (Zhang *et al*., 1990). The benzalkonium chloride concentration in commercial nebulizer solutions has been reduced so that only the rare patient with apparent immunological sensitivity will now react (Ponder and Wray, 1993).

Spearmint

The flavours spearmint (*Mentha spicata*), peppermint (*Mentha piperita*) and menthol (*Mentha labiateae*), used in chewing gum and toothpaste, have been confirmed by challenges in two cases to have triggered

asthma (Spurlock and Dailey, 1990; Subiza *et al*., 1992).

Summary

Carefully designed, well-controlled studies have failed to confirm tartrazine (or other azo and non-azo dye)-provoked asthmatic reactions. Sulphites, on the other hand, have clearly been shown to produce serious, even life-threatening, asthmatic reactions by several proposed mechanisms. Approximately 3–5% of asthmatic patients are sulphite sensitive, most of whom react by inhaling SO_2 generated when sulphites are placed in solution. When the FDA banned sulphites added to fresh foods in 1986, the frequency, and therefore importance, of this problem was greatly diminished. Benzoates and parabens have not been shown conclusively to be a significant problem for asthmatics, even those who are aspirin sensitive. MSG may occasionally produce asthmatic reactions but certainly does not present a problem to the vast majority of asthmatics. BHA and BHT have not been shown to produce asthmatic problems.

Benzalkonium chloride frequently can cause bronchoconstriction when inhaled by asthmatic subjects but the concentration now used is so low that only individuals with immediate hypersensitivity will react; fortunately, such patients are rare. Recently, spearmint flavouring has been shown to trigger asthma in two cases.

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12 Migration of Compounds from Food Contact Materials and Articles

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Introduction

Food comes into contact with a variety of materials and articles during the production process, packaging and storage, and in its final preparation as a meal in the consumer's kitchen. Practical examples of contact materials used in the food industry are metal tanks with or without epoxy coatings, and tubes of rubber or plastic. Typical materials used in large volumes for retail packaging are glass, paper, lacquered metal and plastics. In the consumer's kitchen, a broad selection of household equipment made from plastics, metal coated to various degrees, rubber and lacquered wood is used.

It is clear that extensive use of efficient food packaging materials is indispensable, especially in today's western lifestyle. During the last few decades there has been an increasing demand for retail packagings containing small portions of food which can be kept for long periods. The basic quality of a packaging material is its efficiency in containing the food and in being a barrier against external microbiological and chemical contaminants. Further, the packaging has to market the product, be convenient to use and provide essential information of nutritional value, food additives and the price of the product.

In the case of ready-cooked foods, we expect that a plastic packaging material with all the qualities mentioned above, but with a weight of only a few grams, can be used in hot fill packing of a portion of food under aseptic conditions and maintain an efficient barrier for 6 months in the freezer at –18°C and further during a short heating period to about 100°C in the microwave oven or in boiling water. The packaging industry has developed quite complex materials for such purposes, although these qualities are not visible to the consumer. In many instances, they consist of multilayered structures built up from several types of polymers, adhesives, lacquers and printing inks, as well as a mixture of additives necessary to stabilize the plastic during storage and in the production process.

Consumers are dependent on the safety of these materials and it is no wonder if they become a little nervous when newspaper headlines connect a human health risk to the amount of a certain invisible chemical substance migrating from food contact materials. In the past, there have been several examples of materials showing too high a level of migration of some compound. However, in many instances, such 'food packaging scandals' reflect that the toxic properties of the compound have not been

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related to the actual human consumption. A proper risk assessment requires knowledge of both toxicity and intake. Because of the widespread use of these materials, everybody can agree that it is essential to limit the migration of chemical substances to the food to a safe level.

During the 1980s, the European Commission introduced a series of technical directives on how to control migration, and in 1989 fundamental requirements concerning the inertness and stability of the finished food contact materials were laid down in the so-called framework directive. A detailed regulation of food contact plastics can be expected to be completed in the early 2000s and can be foreseen to cover more than 1000 allowed substances, which have all been through a toxicological evaluation. However, there still remains work for the regulators for the next decades. Important food contact materials such as paper and cardboard, surface coatings, printing inks, rubber, cork and others still need to be regulated in detail.

The food packaging industry traditionally has been quite reluctant to provide information about the composition of their products, and only for the last decades has it been fully recognized by the main producers that the performance of their product constitutes an integrated part of the final safety level of the food product used for human consumption. For the analytical chemist, control of migration from food contact materials is therefore a challenging area since it is often not known what substances to look for and in which concentrations.

Nature of Materials and Compounds

Food contact materials are produced from many different types of materials, ranging from mixtures of anthropogenic substances based on mineral oil to slightly modified natural materials. Some of the more important types of materials are listed in Table 12.1.

It is obvious from Table 12.1 that the bulk of materials used in the production of food contact materials are anthropogenic or natural organic macromolecular substances as well as common inorganic materials. However, such substances constitute only the backbone of the material, which must be modified further depending on the purpose of its practical use. Taking plastics as a first example, the final composition of a packaging material can be made up potentially from thousands of individual starting materials and additives. Beside the monomers themselves, other groups of functional compounds are necessary either in the production of the polymer, in the conversion of the plastic material or for the final performance of the material. Important groups of such compounds are listed in Table 12.2.

When discussing a potential risk of migration of plastic constituents to the food, the compounds of interest are mainly the additives or their breakdown products, which

Table 12.1. Important types of materials used in food contact materials.

Material	Common starting materials	Typical area of use
Plastics	Natural or, more commonly, synthetic monomers converted to polymers	Almost all types of food contact materials
Paper and cardboard	Pulp obtained from plant fibres or recycled paper and cardboard	Bags, cartons, grease-resistant paper, kitchen rolls
Metals	Steel, aluminium, tin	Cans, household utensils, tubes, tanks
Glass	Silica from sand or quartz and carbonates of alkali metals	Bottles, glass containers
Rubbers	Cross-linked natural rubber and polymers based on synthetic monomers	Stoppers, tubes, teats
Lacquers and coatings	A diverse group including waxes, polymers, additives, silicones and others	Surface treatment of many food contact materials

Table 12.2. Chemicals used in different stages in the production of plastic packaging.

Stage of production	Function	Type of compounds	Examples of specific chemicals used
Polymerization of the monomers	Control of the polymerization process	Initiators/catalysts	Peroxides, alkyl-lithium
		Inhibitors/retardants	Substituted phenols
Converting the polymers to a food contact material	Processing of additives	Lubricants	Sterically hindered phenols, aromatic amines
		Heat stabilizers	Calcium–zinc–carboxylic acid complex
	Expanding the material	Foaming agents	Pentane, carbon dioxide
	Plasticizing	Plasticizers	Dialkyl esters of phthalic, citric and adipic acids
			Epoxidized soybean oil
	Laminating	Adhesives	Isocyanates
	Material performance	Anti-dew treatment	Glycerol stearate
		Antistatic agents	Ethoxylated fatty amines
		Colourants/fillers	Titanium dioxide
Protection of polymers from deterioration	Capture of UV photons	Photostabilizers	Alkyl-substituted *o*-hydroxybenzophenones
	Capture of free radicals	Antioxidants	Sterically hindered phenols

are of sufficiently low molecular weight to move by diffusion in the polymer network. For the widely used polyolefins, polyethylene and polypropylene, the additives constitute only a few per cent of the total weight of the plastic. This is in strong contrast to a material such as plasticized polyvinylchloride (PVC) where up to 50% of the final material can be additives, mainly plasticizers.

When chemicals of reasonable purity are used in production, the full composition of an anthropogenic material such as a plastic is well known. In many cases, the toxicology of the pure compounds and often even of their foreseeable reaction/breakdown products has been evaluated by international expert panels. Together, the responsible producer of the polymer, the converter and the end user of such a plastic material in principle have all the necessary information to ensure that the material is safe in its end use.

The situation is somewhat different when we look at another group of food contact materials such as paper and cardboard. The materials are made from renewable resources and some people might consider them safer than plastics because of their 'natural' origin. In general, however, the amount of chemicals used as processing aids during their production and as additives in the final product is significant.

The primary raw material used in the production is plant fibres of natural origin from wood. Compared with, for example polyethylene, the fibres do not have a very well defined polymer backbone since the composition can differ between different types of wood. The main components of the fibres are cellulose, a linear polymer built up from glucose units (~50%), hemicellulose, which is the polymer of a mixture of polysaccharides (~10%), and lignin, a branched alkyl aromatic polymer, constituting the rest.

The first step in the production of paper and cardboard is the pulping process where the fibres are obtained from chips of wood and separated. Depending on the required quality of the paper, the fraction containing lignins and hemicelluloses can be removed partly or totally. The classical methods to obtain a pulp are either by mechanical treatment of the chips of wood or by cooking combined with a chemical treatment (the sulphite and the sulphate methods). In the mechanical method, a pulp with many broken fibres is obtained, but in a process with a high yield. In the chemical

methods, long fibres of the cellulose fraction are obtained, but about 50% of the raw material, lignin and hemicellulose, is excluded. However, in general, a modern paper mill uses a combination of mechanical and chemical treatment to obtain higher yields and a reasonable strength of the final product.

For many packaging applications, it is required that the colour of the paper and cardboard is white, and for that reason the pulp is bleached. The aromatic chromophores of the lignin are responsible for the deviations from white since the natural colour of cellulose and hemicellulose is indeed white. The preferred method today is using peroxides, which, in contrast to the more classical methods involving, for example, chlorine bleaching, does not lead to significant loss in the yield. In some final applications, a certain proportion of recycled fibres are used, alone or in a mixture with virgin fibres. This requires repulping of the used paper and a series of cleaning steps to remove as much as possible of the fraction of fibres which become too small during the repeated recycling process, as well as to remove additives, printing inks and other potential contaminants.

After the pulping and bleaching process, the fibres must be formed for their final use as a food packaging material. This includes the addition of several types of compounds as fillers, colourants, pigments, sizing agents and adhesives, which are all used to improve the functional and visual properties of the final product. Further, in paper and cardboard production, different chemicals are used as processing aids in order to avoid the formation of foam and the growth of microorganisms, or as dispersion agents used to ensure a good distribution of added resins, etc. Finally, a barrier layer of wax or plastics can be applied on the surface.

More complex food contact materials are being developed nowadays for which it can be quite difficult for the controlling authorities to foresee their composition. A current trend is towards development of 'active and intelligent food packaging materials', i.e. packaging materials which – beside protecting the food 'as usual' – can monitor, control or even react to phenomena taking place inside the packaging. The different types of active packaging have been categorized into four groups with regard to their mode of functioning. One group of packaging materials includes ingredients, 'scavengers', which are added for the purpose of absorbing, removing and eliminating substances such as oxygen, ethylene, moisture or taint from the interior of a food packaging with the intention of extending the shelf-life of the foodstuff. Activated charcoal is an example of such a compound, which has been used to remove ethylene in fruit packagings. A second group of packaging contains or produces substances, 'emitters', which are meant to migrate into the food itself or into the packaging headspace in order to produce an effect in the food itself. Sulphite-containing sachets emitting sulphur dioxide in packagings for fresh grapes is a typical example of an emitter. The third group of active packagings include devices, 'indicators', which are able to give information about the food product itself or the storage conditions of the packaging. For perishable products packed in a modified atmosphere free of oxygen, leak indicators containing an oxygen-sensitive redox dye (such as methylene blue) formulated as a tablet or label can be a useful tool to indicate possible spoilage of the foodstuff when a leak occurs. A fourth group includes other categories of active packaging, printed electronic circuits, susceptor packaging for popcorn and pizza being examples. In all probability, new types of active packaging will be seen in the near future (Fabech *et al*., 2000).

The examples above are only used to exemplify the variety of chemicals used in ordinary food contact materials, some of which could potentially migrate to the food and potentially be harmful to humans. Compounds of low and high molecular weight and reactive and non-reactive species are among them; foreseeable as well as not-foreseeable migrants are undoubtedly among them. At present, to some extent, the consumer has to rely on a responsible industry, which takes care that the products they sell are safe in use. The next step is to have suitable regulations to define the desired safety standard and suitable regulations against which to hold the industry standards.

Legislation

The EU legislation in the field of 'materials and articles intended to come into contact with foodstuffs' is expressed in general terms in the Framework Directive 89/109/EEC and is more detailed in specific directives (EEC, 1989). National legislation exists in several countries, often in quite general terms, the legislation in Germany (BgVV, 2002), The Netherlands (Warenwet) (SDU, 2002) and the USA (Code of Federal Regulation) (FDA, 2002) being exceptions containing details that are useful to know when it is necessary to assess materials not covered by specific EU directives. However, in Europe, the joint and developing EU legislation on food contact materials and articles together with resolutions and guidelines from the Council of Europe (COE, 2002) is at present the frame of reference, also taking into consideration that some harmonization with the US legislation takes place. In this context, only the EU legislation will be discussed further.

The Framework Directive

The Framework Directive applies to materials and articles which, in their finished state, are intended to be brought into contact with foodstuffs intended for human consumption. The basic principles of the EU Treaty require the Member States to ensure not only free movement of the goods within the internal market, but also a high level of protection of public health. To fulfil the second aim, article 2 of the directive 89/109/EEC sets the following standard for such materials:

> Materials and articles must be manufactured in compliance with good manufacturing practice so that, under their normal and foreseeable conditions of use, they do not transfer their constituents to foodstuffs in quantities, which could:
> - endanger human health,
> - bring about an unacceptable change in the composition of the foodstuffs or
> - deterioration in the organoleptic characteristics thereof.

For food contact materials, which are not already in contact with food when they are sold, the directive specifies labelling requirements. A 'glass and fork' symbol, introduced by directive 80/590/EEC, can be used to indicate that a material is suitable for such use (EEC, 1980).

It is of paramount importance to note that the responsibility for ensuring compliance with legislation lies with the manufacturer, importer and retailer since no system of governmental approval of food contact materials exists.

These general rules apply to all types of food contact materials with a few exceptions: 'antiques, fixed water supply equipment and covering or coating substances which form a part of the foodstuff and may be consumed'. In the Framework Directive, it is decided further that specific directives should cover all kinds of food contact materials such as plastics, cellulose regenerates, paper and cardboard, rubbers, silicones, etc.

It is a difficult task to produce detailed legislation acceptable for an innovative industry as well as lining up precise restrictions, which enable the controlling authorities to control the legislative measures ensuring consumer safety according to the above standard. For that reason, it has been a long process to develop and agree upon, first, the principles in the framework directive and, secondly, the principles for the regulation of specific types of food contact materials.

So far, the specific legislation on food contact materials is based on a principle of positive lists of compounds which can be used in the production of such materials and which have been evaluated individually by the toxicologists of the EU Scientific Committee for Food (SCF). At present, due to the heavy workload connected with these evaluations, only one single material based on organic polymers – cellulose regenerates (cellophane) – can be considered fully regulated. The directives on regenerated cellulose film (93/10/EEC and 93/111/EC) include such positive lists of individual compounds as well as some compositional limits in the material. Moreover, some specific limits for constituents which may be transferred into the food,

specific migration limits, are given in these directives.

The directives on plastic materials and articles

The completion of specific directives regulating plastics has been on the agenda for a long time, and it seems likely that plastics will be fully regulated in the year 2004.

The plastics directive

The plastics directive (2002/72/EC) was first adopted in 1990, and a series of amendments has followed, mainly adding compounds to the positive lists of the annexes, when they have been toxicologically assessed and specific migration restrictions are laid down (EC, 2002). The new codified directive sets a limit for the maximum amount of plastic constituents allowed to migrate to the foodstuff – the so-called overall migration limit. The limit can be expressed either as 60 mg kg^{-1} foodstuffs or as 10 mg dm^{-2} of plastic surface area, and can be considered as a general hygienic limit independent of the toxicity of the compounds.

The eighth amendment is expected to be adopted in 2004 and, by then, a full positive list of all main compounds which can be used legally in the production of food contact plastics will exist. To many of the monomers and starting substances on the list, a specific migration limit (SML) or a maximum residual quantity in the material (Qm) has been prescribed. SML is expressed in mg kg^{-1} of food or in mg dm^{-2} of surface area, whereas Qm is expressed in mg or µg kg^{-1} of plastic. QmA restrictions were introduced recently (1999/91/EEC) and they are expressed as mg or µg 6 dm^{-2} of surface area.

Technical directives about migration testing

Fundamental agreements about how to test the materials with respect to exposure conditions were laid down already in directive 82/711/EEC with further amendments in directives 93/8/EEC and 97/48/EC (EEC, 1982). The most important message here is that the quantity of compounds migrating from a food contact material to a foodstuff is dependent on the duration and the temperature applied in the period where contact occurs between the foodstuff and the plastic. Table 12.3 shows how the directives translate a situation in practical life into conventionally agreed test conditions.

When selecting the test conditions from Table 12.3, one should consider the worst foreseeable conditions of use for the material in practical applications.

In 1985, it was agreed further that, instead of measuring the migration to the actual foodstuffs, it could be a more practical and standardizable approach to use food simulants (EEC, 1985). The Member States agreed upon the four different food simulants shown in Table 12.4.

Directive 85/572/EEC also contains a list of all types of foodstuffs and the conventionally agreed food simulant(s) assigned to each type. Further, except for pure fats and oils, a reduction factor of from 2 to 5 is assigned to each type of fatty foodstuff. The result of the migration test must be divided by this reduction factor to compensate for the high

Table 12.3. Time and temperature conditions for migration testing (from 82/711/ECC with amendments).

Conditions of contact in actual use	Test condition
Contact time (t)	Test time
≤ 0.5 h	0.5 h
0.5 h < t ≤ 1 h	1 h
1 h < t ≤ 2 h	2 h
2 h < t ≤ 24 h	24 h
> 24	10 days
Contact temperature (T)	Test temperature
< 5°C	5°C
5°C < T ≤ 20°C	20°C
20°C < T ≤ 40°C	40°C
40°C < T ≤ 70°C	70°C
70°C < T ≤ 100°C	100°C or reflux temperature
100°C < T ≤ 121°C	121°C
121°C < T ≤ 130°C	130°C
130°C < T ≤ 150°C	150°C
150°C < T ≤ 175°C	175°C

Table 12.4. Food simulants (from directive 85/572/EEC).

Food simulant	Area of use
Distilled water	Aqueous foodstuffs
3% Acetic acid in water	Aqueous and acetic foodstuffs
10% (15%) Ethanol in water	Aqueous and ethanolic foodstuffs
Olive oil	Fatty foodstuffs
When test with oil is technically inapplicable, use substitute test with isooctane, 95% ethanol and modified polyphenylene oxide	(Reduction factors applicable)

extraction potential of pure olive oil compared with that of most other fatty foodstuffs.

The materials should be tested according to the worst conditions, which can be foreseen in practical use. When a producer of a food contact material sells a product, it has to be labelled with possible restrictions in its use with respect to contact time, contact temperature and types of foodstuff. When no restrictions are given, the product must be able to withstand a 4 h migration test with the food simulants 3% acetic acid and 10% ethanol at 100°C and a 2 h migration test with olive oil at 175°C. However, for some products, it is evident, even without labelling, that they are intended for use at ambient temperature. In such cases, a migration test at 40°C for 10 days with the food simulants is a suitable standard test.

For practical reasons, it is impossible to measure the sum of all migrating species, the overall migration, except in a food simulant. However, for specific compounds, the actual concentration in a foodstuff measured during realistic circumstances will always overrule a measurement in a food simulant.

Analytical methods

The first directives in this area (78/142/EEC, 80/766/EEC and 81/432/EEC) set a Qm restriction of vinyl chloride monomer (VCM) at 1 mg kg^{-1} in the plastic material and an SML in food of 10 µg kg^{-1}. They further specify in detail the laboratory methods that have to be used for this purpose. The development and validation of the analytical methods were organized by the EU Commission, giving rise to a heavy workload. Later, it was recognized that laboratory methods quickly become obsolete and outdated, and for that reason the EU Commission now cooperates with and sustains the European Standardization Organization (CEN) in developing analytical methods in support of the directives. Most of the work takes place in Technical Committee 194, Scientific Committee 1, called 'General chemical methods of tests for materials intended to come into contact with food'.

The standard EN(V) 1186 contains all methods for the determination of overall migration, and some methods for measurement of specific migration can be found in EN(V) 13130 (CEN, 2002).

Toxicological Evaluations are the Basis for the EU Legislation

The compounds in the positive lists in the plastics directive are often connected to a Qm or SML restriction. These restrictions are based on the systematic toxicological evaluations made for all compounds and performed by the EU SCF. In this section, a short description of the general requirements for toxicological studies to be supplied by a petitioner will be given. Also, it will be summarized how the toxicologist uses the results of these studies for evaluations which at a later stage may be used by the legislators to lay down a migration limit or a compositional limit.

Toxicological data required

A precondition for even considering a new compound to be included on the positive list is that it is well characterized with respect to its general physical and chemical properties and that migration data are presented for the compound itself and its eventual transformation or reaction products. The core set of toxicological tests that have to be carried out is shown in Table 12.5. As a general principle,

Table 12.5. General requirements for toxicological studies.

	Migration level observed (in mg kg^{-1} food or food simulant)		
Type of test	< 0.05	0.05–5	> 5
Mutagenicity studies: gene mutations in bacteria, chromosomal aberrations in mammalian cells, gene mutations in mammalian cells	Always required		
Absence of potential for bioaccumulation		Always required	
90-day oral study			
Studies on absorbtion, distribution, metabolism and excretion	Not required but SML restriction < 0.05 mg kg^{-1} or equivalent will be laid down	Under certain circumstances, not all tests may be required	Always required. If any test is omitted, it must be justified by providing appropriate reasons
Data on reproduction			
Data on teratogenicity			
Data on long-term toxicity/carcinogenicity			

the greater the extent of migration into food, the more toxicological information will be required (EU Commission, 2002a).

If the studies mentioned in Table 12.5 or prior knowledge indicates that other relevant biological effects may occur, additional studies may be required.

Evaluations by the EU Scientific Committee for Food

Based on the toxicological data presented, the SCF will evaluate a given compound. When working on a compound for which an acceptable daily intake (ADI), a tolerable daily intake (TDI) or equivalent has already been established by other relevant authorities, the job finishes there. However, on most occasions, the purpose will be to establish a TDI value. The first step in this process is to identify the critical effect of the compound, in principle on the human organism but, in practice, on rodents. The next step is to find the highest concentration of the compound which does not give rise to any negative impact on the most sensitive part of the organism – the no-effect level expressed in amount of compound per kg body weight per day. From here, the toxicologist normally will use a safety factor of 10 to account for the differences between humans and rodents multiplied by another factor of 10 to account for the differences between humans. Although deviations from this procedure can occur, an overall safety factor of 100 will be used most often to obtain a TDI value.

Some of the compounds used in the production of polymers are reactive species that can have a negative impact on human health, some of them even being carcinogenic. For such compounds, a TDI cannot be established, and in general they are allocated restrictions such as not being detectable in the polymer or in food/food simulant. Other reasons not to establish a TDI value for some compounds can be that they are self-limiting because of their organoleptic properties or because the migration limit is set very low (< 0.05 mg kg^{-1}) and the compound is used only in small quantities.

A summary of the evaluations given by the SCF can be found in the so-called 'Synoptic Document', which is updated regularly and can be reached by the Internet (EU Commission, 2002b).

The EU Commission lays down the restrictions

For seriously harmful compounds, the EU Commission will lay down the restriction so

that it is as low as possible in the food contact material or as low as possible in the food. A consideration, though, is to ensure that this detection limit is enforceable, i.e. it can be measured with a sufficient certainty in the laboratories of the Member States.

For less harmful compounds, the Commission in most cases uses a conventional procedure when transforming the SCF opinion to Qm or SML values. Some main conventions are the following:

- The standard reference person has a body weight of 60 kg.
- The standard reference person eats 1 kg of packaged food every day.
- The 1 kg of food is contained in a cube.
- The surface area of the cube is $6 \times 1\ \text{dm} \times 1\ \text{dm} = 6\ \text{dm}^2$.

These conventions are certainly not very precise, but are quite practical for calculation purposes. Not many adult EU citizens have a body weight of 60 kg, and the weight of most children is less. Also, people do not only eat around 1 kg of food per day, they also drink several litres of liquid every day – some from plastic bottles. However, the 60 kg person eating 1 kg of food per day is closely related to the overall migration limit of 60 mg kg^{-1} food simulant. When a given compound has a TDI value of 1 mg kg^{-1} body weight day^{-1} or above, no specific migration limit is needed since the compound will be regulated sufficiently by the overall migration limit. In consequence, a TDI value of 0.5 mg kg^{-1} body weight day^{-1} will result in a specific migration limit of 30 mg kg^{-1} food.

The convention of 1 kg of food being packed in 6 dm^2 of plastic is also far from reality since it is more likely to be more than double that. However, the fixed surface to volume ratio allows for an easy transformation of SML values in the foodstuff to a migration limit from the surface of a packaging material, in this case by division by a factor of 6. In general, the analytical chemist in migration testing uses a sample size of 1 or 2 dm^2.

It can always be discussed as to which safety factors have to be used in risk management. Above, some arguments are given in one direction. An argument in the opposite direction can also be mentioned: most probably several different types of foodstuffs will be packed in several different types of packaging materials and the same migrating species probably will not be present in all materials.

Case Studies where Migration was Found to be (Too) High

Considering the multifarious areas of use and the different conditions of use with respect to contact time, temperature and character of the foodstuffs, surprisingly few food contact materials and articles give rise to serious problems. However, from time to time, new problems appear, either because nobody had even thought of a certain food contact material application as a potential problem before or because new knowledge about individual compounds tells us that we have to be especially aware that they do not migrate to the food. Below, a few examples from history will be treated in a little more detail, but many other cases can be found in the scientific literature.

Lead – an ancient useful but toxic metal

Migration of toxic compounds from food contact materials has been well known from far back in history. Migration of this heavy metal from installations made of lead to the water in the aqueducts used to supply ancient Rome with drinking water has been blamed for the fall of the Roman Empire (Waldron and Stöfen, 1974). The reason for these accusations is that we now know that excessive intake of lead, especially when it concerns infants, among other negative effects, can lead to mental retardation. Nevertheless, new cases of excessive and potentially harmful migration of lead to foodstuffs have continued to emerge during the last decades, for example, from:

- lead in the solder used for soldered cans leached to the foodstuff inside (Jorhem *et al.*, 1995);

- stoppers made from lead being used for wine bottles liberated lead to the wine (Smart *et al*., 1990);
- the bearings used in household blenders gave off lead to the foodstuff (Rasmussen, 1984).

Occasionally, it is still possible to find in the market-place food contact materials made from glass, ceramics or metal which give off lead, especially to more acid types of foodstuffs.

In all examples listed above, it was possible to find alternative solutions by substitution. Today, cans are made without soldering, stoppers for wine bottles are made from plastics, and lead is avoided in alloys and glaze used for food contact purposes. To produce food contact materials free from lead is not a major problem for the responsible manufacturer. Unfortunately, not all manufacturers of ceramics, for example, are sufficiently aware of the regulation in this field or they simply do not act in a responsible way.

Lacquers in cans: another potential source of migration problems

As mentioned above, migration of lead from soldered cans has been a substantial problem. Today, few cans are soldered and almost all types of cans are lined with internal lacquers. The prevalent types of lacquers for this purpose are the bisphenol A-based epoxy resins, which are used mainly to cover the bottom and the cylindrical part of the can, and the organosol lacquers, a dispersion of PVC, which are used especially for the 'easy open' type of lids. The idea of applying lacquers on the inner surface of a can is to protect the packed foodstuff inside the can with an inert material. Both types of lacquers have good product resistance and, whereas the epoxy lacquers are rather brittle, the organosols are a heavier and more suitable flexible support for the stamped lid.

In recent years, some concern has been expressed that migration of the monomer bisphenol A (Fig. 12.1), a compound suspected to exhibit oestrogenic activity, may occur from the epoxy coatings to the food as well as from polycarbonate plastics. However, several investigations have shown that only very limited amounts of bisphenol A migrate compared with the current specific EU migration limit of 3 mg kg^{-1} food (Mountfort *et al*., 1997; Pedersen, 1998; Food Standards Agency, 2001). At present, the epoxy coatings and polycarbonate plastics are therefore considered quite safe in use when produced and applied in agreement with good manufacturing practice.

A series of investigations since 1995 have concentrated on problems with migration of bisphenol A diglycidylether (BADGE) and its reaction products from organosol lacquers. In 1995, BADGE occurred on the EU positive list of monomers and starting substances with a very low SML of 0.02 mg kg^{-1} food (the analytical detection limit). The reason for this low SML was that BADGE seemed to be mutagenic by *in vitro* testing. When BADGE is used as a starting substance in the production of polymers, e.g. epoxy resins (Fig. 12.1), it is not a problem to keep migration below this limit. Therefore, nobody bothered to perform more costly toxicological tests of this compound.

In organosol lacquers, BADGE is not used as a starting substance, but as a heat stabilizer. It is added to the lacquer to stabilize the PVC, which can produce hydrochloric acid when the lacquer is heated for curing purposes and when the food can is sterilized after filling. Free hydrochloric acid in a metal can will give rise to corrosion problems, but BADGE will act as a scavenger of hydrochloric acid and neutralize it. Unfortunately, BADGE, as well as its chlorinated reaction products (Fig. 12.1), moves rather freely by diffusion in the thin layer of cured PVC and migrates easily to the packed foodstuff. If the food is aqueous, BADGE will react with water to produce its hydrolysis products (Fig. 12.1). No toxicological information about the reaction products was available in the mid-1990s.

During 1996–1997, several enforcement laboratories in Europe analysed samples of canned food for BADGE. In these investigations, from 3 to 17% of the samples contained BADGE (without reaction products) in amounts above 1 mg kg^{-1} (van Lierob, 1998). This was certainly not acceptable and, in the following years the migration decreased since

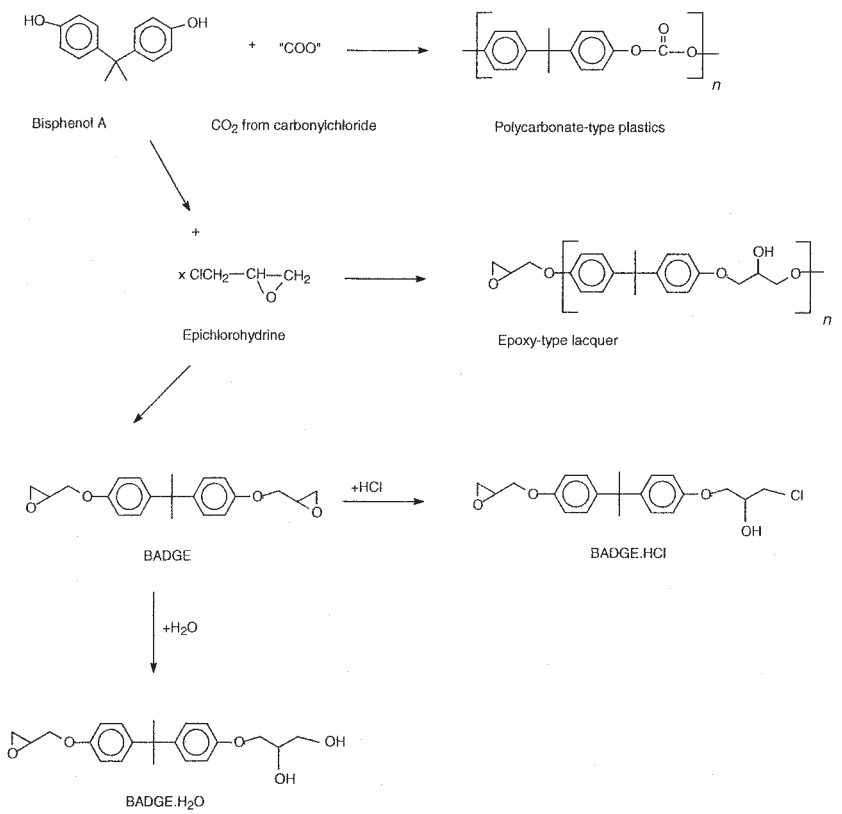

Fig. 12.1. Structural formulae and examples of important reactions of the monomer bisphenol A to: the plastic polymer polycarbonate; a bisphenol A-epoxy resin; and bisphenol A diglycidylether (BADGE). Further, examples of a hydrolysis product (BADGE.H_2O) and a reaction product with HCl (BADGE.HCl) are shown. Also BADGE.2H_2O and BADGE.2HCl (not shown) are, together with BADGE, covered by the EU migration limit.

the industry became aware of the importance of adding a more appropriate amount of BADGE to the organosol lacquer and being more careful in the curing process. Some can manufacturers reacted by changing to products based on bisphenol F diglycidylether (BFDGE), which is chemically less well defined and for which even less toxicological information exists (Grob *et al*., 1999).

One simple solution has been proposed: remove cans with 'easy open' lids from the market and the use of organosol lacquers could be completely avoided in cans for foodstuffs. In fact, most European consumers only eat canned foods a few times a week, and they have not yet forgotten how to use a can opener. However, this proposal was neither popular nor accepted by the food and canning industry, which instead has delivered results of new toxicological studies of BADGE to the EU Commission. *In vivo* studies of BADGE and its main breakdown products did not show any sign of mutagenic activity. Therefore, in 2001, a directive (EC/61/2001) setting a migration limit for the sum of BADGE and its reaction products of 1 mg kg^{-1} food was adopted (EC, 2001b).

Polyvinylchloride plastics – useful but a source of migration problems

Besides being the main ingredient in organosol lacquers, PVC is an important linear halogen-containing polymer, which is used

for many different purposes, including food contact materials. It was among the first plastics to be produced and it is still produced in huge amounts. The polymer can be moulded in almost any form, and at ambient temperature it is hard in its pure form. By adding plasticizers in increasing amounts, the hard material gradually becomes more rubbery and flexible. Soft plasticized PVC occasionally contains as much as 50% plasticizer.

Residual amount of the monomer in PVC

In the middle of the 1970s, it became clear that among workers involved in the production of PVC there was an abnormally high frequency of liver angiosarcoma, a rare form of cancer (Moore, 1975). The immediate reason for this was found to be a high concentration of VCM in the indoor air in factories producing PVC. It was also revealed that significant concentrations of VCM (e.g. 100 mg kg^{-1} plastic) occurred in finished PVC products, such as bottles and foils used for packaging of foodstuffs, and that migration of VCM to PVC-packed foodstuffs, such as oil, butter and liquid foods, could be measured. This was certainly not acceptable, and over a period of a few years the plastics industry succeeded in reducing the residual VCM in the polymer to much lower levels by improved fabrication techniques. The present EU limit is 1 mg VCM kg^{-1} plastics. It has been estimated that the maximum intake per person of VCM from food in the UK was reduced from 1.3 µg to 0.02 µg day^{-1} as a result of the lowered VCM level in PVC (Ministry of Agriculture, Fisheries and Food, 1978).

In the same period, the monomers of other widely used food contact plastics attracted attention because they showed similar adverse effects on human health. It was discovered that frequently used acrylonitrile co-polymers such as acrylonitrile/butadiene/styrene (ABS) plastics contained rather high residual levels of the monomer acrylonitrile. However, in parallel with the example above, industry succeeded in lowering the levels of monomers by modifications of the production methods. As a result, the estimated maximum likely daily intake per person was reduced from 2.5 to 0.2 µg. Moreover, the residual amount of monomers in polystyrene and polyvinylidene chloride plastics was investigated but the level was found to be sufficiently low and safe (Ministry of Agriculture, Fisheries and Food, 1989).

Plasticizers in foods

The most frequently used plasticizers for PVC are the phthalates. Due to the widespread use of plasticized PVC for a vast number of technical purposes and for some food contact materials, the phthalates are produced in huge amounts. The production in Western Europe has been estimated to be close to 1 Mt $year^{-1}$ (European Council for Plasticisers and Intermediates, 2002) and the world production to be several million tons per year (WHO, 1992). Phthalates such as di-(2-ethylhexyl)phthalate (DEHP) can be found in air, water and soil, and they are present in low concentrations in homes, their surroundings and the environment. They show some persistence in the environment, but not to a degree comparable with classical persistent contaminants such as polychlorinated biphenyls (PCBs), an industrial chemical (Chapter 6). A main factor determining the universal presence of phthalates must be considered to be the continuing large and rather constant production.

The migration of phthalates from packaging materials containing these compounds to fatty foodstuffs is a well-known source of food contamination. Today, the intended use of phthalates in food packaging materials is less widespread since these plasticizers or the PVC has been substituted with other compounds or types of plastic. However, processing equipment such as plasticized tubing, surface coatings, gaskets and gloves used in the food industry are other potential sources of food contamination. Examples of more diffuse sources of phthalate contamination with possible implications for foodstuffs are atmospheric deposition on crops, waste water containing phthalates flowing into streams, and vinyl floorings in industry and private homes that may release phthalates during use.

In recent years, the intakes of phthalate plasticizers have attracted some attention because of their possible negative impact on

male reproduction. Milk and milk products can be consumed in rather high quantities by children, which can be considered to be a group particularly susceptible to this possible negative impact. In Table 12.6, a selection of published data in this area are shown.

Several remarkable observations can be drawn from Table 12.6. First of all, it seems that even milk obtained by milking cows by hand contains phthalates in measurable amounts. This is a little surprising since the phthalate esters are expected to hydrolyse to the mono-esters and alcohols on their way through the digestive tract before being absorbed through the intestinal wall and finally excreted by the urine, mainly as glucuronide conjugates.

A second observation is a tendency for the phthalate concentration to be correlated to the fat content of the milk product. A significant example is the DEHP concentration in Norwegian milk products, which all comes from the same investigation by Sharman *et al*. (1994). While skimmed milk (1% fat) contained 0.05 mg DEHP kg^{-1}, the full milk (3% fat) contained 0.11–0.13 mg kg^{-1} and cream (35% fat) contained 1.06–1.67 mg kg^{-1}.

A third observation is to be found by comparing the different DEHP concentrations in hand- and machine-milked Norwegian milks found by Castle *et al*. (1990). A significant DEHP migration from the plasticized PVC milking tubes takes place, and this can be seen by comparing the concentration in the hand-milked product with the concentration in milk from the collection tank. The figures can be compared further with those of Norwegian retail full milk, which has approximately the same percentage of fat. Again a significant increase takes place, probably due to further migration to the milk of plasticizers from food contact materials such as rubber tubes used during transport to the dairy and possibly from gaskets in dairy equipment.

A fourth observation can be based on the Canadian investigation by Page and Lacroix (1995). Whereas in most investigations DEHP is the most dominant plasticizer present in milk and milk products, these authors found rather high concentrations of dibutylphthalate (DBP) and butylbenzylphthalate (BBP) in butter. The origin of these plasticizers was traced to the printing inks and wash coat used in the production of aluminium/paper laminates used as packaging material.

A final remark must be made regarding the column 'Total phthalates', where the reported data are based on an analytical method developed by the Ministry of Agriculture, Fisheries and Food in the UK. In this method, all phthalates are converted to dimethylphthalate and determined as a sum. In this way, the phthalates from complex technical mixtures of dioctyl-, dinonyl- and didodecyl phthalates can be determined even when the concentrations of the individual compounds are below the limit of determination. A full explanation of the reasons for the high results found by this method has not yet been published.

Migration of isocyanates and their hydrolysis products

Flexible plastic laminates are used extensively for the packaging of foodstuffs, especially for products with a long shelf-life or products that need to be conserved in a modified atmosphere – an atmosphere different from the surroundings. Even though these films might seem very thin, they are often manufactured from many layers of various polymers. In some cases, such multiple layer materials can be produced by a co-extrusion process, whereby heating alone joins the layers. In other cases, the materials are more incompatible, and it is necessary to join them with adhesives.

The adhesives used are often made from monomers of aromatic isocyanates and polyols called polyurethane in their polymerized (cured) state. It is of paramount importance that this polymerization process is allowed to proceed to completion. This is done by allowing enough time at a suitable temperature for the polyurethane to form a coherent network, bound to the other layers in the plastic laminate. If residues of isocyanate molecules are still present when the laminate comes into contact with the foodstuff, isocyanates could migrate to the food. If isocyanates come into contact with water,

Table 12.6. Phthalates in milk, cream, butter and cheese: selected literature data.

	Concentration (mg kg^{-1})					
Product and country	Dibutylphthalate (DBP)	Butylbenzylphthalate (BBP)	Di-(2-ethylhexyl)-phthalate (DEHP)	Total phthalates	Notes	References
Raw milk						
Germany	0.029		0.130		Hand milking, 1 sample	Gruber *et al.* (1998)
Germany	0.034		0.120		Machine milking, 1 sample	Gruber *et al.* (1998)
Norway			< 0.005–0.01		Hand milking, 3 samples	Castle *et al.* (1990)
Norway			0.03–0.08		Machine milking (from collection tank), 2 samples	Castle *et al.* (1990)
Retail milks						
Norway, 1% fat			0.05	< 0.04–0.6		Sharman *et al.* (1994)
Canada, 2% fat	n.d.[a]	n.d.	0.04		Total-diet samples	Page and Lacroix (1995)
Norway, 3% fat			0.11–0.13	0.36–1.0		Sharman *et al.* (1994)
UK, whole milk			0.035			Castle *et al.* (1990)
Italy, mixed	0.07		0.21		Mean of positive samples (the half of a total of 50 samples)	Cocchieri (1986)
Canada, 3.3% fat	n.d.	n.d.	0.1		Total-diet samples	Page and Lacroix (1995)
Denmark, 3.6% fat			< 0.05–0.1		Samples from 15 dairies	Petersen (1991)
UK mixed (evaporated including cream)	0.003	0.002	0.3	0.5	Different types/total diet DiBP[b] 0.002; DOP[c] 2.6	Ministry of Agriculture, Fisheries and Food (1996)
Switzerland, mixed	0.02		0.015		Different types/total diet	Kuchen *et al.* (1999)

Cream						
Canada, 17% fat	n.d.	n.d.	1.2			Page and Lacroix (1995)
Switzerland, mixed	n.d.		0.25		Different creams (total diet)	Kuchen *et al.* (1999)
Norway, 35% fat			1.06–1.67	2.9–5.1		Sharman *et al.* (1994)
Butter						
Canada, 80% fat	1.5	0.64	3.4		Four samples in paper/ alufoil (total diet)	Page and Lacroix (1995)
UK			2.5–7.4	4.8–56.6	10 different brands	Sharman *et al.* (1994)
Switzerland, mixed	0.19		1.2		Many types mixed (total diet)	Kuchen *et al.* (1999)
Cheese						
UK			0.24–16.8	2.4–112	25 cheeses, many imported	Sharman *et al.* (1994)
Canada, cheddar-type	n.d.	1.6	2.2		4 samples in plastic (total diet)	Page and Lacroix (1995)
Canada, processed cheese	n.d.	n.d.	1.1		3 samples, 17.7% fat (total diet)	Page and Lacroix (1995)
Italy	0.84		1.08		20 samples, mean value	Cocchieri (1986)
Switzerland, mixed	0.30		1.2		Many types mixed (total diet)	Kuchen *et al.* (1999)

[a]Not detected.
[b]Di-iso-butylphthalate.
[c]Di-octylphthalate.

which constitutes a substantial part of most foodstuffs, primary aromatic amines (PAAs) can be formed (Fig. 12.2).

A selection of isocyanates appears on the positive list of monomers and starting materials of the plastics directive. Since the compounds are very reactive and potentially harmful to health, there is imposed the limitation that the sum of isocyanates remaining in the plastic when it comes into contact with food may not exceed 1 mg kg^{-1} plastic (expressed as units active isocyanate groups, NCO). As the similarly toxic PAAs, formed from the allowed isocyanates, are not normally used in the production of plastic, most of the PAAs do not appear on the positive lists. However, in the sixth amendment to the plastics directive adopted in 2001, it was decided that plastics should not release PAAs in measurable quantities (EC, 2001a). In practice, the permitted limit is set at 20 µg PAA kg^{-1} food simulant, which is the estimated analytical quantification limit, including the analytical tolerance, using an agreed analytical method. Such a method is currently under development by the CEN (2002).

During 2000 and 2001, there were several attempts, mentioned in the European newspapers, to throw suspicion on the flexible packaging industry for selling not fully cured plastic laminates. There certainly could be some economic interest for the industry in selling their products as soon as possible after lamination of the plastic instead of keeping them for the full curing period. However, very few reliable data on residual amounts of isocyanate in plastics and of PAA migration into food simulants have been published till now. Surprisingly, the industry does not seem very interested in publishing data from their internal quality control. However, the European lamination industry has published demanding standards for in-house control, which seem to be followed by responsible manufacturers.

The enforcement laboratories have difficulties in performing an efficient control. It is a troublesome task to obtain samples by surprise in a laminating industry immediately after a roll of laminate can be considered ready for sale. Careful planning of sampling and logistics is required in order to take a few 2 dm^2 test samples from a roll containing hundreds or thousands of square metres, to transport them rapidly to the analytical laboratory under conditions which do not accelerate the curing process and to perform the test analyses immediately after (Trier and Petersen, 2001).

At the time of writing, several European enforcement laboratories are working on these problems and undoubtedly results from such investigations will be published soon.

A Systematic Testing Scheme to Ensure Compliance with Legislation

Generally speaking, the chemist only finds the chemicals he or she is looking for. In many cases, a laboratory implements an analytical method to look for a specific compound in a specific sample matrix determined from the start. Sometimes, luckily, the chemist at the end of the scheduled projects looks at other sample matrices because he or she is curious. One striking example of this is the 'Austrian wine scandal', where it was discovered that diethylene glycol (DEG) was added to wine to 'soften' its taste. Several European control laboratories implemented

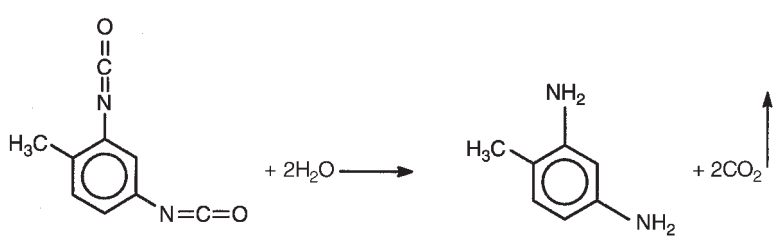

Fig. 12.2. Reaction of 2,4-toluene-di-isocyanate with water to produce the primary aromatic amine 2,4-toluene-diamine.

the analytical method and analysed a large number of wines. When the wine situation was under control, some of the chemists began to analyse foodstuffs packed in regenerated cellulose film containing DEG as a stabilizing agent and found very high amounts of this agent in foods such as fudges, toffees and cakes (Vaz *et al*., 1986).

At present, the single compound strategy is also the concept mainly used in the methods developed by the CEN. However, several attempts to find more suitable systematic approaches to identifying potential migrants from a food contact material have proved successful. An example is the procedure developed by the Food Inspection Service of The Netherlands (van Lierob, 1997), which has also been adopted by other enforcement and industrial laboratories:

1. Identify the polymer(s) using infrared spectroscopy.
2. Extract a small amount of the material with diethyl ether.
3. Add internal standards in known amounts.
4. Inject the extract on a gas chromatograph (GC) with a mass selective detector (MSD).
5. Identify the eluting compounds using dedicated digitalized libraries of mass spectra and GC retention times.
6. Perform a semiquantitative determination of the individual compounds identified.
7. Compare the results with specific migration limits.
8. If necessary, perform a specific migration test using agreed test conditions.

This procedure was developed further by a group of European laboratories taking advantage of supplementary techniques, such as headspace GC for the identification of volatiles, nuclear magnetic resonance, liquid chromatography with UV detection and GC with infrared detection for the identification of potential non-volatile migrants. It was demonstrated further that mutagenicity testing of sample extracts could be a possible tool to ensure that unidentified reaction products and impurities with high toxicity did not occur (Feigenbaum *et al*., 2002). It seems logical to perform mutagenicity testing of such extracts of the final food contact materials since it is a basic requirement that such tests have to be performed for individual compounds on the positive list in the plastics directive. Further, liquid chromatography with mass spectrometric detection has become more common as a routine instrumentation in analytical chemical laboratories and this method will be a suitable supplement to the above-mentioned equipment when identifying the more polar migration species.

The situation is somewhat different when it comes to materials and articles not yet covered by specific directives. As long as we are speaking about materials based on purely synthetic materials made from individually evaluated compounds, procedures such as these mentioned above could possibly be applicable. However, when it comes to materials of natural origin or recycled materials where the starting material is less well defined, it would be helpful to have other tools, at least for research purposes.

As a complementation to the chemical analysis, the idea of using a battery of *in vitro* tests has been used to investigate a series of toxicological effects in extracts of paper of different qualities, containing virgin fibres alone as well as a mixtures of virgin and recycled fibres and further recycled fibres, which has been de-inked or not. The biological testing included a cytotoxicity test using human fibroblasts, a yeast oestrogen assay, the chemical-activated luciferase expression assay (CALUX) Ah receptor assay (sensitive to dioxin-like compounds) and the Ames *Salmonella* assay (mutagenicity test). To some extent, there was a correlation between the contaminant levels found in the chemical analysis and the biological responses. However, especially in extracts of recycled paper, the response in the toxicological tests remains to be explained by identified contaminants (Binderup *et al*., 2002).

Possible Future Instruments in Risk Management

The detailed legislation on food contact plastics has been developed through the last decade and the plastics industry is now faced

with customer demands for compliance testing and the enforcements laboratories are faced with the responsibility to check the in-house control of the industry. The large number of individual restrictions in the form of SMLs or a maximum allowed concentration in the material (Qm and QmA), in combination with actual conditions of use (exposure time and temperature) and the relevant food simulant(s), gives rise to an enormous number of compliance tests.

However, not everything needs to be tested using chemical analysis. Sometimes, analysis can be avoided by using a simple calculation as shown in Fig. 12.3.

Unfortunately, in a significant number of cases, the calculation produces a need for a migration test to be performed. For this reason, the sixth amendment to the plastics directive opens up the possibility for the plastics industry to use the results from application of a scientifically recognized mathematical model as documentation for compliance with legislation. Such models, which are basically built on Fick's second law of diffusion, have been developed, refined and validated, and by now cover homogeneous materials made from the more common polymers. Instead of considering 100% migration in Fig. 12.3, a more realistic situation can be calculated by the model.

A series of other hot topics with possible implications for the food contact materials legislation are to be discussed in the next few years in the European Union.

- The concept of a *threshold of regulation*, or in other words the idea that a compound, known or unknown, present at a concentration below a certain limit in the food or in the food simulant needs neither to be identified nor to be quantified. This concept presents an attractive and a quite relaxing offer to the analytical chemist working with extracts of food contact materials in an enforcement laboratory, but it is not yet accepted by toxicologists.
- The concept of a *food consumption factor* is mainly being marketed by the plastics industry. The idea behind the food consumption factor is that if, for example, nobody eats more than 200 g of fat each day, why should we then consider an

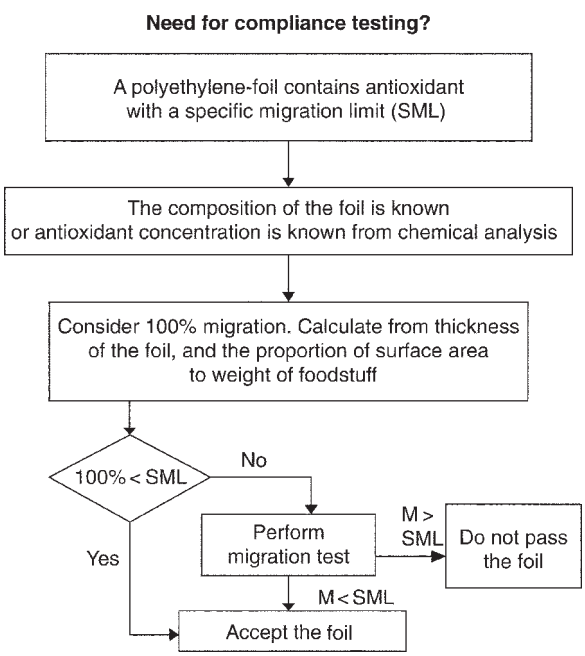

Fig. 12.3. Compliance testing by calculations assuming 100% migration.

SML related to a human intake of 1 kg food every day for migrants only soluble in the fatty food simulant? If this concept is accepted, many compounds having SMLs > 12 mg kg^{-1} food would be covered by the overall migration limit of 60 mg kg^{-1} food. In this way, a substantial number of measurements of specific migration could be saved. An argument against this concept is that, if one fundamental convention is changed, then one has to examine more closely all other conventionally used safety factors. One example is: we drink about 2 l per day, and some of the drinks come in plastic bottles.

- The concept of a *packaging use factor* is driven by the same idea of saving a substantial number of specific migration tests. The fundamental idea is that some positive list compounds are only used for certain types of polymers. When a type of polymer only covers, for example, 10% of the market, then a 'reduction factor' of 10 could be used for these compounds. A logical objection to this concept is that nobody knows exactly how many per cent of the market a given polymer covers. Further, who is going to change the reduction factors when suddenly in one country all milk bottles made of glass are replaced by bottles made of polycarbonate?
- The concept of a *functional barrier* has been discussed for many years and has been defined by the Council of Europe in the following words:

> The functional barrier is any integral layer which under its normal and foreseeable conditions of use reduces all possible material transfers (permeation and migration) from any layer beyond the barrier into food to a toxicologically and organoleptically insignificant and to a technologically unavoidable level.

There are, for the time being, no agreed methods to test a barrier material for functional properties (COE, 2002). An agreement on what performance such a barrier must have is needed when recycled materials, for which no detailed knowledge of their composition exists, are being use in food contact materials.

Conclusions

Food contact materials and articles in thousands of elaborate forms made from a broad selection of different materials are more than ever an integral part of today's lifestyle. The large majority of materials have useful properties and are safe in use. However, many of the starting compounds used in the production of the materials are potentially harmful, and new applications are developed constantly. Therefore, there is a continuing need for the authorities to encourage the industry to act in a responsible manner.

It is of importance to note that the responsibility for ensuring compliance with legislation lies with the manufacturer, importer and retailer, since no system of governmental approval of food contact materials exists. The industry must have reliable in-house controls, and the enforcement authorities should regularly perform a check of the in-house controls as well as supplementary independent analytical control. As is the case for all food products in the food industry, at least the consumers and the enforcement authorities should have easy and full access to all information about all compounds used from production of the polymer to the finished material or article.

Until now, only a few materials are fully covered by detailed regulations, and it must be recommended that frequently used materials such as paper and cardboard, coatings, printing inks, lacquers and rubbers are covered in the not too distant future. Also, an agreement needs to be reached concerning materials made from recycled fibres or plastics. Should such materials of partly unknown composition be allowed in direct contact with foods? If yes, under what conditions?

Acknowledgements

I would like to thank Torsten Berg and Kirsten H. Lund for their help and encouragement.

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13 Veterinary Products: Residues and Resistant Pathogens

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Introduction

As we enter the 21st century, food safety faces a rapidly changing paradigm. As a result of the projected global demand for animal protein due to the increase in populations and expanding international travel and trade, consumers are shifting from a regional commerce to a global environment, and emerging infections and foodborne illness will continue to have a major impact.

Food safety encompasses food science, which involves applying scientific methods to the study of foods. Food science relies on various disciplines such as chemistry, engineering, microbiology, molecular biology, veterinary medicine, epidemiology and public health. This chapter is intended to focus on the scientific aspects of food safety and, in doing so, we have selected several significant veterinary compounds that have broad international importance, from an economic and public health perspective. They include the following: (i) penicillin; (ii) tetracycline; (iii) sulphamethazine; (iv) fluoroquinolones; (v) clenbuterol; and (vi) hormones.

In order to address these veterinary products adequately, they will be discussed from epidemiology, toxicology/pharmacology and risk assessment perspectives. A discussion of public health surveillance as a tool and its application to food safety will be presented. Included in this discussion will be the significance of existing databases to monitor antimicrobial residues and changes in susceptibility to antimicrobial drugs. These tools enhance food safety efforts, by collating data that will, upon analysis, provide information for making future policy decisions.

The emergence and spread of antimicrobial resistance as a result of the use of fluoroquinolones in food animals is examined. Against a backdrop of antimicrobial resistance and a global perspective, drug residues remain a key factor that is often misunderstood and omitted when food safety and public health concerns are considered. Epidemiological data for the drugs penicillin and clenbuterol will be presented to demonstrate clearly existing evidence of acute and chronic health consequences as a result of residues of the parent compound or its metabolites in edible tissues.

Toxic effects may be produced by acute and/or chronic exposure to chemical agents. Acute exposure is defined as a single exposure or multiple exposures occurring within a short time (≤ 24 h). Chronic effects occur when the agent or its metabolites accumulate in the biological system. This occurs when

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absorption exceeds metabolism and/or excretion, when the antimicrobial agent produces irreversible toxic effects or when there is insufficient time for the system to recover from the toxic effect within the exposure frequency interval.

Because of a lack of information regarding the temporal characterization of exposure, it is not always easy to classify all adverse effects reported into acute or chronic categories without some overlap. These factors are important as you read the toxicity and clinical aspects for each drug.

Veterinary compounds are used extensively in food-producing animals for therapeutic effects, prevention of infectious diseases, growth promotion and the promotion of feed efficiency. All veterinary products approved for use in developed countries must meet stringent standards for safety, efficacy and quality. In considering veterinary products for use in food animals, sound scientific evidence must be demonstrated during the drug approval process (see Table 13.1). These agriculture compounds are regulated extensively by the appropriate agency in each country.

It is critical that the prudent use of antimicrobials is emphasized not only to minimize the antimicrobial resistance, but also to ensure the continued efficacy and availability of antimicrobial products for use in food animals and to minimize drug residues and their impact on human health (Table 13.2). These aspects will be discussed in this chapter.

Risk assessment assists in the setting of priorities and tactics for national surveillance activities. It also assists in surveillance design. Surveillance is used to improve risk assessment and risk management decisions. Public health surveillance and risk assessment will be discussed from a food safety perspective. The authors believe that implementation of various on-farm safeguards, such as adequate record keeping, animal identification, herd management practices and promoting the prudent and judicious use of antimicrobials,

Table 13.1. An assessment of hazard and risk management in the drug approval process.

Animal drug application	Risk assessment	Risk management
Laboratory and field studies (observation studies); epidemiological evidence	Hazard identification Does the drug cause an adverse effect, i.e. residue, hypersensitivity?	Development of regulatory options, i.e. tolerances, slaughter withdrawal or milk withheld, time to safe level
Toxicology studies[a] basic toxicology studies metabolism studies toxicology testing in laboratory animals: genetic toxicity, acute toxicity, subchronic toxicity, 2–3 generation reproductive study, carcinogenic studies if genetic toxicity tests are positive residue depletion studies analytical methodology	Dose–response assessment The NOEL is determined from toxicology testing Extrapolation of high to low dose and from animal to man $\text{ADI} = \frac{\text{NOEL}}{\text{safety factor}}$	Evaluate public health, economic, social, political consequences of the regulatory options and harmonization issues
Estimated exposures Characteristics of population Age, sex	Exposure assessment Comparative metabolism study in the toxicology species. A model for human exposure, i.e. number of grams of meat ADI and MRL Risk characterization	Agency decision and action reject approval approve with conditions withdrawal time for drug ban for specific species changes in manufacturing, labelling, etc.

[a]Basic toxicology studies: genetic tests, 90 day feeding studies, two-generation reproductive studies with a teratology component.

NOEL, no observed effect level; ADI, acceptable daily intake; MRL, maximum residue limit.

Table 13.2. Identifiable risk factors for specific drug exposures.

Drug(s)	Adverse health effects
Penicillin	Rash, urticaria and possible anaphylaxis
Tetracycline	Blood dyscrasia
Sulphamethazine	Thyroid hyperplasia
Fluoroquinolones	Antibiotic resistance in foodborne pathogens
β-Agonist/clenbuterol	Ventricular fibrillation
Chloramphenicol	Blood dyscrasia
Aminoglycosides	
Neomycin	Nephrotoxic
Gentamicin	Nephrotoxic

can do much to prolong the lifespan of antimicrobials in animals, while at the same time ensuring that significant human antimicrobial therapies are not compromised or adverse health effects (Table 13.2), attributable to residues in animals, do not occur as a result of using antimicrobials in food animals.

Antimicrobial Agents

The proper clinical use of an antimicrobial agent requires an understanding of the inter-relationships among the pathogen, the host animal and the drug. The following antimicrobial agents, penicillin, tetracycline, sulphamethazine and fluoroquinolones, will be discussed in terms of pharmacokinetics/pharmacodynamics, food safety, toxicity and clinical aspects.

Antimicrobial drugs exhibit selective toxicity in view of their harmful effects to the microorganism without being harmful to the animal or human host. Antimicrobials at the appropriate concentration will demonstrate bacteriostatic or bactericidal effects against the microbial organism.

Antimicrobial mechanisms for the above drugs are as follows:

- Inhibition of the cell wall synthesis: penicillin.
- Inhibition of nucleic acid synthesis: sulphonamides.
- Inhibition of protein synthesis: tetracycline.
- Mutations in the topisomerase genes, decreased permeability of the bacterial cell wall and energy-dependent efflux pumps: fluoroquinolones (Gootz and Bright, 1996).

Penicillin

Pharmacokinetics/pharmacodynamics

The pharmacokinetic properties of a compound include the route of administration, rate of absorption, rate of distribution, volume of distribution, protein-binding capacity of the drug, and the route and rate of elimination, all of which influence the dosing regimen (Davis, 1995; Craig, 1998).

Pharmacodynamics is the study of the biochemical and physiological effects of drugs and their mechanisms of action. Pharmacodynamic properties include the concentration versus time in the tissue and other body fluids, toxicological effects and antimicrobial effect at the site of the infection (this interaction is shown in Fig. 13.1).

A drug is broadly defined as any chemical substance that affects living processes. A therapeutic agent may be defined as a drug which, when given in proper dosages, will produce a beneficial pharmacological or chemotherapeutic (antimicrobial) effect. The process by which a veterinary compound brings about change in some pre-existing physiological function or biochemical process of the living organism is the action of the drug (Vaden *et al*., 1995; Benet *et al*., 1996). The part of the body in which the drug acts and thereby initiates the series of biochemical and physiological changes that are characteristic of the drug is referred to as the *site of action*, while the mechanism by which a drug initiates the series of events considered as an effect is its *mechanism of action*. Hypothetically, the mechanism of action of most drugs involves chemical interaction between the drug and a macromolecular tissue constituent called a receptor with which a drug interacts to produce its characteristic biological effect.

Penicillin interferes with the development of bacterial cell walls by interfering with

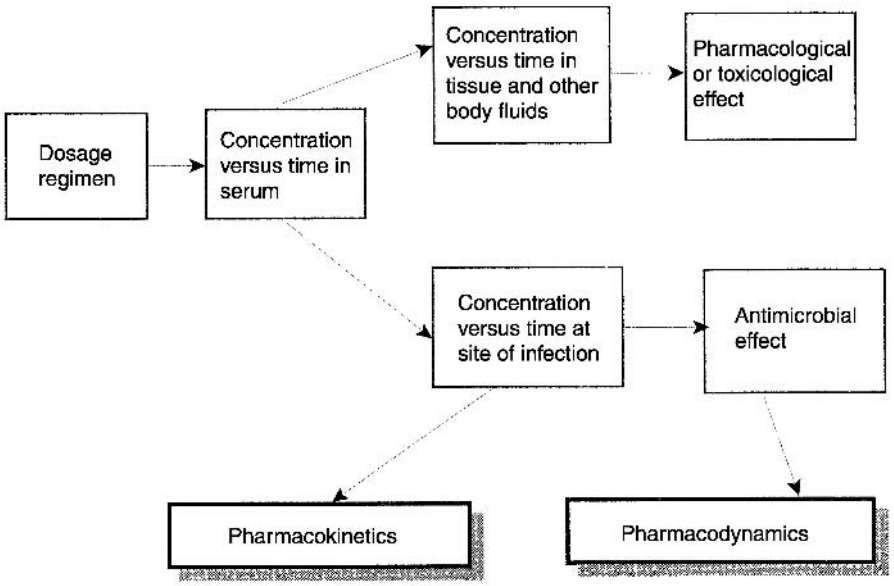

Fig. 13.1. Pharmacokinetic/pharmacodynamic interactions (Craig, 1998).

transpeptidase enzymes, thereby affecting growing cells. The effect of the penicillin is generally characterized as bactericidal to certain organisms during their growth phase.

Distribution in food

The penicillins gained wide agricultural use in the 1940s. Penicillin is approved for use in food-producing animals in most countries as a production agent in cattle, sheep and swine, and as a therapeutic agent for the treatment of bacterial pneumonia in cattle and sheep, erysipelas in swine and strangles in horses. Penicillin is a potent antibacterial compound that is effective against a variety of Gram-positive and Gram-negative organisms with relatively low to no toxicity in animals.

The penicillins are organic acids that are generally available as the sodium or potassium salt of the free acid. In the dry crystalline form, penicillins are stable but lose their activity rapidly when dissolved. Penicillins have short half-lives (0.5–1.2 h) (Sundlof, 1994) in all species of domestic animals. After absorption, the penicillins are widely distributed in the extracellular fluids of the body, but they cross biological membranes poorly because they are ionized and poorly lipid soluble.

Diffusion of penicillin into the tissues and fluids occurs as long as the unbound plasma concentration exceeds that of the tissue and fluids. Tissue residues of penicillin in slaughter animals are considered a public health hazard because of the potential for hypersensitivity reactions in people. Based on available data, the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), with approval by the Codex Alimentarius Commission (Codex), established the maximum residue limits (MRLs) for muscle, liver and kidney in cattle, pigs and chickens at 50 mg kg^{-1} and 4 μg l^{-1} for milk.

Toxicity and clinical aspects

ACUTE An assessment of toxicity has many facets related to the characteristics and conditions of exposure. Generally, scientists are concerned with the frequency of exposure, the route by which exposure occurs and the dose. With acute exposure, the dose is usually delivered in a single event and absorption is rapid. Acute exposure effects are defined as those that occur or develop rapidly after

administration of chemical substances. The ability of penicillin to provoke hypersensitivity reactions in animals and humans is a problem that requires consideration. The prevalence of such reactions is a function of both route of administration and formulation of the drug. In addition, changes in the intestinal flora induced by penicillin administration can contribute to diarrhoea (Kidd, 1994).

Hypersensitivity, damaging immunological reactions, may manifest itself in many ways, from life-threatening anaphylactic reactions to lesser reactions, such as rashes (Table 13.2). Reportedly, there are documented adverse reactions in persons consuming foods contaminated with drug residues. The most frequently cited reactions are allergic reactions to the β-lactam drug penicillin (Riviere, 1992; Sundlof, 1994; Paige *et al*., 1999b). Other drugs including aminoglycosides, sulphonamides and, in a few cases, tetracycline, may cause allergic reactions in sensitive persons. Reportedly, both epidemiological and experimental data indicate that levels of penicillin as low as 5–10 IU are sufficient to produce allergic reactions in previously sensitized individuals (Sundlof, 1994). Adverse human reactions are manifested as severe swelling of the skin, serum sickness, shock and less serious reactions such as skin rashes, asthma and fever. Since these reactions are not dose related, it is highly probable that individuals allergic to the drugs enumerated above, when exposed via the food, could suffer an allergic reaction. Possibly, the reason that few cases are documented is that many cases might be masked by other health conditions, especially in elderly populations, as well as problems with under-ascertainment and under-reporting.

For penicillin, the incidence of death due to anaphylaxis is 0.02 per 1000 courses of therapy (an undetermined number of doses used in treatment of bacterial infections).

CHRONIC A malfunction of the mechanisms for terminating action of the agent is a primary cause of toxicity. Normal doses are thereby present for a longer period of time in the animal and repeated doses are likely to result in accumulation of the drug to toxic levels. The end result can be an accumulation in the kidney and, in some cases, death.

In most countries, no residue of an animal drug is permitted in foods intended for human consumption if the animal drug or its residue is found to induce cancer when ingested by man or animals. Most residues of veterinary compounds or chemicals occur in food at such low concentrations that they rarely pose a chronic or long-term health hazard to consumers. The pre-approval process, with its human food safety evaluation and tolerance-setting procedures, represents a very strong safeguard against these hazards. This process will be discussed later in this chapter. However, chronic or long-term effects such as carcinogenesis as a result of exposure to drug residues in food are particularly difficult to detect and are certainly under-ascertained as well as under-reported (Paige *et al*., 1999b).

Tetracycline

Pharmacokinetics/pharmacodynamics

Tetracycline is produced semi-synthetically from chlortetracycline. Chlortetracycline and oxytetracycline are elaborated by *Streptomyces aureofaciens* and *Streptomyces rimosus*, respectively.

Shortly after its introduction in 1948, tetracycline was determined to have a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria. However, resistance has reduced its usefulness over the last decade. Tetracycline is a bacteriostatic antibiotic that inhibits bacterial protein synthesis, with its site of action being the bacterial ribosome. Tetracycline enters the bacterial cell through passive diffusion, or by an active transport system that pumps the tetracycline through the inner cytoplasmic membrane.

Bacteria resistant to one tetracycline frequently exhibit resistance to the others (chlortetracycline and oxytetracycline). The tetracyclines are bound to plasma proteins to various degrees. The approximate values are tetracycline, about 65%, and oxytetracycline, 20–40% (Prescott and Baggot, 1993; Benet *et al*., 1996).

Oxytetracycline and tetracycline are incompletely absorbed in man and, after a single oral dose, peak plasma concentrations are attained in 2–4 h. They have half-lives in the range of 6–12 h, and they are frequently administered two to four times daily.

The long-acting formulation of oxytetracycline available for intramuscular (i.m.) administration in food animals exhibits their long-acting effect because of both the high dosage used and the prolonged drug persistence at the site of i.m. injection.

All of the tetracyclines are concentrated in the liver and excreted, by the bile, into the intestine, where they are partially reabsorbed. Because of their enterohepatic circulation, they may be present in the blood for a long time after therapy is discontinued. Tetracyclines account for a relatively high frequency of illegal residues found in animal tissue.

The evaluation of chlortetracycline, oxytetracycline and tetracycline by the JECFA led to the following recommendations regarding MRLs. These are expressed as parent drug, singly or in combination: 100 μg kg^{-1} in muscle; 300 μg kg^{-1} in liver; and 600 μg kg^{-1} in kidney of cattle, pigs, sheep and poultry; 100 μg l^{-1} in cattle and sheep milk; and 200 μg kg^{-1} in eggs (Goodman Gilman *et al*., 1991; Sundlof, 1994; Roestel, 1998).

Distribution in food

Systemic availability of tetracyclines can vary widely among the different oral preparations. The absorption of tetracycline is decreased by the presence of food in the stomach. In adult ruminants, it is believed that oral dosing with tetracycline interferes with the fermentation process in the rumen. Because of the enterohepatic circulation, the tetracycline may be present in the body for a long time after cessation of therapy. This has been attributed to high tissue residue levels.

Toxicity and clinical aspects

ACUTE From a pharmacological viewpoint, the tetracyclines are relatively safe. However, adverse events, as a result of the irritant nature of the drug, include nausea, vomiting and diarrhoea after oral administration and tissue damage at the injection site. Fatal anaphylaxis has been reported occasionally in dogs administered intravenous (i.v.) injections. Unless administered slowly, i.v. injection of a tetracycline is likely to cause an animal to collapse (Prescott and Baggot, 1993).

Tetracycline may produce gastrointestinal irritation to varying degrees in some individuals. Although rare, various skin reactions, including rashes and urticaria, may follow the use of tetracycline. Angio-oedema and anaphylaxis are among the more severe allergic responses. Tetracycline undergoes enterohepatic circulation, with much of the compound excreted in bile being reabsorbed from the intestines. This process contributes to the half-life of 6–10 h, which is long for drugs that are eliminated mainly by renal excretion.

CHRONIC Hepatotoxicity may result from accumulation of tetracyclines when they are not eliminated quickly enough by the kidneys or by administration of frequent and/or large doses above recommended therapeutic dosages. Tooth moulting/discoloration occurs when tetracyclines are administrated during pregancy.

The toxicology of tetracycline has failed to show any evidence of carcinogenicity in rats fed 0, 12,500 and 25,000 ppm of tetracycline for 2 years. However, liver and kidney toxicity have been reported. Most toxic and clinical side effects of tetracycline are due to the direct toxicity of the drug or to alterations of microbial flora.

Sulphamethazine

Pharmacokinetics/pharmacodynamics

The sulphonamides represent one of the oldest groups of antimicrobial compounds in veterinary use today. Sulphanilamide, an amide of sulphanilic acid, was the first sulphonamide used clinically. Sulphonamides have been used in clinical veterinary medicine for nearly 50 years, and widespread resistance has developed against most of these compounds. The emergence of

Table 13.3. Classification of sulphonamides based on plasma concentration vs. time profiles.

Short-acting sulphonamides	Intermediate-acting sulphonamides	Long-acting sulphonamides	Enteric sulphonamide[a]
Sulphamethazol	Sulphamimethoxine	Sulphamethazine	Sulphaquinoxaline
Sulphathiazole	Sulphamethoxazole	Sulphadimethoxine	
Trisulphapyrimidine	Sulphamethazine		
	Sulphadiazine		

[a]This class is not absorbed (or minimally absorbed) from the gastrointestinal tract after oral adminstration but acts locally within the lumen of the gastrointestinal tract (Spoo and Riviere, 1995).

resistance has limited their use. In addition to microbial resistance, there is concern regarding possible carcinogenicity of some sulphonamides in laboratory animals (Littlefield *et al*., 1989).

Many derivatives of sulphanilamide with differing pharmacokinetics and antimicrobial spectra are used in veterinary medicine. The most commonly used veterinary compounds include sulphamethazine, sulphadimethoxine, sulphadiazine, sulphanilamide and sulphamethoxazole. Sulphonamides are used primarily to treat microbial infections of the urinary, gastrointestinal and central nervous systems. Sulphonamides are often given in combination with each other to obtain high blood or urine levels. The antimicrobial effect is additive. Sulphonamides act by interfering with the normal production of RNA, protein synthesis and microbial replication mechanisms.

Sulphonamides have a broad spectrum of activity, affecting Gram-positive, Gram-negative and many protozoal organisms. Sulphonamides are bacteriostatic rather than bactericidal. Therefore, the cellular and humoral defence mechanisms of the host are essential for the final eradication of the infection.

Distribution in food

Sulphonamides are distributed throughout all tissues of the body. They are major contributors to antimicrobial residues. Sulphonamides are eliminated from the body partly as the unchanged drug and partly as metabolic products. Clinical application of sulphonamides in swine and cows has led to the presence of sulphamethazine residues in these livestock classes.

Sulphonamides in general are absorbed rapidly from the gastrointestinal tract when administered orally (Prescott *et al*., 2000). Sulphonamides are classified as short, intermediate or long acting according to the plasma concentration and time profile, which is essentially the rapidity with which compounds are absorbed and excreted (see Table 13.3).

Sulphonamides are considered short acting if blood concentration levels remain higher than 50 g ml^{-1} for less than 12 h after a single therapeutic dose, intermediate if these plasma levels are obtained between 12 and 24 h, and long acting if obtained 24 h after dosing. Acetylation occurring in the liver and lungs is the major pathway by which sulphonamides are metabolized in most species. All sulphonamides, with the exception of the enteric compounds, are excreted by the kidneys as parent compounds or as metabolites by way of glomerular filtration. Ruminants metabolize sulphonamides by acetylation pathways, and apparently sulphonamides are the major urinary metabolites in cattle, sheep and swine. There are additional metabolic pathways, beyond the scope of this chapter, but they all display metabolites of reduced therapeutic activity and may be therapeutically inactive.

Toxicity and clinical aspects

ACUTE Sulphonamides may produce a variety of side effects either of an allergic nature or by direct toxicity (Table 13.2). In a small population of humans, sulphonamide therapy has been known to produce idiosyncratic drug reactions (unpredictable rare events dependent upon the individual response to the drug). These reactions may include drug fever and urticaria. These reactions are usually reversible in nature.

Aplastic anaemia and thrombocytopenia have been reported as being induced by drug therapy with trimethoprim–sulphadiazine.

Acute toxic effects, although rare, are most commonly associated with overdosage or too rapid rates of i.v. drug administration. For example, dogs receiving large doses of sulphanilamide (1 g kg^{-1} of body weight (BW)) have exhibited increased salivation, vomiting, diarrhoea, hyperpnoea, excitement, muscular weakness, ataxia and spastic rigidity of the limbs.

In cats given large doses of sulphanilamide, spasticity of the limbs and dyspnoea have been observed. Oral doses of 300–500 mg kg^{-1} of sulphamethoxypyridazine produced transient toxic symptoms in pigs (Booth, 1977).

CHRONIC Sulphonamide drugs are commonly administered in a wide variety of dosage forms including i.v. solutions, oral boluses, sustained-release boluses (which contribute to many of the residue problems) and crystalline powder.

Although relatively safe compounds, disorders of the haematopoietic system have been observed following the use of sulphonamide drugs for the treatment of diseases in animals. Transient agranulocytosis and mild haemolytic anaemia and vesicle haemorrhage have been associated with treatment in calves and mink, respectively.

The Food and Drug Administration (FDA) is concerned about thyroid toxicity (Table 13.2) as the major human safety concern associated with this drug. In chronic feeding studies conducted in 1989–1990 at the National Center for Toxicological Research (NCTR), high doses of sulphamethazine in the diet were associated with significant increases of thyroid follicular cell adenomas in both male and female B6C3F1 mice and of thyroid follicular cell adenomas and adenocarcinomas in male and female F344 rats (Littlefield *et al*., 1989, 1990). These neoplastic lesions in rats appeared only in the 4800 ppm dose group.

These findings resulted in agency concern regarding the continued use of sulphamethazine. The primary focus was whether the Delaney Clause was being compromised. The Delaney Clause under the US Federal Food, Drug, and Cosmetic Act (FFDCA) prohibits the use of veterinary compounds found to induce cancer when ingested or found to induce cancer in man or animals. However, FDA scientists during this time felt that thyroid tumours produced in rodents ingesting high doses of sulphamethazine were attributable to enzyme inhibition resulting in elevated levels of the thyroid-stimulating hormones (TSH) that do not occur at low doses of sulphamethazine.

Fluoroquinolones

Pharmacokinetics/pharmacodynamics

Fluoroquinolones are one of the most valuable antimicrobial drug classes available to treat human infections because of their broad spectrum of activity, pharmacodynamics, safety and ease of administration. This class of drugs is effective against a wide range of human infections, including those that are resistant to other drugs, and has been particularly important in the treatment of foodborne bacterial diseases (Petruccelli *et al*., 1992; Moellering, 1993; Hooper, 2000).

Selective pressure exerted by fluoroquinolone use is the driving force behind the dissemination of resistance determinants to fluoroquinolone compounds. The association between fluoroquinolone use and resistance has been documented in various settings (McGowan, 1983; Anon., 1995; Piddock, 1996).

Fluoroquinolones are totally synthetic antimicrobials, with no known natural analogues. Therefore, resistance to the fluoroquinolones is due only to use of the drug. Resistance to any of the fluoroquinolones confers resistance to all members of the drug class (Acar and Goldstein, 1977; Piddock, 1998). The fluoroquinolone drugs used in human and veterinary medicines are either identical or very similar (Kidd, 1994; Acar and

Goldstein, 1997). The primary targets of fluoroquinolones are DNA topoisomerase II (DNA gyrase) and DNA topoisomerase IV (Hooper and Wolfson, 1993; Ascar and Goldstein, 1997). Topoisomerase is an essential enzyme found in all cells; topoisomerase II and IV catalyse negative supercoiling of DNA, a vital process in cellular metabolism (Drlica and Zhao, 1997).

Fluoroquinolone resistance is mediated by several mechanisms: (i) target mutations in the topoisomerase genes; (ii) decreased permeability of the bacterial cell wall; and (iii) energy-dependent efflux pumps. Resistance due to a decreased drug influx is generally low level resistance. Each of the three fluoroquinolone resistance mechanisms can occur simultaneously within the same cell, leading to very high resistance levels. Frequent exposure to the drug is likely to lead to higher levels of resistance (Piddock, 1999).

Food safety concern

In industrialized countries, the major foodborne pathogens, *Campylobacter* and *Salmonella*, are transferred infrequently from person to person (Tauxe, 1992; Angulo *et al*., 1998). In these countries, epidemiological data have demonstrated that the primary source of antibiotic-resistant foodborne infections in humans is the acquisition of resistant bacteria from animals via food (Harris *et al*., 1986; Tauxe, 1992; Threlfall, *et al*., 1996). Evidence for the transfer of resistant pathogens from animal-derived food to humans comes from several different types of foodborne disease follow-up studies, including laboratory surveillance, molecular subtyping, outbreak investigations and examination of infectious dose and carriage rates (Holmberg *et al*., 1984; Tacket *et al*., 1985; Spika *et al*., 1987; Smith *et al*., 1999, 2000).

When antimicrobial drugs such as the fluoroquinolones are administered to food-producing animals, they promote the emergence of resistance in bacteria that may not be pathogenic to the animal but are pathogenic to humans (Endtz *et al*., 1991; Bates *et al*., 1994; Piddock, 1996; WHO, 1997, 1998; Glynn *et al*., 1998). For example, *Salmonella* and *Campylobacter* are ubiquitous and can exist in the intestinal flora of various food-producing animals without causing disease. However, these bacteria can cause severe, even fatal, foodborne illness in humans. If using an antimicrobial in a food-producing animal causes resistance to occur in such bacteria and the resistant bacteria cause an illness in a consumer, who needs treatment, that treatment may be compromised.

Reports from the scientific and public health communities have identified a relationship between the approval of fluoroquinolones for therapeutic use in food-producing animals and the development of fluoroquinolone resistance in *Campylobacter* in animals and humans. The approval and use of these drugs in food-producing animals in The Netherlands (Endtz *et al*., 1991; Jacobs-Reitsma *et al*., 1994), the UK (Piddock, 1995), Spain (Velazquez *et al*., 1995; Perez-Tallero *et al*., 1997) and the USA (Smith *et al*., 1999; Rossiter *et al*., 2000) temporally preceded an increase in resistance in *Campylobacter* isolates from treated animals and ill humans.

In The Netherlands, *Campylobacter* isolates from humans and poultry were examined for resistance to the human fluoroquinolone ciprofloxacin between the years 1982 and 1989 to determine the influence of licensing of enrofloxacin for veterinary use in 1987 (Endtz *et al*., 1991). Initially, none of the *Campylobacter* isolates from either human or poultry sources was resistant to ciprofloxacin. From 1987 to 1988, resistance levels rose to 8% in humans and 8.4% in poultry, and in 1989 fluoroquinolone resistance among the *Campylobacter* isolates was 11% in humans and 14% in poultry (Endtz *et al*., 1991).

Despite several restrictions placed on the use of poultry fluoroquinolone products in the USA, fluoroquinolone-resistant *Campylobacter* has been isolated repeatedly from domestic retail chicken products sampled. From January to June 1999, public health laboratories in Georgia, Maryland and Minnesota, under the direction of the Centers for Disease Control and Prevention (CDC), tested 180 chickens with 23 distinct brand names that

were purchased from 25 grocery stores. *Campylobacter* were isolated from 80 (44%) of the chickens. Nineteen (24%) of the samples had *Campylobacter* isolates resistant to fluoroquinolones and 25 (32%) were resistant to nalidixic acid, a quinolone antimicrobial drug that serves as a precursor to fluoroquinolone resistance development (Rossiter *et al*., 2000). These retail chicken findings are consistent with those from an earlier, independent study in the USA conducted by the Minnesota Department of Health.

Researchers at the Minnesota Department of Health studied quinolone resistance among Minnesota residents, and evaluated chicken as the source of the resistance. They found that the proportion of quinolone-resistant *Campylobacter jejuni* isolates from humans increased from 1.3% in 1992 to 10.2% in 1998 (Smith *et al*., 1999). The proportion of resistant *C. jejuni* collected from all reported cases of illness increased only slightly from 1992 to 1994. Researchers found that subsequent increases, between 1996 and 1998, were predominantly associated with foreign travel. More importantly, the percentage of resistant infections that were acquired domestically also increased from 0.3 to 4.2% between 1996 and 1998 (Smith *et al*., 1999).

As part of the study, the Minnesota Department of Health, in cooperation with the Minnesota Department of Agriculture, collected 20 different brands of retail chicken products from 18 markets in the Twin Cities metropolitan area in 1997. *Campylobacter* were isolated from 88% (80/91) of the samples; 20% of these were resistant to fluoroquinolones. The products with resistant strains had been processed in five states (Smith *et al*., 1999).

Molecular subtyping revealed a strong association between resistant *C. jejuni* strains from chicken products and *C. jejuni* strains from the domestically acquired human cases of campylobacteriosis. The study used polymerase chain reaction with restriction length polymorphism flagellin gene typing to identify strains of fluoroquinolone-resistant *C. jejuni* among isolates from the domestically acquired human cases and locally available retail chicken products. The investigators attributed the 1996–1998 increase in resistant domestic cases among humans to poultry treated with fluoroquinolones, which were approved in the latter part of 1995 (Smith *et al*., 1999, 2000).

To assist in establishing the magnitude of the human health impact of fluoroquinolone use in animals, the US FDA contracted with a risk analyst to develop a risk model. The risk assessment was intended to estimate the extent of the risk to human health from resistant *Campylobacter* pathogens attributed to the use of fluoroquinolones in chickens in the USA. The risk assessment addressed that portion of the risk that was quantifiable, which is that related to the consumption of chicken. The unquantifiable portion, that portion due to the spread of the pathogen from chicken to other foods through contamination during food preparation or from secondary spread to other animals, was not considered in the risk assessment.

An assumption made in the risk assessment was that the presence of fluoroquinolone-resistant *Campylobacter* on chicken carcasses results from the use of fluoroquinolones in chickens. This does not mean that every chicken carrying resistant *Campylobacter* had to have been treated with a fluoroquinolone. Resistant organisms could have been acquired from a contaminated environment due to fluoroquinolone drug use in a previous flock, through contact with other chickens during transportation to the slaughter plant and ante-mortem processing, or through contamination of other foods in the home by raw chicken meat.

The number of *Campylobacter* culture-confirmed human cases in the US population was used to estimate the total burden of campylobacteriosis, which was 1.7 million cases of campylobacteriosis with a 90% confidence distribution of 1.1–2.7 million cases for 1999 (US Food and Drug Administration, 2000).

The model also estimates the number of fluoroquinolone-resistant *Campylobacter* cases attributable to chickens. This estimate excludes travellers to countries outside the USA, those patients who were prescribed a fluoroquinolone prior to stool culture, and those patients who were unsure of the timing

of their treatment in relation to stool culture. For 1999, the mean number of the domestically acquired fluoroquinolone-resistant *Campylobacter* cases attributable to chickens is approximately 190,000 (US Food and Drug Administration, 2000). For 1999, the estimated mean number of people infected with fluoroquinolone-resistant *Campylobacter* from consuming or handling chicken and who subsequently received a fluoroquinolone as therapy is approximately 11,500 (US Food and Drug Administration, 2000). These people received less effective or ineffective therapy for their infections, resulting in adverse health effects. The adverse health effects also have a negative impact on productivity in terms of lost working days and increased cost of medical care.

Toxicity and clinical aspects

ACUTE Fluoroquinolones are considered to be relatively safe antimicrobial drugs and, when administered at the therapeutic doses, toxic effects are mild and limited. The most common complaints are nausea, vomiting, diarrhoea and other upper gastrointestinal discomfort, headache and dizziness (Prescott *et al*., 2000).

Fluoroquinolone use in dogs has been associated with canine toxic shock syndrome caused by *Streptococcus canis*.

CHRONIC At very high doses (50 mg kg^{-1}), the fluoroquinolones have been associated with arthropathies in young experimental animals. Photosensitivity in humans occasionally has been associated with the use of fluoroquinolones. Renal toxicities ranging from acute renal failure to mild interstitial inflammation of the kidney tubular walls have been reported in humans. Reportedly, most cases of renal toxicity have been associated with the use of fluoroquinolones at doses greater than the therapeutic range.

Most adverse effects with fluoroquinolones are associated with the administration of higher doses. Because of cartilage erosion, fluoroquinolones are not recommended for use in young, growing animals. It is hard to classify these events as acute or chronic, as cartilage damage can occur as quickly as 12–24 h after a single large dose of a quinolone.

Production Drugs

Clenbuterol

Pharmacokinetics/pharmacodynamics

The nervous system is divided into the central nervous system (CNS) and the peripheral nervous system, which is divided further into the somatic and the autonomic nervous systems. The autonomic nervous system is separated into two main divisions, the sympathetic and the parasympathetic. The autonomic nervous system is an important part of the complex machinery by which the body maintains its internal environment constant. These aspects are important and have direct relevance to the clinical use and application of the drug clenbuterol.

Amine compounds that cause physiological responses similar to those evoked by the endogenous adrenergic mediators adrenaline and noradrenaline are called adrenergic drugs. The pharmacological effects of these amines are to mimic the sympathetic nervous system, thereby resulting in activation of adrenergic receptors of effector cells. Most adrenergic drugs affect both α and β receptors (Levine, 1983; Adams, 1995).

One of the most popular illegal drugs used in food-producing animals in the 1990s was the β-agonist compound clenbuterol. The β-agonists are a class of compounds that have profound pharmacological and physiological effects. These compounds evoke specific responses in a variety of tissues by binding with affinity and high specificity to β-adrenergic receptors. The β-adrenergic receptors are classified as either β_1 or β_2, based on their pharmacological response. Most tissue possesses both receptors in varying proportions (Levine, 1983; Adams, 1995). β-Agonists are members of a pharmacological class of drugs that have demonstrated effectiveness as bronchodilators and growth-promoting and repartitioning agents

in many species, including cattle, sheep, pigs, poultry and man.

Distribution in foods

Clenbuterol is a member of a class of drugs known as β_2-adrenergic agonists. It is approved in the UK for therapeutic use in cattle and horses. It is approved in the USA as a bronchodilator for treating chronic respiratory disease (heaves) in horses. It has been used illegally in Europe and the USA by some livestock producers to increase carcass leanness. When used illegally, it is generally used in the feed.

Animal drugs are regulated on the basis of the total residue present in an edible tissue because of the possibility that metabolites might also cause residues.

Studies were conducted to determine the distribution and the concentration of parent clenbuterol in tissues 48 h after dosing. A single oral dose of [^{14}C]clenbuterol HCl (1.59 μCi mg^{-1}, 3 mg kg^{-1} BW) was administered to Holstein calves weighing 74–96 kg. This concentration is greater than that required for therapeutic purposes (0.8 μg kg^{-1} BW) or growth-promoting purposes (Smith and Paulson, 1997).

Residues of clenbuterol remaining in edible tissues of animals are composed of parent clenbuterol, metabolites and perhaps bound residues. Human effects after illegal use of clenbuterol are attributable to parent clenbuterol remaining in edible tissues. However, the role of clenbuterol metabolites remains unknown.

Studies were done to examine the pharmacokinetics of clenbuterol after effective anabolic dosages of 5 μg kg^{-1} BW given twice daily for 3 weeks (Heinrich *et al*., 1991) (note: the anabolic dosage in such studies is generally 5–10 times higher than that required for therapeutic purposes). The analysis was done at 0, 3.5 and 14 days after withdrawal. The data revealed that clenbuterol concentrations in the lung dropped from a mean of 76 to less than 0.8 ng g^{-1} after 14 days, and the liver concentrations decreased from 46 to 0.6 ng g^{-1}. The highest levels were always found in the eye: 118 ng g^{-1}, which dropped to 15 ng g^{-1} after 14 days (Heinrich *et al*., 1991). The peak absorption of clenbuterol is rapid and measured as between 2 and 7 h.

Toxicity and clinical aspects

Clenbuterol is one of a few residue-producing animal drugs that has been shown to cause an immediate health concern in consumers. Residues of β-agonists in animal tissues used for food constitute a potentially serious human health risk. Many harmful effects to humans have been demonstrated for these drugs due to their bronchodilator effects, muscle tremors and tachycardia. Meat from clenbuterol-treated animals has been found to cause illness and even death in humans. The acute toxic effects are clear and predictable from the pharmacological mode of action of β-agonist compounds. It is felt that residues of clenbuterol remaining in the edible tissues of animals are composed of parent clenbuterol, metabolites and perhaps bound residues.

The main indications in human medicine for clenbuterol are for treatment of chronic obstructive airway disease such as asthma and chronic obstructive bronchitis. Like some bronchodilators, the drug expands the tiny air passage in the lungs and lets air flow more freely.

Clenbuterol has been implicated in several outbreaks of foodborne illness in Europe. In 1990, a Spanish epidemiologist reported that 135 people became ill after consuming beef liver containing clenbuterol residues (Martineaz-Knavery, 1990; Pulce *et al*., 1991). Clinical symptoms consisted of muscle tremors, heart palpitations, nervousness, general myalgia, fever, nausea, chills and vomiting. These symptoms are of particular concern because toxicity can appear suddenly following the consumption of clenbuterol residues (Table 13.2).

A second episode of clenbuterol food poisoning was reported in France in 1991 and affected 22 people in eight different families who consumed bovine liver. The patients suffered tachycardia and muscle tremors for 2 or 3 days. The infective dose was calculated as 1–2 μg kg^{-1} day^{-1} (Salleras *et al*., 1995). In 1994, calf liver was again implicated in a case involving 16 people in Italy. The significant

aspect of these cases is that veal liver was the main source of exposure, although a recent response implicated veal muscle (Sporano *et al*., 1998) (see Table 13.4 for a list of clenbuterol cases).

In 1996, 62 persons in Italy were involved in an outbreak and sought help at the emergency room in two hospitals. Tremor, tachycardia, palpitations and nervousness were the predominant symptoms, lasting between 10 and 48 h. The unique aspect of this episode of foodborne poisoning by clenbuterol is that clinical symptoms appeared after consumption of non-organ meat. All of these people purchased beef meat from a common source and reported consuming the meat from 10–30 min and up to 2–3 h before symptoms appeared. It was concluded that therapeutic dosages (0.8 μg of clenbuterol kg^{-1} BW) were ingested by patients that ate 20 g of meat; however, a normal meal is 100 g of meat (five times the therapeutic dose), 4.0 μg of drug kg^{-1} BW; it has been established that cardiovascular signs appear at this level (Salleras *et al*., 1995; Sporano *et al*., 1998; Paige *et al*., 1999b).

ACUTE Clenbuterol has high β_2-agonist activity and relatively less β_1-agonist activity. There are indications that acute heart rate increases but usually for less than 2 min. This brief tachycardia may be mediated reflexly due to transient β_2 vasodilation and hypotension (Adams, 1995).

CHRONIC Chronic use can result in refractoriness due to down-regulation (i.e. reduced numbers) of β receptors and can cause β_1 side effects at high doses.

Hormones

This section discusses the FDA's health risk assessment procedures for residues of hormones and the European Community Directives prohibiting the use of anabolic agents for growth promotion.

There are six hormonal agents in the USA approved for growth promotion purposes. The natural hormones supplied exogenously for growth promotion purposes are 17β-oestradiol, testosterone and progesterone. The approved synthetic hormones are trenbolone acetate, melengestrol acetate (MGA) and zeranol; these compounds mimic the actions of testosterone, progesterone and 17β-oestradiol, respectively. With the exception of MGA, the hormones are approved either alone or in combination as components of an ear implant. The sixth hormone, MGA, is approved only as a feed additive and also functions to suppress oestrus in feed-lot heifers. Meat from animals treated with any one of the six agents for growth promotion purposes cannot be imported into the European Community (EC) (Leighton, 1999).

Table 13.4. Foodborne outbreaks associated with clenbuterol.

Year	Case history
1990	Spanish epidemiologist reported 135 people became ill consuming beef liver containing 0.16–0.30 ppm residues
1991	Report in France of 22 affected people in eight different families that consumed calf liver. Contained 0.375–0.500 μg g^{-1}
1994	Reported illness in 127 people in Spain, calf liver implicated
1995	Clenbuterol (0.5 ppm) was isolated in beef fillet and rump steaks associated with an outbreak involving 16 people in Italy
1996	62 persons in Italy sought help at the emergency room in two hospitals. Consumption of non-organ meat. All cases purchased meat from a common source. Treatment doses = 0.8 mg (in 20 g of meat) 5 × treatment dose = 4.0 mg = cardiovascular signs
1998	Foodborne illness in China, where 9/14 reported illness from consuming lung soup from pig lung tissue. Reportedly pig given clenbuterol for weight gain

Whereas the FDA sets food safety standards for animal drugs within the USA, international food safety standards are established by Codex. In 1987, JECFA conducted a safety assessment of five hormones used for growth promotion purposes in cattle. JECFA functions as a scientific advisory body of the Codex. The purpose of JECFA's safety assessment was to establish a safe level of residues of hormones (an acceptable daily intake, or ADI) in animals treated with these agents. Their recommendations subsequently were adopted by Codex, which is responsible for the execution of the Joint FAO/WHO Food Standards Programme (Leighton, 1999).

Naturally occurring hormones

Oestradiol, progesterone and testosterone are naturally occurring hormones produced throughout the lifetime of every man, woman and child, and are required for the proper physiological functioning and maturation of every mammal. Because these compounds are naturally occurring and identical in man and in food-producing animals, the consumer is exposed throughout his/her lifetime to rather large quantities of these hormones through his/her own daily production and to much lesser quantities from food from unmedicated animals. All products marketed in the USA for livestock are granulated as pellets and all are designed to deliver the hormones at a constant sustained rate when injected subcutaneously under the skin of the animal's ear. Numerous scientific studies have demonstrated that, when these drugs are used in accordance with good husbandry practices, concentrations of the hormones in meat remain within the normal physiological range that has been established for untreated animals of the same age and sex. Because of the slow release of the hormone and because the half-life of these endogenous hormones is extremely short (10 min), no pre-slaughter withdrawal time is necessary to protect public health (Leighton, 1999). Therefore, consumers will not be at risk by eating meat from animals treated with these compounds since the amount of added hormones is negligible compared with the consumer's own daily production rate.

Synthetic hormones

Unlike naturally occurring hormones, there are no daily production rates from the synthetic compounds trenbolone acetate, MGA and zeranol. These compounds are not metabolized as quickly as the naturally occurring steroids. Therefore, the FDA required extensive toxicological testing in animals to determine a safe level in meat for these compounds. Furthermore, the FDA has required that the manufacturers demonstrate that the amount of hormone left in the meat after treatment is below this safe level (Food and Drug Administration, 1966; Code of Federal Regulation, 1999).

As with antimicrobials and antibiotic drugs, the Center for Veterinary Medicine (CVM), in assessing the safety of any product, reviews information on the specific product in question under conditions of use. As previously stated, an assessment of risk is incorporated into the approval process and a post-approval monitoring programme along with risk management tools.

The Use of Epidemiological Methods in Food Safety

Epidemiological concepts and methods can be used easily with other scientific disciplines to study food safety. Diseases, residues and chemical hazards in populations are not randomly distributed. The methods of epidemiology are used to describe how age, time trends, geographical trends, husbandry practices, lack of prudent drug use and other variables affect the distribution of foodborne illness, residues, resistance and other food safety hazards. Epidemiology can be used to investigate many different types of health outcomes where the causes are either unknown or poorly understood. The specific aims and objectives are to describe, explain and predict events, for the purpose of implementing some type of intervention.

Surveillance is the systematic collection of data pertaining to the occurrence of specific events, the analysis and interpretation of these data and the dissemination to those who need

to know (Lwanga, 1978; Friis and Sellers, 1996; Gordis, 2000). Surveillance systems provide the means for collecting, testing and monitoring trends in the prevalence of foodborne pathogens and the antimicrobial resistance profiles of these bacteria. Well-designed surveillance systems for antimicrobial resistance can identify outbreaks of foodborne illness and epidemics of multi-drug-resistant pathogens in animals that have the potential to become foodborne pathogens.

Risk has been termed the probability of injury, disease or death under specific circumstances. Risk may be expressed in quantitative or qualitative terms. In the former, it takes values from 0 to 1 (probability that harm will not occur to probability that it will). In the latter, risk can be described as high, low or trivial.

Hazards are evaluated in terms of acceptable levels of risk. Safety in its common usage means without risk; thus, the safety of chemical/drug residues in food is a condition of exposure under which there is relative certainty that no harm will result in the exposed population. This premise is based on the fact that inherent in the drug approval process are the elements of risk assessment.

In determining the impact of any chemical or drug on human health, two distinct elements have been identified, risk assessment and risk management. These two aspects will be discussed as they pertain to food safety.

Assessment of hazard and risk

In today's environment, the public, media, politicians and our global partners are demanding that our food supplies are free of risk. While scientists have disagreements about the nature and magnitude of food-related risk, there is general agreement on what constitutes food-related risks and their relative importance.

Pre-market testing requirements and tolerance setting by regulatory authorities and strict monitoring of residue levels have helped to ensure that risk from residues in food remains low. Tolerance setting involves utilizing risk assessment and the concepts of hazard identification, dose–response assessment, exposure assessment and risk characterization in the approval process to ensure food safety for those drugs approved for food-producing animals (see Table 13.1).

A tolerance represents the maximal level or concentration in or on animal feed ingredients or animal tissues at the time of slaughter (e.g. the tolerance for penicillin is 0.05 ppm mg^{-1} kg^{-1} (Freidlander *et al*., 1999)). A violative residue is the occurrence of a drug or chemical residue above the tolerance level found in edible tissue, fat, kidney, liver, muscle, meat byproducts or skin of a food-producing animal.

The human food safety evaluation of a new animal drug involves an independent evaluation of its residue chemistry characteristics and its toxicology. Information generated from the toxicology review is used to establish the safe concentrations for total residues in the edible tissues of food-producing species. Information obtained from the residue chemistry review is used to establish the tolerance and set withdrawal times for the new animal drug. The primary objective of these activities is to ensure that potentially harmful residues of the drug will not occur in food derived from treated animals (Freidlander *et al*., 1999) (see Table 13.1).

The initial assessment involves hazard identification, the examination of the structure of the compound for potential biological activity or carcinogenicity based on available information about the veterinary compounds or its chemical class.

The first step in assigning a safe concentration for a residue is to determine the ADI. The ADI is calculated using data generated from the toxicology studies. To calculate the ADI, a no observable effect level (NOEL) is selected from one of the oral toxicity studies. Depending on which toxicity study is used, a safety factor of 100 or 1000 is applied to the calculation.

$$\text{ADI} = \text{NOEL}/\text{safety factor}$$

A safe concentration for total residues in meat is then calculated:

$$\text{Safe concentration} = \frac{\text{ADI } (\mu\text{g kg}^{-1}\text{ day}^{-1}) \times 60\text{ kg}}{\text{g consumed day}^{-1}}$$

In the USA, the safe concentration of residues is based on the assumption that edible muscle and organ tissues comprise 500 g of a 1500 g solid food diet and that organ meats and fat are consumed in lesser quantities than muscle. The 500 g of edible muscle and organ tissue consumption values are given as follows: muscle, 300 g consumed; liver, 100 g consumed; kidney, 50 g consumed; and fat, 50 g consumed. Milk and egg products are regulated as independent commodities as they are not considered as components of the 500 g of muscle and organ tissues (Freidlander *et al*., 1999).

General residue chemistry requirements include a total residue depletion and metabolism study, a comparative metabolism study between the target species and the laboratory toxicological species. If results indicate a comparable metabolism in both species, then the human population would be exposed to the same residues when consuming meats, milk or eggs derived from the treated target species. Any metabolite identified as being of toxicological concern will undergo further toxicological examination. Further chemistry requirements include the development of a validation or determinative method, a confirmatory analytical method for identification, a cold residue depletion study to set the tolerance for the marker residue in the target tissue, and a marker residue depletion study.

The drug sponsors of veterinary compounds are required further to measure the depletion of total residues of toxicological concern from the edible tissues until their concentration is at or below the safe concentration. The tissue or organ that requires the longest time to deplete to the safe concentration is referred to as the target tissue. A marker residue is selected from the target tissue; this is the parent compound or one of the metabolites. The marker residue will exist in a known relationship to the total residue in that tissue (e.g. 60% of total, 85% of total, etc.) (Freidlander *et al*., 1999). The concentration of the marker residue in the target tissue when the total residue has depleted to the safe concentration is called the tolerance.

The withdrawal period is the amount of time it takes for the marker residue to deplete to the tolerance in the target tissue.

Public health surveillance and its role

Database systems are diverse and unique in capturing large amounts of data that are reliable and can be analysed to allow the scientist ultimately to generate information useful in hypothesis testing and in facilitating epidemiological or laboratory research. Data captured in these databases can serve a function in public health surveillance, which involves the collection, analysis and interpretation of data on the frequency of occurrence and distribution of health events in the population.

One of the major steps in surveillance is the timely dissemination of information to others in the public health systems that need to know. Four such databases in the USA and one in Denmark, and their relevance to food safety initiatives, are discussed in this section.

A final link in the surveillance chain is the application of these data from these systems to prevention and control. In a larger sense, surveillance is concerned with the ongoing systematic collection, analysis and interpretation of health data essential to the planning, implementation and evaluation of public health practice.

FoodNet

In the early 1990s, the National Academy of Science Institute of Medicine published a report emphasizing the ongoing threat of emerging infectious diseases. The CDC developed a strategy to respond to this threat. The main feature of this strategy was to establish the Emerging Infections Program (EIP) in seven sites across the USA (California, Connecticut, Georgia, New York, Maryland, Minnesota and Oregon). The goals of the EIP network are to improve national surveillance for new and emerging diseases, conduct applied epidemiological and laboratory research, develop prevention and control measures, and strengthen the national public health infrastructure (Institute of Medicine, 1992).

The CDC / US Department of Agriculture (USDA)/FDA Foodborne Disease Active Surveillance Network (FoodNet) is the foodborne disease component of CDC's EIP. It is a collaborative project of the CDC, FDA and USDA with the aim of collecting more precise information on the incidence of foodborne disease in the USA. FoodNet works with state and local health departments to provide active surveillance for foodborne diseases and related epidemiological studies designed to help public health officials better understand the epidemiology of foodborne diseases in the USA (Foodborne Disease Active Surveillance Network, 1998–1999).

NARMS

There is increasing concern that the use of antibiotics in food animals may lead to development of resistant strains of bacteria that could cause illness in people. The increase in resistance has led to re-examining the role that antimicrobial drugs used in food-producing animals play in the emergence of antimicrobial drug-resistant bacteria.

For public health reasons, there is a need to monitor carefully the development of resistance or changes in susceptibility to antibiotics. In order to accomplish this task, the USA developed a system modelled after the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) to identify when foodborne bacteria that cause disease in humans begin to develop resistance to antimicrobials used in food animals. This programme is called the National Antimicrobial Resistance Monitoring System–Enteric Bacteria (NARMS–EB) (Tollefson *et al*., 1998). The programme alerts public health agencies to changes in susceptibilities of enteric organisms (intestinal) from both animal and human sources to several antimicrobials. The food animal specimens are gathered from healthy farm animals, animal clinical isolates and carcasses of food animals at slaughter. The human origin samples are submitted by 17 state and local Departments of Health and are tested at the National Center for Infectious Diseases, (CDC), in Atlanta, Georgia. NARMS tests non-typhoid *Salmonella*, *Shigella* (human isolates only), *Escherichia coli*, *Campylobacter*, enterococci and *Salmonella typhi* (human isolates only) for susceptibility to a variety of different antibiotics. Data can be compared from previous years to look for evidence of changes in resistance of the organisms to these drugs. Data are available on the FDA Center for Veterinary Medicine home page at www.fda.gov/cvm.

These data will prove useful for public health officials, regulatory agencies, responsible animal producers, drug manufacturers and veterinarians in developing mechanisms for the prudent use of antibiotics and for the protection of public health by ensuring that significant antimicrobial therapies are not lost due to the use of antimicrobials in food-producing animals.

RVIS/TRIMS

Numerous surveys conducted during the 1980s indicated that the consumer was concerned about the potential risk of chemical residues in foods of animal origin. It was thought that residual chemicals in the edible tissues of food animals over the established tolerance level posed greater risk than other hazards associated with foods.

In response to consumer concerns in the USA, the USDA, the Food Safety Inspection Service (FSIS) and the FDA created the Residue Violation Information System (RVIS) to share pertinent data for regulatory enforcement on a regular and open basis. For the benefit of others associated with chemical residue control in food animals and animal products, helpful features of this system are elaborated here. The RVIS has proven to be an excellent tool for supporting residue control measures in meat and poultry because it allows exchange of information among participating agencies regarding regulatory enforcement.

The RVIS database is a nationwide, interagency computer information system designed to share pertinent data for regulatory enforcement on an open and regular basis (Paige *et al*., 1999a). The system operates 24 h a day to provide information on residue violations in livestock and poultry slaughtered in the USA. It also includes residue testing data for processed eggs.

The Tissue Residue Information Management System (TRIMS) was developed in 1988, and is now linked with the RVIS database. The descriptive data collected by TRIMS can be used to generate hypotheses relative to identifying risk factors for drug residues. Data from this database have been used to write articles that have appeared in veterinary journals and agricultural trade journals (Paige *et al*., 1999a; Sundlof *et al*., 2000). Information has proved useful in determining whether patterns of violation can be related to specific slaughter class pairs and pharmacological drug classes and, if so, what preventive measures can be implemented.

DANMAP

One such programme that has gained international recognition in this area is DANMAP, which was set up in 1995 (Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, 1997). Specifically, DANMAP monitors antimicrobial resistance in bacteria from food animals, foods and humans and it has been designed to provide a basis for comparison of the occurrence of resistance in these three reservoirs. In addition, DANMAP reports data on the consumption of antimicrobials in animals and in humans, and on associations between the use of antimicrobials and trends in antimicrobial resistance.

Consumption data include the use of antimicrobials in food animals, including therapeutic use, growth promotion use and use as a coccidiostat. For humans, consumption consists of a comparison of consumption within Denmark and an analysis of the trends over time. The resistance data are from three sources matched with the three reservoirs. The pathogens consist of zoonotic bacteria, indicator bacteria (*E. coli* and *Enterococcus faecium/Enterococcus faecalis*) and bacteria isolated from diagnostic laboratories.

Conclusion

Regulatory agencies are engaging in risk assessment as a mechanism for making policy decisions on food safety. Since antimicrobial resistance and residues represent a global problem, we must engage in international harmonization as the emergence of resistance to antibiotics has gained global prominence. We have tried to emphasize that to confront food safety issues of resistance and residues requires a scientific and public health strategy. As indicated, such safety assessment criteria are inherent in the veterinary drug approval process.

The presence of a strong science-based approach by regulatory agencies will do much to assure our consumers both domestically and internationally that they are receiving a safe animal product. We must continue to encourage veterinarians to engage in prudent drug usage, and to improve farm management practices, which are viewed as promising ways of preventing foodborne illness. In addition, there is also a need to continue to improve surveillance and applied research to elucidate the mechanism of new control and preventive methods.

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14 Prion Diseases: Meat Safety and Human Health Implications

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Introduction

Prion diseases are rare in human beings; however, they are now often the subject of front page newspaper headlines and have had a profound effect on international trade, the food industry, pharmaceuticals and hygiene regulations in hospitals. This group of diseases, also known as the transmissible spongiform encephalopathies (TSEs), includes scrapie in sheep and goats, chronic wasting disease (CWD) of deer and Creutzfeldt–Jakob disease (CJD) and its bovine spongiform encephalopathy (BSE)-related version (variant CJD (vCJD)) in humans (Table 14.1). There is around one new case of CJD per million of the world population per year, but vCJD has been reported in just over 100 cases to date, the vast majority in the UK. TSEs are transmissible between individuals, either by direct injection – deliberate in the case of laboratory animals or accidental in the case of iatrogenic infection of humans – or by as yet unknown routes in the natural infections in animals. It is thought that the vCJD agent is ingested with food (possibly meat); however, it is not at all clear how the 'normal' or sporadic form of CJD is acquired. TSEs have no cure and are characterized by the presence of an abnormal form (PrP^{SC}) of a membrane protein (PrP^{C}) in infected organs of the body. PrP^{SC} is considered by many to be itself the infectious TSE agent and is known as the prion protein. Other researchers remain unconvinced of this hypothesis, or feel it remains unproven; however, at the very least, PrP^{SC} is a reliable marker for the presence of TSE infectivity.

PrP Protein and the Nature of the TSE Agent

The normal form of the PrP protein (PrP^{C}) is attached to neuronal cell surfaces via an anchor made up of sugar molecules. The main differences between the two isoforms of PrP are listed in Table 14.2. The function of PrP^{C} is not known; however, there are several intriguing hints that it may have a role in the activation of T cells in the immune system (Mabbott *et al*., 1997), in the electrophysiology of nerve cells (Collinge *et al*., 1994; Manson *et al*., 1995) and in the maintenance of sleep continuity (Tobler *et al*., 1997). The aggregated form of PrP (PrP^{SC}) is partially resistant to proteases and is very closely associated with infectivity. In one of the major hypotheses on the nature of the TSE agent, PrP^{SC} is itself the infecting entity agent or

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Table 14.1. Transmissible spongiform encephalopathies (prion diseases).

Human diseases	Acronym	Types	Aetiology
Creutzfeldt–Jakob disease	CJD	Sporadic	Unknown
		Familial	Linked to PrP gene mutations
		Iatrogenic	Contamination during surgery or of growth hormone
Gerstmann–Straussler–Scheinker syndrome	GSS	Familial	Linked to PrP gene mutation, e.g. codon 102
Fatal familial insomnia	FFI	Familial	Linked to PrP gene mutation, e.g. codon 178
Kuru	–	Acquired	Associated with funeral rites
Variant Creutzfeldt–Jakob disease	vCJD	Acquired	?Diet, related to BSE

Animal diseases		Acronym	Types	Aetiology
Scrapie	Sheep, goats	–	Natural	Infection, unknown mode of transmission
Chronic wasting disease	Deer	CWD	Natural	Infection, unknown mode of transmission
Transmissible mink encephalopathy	Farmed mink	TME	Acquired	Contaminated feed
Bovine spongiform encaphalopathy	Cattle	BSE	Acquired	Contaminated feedstuff
Feline spongiform encephalopathy	Cats	FSE	Acquired	Diet, related to BSE
Spongiform encephalopathies	Zoo animals	SE	Acquired	Diet, related to BSE

Table 14.2. Differences between PrP^{C} and PrP^{SC}.

Characteristic	PrP^{C}	PrP^{SC}
Action of proteinase K enzyme (PK)	Degraded	Partially resistant
Molecular weight	33–35 kDa	33–35 kDa
Molecular weight after PK treatment	Degraded	27–30 kDa
Detergent	Soluble	Insoluble
Present in normal brain?	Yes	No
Present in TSE brain?	Yes	Yes
Infectivity	Does not co-purify	Does co-purify

prion. In this 'protein only' theory, PrP^{SC}, arising from an infection or from a mutant PrP gene, acts as a catalyst in the conversion of endogenous PrP^{C} into yet more PrP^{SC}, thus either destroying the normal function of the protein or poisoning the nerve cells and resulting in degenerative disease (Prusiner *et al*., 1990). Natural scrapie in sheep tends to be familial in appearance and has been said to result from a recessive gene, the protein product of which causes disease (Parry, 1984); however, as described later, this hypothesis has now been discounted. Around 15% of the human TSEs also show a familial pattern with a dominant pattern of inheritance (Brown *et al*., 1987), and are considered to be genetic diseases (resulting directly from a mutation). However, genetic disease or not, once a TSE does occur, it is often transmissible experimentally to laboratory animals, not the case with simple genetic diseases, for example thalassaemia (Rund *et al*., 1991), and so additional explanations for disease spreading mechanisms are required.

Because of the heretical nature of the 'protein only' hypothesis, in that it invokes an infecting agent carrying genetic information embedded in protein and not in DNA or RNA, the prion theory (Prusiner, 1982) was difficult for many to accept (Chesebro, 1998). TSEs have been shown to exist in many different strains, and so one of the main opposing ideas involves PrP^{SC} protein as part of a two-component structure (the virino), including a

nucleic acid which specifies strain information (Farquhar *et al*., 1998). The virino hypothesis is still tenable as the prion hypothesis remains to be completely proven.

Strains of TSEs

The relatively conventional concept of strains, biologically similar to strains of viruses, has also been applied to the many identifiable types of TSEs. For example, around 20 strains of scrapie have been isolated from natural sheep scrapie following injection into laboratory rodents, where they produce precise incubation periods and brain region pathology in affected animals (Dickinson and Meikle, 1971; Bruce *et al*., 1991). TSE strain characteristics have been used most recently to great effect in the identification of BSE-like infections of mammals other than cattle (Bruce *et al*., 1994), particularly vCJD in humans (Bruce *et al*., 1997). vCJD transmits very easily to mice, producing a BSE-like pattern of incubation periods and pathology, and is clearly different from sporadic CJD, which does not transmit at all well to mice (Bruce *et al*., 1997). Distinct strains of natural scrapie may also exist in sheep (Hunter *et al*., 1997b). Strains of TSEs are explained, depending on the hypothesis adopted, in terms of the necessity for a scrapie-specific informational molecule in addition to PrP (the virino hypothesis), or as the result of reproducible three-dimensional structures of the PrP^{SC} protein molecule (the prion hypothesis), which would act as strain-related templates for the conversion of PrP^{C} molecules to the aberrant way of folding typical of PrP^{SC}. There is evidence from test-tube experiments that such a PrP^{C}/PrP^{SC} conversion, promoted by added PrP^{SC} from various TSE sources, can happen in a strain-specific manner (Caughey *et al*., 1998). Alternative means of holding information within protein molecules could be related to the degree of glycosylation (complex sugar side chains) of PrP^{SC} molecules. These, as revealed by antibody staining (Western blots), form characteristic three-banded patterns which in some cases can be related to the strain of the infection (Collinge *et al*., 1996b) (Fig. 14.1).

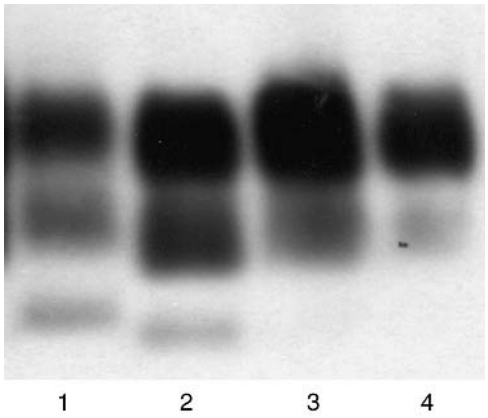

Fig. 14.1. Patterns of PrP^{SC} protein on Western blots. Lanes 1 and 2 are prepared from sheep infected with different sources of natural scrapie; lanes 3 and 4 are from sheep infected experimentally with BSE.

Genetics of Prion Diseases

In humans, occurrence of disease has been linked to the precise sequence of amino acids specified in an individual's PrP gene. In sheep, goats, deer and mice, PrP amino acid codon variation has also been linked to TSE disease and to the length of the incubation period, but cattle have shown no such association with BSE. Mouse genetics will not be discussed here as extensive reviews are available elsewhere and mice do not represent a substantial food source for humans!

Genetics of human TSEs

Each species in which linkage has been demonstrated between PrP genotype and TSE has had its own set of disease-linked amino acids. Humans are no exception, although CJD and Gerstmann–Straussler–Scheinker syndrome (GSS), which are distinguishable on clinical and pathological criteria, are each linked to several different

PrP gene sequence changes (polymorphisms or mutations) in different affected families (Table 14.3). The sporadic forms of CJD in humans are not linked to any mutations of the PrP gene and, instead, a codon 129 polymorphism, methionine (M) or valine (V), is associated with differences in susceptibility to disease in that homozygous individuals (either MM or VV) are over-represented in CJD cases and heterozygosity (MV) seems to confer some protection (Palmer *et al*., 1991). At the time of writing, all reported vCJD cases are of MM PrP genotype (Collinge *et al*., 1996a), which occurs in around 37% of Caucasian populations.

Other forms of TSEs in humans appear to be genetic diseases, resulting directly from a mutation in the PrP gene, for example one form of GSS which is linked to a codon 102 proline to leucine mutation (Hsiao *et al*., 1989). This mutation, when introduced into the mouse PrP gene in transgenic (Tg) mice and expressed at extremely high levels, resulted in a spontaneous scrapie-like disease which, although no PrP^{SC} was detectable by standard methods, transmitted infection to hamsters and other Tg mice and not to normal mice (Hsiao *et al*., 1994). This experiment supports the 'protein only' hypothesis because apparently the only requirement for disease to develop is a single amino acid mutation. However, the interpretation of the results has been disputed on the grounds of the lack of PrP^{SC}, the odd transmission characteristics and the high levels of expression needed to see the effect – single-copy transgenes do not make the mice ill (Chesebro, 1998). It is known that high levels of protein produced from normal PrP genes can also result in illness in Tg mice (Westaway *et al*., 1994) and so PrP poisoning is a possibility.

There are several other human PrP gene mutations associated with disease, and various forms of human PrP protein expressed by naturally occurring mutant genes have been studied in cell lines in culture and have been found to be both abnormally processed, for example not appearing on the cell membrane, and to acquire characteristics of the disease-associated PrP^{SC} protein isoform (Lehmann and Harris, 1996; Daude *et al*., 1997). This suggests that mutations in the PrP gene may cause illness directly through loss of function of the PrP protein by misprocessing or that the mutant protein forms deposits and poisons the surrounding cells. In the familial, or genetic, forms of human TSEs, there is thought therefore to be no need to look for a source of disease other than the aberrant PrP gene itself; however, with sporadic CJD, there is no easy explanation related to genetics, and an environmental source of infection, for example contaminated foodstuffs, may eventually be found to be a risk factor.

Genetics of sheep TSEs

The means by which scrapie transmits between sheep is not well understood, although there is good evidence that the most common means of entering the body is by the oral route (Hadlow *et al*., 1982; Van Keulen *et al*., 1999). Studies of natural scrapie in sheep have confirmed the importance of three amino acid codons in the sheep PrP gene (136, 154 and 171) (Belt *et al*., 1995; Clouscard *et al*., 1995; Hunter *et al*., 1996). (A diagram of the sheep PrP gene structure (similar in all species) is shown in Fig. 14.2 and sheep genotypes are usually

Table 14.3. Examples of human PrP gene mutations associated with familial TSEs.

Disease	Amino acid number	Change	Codon 129[a]
CJD	178	Aspartic acid → asparagine	Valine
	200	Glutamic acid → lysine	Methionine
FFI	178	Aspartic acid → asparagine	Methionine
GSS	102	Proline → leucine	Methionine

[a]Codon 129 polymorphism: the amino acid at this position influences disease type that occurs in combination with mutation.
FFI, fatal familial insomnia.

represented giving each of the three codons in turn for each allele in turn.) The most resistant genotype is ARR/ARR and the most susceptible is VRQ/VRQ, with a range of other genotypes of varying degree of disease risk in between these two extremes (Dawson *et al*., 1998). Not all sheep breeds are the same, however, as Suffolk sheep have a very simple linkage with disease: this breed does not have the VRQ allele and scrapie occurs in ARQ/ARQ sheep. Other breeds with the additional VRQ allele, such as Cheviots and Swaledales, are much more complex and, when the highly susceptible VRQ/VRQ genotype occurs, as it is almost always in scrapie-affected sheep, it has been suggested that scrapie may be simply a genetic disease (Ridley and Baker, 1995). However healthy VRQ/VRQ animals can live to more than 8 years of age, well past the usual age-at-death from scrapie (2–4 years) (Foster *et al*., 1996a), and susceptible sheep genotypes are easily found in countries that are free of scrapie clinical cases (Hunter *et al*., 1997a). The genetic disease hypothesis seems less likely, therefore, than a disease process which involves an infecting agent (prion or virino) causing disease only in susceptible sheep. However, in order to eliminate TSE diseases from sheep in countries where scrapie is endemic, breeding for resistant genotypes currently is being promoted, rather than elimination of infection – the route used with cattle BSE.

A great deal of information on the details of PrP genotype linkage with disease in sheep has come from experimental infections of sheep with scrapie or with BSE under controlled conditions (Goldmann *et al*., 1991a, 1994). The genotypes of sheep targeted by BSE are quite distinct, with the shortest incubation periods in ARQ/ARQ sheep and longer incubation periods in ARQ/ARR sheep. Although some sources of experimental scrapie (e.g. CH1641) also target these genotypes, SSBP/1 is different and affects Cheviot sheep encoding the VRQ allele (Goldmann *et al*., 1994). It is possible, therefore, that there are also various types or strains of natural scrapie which target either particular sheep breeds and/or different PrP codons. The best way to investigate this at the moment is by passage of natural scrapie into a panel of mouse strains where characteristics of incubation periods and the brain areas which become damaged give distinct profiles or patterns (Bruce *et al*., 1994). It is also possible that scrapie strains may produce PrP^{SC} proteins with distinct patterns on Western blots – a method currently under investigation for strain typing in several laboratories throughout the world (e.g. Hope *et al*., 1999).

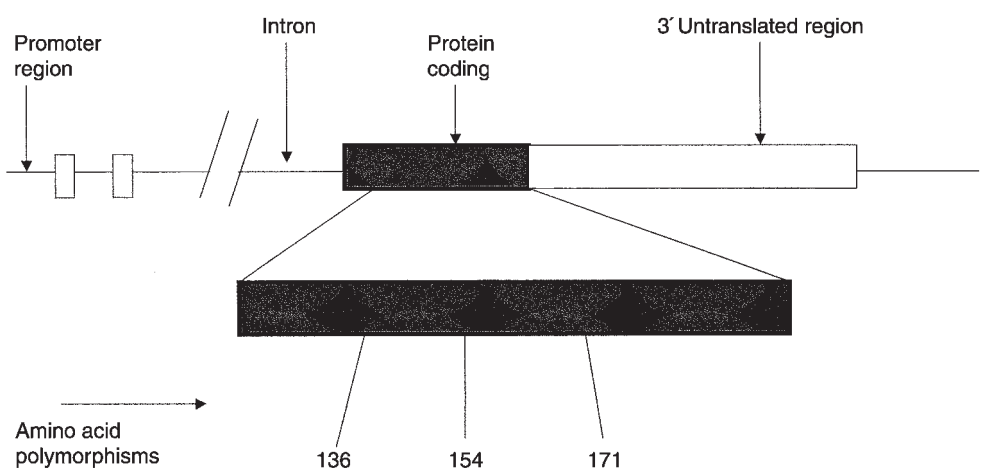

Fig. 14.2. Diagrammatic representation of the structure of the sheep PrP gene with the positions of the three disease-linked amino acids. Black boxes represent the protein-coding region of the gene.

Genetics of BSE in cattle

It is generally accepted that BSE in cattle was the result of feeding of infected meat and bone meal-derived protein supplements. Several other captive animals succumbed to BSE-like infections (Bruce *et al*., 1994), including domestic cats, although the domestic dog has remained unaffected for reasons which are not understood. When BSE was found in cattle, the cattle PrP gene was searched for markers of resistance or susceptibility to disease similar to those that had been found in mice, sheep and humans. The cattle gene, however, is remarkably invariant compared with that of sheep and humans and has so far shown no linkage with disease (Goldmann *et al*., 1991b; Hunter *et al*., 1994). Cattle therefore appear to be unusual in not (so far) demonstrating a PrP-related link with TSE incidence. It may be that all cattle would be susceptible to BSE if they received a high enough dose of infection, but clearly breeding for resistance to BSE in cattle at the moment is not an available option. The best method for control is to eliminate the infection from cattle feed both in the UK and in other countries throughout the world.

Distribution of Infectivity in Body Tissues

Methods of detection

It is important to establish which tissues of TSE-affected animals or people are actually infected because, particularly with reference to BSE, this dictates which tissues or products can be safely eaten or used in pharmaceuticals. Demonstration of infection by means of mouse transmission studies takes a long time (up to 2 years) but is, when positive, unambiguous – there is infection present and it will transmit to another individual. Negative results, when no disease is transmitted to the mice, could mean absence of infection but could also mean that there is simply too low a level (titre) of infection to be detected within the test animals' lifespan, or that the sensitivity of the test is not high enough. The latter point relates to what is known as the species barrier, which occurs often (but not always) when a TSE is transmitted from one species to another (Bruce *et al*., 1994). Because the species of animal is changed, the TSE infection seems to have to work harder to produce disease, giving long incubation periods and/or lower detectable titre. When cattle BSE is titred in cattle, it is possible to detect 500-fold greater levels of infection than in mice (Wells *et al*., 1998). However despite this problem, the mouse assays have been of immense importance and are still used as the main means of demonstrating infection, largely because of the fact that BSE gives a clear 'fingerprint' of lengths of incubation period and patterns of damage produced in mouse brain tissue, and allows the positive identification of the BSE agent in cattle and other species – most notably in humans affected by vCJD (Bruce *et al*., 1997). Transgenic mice, which are created in laboratories and produce the PrP proteins of other species, are being developed in the hope of improving the mouse assay system.

The other means of demonstration of the presence of infection is by detection of PrP^{SC} protein either by extraction and reaction with anti-PrP antibodies (Western blotting) (Hope *et al*., 1999) or by antibody detection within sections of tissue examined under the microscope – immunohistochemistry (Van Keulen *et al*., 1999). Using PrP^{SC} as a marker of infection is an excellent alternative to mouse bioassay, although it is subject to the same potential problems: a positive result is clear, but a negative result may simply mean that the detection limit of the method is not sensitive enough. There are examples of infection detected in mice without concomitant detection of PrP^{SC}, however, and so it is always advisable to retain some caution in the interpretation of results (Lasmezas *et al*., 1997; Manson *et al*., 1999).

Species used in food production: sheep and cattle

Using bioassay in mice, natural scrapie infection has been studied in diseased animals and

also during the preclinical phase of the disease during which animals appear perfectly healthy. In one study during development of scrapie, infection was detected first (at 10 months of age) in sheep gut and lymphoid tissue, then much later in the central nervous system (CNS) and brain, the latter of which has greatest amounts of infectivity as the clinical signs develop and the animal becomes ill. In this study, no infection was found in milk, udder or muscle (Hadlow *et al*., 1982). However, in a more recent report, peripheral nerves in scrapie sheep muscle were found to be infected (Groschup *et al*., 1996). Sheep experimentally infected with BSE have infection present in both brain and spleen (Foster *et al*., 1996b), suggesting that, should BSE infect sheep naturally, it would have a similar distribution throughout the body to that found with scrapie. Using blood transfusion between sheep (no species barrier), BSE infection has also been detected in blood removed from experimentally infected animals during the preclinical phase of disease (Houston *et al*., 2000). In contrast, cattle naturally infected with BSE have shown signs of infection only in the brain and spinal cord, and even experimentally infected cattle have only shown additional sites of infection in part of the intestine (distal ileum) (Wells *et al*., 1998) and, specifically, milk from BSE cattle was also negative (Taylor *et al*., 1995).

Using PrP^{SC} as a biochemical marker for infectivity, signs of infection can be detected in sheep tonsil as early as 3 months of age in animals destined to develop scrapie at about 2 years of age (Schreuder *et al*., 1998). PrP^{SC} is found throughout the body of scrapie sheep, with some tissues, such as the liver, being relatively spared and others having higher amounts (spleen, parts of the intestine); however, up to and during the clinical phase, PrP^{SC} is found in increasing, and very much higher, amounts in brain and CNS tissues (Van Keulen *et al*., 1996). PrP^{SC} has also been found in placental tissue, although any involvement of placenta in natural transmission of scrapie from mother to offspring may not be *in utero* but could result from ingestion of discarded placental tissue by the ewe herself or by other sheep. In sheep experimentally infected with BSE, lymphoid tissues show signs of infection early in the incubation period (Jeffrey *et al*., 2001) and, by terminal stages of disease, PrP^{SC} deposits are found widespread throughout the body (Foster *et al*., 2001b). In BSE-affected cattle, it is more difficult to find evidence of PrP^{SC} outside the CNS (Wells *et al*., 1998). Clearly the pathogenesis of BSE in cattle is different from that in sheep, with more limited involvement of non-CNS tissues. It follows from this, therefore, that in thinking about the risks of infection of humans with BSE, high risk cattle tissues are liable to be fewer in number and easier to eliminate from the food chain than would be the case if BSE in sheep were to become endemic.

Although there is no evidence for the occurrence of BSE in the USA, another related disease does occur: chronic wasting disease (CWD) of mule deer and elk (Williams and Young, 1992; Spraker *et al*., 1997). This prion disease occurs in both farmed and wild populations. Although there are precautions in place which test the brain tissue of hunted animals for the presence of PrP^{SC} protein (Laplanche *et al*., 1999), there are concerns about the occurrence of CJD in a small number of individuals known to have consumed wild venison. Recent epidemiological studies have judged that there was no link between the two diseases but, if more CJD cases occur in hunters, there may be a change of opinion. There is, at the time of writing, no published evidence from outside North America for CWD occurrence on venison farms or in the wild.

Humans

In sporadic CJD cases, signs of infection are not widespread throughout the human body; however, from early studies of vCJD, it was apparent that this new disease was behaving differently. PrP^{SC} was detected in tonsils of a number of vCJD victims and in the appendix tissue of another case (Hill *et al*., 1997, 1999). Further studies have since been set up to find out which tissues represent a risk of infection for humans and for contamination of surgical instruments. Infectivity was found in mouse bioassays of vCJD brain, as expected, but also in tonsil and spleen at levels between 100

and 1000 times lower than in brain (Bruce *et al*., 2001). Blood fractions (buffy coat and plasma) were negative. In addition, PrP^{SC} has also been found in lymph nodes, retina and optic nerve, and at low levels in one vCJD case in the rectum, adrenal gland and thymus – with obvious implications for contamination of surgical instruments. Other vCJD tissues tested for PrP^{SC} were negative, including blood buffy coat preparations (Wadsworth *et al*., 2001).

How are TSEs Contracted?

The route of transmission of natural scrapie in sheep is not known with certainty but, because of the early involvement of the alimentary tract (PrP^{SC} staining), the oral route is implicated (Van Keulen *et al*., 1995, 1996, 1999). Other routes of infection through wounds on the skin or in the mouth are also possible. Infection may be picked up from pasture contaminated with infected placental tissue, or by simple contact with other infected animals. BSE in cattle is most likely to have been spread by oral infection through eating contaminated meat and bone meal (MBM) (Wilesmith *et al*., 1991) although in this case the infection seems to by-pass the peripheral tissues and travel straight to the brain.

In humans, the route of infection in sporadic CJD is not known. Epidemiological studies sometimes show connections with diet or lifestyle, but these have been thought to be artefactual due to the relatively small numbers of affected individuals (Wientjens *et al*., 1996). One study suggested surgery was a risk factor (Kmietowicz, 1999), and there are clear instances of iatrogenic infection following surgical procedures (Shimizu *et al*., 1999) or following injection with contaminated growth hormone used to treat undersized children (d'Aignaux *et al*., 1999). There are forms of CJD which appear to be genetic in origin and are linked to the occurrence of specific mutations of the PrP gene (Ghetti *et al*., 1995). If this view is correct, no further route need be sought for the familial human TSEs; however, it has been shown beyond any doubt that the vCJD infectious agent is identical to BSE (Bruce *et al*., 1997) and it has been assumed that the disease is picked up via the oral route and eating BSE-infected cattle products.

As it is not known how many people are already infected with vCJD and quietly incubating the disease, it is impossible to judge the risk to others from potentially contaminated blood products. Sporadic and genetic forms of CJD are not thought to have infectivity in peripheral tissues, and there is no epidemiological evidence linking blood transfusion with incidence of sporadic CJD. As vCJD involves infection of peripheral tissues and to deal with the potential risk, UK blood supplies are depleted of white blood cells and new US Food and Drug Administration rules will forbid donations from anyone who spent 3 months in Britain from 1980 to 1996 or those who have spent 5 years or more in France since 1980. The rules will also ban donations from anyone who received a blood transfusion in Britain since 1980 and from American military personnel who spent 6 months or more on a European base from 1980 to 1996, when British beef was sold to bases there (Cimon, 2001). Only time will tell if these precautions are justified.

Risk Factors

There have been more than 177,000 cases of BSE confirmed in cattle in Great Britain since the outbreak started in the late 1980s, peaking in 1992 with 36,682 recorded cases. Between 1991 and 1995, there were more than 10,000 cases per year; however, in 2000, the numbers had dropped to 1270. Other countries are also affected, although at a much lower rate: Northern Ireland (total to December 2000 = 1810, peaking in 1993 with 459); Republic of Ireland (total = 567, peaking in 2000 with 132); France (total = 233, with 153 in 2000); Portugal (total = 489, peaking in 1999 with 159) and Switzerland (total = 364, peaking in 1995 with 68) (data from the UK Department for the Environment, Food and Rural Affairs (DEFRA)). Many, but by no means all, cases in countries outside the UK are in cattle imported from the UK. Some animals are

home bred, but it should be remembered that MBM has been exported to many countries throughout the world by UK manufacturers and may have caused disease by ingestion.

It is assumed that the source of BSE infection in humans is through consumption of infected meat products. In examining the eating patterns of vCJD patients, however, no obvious common factor emerges which makes these people different from the rest of the UK population. The highest risk factor for development of vCJD has been said simply to be residence in the UK. However, as the outbreak of vCJD continues, intriguing connections are being made. Sporadic CJD has a uniform distribution related to local population levels (Cousens *et al*., 1997), but there is a slightly higher risk of contracting vCJD in northern parts of the UK, where consumption of meat products, rather than beef itself, is thought to be more common (Cousens *et al*., 2001). Meat products (pies, sausages, etc.) may have contained infected cattle CNS tissues but it remains difficult to demonstrate that vCJD patients' diets were much different from those of non-affected individuals within the same family. One small cluster of vCJD cases in England has been attributed to local butchery practices, which involved splitting of cattle heads (Anon., 2001) and potential contamination of meat with brain tissue.

It is known that sheep can be infected with BSE experimentally by consumption of as little as 0.5 g of BSE cattle brain (Foster *et al*., 1993). As UK sheep were also fed supplements containing MBM, although in greatly reduced amounts compared with cattle, it is a theoretical possibility that sheep were also infected with BSE. Various studies are under way to look for signs of BSE infection in sheep by identification of the characterisitic BSE incubation period pattern in mouse transmission experiments and by looking in scrapie cases for PrP^{SC} protein which seems to adopt a particular form associated with BSE. In order to increase the chances of finding BSE should it exist in sheep, and since in experimental studies using BSE infection the sheep which become sick with the shortest incubation period are of ARQ/ARQ PrP genotype (Foster *et al*., 2001a), ARQ/ARQ sheep with clinical TSE signs have been selected for further study. Natural scrapie can also occur in ARQ/ARQ sheep, however, and, at the time of writing, no firm evidence of BSE has been found in sheep.

Clinical Symptoms and Signs

Symptoms in humans

Sporadic CJD patients have rapidly advancing dementia with development of movement disorders, difficulty in walking, cortical blindness and mutism, with median survival time between onset of symptoms and death being 4.5 months. In contrast, vCJD has a longer clinical phase (median survival time ~10 months longer than with sporadic CJD). Patients with vCJD tend to be younger on average than sporadic CJD cases and have psychiatric symptoms of withdrawal and depression. Early symptoms of vCJD also include painful and persistently cold feet and legs, and neurological signs do not develop until later in the clinical course, when movement disorders, complete dependence and mutism begin and progress until death. The neuropathology of vCJD is also distinct from that of sporadic CJD in that the former exhibits very large plaques, or deposits, of PrP protein surrounded by vacuoles (so-called 'florid' plaques) in the cerebral and cerebellar cortex of the brain (Stewart and Ironside, 1998).

Signs in sheep

Clinical signs of scrapie in sheep (Dickinson, 1976) start with mildly impaired social behaviour followed by locomotor incoordination or ataxia with trembling. Pruritis and wool loss can result from the animal attempting to relieve what seems to be an intense itching by scratching against fence posts or by biting the affected area; however, these clinical signs are highly variable and can last from 2 weeks to 6 months. Lesions in the brain include neuronal degeneration with the formation of vacuoles (holes), proliferation of astroglial cells but no demyelination or other overt inflammatory responses. These

features develop in the later stages of the incubation period and it is very difficult to detect (by histopathology) those animals with scrapie which are not yet visibly affected by the disease.

In experimental studies of BSE in sheep, the clinical signs are very similar to those of scrapie (Foster *et al*., 2001a), and in a field situation it is expected that, should BSE have infected sheep, it would be impossible to use these to differentiate scrapie (presumed to be non-pathogenic to humans) from BSE.

Treatments

There are as yet no approved treatments which cure TSE diseases. There are possible candidates amongst drugs which prolong the incubation period in animal studies, for example pentosan polysulphate (PS), which if administered 7 h after injection with ME7 scrapie prolonged the incubation period in mice by up to 66% (Farquhar *et al*., 1999). This is not a cure, but is one example of current studies which are aimed at understanding how therapeutics might work. It has also been suggested that anti-PrP antibodies could prevent the conversion of PrP^{C} to PrP^{SC} in the body of affected individuals as it seems to do in cell culture (Peretz *et al*., 2001). An interesting recent development involves trials in humans clinically affected with TSE disease of the anti-malarial drug quinacrine, not used widely in the UK since the 1970s, and the related chlorpromazine, which carry additional worries of side effects. At present, the only real defence against these diseases in humans is avoidance of infection, and legislators have been busy trying to ensure that infection is removed from the human food chain.

Legislation and Regulatory Issues

The first laws designed to prevent the spread of BSE in cattle by banning the use of feeding ruminant tissues to ruminants in the UK came into force in 1988, and the rules have continued to be tightened since then. The European Commission took the first steps in limiting cattle exports from the UK in 1989, which culminated in a complete ban in 1996. In 1989, there was also the banning in human food produced in England and Wales of cattle tissues expected (from previous studies in sheep scrapie) to be infectious. The same legislation was applied in Scotland and Northern Ireland in the following year. For 2 years, from late 1997, meat on the bone was also banned from human consumption.

It was clear, however, that the 1988 ruminant feed ban had not been completely effective as cattle born after the ban also developed BSE; however, greater control over abattoirs and rendering plants has resulted in a continual drop in numbers of BSE cases in the UK. It is disturbing that the very stringent rules imposed after the announcement of the occurrence of vCJD in 1996 do not seem to be totally effective, as animals born after that date are now showing signs of BSE, albeit in very low numbers. Laws relating to the age of animals allowed into the human food chain (1996) mean that UK cattle over 30 months cannot be used as a source of meat or mechanically recovered meat (MRM). MBM and ruminant-derived products have been subject to a plethora of regulations in the UK and in Europe about what they can contain and what they can be used for. For example, in 1998, the sale of MBM derived from mammalian tissues was prohibited for use as a fertilizer on agricultural land, and the use of certain ruminant tissues in cosmetics was banned in the UK in 1997. As sheep also became suspect in the BSE audit trail, heads of sheep and goats were prohibited for human consumption in the UK in 1996. However, attempts by the European Commission to agree on legislation to control the use of high risk tissues in food and food products have been greatly hampered by the relative difficulty of removal of the spinal cord from sheep and goats compared with the much larger cattle. It also appears that, if BSE does occur in small ruminants, it may be more widespread in body tissues than it is in cattle. Since the introduction of a reliable means of cattle identification by ear tagging and individual cattle passports with a tracing system, coupled with all of the other control measures in place in the UK, the export market is

gradually opening up again for cattle and meat products (data from DEFRA, UK).

Conclusions

It is too soon to predict accurately the final numbers of people who will become affected by vCJD; however, it is likely that the measures in place to protect humans and animals will mean that, if an epidemic of vCJD does occur, its time span will be limited. Intense efforts are also being made to understand the disease, how it spreads and how it can be treated or prevented in susceptible individuals. It seems, however, that a great deal of the dangerous BSE-infected MBM was exported from the UK to other parts of the world and may have been fed there to indigenous ruminants. Organizations such as the World Health Organization, the European Union, the Office Internationale d'Epizootiques and the World Trade Organization have been trying to raise the issue of the dangers of BSE in regions outside Europe and the USA, and it is to be hoped that BSE and vCJD will not become major problems for the rest of the world population.

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15 The Safety Evaluation of Genetically Modified Foods

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Introduction

The use of gene technology in food production is the cause of significant controversy that has been fuelled by the activities of various pressure groups and by the nature of some media reporting. This heightened concern has led to a complex situation where the discussion of genetically modified (GM) food raises a whole series of social issues that go well beyond the strictly scientific assessment of safety. This chapter is intended to highlight those safety issues that are addressed when an objective scientific evaluation of GM foods is undertaken. Discussion is focused on health risks to the consumer, and issues that are associated with agricultural practice are not considered. In addition, the frequently raised question of benefit versus risk and the subtleties of risk perception are not covered. In this regard, the Agriculture and Environment Biotechnology Commission Report *Crops on Trial* (AECB, 2001) provides a useful perspective on the ethical and social impact of and public attitudes towards GM technology.

The safety assessment of GM food addresses a series of well established and internationally accepted questions. However, there are differences in implementation, most notably between Europe and the USA. In Europe, GM food legislation is dominated by EC Regulation 258/97 on 'Novel foods and ingredients'. This legislation (EC, 1997a) demands a formal process of pre-market approval that draws on the opinions of independent scientific committees in each Member State. In practice, the competent authority of the country where a product is first intended to be marketed undertakes the initial safety assessment. All other European Member States then have an opportunity to comment on the initial opinion. Where safety concerns are raised, scientific evaluation is passed on centrally to the Scientific Committee for Food. Some novel foodstuffs, including GM soybean derivatives, were already marketed in Europe before this legislation came into force, but they were subject to earlier evaluation by national safety committees, notably by the Advisory Committee on Novel Foods and Processes (ACNFP) in the UK (ACNFP, 1994).

In the USA, the Food and Drug Administration (FDA) holds the prime responsibility for GM food safety under the Federal Food, Drug and Cosmetic Act. The FDA policy on foods developed by biotechnology is outlined in a policy statement made in 1992 (FDA, 1992). While US regulatory involvement is less hands-on, the scientific principles used for safety evaluation have much in common with those of the EU. A recent review of the

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regulatory process in the USA has led to a requirement for data submission 120 days prior to the marketing of bioengineered foods (FDA, 2001a), and updated extended guidelines have been announced (FDA, 2001b).

Application of GM Technology in Food

GM technology is applied to food in a wide variety of different ways. Food production involves the use of many ingredients, additives and enzyme preparations that are derived from a diversity of sources. These include microorganisms that are often exploited as 'cell factories' for the production of food ingredients and processing aids. Microorganisms used in this way are often subject to genetic improvement, but they are used to produce purified food components that are often nature identical. Calf chymosin, the active ingredient of rennet, is used extensively by the dairy industry in cheese making and provides a good example of a GM cell factory application. A gene for the chymosin enzyme has been introduced into the yeast *Kluveromyces lactis*, the filamentous fungus *Aspergillus niger* and the bacterium *Escherichia coli*, and its expression in these heterologous hosts provides an alternative source to traditional rennet extracted from the stomachs of slaughtered calves. This represents one of the earliest commercial applications of GM food technology and it has been subject to detailed safety evaluation both in the USA and in Europe.

Another example of a microbial cell factory that currently is awaiting EU safety approval is the use of a GM strain of *Bacillus subtilis* for the manufacture of the vitamin riboflavin (ACNFP, 1997). This is distinct in that the vitamin is a metabolite rather than the direct protein product of the introduced gene, and it is representative of another generic application of GM technology in metabolic engineering.

Traditional biotechnology includes the exploitation of microorganisms for food fermentation, and this has evolved into a major sector of the food industry. This covers the use of yeasts in brewing and bread making, and the use of lactic acid bacteria in dairying and the production of fermented vegetables and meats. GM technology has been used for the development of improved strains of these food fermentation microorganisms. Examples include a bread-making strain of *Saccharomyces cerevisiae* with improved maltose metabolism and a brewing strain of the same species that expresses an amylase gene derived from another closely related *Saccharomyces* species. In both cases, safety evaluation has been undertaken by the ACNFP (ACNFP, 1994), but food products based on the use of these GM yeast strains have never been marketed in Europe. Because of the manufacturing methods used for bread and beer, the derived food products would not contain viable GM yeast cells or, in the case of a live beer, only very low numbers. In contrast, some dairy products, such as yoghurt, contain high numbers of viable lactic acid bacteria and, as a consequence, viable GM derivatives of these microorganisms would also be present. These distinctions are important for safety evaluation, and the particular issues raised by viable GM microorganisms in food have been the subject of detailed consideration by a variety of organizations including ILSI (1999) and, most recently, FAO/WHO (2001a).

Crop plants account for the vast majority of current GM food applications. GM plant material might be marketed as an intact food, as in the case of fresh fruits, or it might be subject to processing, as in the case of canned tomato paste. The safety evaluations of both fresh GM tomato fruit and canned GM tomato paste have been undertaken (ACNFP, 1995, 1996). Genetic modification involved the introduction of an 'antisense' or 'sense' gene for the natural tomato pectinase enzyme. Expression of the engineered gene causes reduced pectinase activity during the fruit ripening process with consequent reduced texture change. This lessens mechanical damage to the ripe fruit and reduces the associated spoilage. In addition, altered processing characteristics improve the yield of tomato paste, with associated economic benefits.

Commodity crops, such as soybean, are used for the production of a range of purified or semi-purified derivatives that are added to processed foods. The extraction of oils, flours

and other food ingredients from commodity crops has implications for safety evaluation in that transgene DNA and expressed proteins are subject to varying degrees of inactivation and removal. In addition, it is pertinent that these derivatives are used extensively in processed foods, making consumer exposure very widespread.

In many examples of GM food, the technology has been used to improve agricultural performance. The most widespread applications are the introduction of genes that confer tolerance to otherwise non-specific herbicides and the gene for a *Bacillus thuringiensis* insecticidal protein that provides in-built protection from pest damage. While GM food technology currently is dominated by these 'first-generation' agronomic applications, there is a considerable effort devoted to 'second-generation' traits that are intended to benefit the food manufacturer or the consumer. Examples of second-generation traits include improvements to seed storage proteins, oil content and starch, the removal of allergens and the fortification of micronutrients and antioxidants. A good example is the development of GM yellow rice in which three genes for the biosynthesis of β-carotene were introduced into a conventional rice cultivar. As β-carotene is the provitamin for vitamin A, this has the potential to address a serious nutrient deficiency that causes blindness in many children in developing countries.

This very brief overview of GM food applications inevitably will be incomplete, but critically it serves to emphasize the diversity of GM technology uses in food. It establishes the need for case-by-case consideration of safety, and this is already well established as a key guiding principle in safety assessment. It is particularly relevant that purification and processing, such as heat treatment, may inactivate or remove introduced foreign genes and their expressed proteins, with obvious implications for safety assessment. Some examples of the diversity of GM food applications that have been the subject of safety evaluation are listed in Box 15.1, and a more complete position is readily available from the EC release, *Facts on GMOs in the EU* (EC, 2000).

GM Technology

The *in vitro* manipulation of DNA is common to all applications of GM technology. For each target material, there is a need to use a specific technology for DNA delivery and to ensure its subsequent maintenance. This can vary depending on the target species that is the subject of genetic modification and it has implications for safety evaluation. One of the central safety assessment criteria is an analysis of the DNA that has been introduced as well as the methods that have been employed during transgene delivery. In bacteria, gene technology is generally more advanced and controllable than is the case for plants. The relatively small genome size and the availability of an increasing number of whole bacterial genome sequences provide a valuable pool of detailed information. An important advantage is the ease with which homologous recombination can be used to facilitate the directed delivery of transgenes to specific genome sites. In general, it is possible to devise food-compatible genetic systems for the exploitation of GM technology, using both plasmid-based and chromosome integration systems.

In contrast, plants suffer from the fact that the exploitation of homologous recombination remains a challenging objective. Generally, DNA delivery does not involve control over the genome site into which a transgene is integrated and, in many cases, the delivered DNA becomes rearranged during genetic modification. Transgene delivery in plants involves three technologies: protoplast transformation; microparticle bombardment or biolistics; and *Agrobacterium* binary vectors. The latter two processes are the most widely used. As its name suggests, microparticle bombardment involves the penetration of plant tissue by tungsten particles coated with transgene DNA. *Agrobacterium* technology exploits the disease features of the pathogen *Agrobacterium tumefaciens*, which has evolved a natural process for the delivery of bacterial genes to the plant genome. Using a process related to bacterial conjugation, a specific tract of *Agrobacterium* DNA is transferred to the plant genome, where its expression leads to

Box 15.1. Examples of the safety assessment of GM foods in Europe.[a]

Approvals made by ACNFP prior to Regulation (EC) 258/97

Calf chymosin expressed in the microorganisms *Aspergillus niger*, *Kluveromyces lactis* and *Escherichia coli*. (Widely used as a milk-clotting agent in cheese manufacture.)

Baker's yeast *Saccharomyces cerevisiae* expressing maltose permease. (Approved but never commercialized.)

Brewer's yeast expressing an amylase gene to facilitate starch breakdown with improved fermentation and carbohydrate conversion. (Approved but never commercialized.)

Paste prepared from Zeneca tomato engineered for reduced expression of the polygalacturonidase gene and altered ripening characteristics giving improved yields and reduced spoilage. (Product marketed successfully by two UK supermarkets but now withdrawn.)

Processed derivatives of Monsanto GM soybeans engineered for tolerance to the glyphosate herbicide Roundup. (Imported and used widely in processed foods.)

Processed derivatives of Ciba Geigy GM maize engineered to produce *Bacillus thuringiensis* insecticidal protein.

Some examples of applications pending approval under Regulation (EC) 258/97

AgrEvo UK processed oil derived from oilseed rape (TOPAS 19/2) tolerant to glufosinate ammonium herbicide.

Flour, gluten, semolina, starch, glucose and oil derived from Monsanto maize (MON810) expressing the *B. thuringiensis* insecticidal protein gene *cry*IA(b).

Starch, oil, heat-processed and fermented products derived from AgrEvo maize (T25) tolerant to glufosinate ammonium herbicide.

Food and food ingredients produced from Pioneer maize (MON809) expressing the *B. thuringiensis* gene *cry*IA(b).

Processed oil from Plant Genetic Systems male-sterile (DBN230-0028) and fertility restorer (DBN212-0005) oilseed rape lines.

Riboflavin produced by Hoffman La Roche GM *Bacillus subtilis*.

Some examples of food-relevant approvals under Directive 90/220/EEC

Plant Genetic Systems male-sterile swede rape resistant to glufosinate ammonium herbicide. (Used for breeding activities.)

Monsanto soybeans tolerant to glyphosate herbicide. (Approved for import and processing.)

Bejo-Zaden BV chicory male-sterile and tolerant to glufosinate ammonium herbicide. (Used for breeding activities.)

AgrEvo swede rape tolerant to glufosinate ammonium herbicide. (Approved for import and processing.)

AgrEvo maize (T25) tolerant to glufosinate ammonium herbicide. (Approved for import and processing.)

Novartis maize (Bt-11) tolerant to glufosinate ammonium herbicide and expressing the *B. thuringiensis* insecticidal protein gene *cry*IA(b). (Approved for import and processing.)

Some examples of applications pending approval under Directive 90/220/EEC

DLF-Trifolium, Monsanto and Danisco Seed fodder-beet tolerant to glyphosate herbicide.

Zeneca processing tomato engineered for reduced expression of the polygalacturonidase gene.

Amylogene potato with altered starch composition.

Novartis maize (Bt-11) tolerant to glufosinate ammonium herbicide and expressing the *B. thuringiensis* insecticidal protein gene *cry*IA(b) for cultivation.

Pioneer maize (T25 + MON810) tolerant to glufosinate ammonium herbicide and expressing the *B. thuringiensis* insecticidal protein gene *cry*IA(b).

Monsanto maize (GA21) tolerant to glyphosate herbicide.

[a] Across Europe, the approval of GM food is controlled by EC Regulation 258/97 on 'Novel foods and novel food ingredients'. At present, all applications under this Regulation are pending. Prior to this Regulation, the ACNFP made several approvals of GM foods for commercialization within the UK. Deliberate releases into the environment, including the agricultural use of GM crops, are controlled at a European level by Directive 90/220/EEC. The table gives examples of these approvals and pending applications. A more complete data set is available in the EC release *Facts on GMOs in the EU* (EC, 2000).

the development of a gall. Substrates that support the growth of *Agrobacterium* are produced within the gall. By disarming this disease process, it is possible to engineer *Agrobacterium* vectors that will carry trait genes into GM plants. In addition to the integration of trait genes within the plant chromosomes, it is possible to integrate transgenes within the genomes of plant chloroplasts. Because these organelles have genetic features that are related to those of the prokaryotic bacteria, this involves the use of homologous recombination to 'place' transgenes in a predetermined location. A diagrammatic overview of the various techniques used in the construction of GM plants is included in Fig. 15.1.

An important part of the safety evaluation process is the provision of molecular data that demonstrate the nature of foreign DNA that has been inserted as a result of genetic modification. The method used to transform a GM plant has a bearing on this. It is well established that biolistic delivery often leads to extensive structural rearrangement of the integrated DNA. In some cases, this is so extensive that it is extremely difficult to unravel its DNA sequence. This has led to a preference for the use of *Agrobacterium* delivery, which can also cause the integration of multiple DNA copies but is less prone to causing structural rearrangement of the transgeneic DNA.

One limitation of *Agrobacterium* had been its restriction to dicotyledonous plants, but recent developments have extended its use to also include monocotyledonous plants such as rice and maize (Ishida *et al*., 1996).

Another distinct issue that has been of concern to regulatory authorities is the realization that small fragments of transgene DNA can sometimes be integrated at secondary genome sites following biolistic delivery. Recently, this was found to have taken place in the GM soybean line that is widely used commercially. Careful analysis of this specific case eliminated any safety concerns, but this observation does serve to emphasize the need to investigate unintended secondary integration of potentially small fragments of transgenic DNA.

Further options that influence safety and containment are:

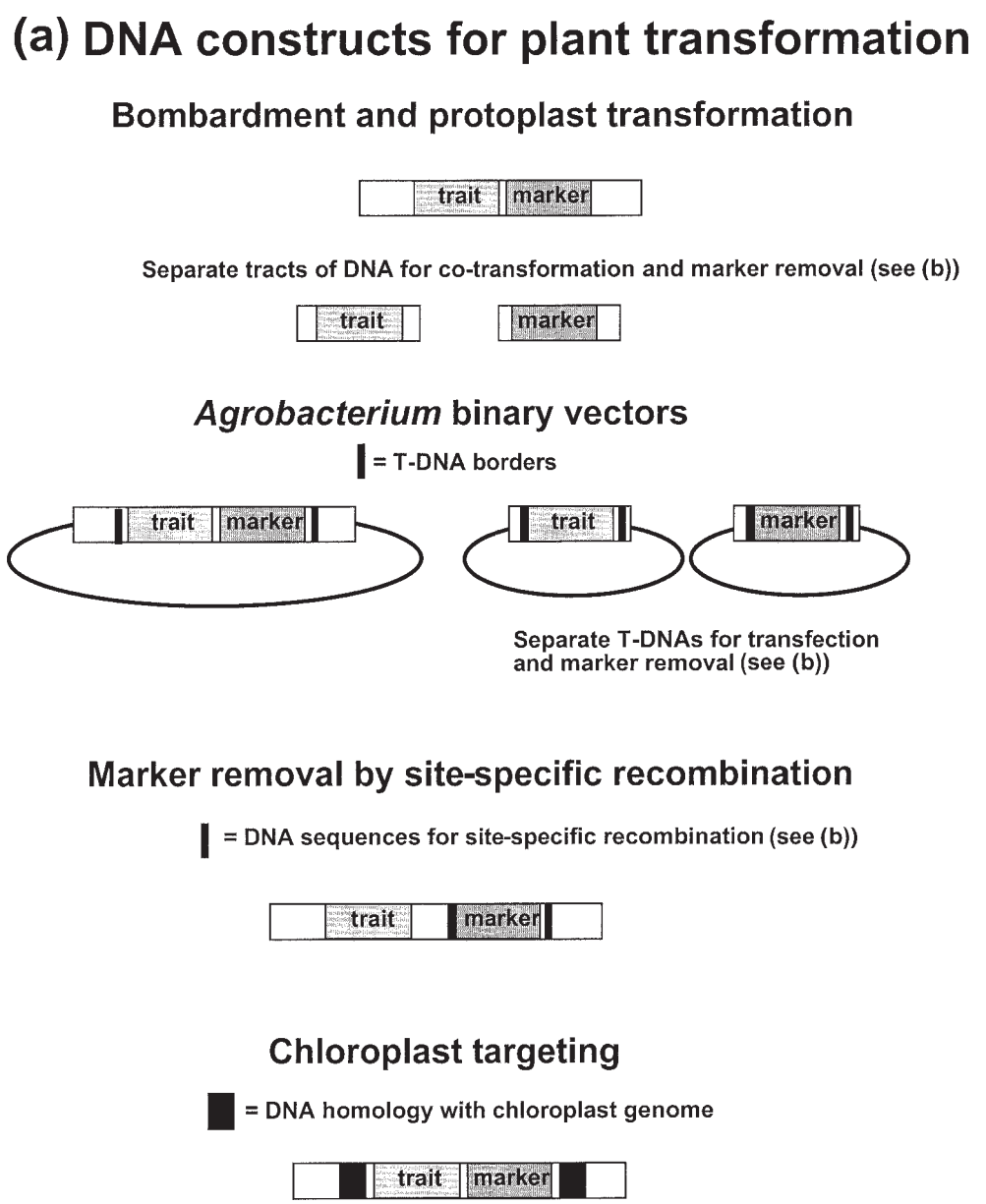
- The use of a vector designed to deliver transgenic DNA into the plant chloroplast genome by homologous recombination provides controlled integration at a known site. In addition, the lack of chloroplasts in pollen provides environmental containment of transgenes.
- The separation of trait and plant selection genes on distinct DNA fragments facilitates their unlinked integration in the plant genome. Alternatively, the plant selection gene can be flanked by sequences that are recognized by site-specific recombinases (e.g. *cre*/*lox*). In both cases, elimination of the selection gene is possible using conventional plant crosses.
- The elimination of unnecessary DNA that was used during bacterial stages is very straightforward, but this has not always been undertaken, leading to problems with antibiotic resistance genes and more complex rearrangement of trait DNA.
- The marker elimination process described in Fig. 15.1(b) is realized by conventional crosses that facilitate the segregation of trait and selection genes. For site-specific recombination, the recombinase gene is introduced from a separate GM plant to effect marker deletion, and this gene can be removed by a subsequent conventional cross.

The Use of Selectable Marker Genes

In order to effect the introduction and expression of transgenic DNA, there is a universal need to use some form of selection to differentiate the transformed cells. This usually involves the use of a selectable marker gene that may be physically linked to the chosen trait genes. For the primary transformation of GM plants, antibiotic resistance genes have often been exploited as convenient selection markers. The general importance of antibiotics for human and veterinary medicine and

the high profile of microbial drug resistance have made this practice controversial. The nature of the selection system used in GMO construction is a particular focus of safety assessment.

The *npt*II gene, originally derived from *E. coli* transposon Tn*5*, is used frequently as a plant selection marker, and to this end it has been equipped with a plant-specific promoter to facilitate its expression in plant cells. A comprehensive argument about the safety of *npt*II used as a plant-selectable marker has been developed, and this was first formally presented by Calgene (1990) and accepted by the US FDA and other regulatory bodies. A significant factor is the limited importance of



kanamycin and neomycin in the treatment of bacterial infections in humans mainly as a consequence of their relative toxicity. In addition, it is recognized that antibiotic resistance is already widespread in bacteria, and rare gene transfer from a GM food source is unlikely to be of practical consequence (Nap *et al.*, 1992).

Alternative selection markers that avoid the use of antibiotic resistance genes are becoming available, and mechanisms, such as the *cre*/*lox* system (Dale and Ow, 1991),

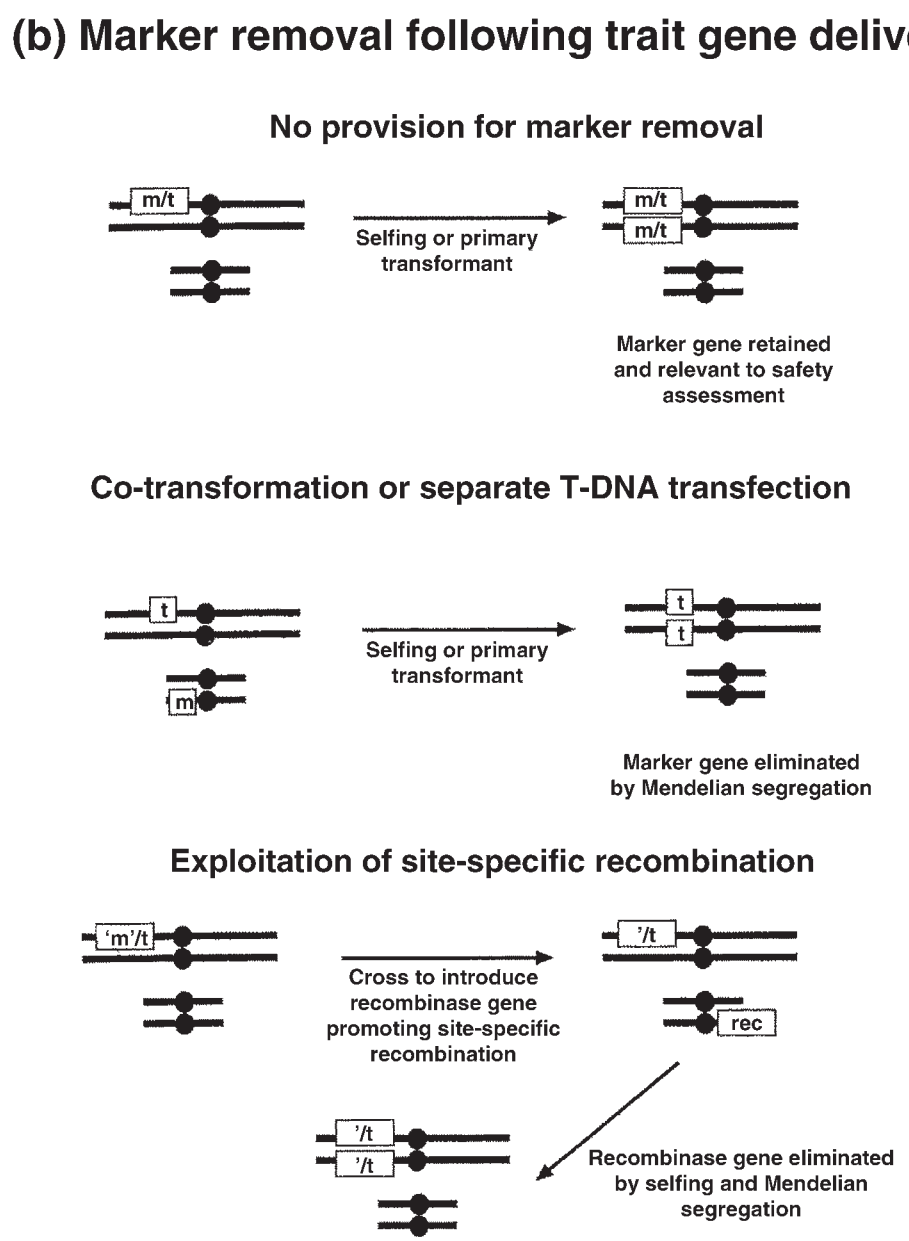


Fig. 15.1. Overview of techniques used in the construction of GM plants; adapted from Gasson and Burke (2001).

have been developed to facilitate the removal of selection markers after GM plant construction. Recently, Novartis Seeds announced their intention to phase out the use of antibiotic resistance genes. Their 'Positech' marker system uses a selection for growth on mannose and relies on a gene for phosphomannose isomerase (Anon., 2000). Other approaches include the use of co-transformation of trait and selection genes followed by segregation of the latter. This has been effective in both *Agrobacterium* transformation (Komari *et al*., 1996) and biolistic transformation (ACNFP, 1996). Figure 15.1 includes a schematic representation of these alternative selection methods and strategies to eliminate selection markers from the final GM plant constructs. Despite these developments, GM plant material carrying antibiotic resistance genes continues to be put forward for consideration by regulatory authorities who need to assess safety on the basis of objective scientific criteria.

In contrast to *npt*II, several GM plants have been developed in which other antibiotic resistance genes have been introduced. In these cases, the antibiotic resistance genes have not been engineered as plant selection markers and they retain their original bacterial promoter. Genes in this category include *bla*, *aad* and *npt*III, which confer resistance to ampicillin, streptomycin/spectinomycin and amikacin, respectively. All of these antibiotics have greater use in clinical medicine than kanamycin and neomycin. The most common reason for the presence of these genes in GM plants is that the trait gene was first engineered using a bacterial vector and *E. coli* cloning techniques where these bacterial selection markers would be of value. Subsequently, the complete bacterial vector has been delivered to the GM plant without first removing these now redundant DNA sequences. The bacterial marker genes are not directly selectable in plants, and there is no good reason for their presence in GM material destined for use as food. Despite this, there are scientifically valid arguments that they do not pose a safety hazard. Most persuasive is the fact that drug resistance is already widespread in bacteria as a direct consequence of the very extensive use of antibiotics in human and veterinary medicine and as animal growth promoters. Also, the process of gene transfer from a GM plant to a microorganism is likely to be a very rare event, and this is discussed in more detail below. This issue has been controversial for regulatory authorities, with somewhat different views emerging on the use of antibiotic resistance genes in GM plants. The ACNFP has adopted a cautious position (ACNFP, 1995) and, in general, the inclusion of antibiotic resistance genes in GM plants is widely discouraged.

The Safety Assessment Process

Despite differences in safety administration, notably between the USA and Europe, the scientific issues that are addressed during the safety assessment of GM food are very consistent. Details have been published by both the FDA (1992) and the EC (1997b), and an overview of the main features is presented in Box 15.2. A key principle is that an integrated, stepwise and case-by-case approach is required. Safety assessment is aided by the use of decision trees that give guidance on the specific points that need to be addressed for an individual case. Substantial equivalence plays a key role in identifying differences between a GM food and its conventional counterpart, and these become a focus for further consideration. The role of substantial equivalence in safety assessment is discussed in detail below. The information that is required for the safety assessment includes: details of the genetic modification; the stability of the modification and potential for its transfer; protein expression and its effect on function, allergy and toxicity; potential secondary effects; composition; intended uses and effects of cooking and processing; and potential intake and dietary impact.

The Role of Substantial Equivalence

The concept of substantial equivalence plays an important role in the safety evaluation of

Box 15.2. Overview of GM food safety assessment.

Major features of safety evaluation:

- Use of an integrated, stepwise and case-by-case approach
- Intended and unintended differences between GM and conventional counterpart identified using the substantial equivalence approach

Specific data required for:

- Details of the genetic modification
- Stability of the modification and possibility of transfer of the modified genetic material
- Potential secondary effects of the genetic modification
- Composition of the GM food or food ingredient
- Intended use and effects of processing or cooking
- Potential intake and dietary impact

GM food. First, it is important to stress that this concept is not in itself a safety assessment process and, in particular, it is not intended to identify hazard. Rather, it acts to identify key issues that require detailed safety evaluation. Substantial equivalence was developed under the guidance of the World Health Organization (WHO), the Organization for Economic Cooperation and Development (OECD) and the Food and Agriculture Organization of the United Nations (FAO) following on from the realization that conventional approaches to safety assessment, as used for pharmaceutical products, have serious limitations (FAO/WHO, 1991, 1996; OECD, 1993). These limitations also explain why, in contrast to pharmaceuticals, animal feeding experiments are less prominent in the safety evaluation of novel foods. The general approach to safety testing using animal feeding involves the consumption of increased amounts of the test substance until an adverse effect is detected. The sheer bulk of many whole foods prevents this increased exposure, and data interpretation is compromised by the fact that foods are complex mixtures of many different chemicals. Another critical point is that food contributes to nutrition. Toxicology testing using animals depends on the establishment of optimal nutrition to provide a controlled background against which to evaluate any effect from the fed test substance. In the case of whole GM food material, it is self-evident that any observed negative effect is as likely to arise from disturbed nutrition as it is to be caused by novel technology. These limitations of conventional animal testing approaches for the safety evaluation of biotechnology products were emphasized during the safety assessment of other novel technologies: food irradiation and mycoprotein. Thus there is a well-established scientific basis for seeking new approaches to the safety evaluation of novel foods.

The substantial equivalence concept recognizes that many people have eaten conventional foods over a very long period of time and this establishes an accepted level of safety. Genetic modification involves the introduction of only small changes, and thus a comparative approach can be used to reveal any intended and unintended differences between GM material and its conventional counterpart. The OECD (1993) described substantial equivalence as embodying 'the idea that existing organisms used as food, or as a source of food, can be used as the basis for comparison when assessing the safety of human consumption of a food or food component that has been modified or is new'. By concentrating on the safety assessment of the differences between a GM derivative and its conventional counterpart, it can be concluded that the established and accepted safety of the conventional food has not been compromised. The comparative approach involves the evaluation of a large body of phenotypic data that include agronomic traits and details of chemical composition. Typically, the latter includes fats, proteins, solvent-extracted hydrophilic

matter, fatty acids, amino acids, micronutrients, antinutrients, crude fibre, ash and moisture content. Particular attention is given to any known toxins or allergens.

Substantial equivalence has been debated widely in recent years, and it has attracted some vigorous criticism (Millstone *et al*., 1998; RSC, 2000) and equally robust defence (Burke, 1999; Kearns and Mayers, 1999; Trewavas and Leaver, 1999). Recently, the FAO/WHO (2000) report, *Safety Aspects of Genetically Modified Foods of Plant Origin*, addressed criticism of the application of the concept of substantial equivalence and reaffirmed its usefulness. In particular, it emphasized that the determination of substantial equivalence is not in itself an end point but rather the starting point for safety evaluation. The substantial equivalence concept is likely to be challenged further as genetic manipulation technology advances. The stacking of multiple traits and the engineering of some second-generation traits that may deliberately alter metabolic flux are examples of some areas where the application of substantial equivalence may prove complex.

One major concern is the capacity of substantial equivalence to reveal unintended consequences of genetic modification. In this regard, it is especially pertinent that conventional breeding is as likely as GM technology to generate unintended effects. Conventional breeding can exploit techniques, such as mutagenesis and induced polyploidy through the use of colchicines, that might be expected to lead to unpredictable genetic changes. It is curious that there is relatively little concern with respect to this very similar risk issue. Overall, the controversy surrounding substantial equivalence serves to highlight the fact that its role in safety evaluation often is misunderstood. Also, it is clear that substantial equivalence does indeed have limitations.

A current area of research activity concerns the possibility of exploiting new methodologies such as molecular profiling techniques to provide a more detailed analytical comparison. Currently, substantial equivalence involves an analysis of composition and phenotypic parameters that is undertaken with a targeted approach. In contrast, molecular profiling is non-targeted and more holistic. Molecular profiling encompasses three distinct technologies: metabolic profiling; proteomics; and DNA microarrays. These technologies interrogate sequential steps in the expression of genes through mRNA, proteins and metabolism, as is illustrated in Fig. 15.2.

Metabolite profiling exploits a variety of chemometric technologies to gather gross data on the distribution of individual metabolites. Critically this does not involve the prior selection of individual target molecules for quantification. In particular, gas chromatography–mass spectroscopy (GC–MS), nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC) techniques are capable of detecting, resolving and quantifying a wide range of compounds in a single sample.

Proteomics involves the use of two-dimensional gel analysis to separate individual proteins that are present in a particular tissue. An example of this technique applied to four different conventional varieties of tomato fruit is given in Fig. 15.3. While these profiling techniques are intended to reveal differences using a holistic analysis, there is a need to follow up with an assessment of the safety implications for any differences that are found. Proteomics has a valuable dimension in this regard in that it is possible to identify individual protein spots by relating them to the genes by which they were encoded. This relies on the availability of genome sequence data and thus the ease of application varies for individual species. The identification process involves the use of MALDI-TOF mass spectrometry to determine the mass of protein fragments generated by specific proteolytic cleavage. Because the cleavage pattern is predictable from the amino acid sequence of a given protein, identification is possible. Alternatively Q-TOF mass spectrometry can be used to gain sequence data directly from a protein spot, and this can be matched to information available in sequence databases.

In our own laboratory, this latter approach has been used successfully to identify an unknown protein that appeared in a GM tobacco plant. In this case, an experimental transgenic line with a very severe morphological abnormality was being studied. The

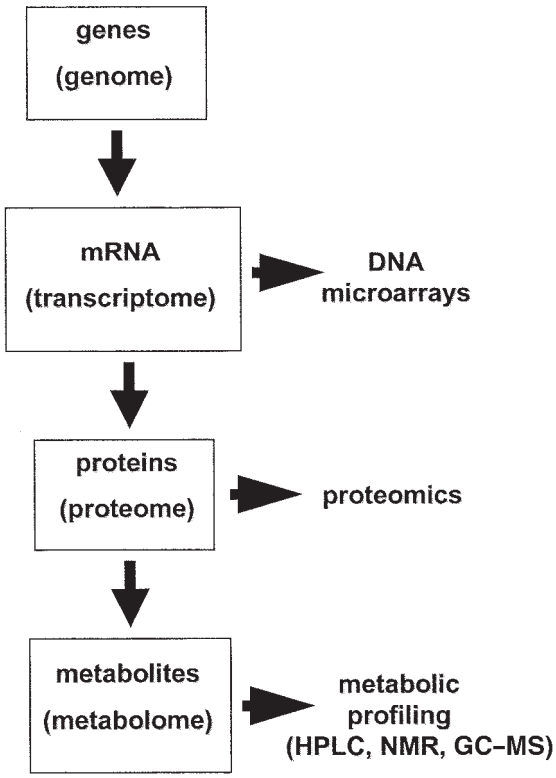


Fig. 15.2. Targets for molecular profiling.

gross proteome was remarkably similar to that of a normal non-GM syngenic plant but two new protein spots were detected in the GM plants. These were both identified as the same plant lectin, suggesting that a plant defence mechanism had been stimulated in response to the genetic modification. The fact that one protein generated two protein spots is important as it emphasizes the complexity that can arise where post-translational modification of a protein takes place. Proteomics is a powerful technique that couples gel electrophoresis with mass spectrometry and permits the visualization and possible identification of several thousand proteins representing the detectable part of the cell's total complement of proteins (the proteome). The technical limitation is associated with the relatively slow and demanding gel electrophoresis process, but alternatives to this are currently being developed.

DNA microarrays with many thousands of gene sequences arrayed on nylon or glass substrates permit simultaneous examination of the steady-state levels of the cell's mRNAs (the transcriptome). Differential labelling of mRNA extracted from a GM plant and its conventional counterpart with fluorescent dyes, and their hybridization to a DNA microarray generates data on the relative abundance of individual mRNA molecules for each individual gene. This approach depends on the availability of sequence data with which to design the probes used in microarrays and it gives instant data on the identity of any differences that are revealed.

Molecular profiling techniques generate a very large volume of data, but the point of interest is detecting biological components that are changed in the GM food. Determining the significance of the changes and their relevance to safety assessment is difficult without a background context based on the natural variation in the levels of the various biological components in conventional varieties. While the analytical power of molecular profiling

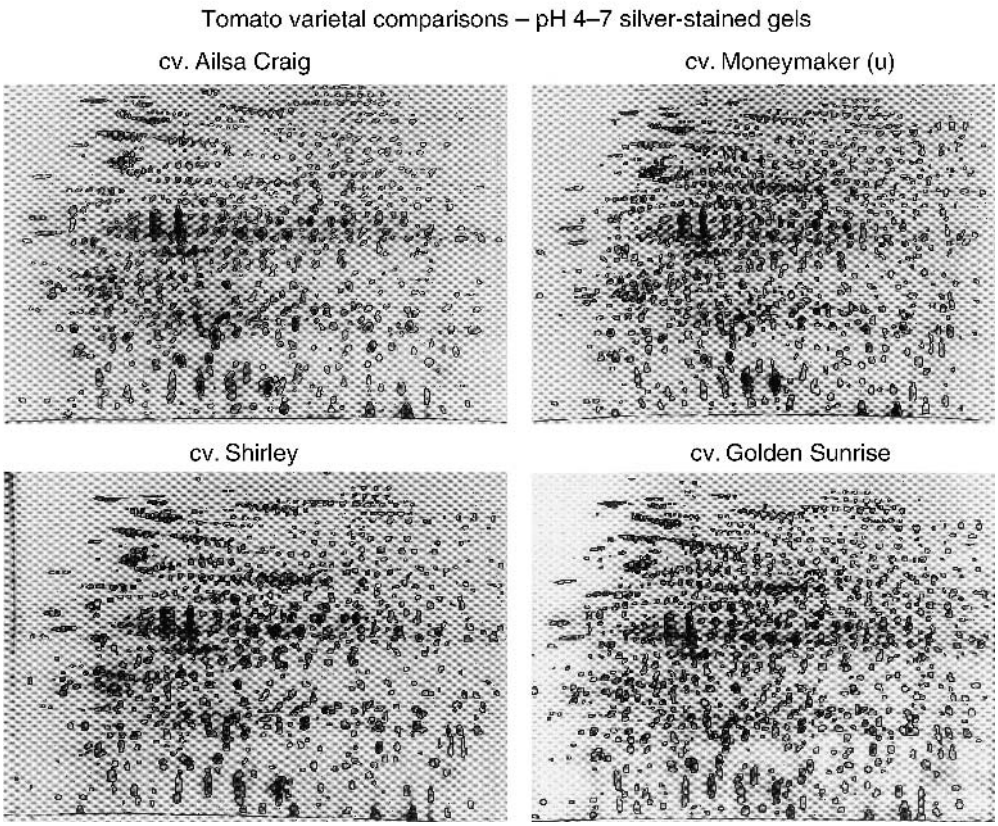


Fig. 15.3. Examples of proteomic analysis of tomato fruits.

approaches is without question, it remains to be seen how useful they will be in practice for safety evaluation. The new techniques will need to be validated and, in addition, the significance of any observed differences will need to be determined. Clearly, gene expression under normal circumstances is dynamic, and any differences seen in a GM derivative will need to be considered against this background. The potential of molecular profiling was highlighted by the Royal Society of Canada (RSC, 2000) and has been debated by FAO/WHO (2000). In Europe, a significant programme of research supported by the EC and the UK Food Standards Agency is under way with the intention of investigating the potential of molecular profiling and the relative merits of the different techniques in the context of safety evaluation.

The Impact of the Introduced Trait

Clearly, in most cases, genetic modification involves the introduction of a new intended trait to a conventional food material. In the context of substantial equivalence, it is thus likely that an intended difference will be present at least at the level of the primary food material. This last point is significant because the processing of a food material may, to a greater or lesser extent, eliminate the difference. A good example is purified oils prepared from GM plants such as oilseed rape or maize where it is difficult to differentiate these products from their conventional counterparts.

The introduced trait is often readily amenable to safety assessment using conventional toxicology approaches. For example,

the insecticidal protein produced by *Bacillus thuringiensis* has been evaluated extensively in isolation from GM plants that have been transformed with its gene. One frequently expressed concern is that expression of the same gene in bacteria and a GM plant may generate different protein products and this needs to be addressed. An obvious possibility is that glycosylation may occur in plants when it is not found in bacteria.

In addition to potential toxicity or other physiological effect, there is a need to assess the potential that a newly expressed protein might be allergenic. This involves an assessment of the stability of the protein and database searching to determine homology with known allergens. Known allergens share certain common properties that include resistance to heat, stomach acid and degradation by gastrointestinal tract enzymes, all of which can be assessed.

FAO/WHO have undertaken several recent reviews of GM food safety assessment that have addressed the question of allergenic potential, and this has culminated in the report of an expert evaluation that took place very recently in Rome during 2001 (FAO/WHO, 2001b). The FAO/WHO report from the Rome meeting updated a widely used decision tree that was designed to guide the assessment of allergenic potential, and this new version is reproduced as Fig. 15.4. This most recent report drew a distinction between expressed proteins that were derived from sources with known problems of allergenicity and sources with no known allergenicity. In the former case, initial investigation is recommended to be based on an analysis of sequence homology to known allergens present in the source material. A negative result is followed up by immunoassays to investigate possible immunoglobulin E (IgE) binding and possibly *in vivo* studies using patients allergic to the source food. It was recognized that the use of human *in vivo* methods would raise ethical issues and that their use would need to be considered on a case-by-case basis. Where the expressed protein is derived from a source with no known allergenicity, the initial investigation is also focused on database searches. A negative result is followed by targeted serum screening using samples containing high levels of IgE antibodies broadly related to the gene source. A positive result would suggest that the protein was potentially allergenic. Following a negative result, further studies of pepsin resistance and the use of suitable animal models are recommended. The FAO/WHO consultation emphasized the importance of maintaining and constantly updating an allergen database. Also it recognized that animal models had not been evaluated for all food allergens but sufficient scientific evidence was available to suggest that animal models will contribute valuable information regarding the allergenicity of foods derived from biotechnology.

Fate of Consumed DNA

Following the consumption of GM food, the fate of any novel introduced genetic material is pertinent. Concern extends to the possible uptake by host cells and by microorganisms that inhabit the gastrointestinal tract. The general sensitivity of consumed DNA to inactivation and degradation provides one of the best established barriers to the transfer of transgenes. The enzyme deoxyribonuclease I produced by the salivary glands, pancreas and small intestine is a potent degradative enzyme, and the low pH of the stomach removes adenine and guanine residues from DNA, thereby eliminating its biological activity (Beever and Kemp, 2000).

Several recent studies add to our understanding of DNA survival following its consumption. An *in vivo* study by Mercer *et al*. (1999) investigated the effect of human saliva on DNA survival using competitive polymerase chain reaction (PCR) and tested biological activity by measuring transformation into the naturally competent oral bacterium *Streptococcus gordonii*. Despite evidence of DNA degradation, sufficient biologically active DNA survived exposure to saliva to generate transformants, albeit at a reduced frequency. Duggan *et al*. (2000) investigated DNA degradation by ovine saliva and ovine rumen fluid, measuring biological activity using *E. coli*

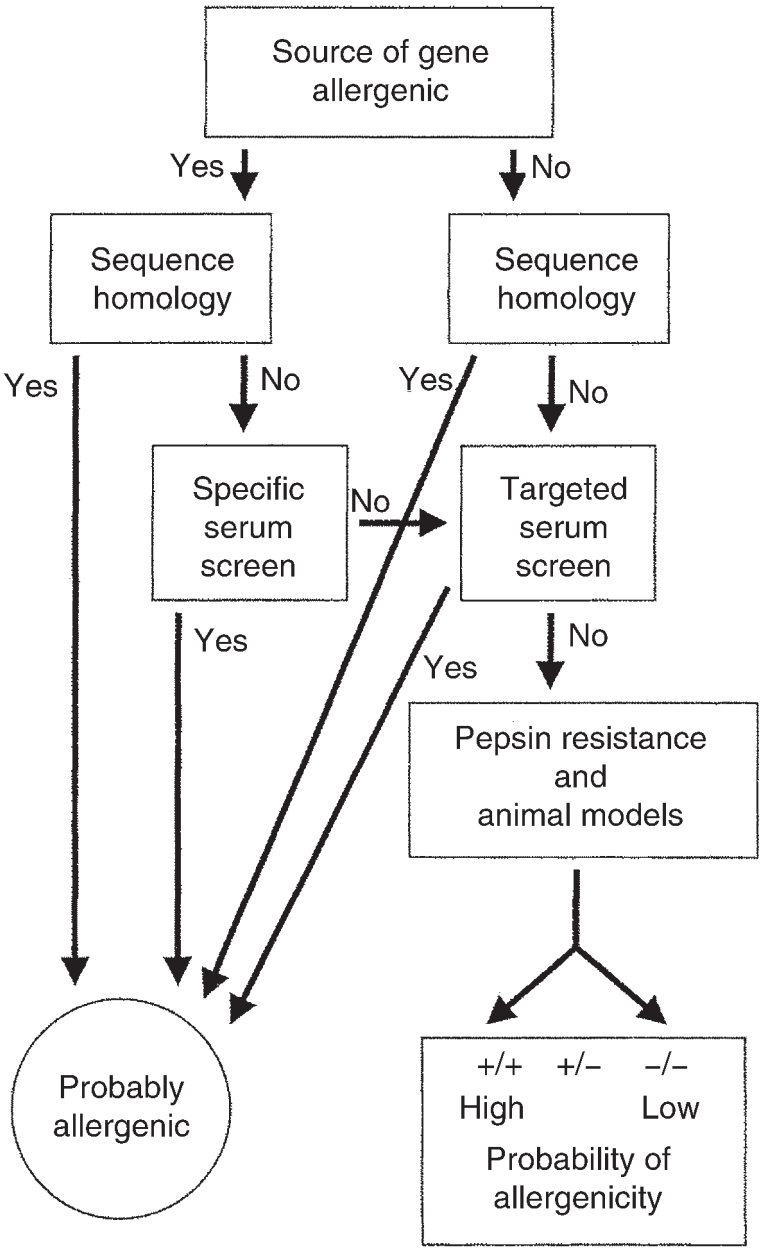


Fig. 15.4. FAO/WHO decision tree guiding the assessment of allergenicity.

transformation. PCR amplification of DNA was possible for 30 min after exposure to rumen fluid, but transforming ability was lost within 1 min. In contrast, the ability to transform *E. coli* was retained even after 24 h exposure to saliva.

These studies suggest that, although degraded, DNA remains available for transformation in the oral cavity. In contrast, it is inactivated rapidly and loses biological activity further down the gastrointestinal tract. Chambers *et al*. (2000) investigated the

fate of the pUC18 ampicillin resistance gene *in vivo* using chicken feeding experiments, and their findings reinforce this view. Both bacteria carrying pUC18 and transgenic maize carrying the *bla* gene were studied. PCR–restriction fragment length polymorphism (RFLP) was used to differentiate the test *bla* gene from naturally occuring *bla* genes that may have been present in the gastrointestinal tract microflora. The pUC18 gene lacks a *Pst*I site that is present in the natural gene. While the maize-derived gene could be detected in the crops of experimental chickens, it did not persist further down the intestinal tract. In contrast, bacteria carrying pUC18 provided protection, allowing *bla* gene detection throughout the intestinal tract.

A somewhat different picture has emerged from the work of Schubbert *et al*. (1994, 1997). These authors fed mice with bacteriophage M13mp18 DNA, and the fate of this foreign DNA in the animals was followed by several methods. Fragments of M13mp18 DNA were detected in the contents of the small intestine, the caecum, the large intestine, the faeces and in blood. It was calculated that 2–4% of orally administered DNA was detected in the gastrointestinal tract and 0.1–0.01% was retrieved from blood. M13mp18 DNA fragments were traced by PCR to peripheral leucocytes and located by fluorescence *in situ* hybridization (FISH) in about 1 of 1000 white cells between 2 and 8 h after feeding and in spleen or liver cells up to 24 h after feeding. M13mp18 DNA could be traced by FISH in the columnar epithelial cells, in the leucocytes, in Peyer's patches of the caecum wall, in liver cells, and in B cells, T cells and macrophages from spleen. These findings suggest transport of foreign DNA through the intestinal wall and Peyer's patches to peripheral blood leucocytes and into several organs. Upon extended feeding, M13mp18 DNA could be cloned from total spleen DNA into a λ vector. Schubbert *et al*. (1998) extended this study and obtained similar results using a plasmid expressing the gene for green fluorescent protein. The broad conclusion from this work is that consumed DNA may survive, cross the gut epithelium, enter the bloodstream and interact with mammalian cells. However, the significance of these results has been questioned. The experiments were artificial in the sense that mice were exposed to large amounts of pure prokaryotic DNA. Beever and Kemp (2000) questioned the fact that the DNA used in these experiments was unmethylated and contained sequences likely to cause up-regulation of inflammatory cell activity and to stimulate a significant immune response. It is possible that this induced response contributed to the detection of DNA in white blood cells.

Overall, there is substantial evidence to suggest that DNA degradation is very efficient in the gastrointestinal tract but some may remain biologically active in the mouth for sufficient time to be taken up by naturally competent oral bacteria. The implications of the observations made by Doeffler and colleagues are less clear, but suggest that, despite its hostile environment, some DNA may survive in the gastrointestinal tract.

DNA Transfer from GM Plant Material to Bacteria

Concern about gene transfer from GM plant material into microorganisms needs to be related to the nature of the genes that are involved. As already discussed, the use of antibiotic resistance genes has been a particular concern. The most likely mechanism by which microorganisms might acquire transgene DNA is by its release from GM food and its subsequent uptake by naturally competent bacteria. This is a real possibility in the oral cavity, as has been demonstrated by the work of Mercer *et al*. (1999) that is described above. In general, the status of bacterial gene transfer by natural genetic transformation has been reviewed extensively by Lorenz and Wackernagel (1994). A few studies have investigated DNA transfer from GM plant material to microorganisms, and these studies suggest that such an event would be extremely rare.

Schluter *et al*. (1995) used a model system based on the plant pathogenic species *Erwinia chrysanthemi*. A transgenic potato carrying an integrated pBR322 plasmid was used, and the plant pathogenic property of

Erwinia provided an intimate association between the plant material and the potential bacterial recipient. *Erwinia* causes soft rot by lysing plant tissues, and it is able to support the replication of the pBR322 plasmid and the expression of its antibiotic resistance genes. Evidence for plant to bacterium transfer was not detected. Related *in vitro* experiments were undertaken to provide quantitative data on gene transfer. This was estimated to have a maximum probability of 5.8×10^{-14} for an experiment using 0.9 g of potato tuber and 6.4×10^{8} bacteria.

DeVries and Wackernagel (1998) used naturally competent *Acinetobacter* and focused on the plant selection marker derived from the *npt*II kanamycin resistance gene. In these experiments, the recipient *Acinetobacter* strain carried an inactive homologue of the *npt*II gene that was under the control of a bacterial promoter, and this strategy increased the likelihood of detecting a positive result. In this case, transforming DNA did not need to be capable of autonomous replication or illegitimate recombination to be detected. Homologous recombination between the plant-derived *npt*II gene and the mutant resident gene would repair the defect in the latter gene, leading to the recovery of kanamycin-resistant transformants by marker rescue. This event was detected readily at a frequency of 0.9×10^{-4} per *npt*II gene. However, when artificial homology between the transgene and the recipient genome was absent, transformation frequency fell below the 1.3×10^{-13} limit of detection. The marker rescue data suggest an efficient mechanism for DNA transfer in which as few as 2.5×10^{3} transgenic potato cells could generate a transformant, and rescue of the kanamycin resistance marker was effective even in the presence of a more than a 6×10^{6}-fold excess of plant DNA. It is important to emphasize that this process depends on the provision of artificial homology and represents marker rescue rather than the recovery of unique DNA from the transgenic plant. However, it clearly demonstrates that naturally competent bacteria are very effective at taking up transgene DNA and, in the event of DNA homology being available, transformation is likely to take place. Gebhard and Smalla (1998) obtained similar results using marker rescue by *Acinetobacter* and DNA from GM sugarbeet.

As has been emphasized by Nielsen *et al*. (1998), selective pressure is of critical importance in assessing the consequences of gene transfer. The occurrence of a gene transfer event in itself is unlikely to be of any great significance unless it led to selective advantage in the recipient. Conversely, the existence of selective advantage could make even a very rare genetic event important.

Post-market Monitoring

Post-market monitoring of GM foods is considered by many to be an important component of safety assurance. While this a desirable objective, it is far from easy to realize. It is difficult to gather data on which members of the population were eating particular GM foods. A proposal in the UK to use supermarket 'loyalty' cards was rejected on grounds of the invasion of privacy. Also, GM foods, if they are present in the diet of any population, will be derived from such a range of crops and processes and present in such a large range of foods that it will be extremely difficult, if not impossible, to correlate consumption of any particular GM food to any recognizable syndrome. Experience in the USA, where a large number of people have been eating products containing GM soya and GM maize for a number of years without any ill effects, suggests that any effects will be very small. Despite the difficulties, post-market monitoring continues to be evaluated as a potentially valuable approach. Indeed the recent FDA/WHO report on allergenicity recommended:

> Post-market surveillance is a valuable tool in the monitoring of adverse effects and long-term sequelae of foods derived from biotechnology and the Consultation recognized that the feasibility of certain aspects of its implementation would need further investigation. (FAO/WHO, 2001b)

Conclusions

GM food includes a range of distinct applications of modern biotechnology, ranging from cell factories for the production of nature-identical ingredients through to the provision of GM fruits to be eaten fresh and unprocessed. The safety evaluation of GM food involves well-established procedures that have been developed extensively over the past decade, and these procedures are subject to ongoing review and updating. There is broad consensus on the relevant risk issues, and structured approaches have been designed to focus on those issues that are relevant to individual cases. To date, these processes have proved effective in dealing with GM food innovations but, as more complex trait development is undertaken, it will be important that safety assessment continues to meet the challenge.

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16 Genetically Modified Foods: Potential Human Health Effects

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Introduction

The scope of this review is restricted to data-based considerations about the safety of genetically modified (GM) foods of plant origin for health. No opinions unless supported by experimental results will be discussed. The emphasis will be on papers published in peer-reviewed journals. A few articles will be mentioned from non-peer-reviewed journals but only if they influenced the development of science-based ideas for the regulatory process. Environmental issues will not be dealt with.

Safety evaluation of whole foods derived from crops with considerable natural variability is more difficult than that of a single chemical, pharmaceutical or food additive, or defined mixtures of them. Published results of tests for toxicity and nutritional wholesomeness of complex foodstuffs are therefore few and far between. A recent comment in *Science* described this in its title: 'Health risks of genetically modified foods: many opinions but few data' (Domingo, 2000). Even a cursory look at the list of references of a recent major review on food safety issues (Kuiper *et al.*, 2001) showed that most of the publications referred to were non-peer-reviewed institutional opinions or envisaged future scientific and methodological developments for safety assessments, but were short on actual published scientific papers on which a reliable database of safety could be founded. Judging by the absence of published data in peer-reviewed scientific literature, apparently no human clinical trials with GM food have ever been conducted. Most attempts to establish the safety of GM food have been indirect. At best, inferences have been drawn from animal trials, but the preferred approach is to use compositional comparisons between the GM foodstuff and its traditional counterpart. If these results show no significant differences, the two foodstuffs are 'substantially equivalent', meaning that the GM food is as safe as the non-GM food. Thus, as the regulation is almost exclusively dependent on 'substantial equivalence', the published results of GM food analyses and inferences drawn from them for health will be examined critically in this review.

In genetic modification, the intended gene is incorporated into the genome of a crop using a vector containing several other genes, including, as a minimum, viral promoters, transcription terminators, antibiotic resistance marker genes and reporter genes. Although in GM food safety the role of the intended gene is very important, the potential

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effects of these other genes need also to be taken into account because other parts of the construct or the insertion of the vector could contribute substantially to the overall effect (Ewen and Pusztai, 1999a). There is in fact some evidence that some of the other genes of the vector may have an effect on safety. This is particularly so as it is now known that DNA does not always break down in the alimentary tract (Schubbert *et al*., 1994, 1998; Hohlweg and Doerfler, 2001). This opens up the possibility that the antibiotic resistance marker gene, in addition to others, may be taken up by bacteria in the digestive tract and contribute to the spreading of antibiotic resistance via human gut bacteria. In this context, one potentially important observation was that a substantial proportion (6–25%) of a genetically engineered plasmid survived a 1-h exposure to human saliva (Mercer *et al*., 1999). Partially degraded plasmid DNA also successfully transformed *Streptococcus gordonii*, a bacterium that normally lives in the human mouth. Saliva also contains factors which increase the ability of bacteria to become transformed by naked DNA. Therefore, the prospect of the uptake of undegraded or partially degraded vector genes, including the antibiotic resistance gene, will have to be seriously considered. However, the main concern in GM food safety is what are the direct effects of the expression of the main intended gene after its insertion into the plant genome via a gene construct. An additional concern is that this may also cause significant, indirect and unintended effects on the expression and functionality of the plant's own genes. The number of copies of the construct inserted and their location in the plant genome (pleiotropic effect) are of particular importance in this respect, with the possibility that many unexpected changes may occur. This possibility is in fact generally accepted, and the inadequacy of the currently used methods to detect them is frequently acknowledged (Kuiper *et al*., 2001). Pleiotropic effects always occur with both conventional cross-breeding and genetic engineering, and their unwanted consequences usually are eliminated by empirically selecting for the desired trait and discarding the potentially harmful ones. Some of these changes are unpredictable and therefore we can only compare the known properties and constituents but cannot look for, or even less analyse, unknown components. This imposes limitations on our selection criteria. Reliance based solely on chemical analysis of macro/micronutrients and known toxins is at best inadequate and, at worst, dangerous. More sophisticated analytical methods need to be devised, such as mRNA fingerprinting, proteomics and secondary metabolite profiling (Kuiper *et al*., 1999). However, and most importantly, there is an urgent need to develop comprehensive toxicological/nutritional methods to screen for the unintended potentially deleterious consequences for human/animal health of genetic manipulation to pinpoint the problems in advance of the incorporation of the GM foodstuff into the food chain (Ewen and Pusztai, 1999b). Although some limited animal tests have been done, only a few of these have been published. However, data from some of these studies recently have been placed on the Internet. Although they were not peer reviewed, they were incorporated into this review because of their potential importance for other scientists.

Non-peer-reviewed Safety Tests on Commercial GM Crops in the Public Domain

FLAVR SAVR™ tomatoes

The first example of official safety evaluations of a GM crop, Calgene's FLAVR SAVR™ tomato, including a 28-day rat feeding trial, was commissioned by Calgene for the Food and Drug Administration (FDA) before its general release. Although the details of this study have never been published properly, because this work had such an extraordinarily major effect and influenced GM food regulation in the USA and elsewhere, there is a compelling need to analyse the methods used and the conclusions reached. Fortunately, as a result of a court case in the USA (Alliance for Bio-Integrity *et al*. vs. Shalala *et al*.), most data in the FDA's files are now on the Internet in the public

domain and can therefore be evaluated (Alliance for Bio-Integrity, 1998).

This GM tomato study shows most of the problems which may be encountered in GM food safety evaluation, particularly if, like the tomato, they are fruits rather than foodstuffs and their protein and energy contents are insufficient for supporting the growth of young animals. The methods used and results obtained in this study are important not only for their own sake but also for their influence on the process of regulation.

Substantial equivalence

As 'substantial equivalence' features so prominently in GM food regulation (Kuiper *et al*., 2001), including in this GM tomato study, there is a need to look more closely at this concept. This issue has been dealt with in some depth by a recent article (Millstone *et al*., 1999) in which the problems with this concept were highlighted, such as that 'substantial equivalence' has never been defined properly and that there are no legally binding rules on how to establish it in practice.

Differences in growth conditions can have a serious impact on composition and, therefore, in the absence of specification of the origin and the conditions of cultivation of the different GM and non-GM samples, strict scientific comparisons cannot be made. These are not valid unless the parent line is grown side by side with the GM line. Comparisons with historical or literary values have only limited scientific validity.

'Substantial equivalence' is a crude, non-scientific concept. It provides a loophole for the GM biotechnology companies not to carry out nutritional and toxicological animal tests to establish whether the biological effect of the GM crop-based foodstuff is substantially equivalent to that of its non-GM counterpart. It therefore allows them to claim that there is no need for biological testing because the GM crops are similar to their conventional counterpart, while on the other hand, because they contain novel genes from other organism(s), they are patentable. However, unintentional and unpredictable changes can occur in plants because of the incorporation and positioning of the vector in the plant genome. It cannot therefore be known which of the hundreds of components of the GM crop may carry toxic or allergenic properties. As most of these are unknown, by definition, they cannot be included in analytical comparisons. Determination of the amounts of protein, carbohydrates, fats and other nutrients can only be a starting point. The consumption of minor and unexpected constituents of potentially high biological activity may have considerable and disproportionately large effects on the digestive tract. Their presence, therefore, can only be revealed from animal studies, and this makes it imperative that these are performed with a flawless design and experimentation.

The FLAVR SAVR™ tomato was produced by 'antisense' GM technology. As part of its safety evaluation, it was subjected to compositional analysis for total protein, vitamins and minerals to establish whether any unexpected changes in gross fruit composition had arisen as a result of the integration of the FLAVR SAVR™ and *kan*^{r} genes into the tomato genome. It was claimed that no significant changes were found and that the contents of potentially toxic glycoalkaloids, particularly tomatine, and to a lesser extent solanine and chaconine, were also similar (Redenbaugh *et al*., 1992) and therefore this GM tomato was substantially equivalent to other non-GM tomato lines. However, to supplement these, several feeding studies were also performed by commercial laboratories at the request of the FDA.

Acute toxicity

First, range-finding, limit acute oral toxicity tests of the processed tomatoes in rats were carried out by the IIT Research Institute of the Life Sciences Department (Chicago, Illinois, USA). A single dose of the homogenates prepared from about 80 g of various GM and control tomatoes, respectively, was administered (15 ml kg^{-1}) by gavage to groups of Harlan Sprague–Dawley rats (five male or female rats per group) fed *ad libitum* on rat chow for 14 days to establish whether the GM tomatoes were toxic or not. As claimed, no test substance-related mortalities occurred and increases in mean body weights were not

significantly different between GM and control groups. However, as the range of the rats' starting weights was unacceptably wide (female rats weighed 131–186 g (± 18%) and male rats 159–254 g (± 23%)), in such a short (14 day) study with five rats per group, it would have been difficult for significant differences to develop. For comparison, only a few per cent variation in starting weights is permitted in papers published in high-quality nutritional journals. Thus, the poor design of this feeding study largely invalidated the conclusions that GM tomatoes were not toxic. To supplement these, three more rat feedings studies of similar design were carried out by International Research and Development Corporation (Mattawan, Michigan, USA).

Twenty-eight-day toxicology/histology study

Of the three studies, the most complete set of data is available for the second. In this, four groups of rats (20 males and 20 females per group) fed standard rat chow for 28 days were gavaged twice daily with homogenized tomatoes (15 ml kg^{-1}). Two groups were given GM tomatoes, CR3–613 or CR3–623 (CR3–623 is the commercial FLAVR SAVR™ tomato). There were two control groups, one of which was gavaged with the parent CR3 tomato homogenates and a second control group in which the rats were gavaged with water even though the relevance of this group is somewhat questionable. At the request of Calgene, an expert panel was retained (ENVIRON Corp., Arlington, Virginia, USA) to evaluate the data. They concluded that gavaging rats with GM tomato purée resulted in no significant changes in body weight, food consumption and clinical chemistry or haematology parameters in comparison with control tomatoes. However, there was a possible treatment-related increase in glandular stomach erosion/necrosis in four out of 20 female rats but none in the controls or in male rats at the end of the 28-day feeding period. The number of four female rats was increased to seven when the histology slides were re-scored by PATHCO, an independent pathology working group. This prompted a repeat study in which the dose of the tomato purée was increased by twofold. Unfortunately, in this study, some of the CR3 control and CR3–623 GM tomato lines were grown at different locations and harvested at different times from those in the second experiment. However, this was not regarded as important by the expert panel even though, when the same tomatoes were used as in the second experiment, the results appeared to show similar tendencies; two out of the 15 females developed stomach glandular erosions with the GM tomatoes, while none were found in the control females. However, in a not clearly understandable way, the ENVIRON panel concluded that the lesion of glandular erosion was not related to the administration of GM tomatoes. According to them, such lesions occur spontaneously in animals that are stressed or given mucolytic agents, when food is restricted or when animals are restrained in cages, even though these parameters have not been investigated systematically. Moreover, none of these circumstances applied, since tomatoes contain no mucolytic agents, food was provided *ad libitum* and the rats were not restrained. It was also suggested that, because the lesions were possibly of short duration, they were incidental, not related to the test material and would have healed spontaneously. Unfortunately, none of these assumptions was confirmed by further experimentation as no samples other than those at the end of the 28-day experiment were taken to probe into the timing and reversibility of the incidence of the stomach lesions. Clearly, the results of these three studies should have prompted more experimentation to investigate in more detail the effect of GM tomatoes on stomach histology and, what is even more important, these studies should have been extended to include the possible effects of GM tomatoes on both the small and large intestines.

The red or dark red pin-point lesions present in the stomach of female rats which were described as necrosis would be termed 'erosion' in human pathology, which may have sequelae, such as life-endangering

haemorrhage. Erosions cannot be termed 'mild', as unpredictable haemorrhage can occur in the elderly human, particularly on low dosage aspirin to prevent thrombotic events, and synergy with transgenic tomatoes may occur. The assumption that the lesions are related to stress does not explain the low incidence in other groups, particularly in the second study. The relevance and significance of gastric erosions in the human may be a matter of life and death in the older age groups. It has been implied that pathologists in general might not report such a lesion but, in the present era of vexatious litigation, mention would have been made in any human pathologist's report to avoid an accusation of negligence. This may not be required in veterinary pathology but these rat studies were done with humans in mind and therefore the pathology findings must be put in this human context. It is probably true to suggest that these lesions are of short duration, but the serious nature of erosive lesions should not be trivialized. This is the more serious because seven out of 40 rats eating GM tomatoes died within 2 weeks. The nature of these deaths was not specified and the evidence that they were not related to the ingestion of transgenic tomatoes was inconclusive.

In a further development, the Scientific Committee on Food of the European Commission Directorate C (2000) concurred with the conclusion reached in the US Food and Drug Administration (1994) memorandum. In their opinion, although the results showed an unexplained disparity, they were not supportive of a substance-related effect of the FLAVR SAVR™ tomato. However, it is likely that the EU Committee may not have seen all the primary data and their opinion would therefore have been based on incomplete evidence. It is also regrettable that, by ascribing the gastric erosions in rats to 'an artefact of gavage studies', the EU Committee has in fact labelled the scientists carrying out the work as incompetent. As these erosions were found at the end of a 28-day study during which 160 rats were gavaged twice daily with tomatoes, it is unlikely that even poorly trained workers would not have become more competent, so as to avoid causing such an anomaly.

Effects on body weight, food intake and organ weights

The conclusion of the ENVIRON panel that feeding rats on GM tomatoes (CR3–623) for 28 days had no effect on weight gain, feed intake and organ weights could not be justified because the starting weights of the rats were so widely different – a range of 130–258 g (± 33%) for males and 114–175 g (± 21%) for females – that finding significant differences in weight gain, feed intake and organ weights was not likely. Indeed, weight gains varied between wide limits (102–230 g for males and 46–127 g for females) in 28 days. Even under these conditions, although the average starting weight of the male rats gavaged with CR3–623 GM tomatoes was the highest (148.1 g), their final weight (316.5 g) was the lowest. Accordingly, the rats gavaged with GM tomatoes grew the least of the four groups of rats. The feed intake of the different groups also varied between wide limits; 133–203 g for males and 102–153 g for females. Not surprisingly, the feed conversion efficiency (weight gain/total feed intake) of female rats on GM tomatoes (0.152) was significantly ($P < 0.05$) less than that (0.167) obtained for female rats on control non-GM CR3 tomatoes.

The large range of starting weight differences also excluded the possibility of finding significant differences in the organ weights of the four groups of rats. The standard deviations of mean values were very large, in some instances more than 20%. It is the more remarkable that, even under these conditions, some differences in organ weights were found, including the testes for males and the thyroid/parathyroid for females. Finding no significant differences in biochemical, haematology and ophthalmology parameters between GM and non-GM tomatoes was not unexpected either, because of the large initial body weight differences.

Overall, it is regrettable that these rat toxicological feeding studies were poorly

designed, as a great deal of effort, work and money must have been spent on them and so much rested on the outcome. The FDA's conclusion that FLAVR SAVR™ presented no more dangers to consumers than ordinary tomatoes does not therefore appear to rest on good science and evidence which could stand up to critical examination. Rather tellingly, the results of these studies have never been published in peer-reviewed journals. The study as described not only raises questions about the design, methods and conclusions for this study but also whether they could have any general validity for other GM foods. In this light, it is the more surprising that after these studies the FDA has required no nutritional/toxicological testing of other GM foods.

Aventis's Chardon LL herbicide-resistant GM maize

Due to the UK government's attempt to place Chardon LL seed on the National List, a part of the supporting evidence submitted by Aventis contained data on the composition of two lines of seed to establish their substantial equivalence to the conventional parent maize line. The evidence also included the results of a 14-day rat feeding study. All this is to be found in a file deposited by the Ministry of Agriculture, Fisheries and Food (MAFF) with the British Library (British Library File, 1997).

Compositional analysis

In the absence of specifying the origin and conditions of cultivation of the different GM and non-GM samples, strict scientific comparisons could not be made between them. However, even under these conditions, the composition of T14 and T25 GM maize expressing phosphinothricin acetyltransferase enzyme (PAT-PROTEIN) showed many statistically significant differences in fat and carbohydrate contents in comparison with non-GM grain samples, and fat, protein and fibre between silage samples from GM and non-GM maize. Thus, the conclusion that GM maize is not 'materially different' from current commercial varieties cannot be regarded as valid.

Repeated dose oral toxicity (14-day feeding) study in rats

The rationale for this study was to assess the cumulative toxicity of PAT-PROTEIN given to rats in their diet for 14 days and to provide a rational basis for toxicological risk assessment in man. Although testing of the PAT-PROTEIN can be commended, this study was no substitute for the nutritional testing of the entire GM plant, seeds, vegetative parts and silage in all target animal species. Without these, the potentially harmful, unintended and unpredictable effects of the gene transfer, other components of the vector and gene insertion (positioning effect) cannot be established or excluded.

Unfortunately, as the design of the experiment was faulty, it is difficult to draw valid conclusions from a feeding study, carried out with five rats per group, in which the starting weight of the rats varied by more than ±20% (53–82 g for males and 50–74 for females) rather than the usual ±2%. For any differences to reach significance, they needed to exceed ± 20%, and to achieve this in a 14-day study would have required catastrophic experimental conditions. The five rats per group were not housed singly and therefore their individual feed intakes could not be monitored even though the huge differences in the starting weights should have led to major differences in the feed intakes of the individual rats. Moreover, the group feed intakes were not measured continuously. There were four groups of rats (five male/female rats per group) in the experiment. However, rats in group 1 were fed a different diet (full rat chow) from the other three groups and therefore group 1 was not appropriate for (statistical) comparisons. The diet of the second group contained 5 g kg^{-1} and the third group had 50 g kg^{-1} PAT-PROTEIN mixed in with 45 and 0 g kg^{-1}, respectively, of commercial (SOJAMIN, KLIBA Muhlen AG) low soybean protein diet (11% raw protein). The diet of the fourth group contained 50 g kg^{-1} SOJAMIN but no PAT-PROTEIN. Thus, for statistical analysis, the second and third groups ought to

have been compared with rats in the fourth group. Curiously, although the main target organ of the PAT-PROTEIN fed to rats was the digestive tract (and pancreas), the weights of these were not measured. This is a major experimental design fault.

The starting weight and the feed intake of the third group (high PAT-PROTEIN) were the highest, but they ended up with the lowest final body weight. This indicated an elevated metabolic activity, probably induced by the PAT-PROTEIN. Our analysis of variance (ANOVA) shows that the weight gain for both male and female rats on the high PAT-PROTEIN diet (group 3: 65.2 and 43.6 g for males and females, respectively) was significantly ($P < 0.05$) less than that of either the fourth group (control: 72.8 and 48.8 g for males and females, respectively) or group 2 (low PAT-PROTEIN diet: 73.4 and 44.4 g for males and females, respectively). As PAT-PROTEIN reduced feed conversion efficiency, it is potentially harmful. The conclusion that 'there were no differences which could be attributed to treatment with the test article' was therefore not valid. Similarly, that 'there were no changes on … clinical biochemistry and urine analysis after 14 days' is not valid either as the authors' own results described differences between the groups in glucose, cholesterol, triglyceride and phospholipid levels, indicating an increased metabolic functional load in the rats. It is unexplained why these differences were dismissed by the authors as incidental and unrelated to the treatment. Our ANOVA analysis revealed that the urine output in rats on the high PAT-PROTEIN diet was significantly ($P < 0.05$) reduced, indicating treatment-related effects (urine output of 5.4 and 4.4 ml for males and females in group 3 vs. 7.1 and 6.5 ml for males and females, respectively, in control group 4).

The large differences in the starting weight of the rats probably prevented finding significant differences in organ weights. However, even under these conditions, rats fed the high level PAT-PROTEIN diet (third group) had the lowest liver, thymus and spleen weights of all groups (even though the differences with controls were not significant). This is of particular importance because the macroscopic findings indicated thymus foci in 20–40% of the animals fed diets containing the PAT-PROTEIN.

In conclusion, the design and execution of this feeding study were poor and, contrary to the authors' conclusions, the results indicated treatment-related effects induced by PAT-PROTEIN (of unspecified origin). The results therefore could not be taken as evidence that the transfer of its gene into maize represented no risk for the rat and, by inference, for humans, particularly as no gut histology studies have been completed so far. Finally, a recent publication (Chiter *et al*., 2000) showed that DNA survived in intact form or slightly fragmented unless the GM maize was heat processed extensively. Therefore, the possibility exists that with underprocessed maize products humans and animals might be exposed to the DNA used in the genetic engineering.

Compositional Studies Published in Peer-reviewed Journals

Herbicide-resistant soybean

Befitting its importance in both human and animal nutrition, a great deal of attention has been given to the compositional analysis of herbicide-resistant and other GM soybeans. Several publications appeared in nutritional and other journals demonstrating the compositional 'substantial equivalence' of GM and non-GM soya. Thus, it was claimed that the macronutrient composition of glyphosate-tolerant soybean (GTS) seeds resulting from the transformation of conventional soybean with a gene encoding 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium*, to make the soya herbicide resistant, was equivalent to that of conventional soybeans. This applied equally to GTS unsprayed with glyphosate (Padgette *et al*., 1996) or sprayed with this herbicide (Taylor *et al*., 1999). It was claimed that the results of proximate chemical analyses of the contents of crude protein, oil, ash, fibre, carbohydrates and amino acids of solvent-extracted and toasted or untoasted soybean meals of unsprayed GTS and control soybean had shown that all these lines were

substantially equivalent (Padgette *et al*., 1996). Similar findings were described for sprayed GTS (Taylor *et al*., 1999). Although this appeared to be true for most macronutrients, several significant differences between GM and control lines, such as in ash, fat and carbohydrate contents, were also found (Padgette *et al*., 1996). However, these were not regarded as having biological significance.

A closer inspection of the data in the papers, however, revealed that the statistical comparison of the macronutrients of GM and non-GM lines was not scientifically valid. Instead of comparing their amounts in a sufficiently large number of samples of each individual GTS with its appropriate individual parent line grown side by side at the same location and harvested at the same time to establish whether they were 'substantially equivalent', what the authors compared was a large number of different samples from different locations and harvest times. As growth conditions have a major influence on seed composition, the range of the amounts of constituents in the different samples, regardless of whether they were GM or non-GM, was so great (±10% or more) that the chances of finding statistically significant differences were unreal. It is possible that from a practical point of view the variation in protein concentration of samples of the three lines of between 36.8 and 45% would fall into the normal range of agronomic variability of soybeans and therefore may not be of major concern for agronomists. However, this comparison is not strict enough to establish whether the genetic modification introduced any unintended compositional changes. What is remarkable is that, even with this approach, many significant changes in macronutrient levels were found. Thus, the claim of 'substantial equivalence' of GTS lines with non-GM soybean is not supported by rigorous scientific evidence.

The potential importance in human health of natural isoflavones, such as genistein, daidzein and coumestrol present in soybeans, is generally recognized. It was, therefore, of considerable interest whether any changes occurred in these components as a result of genetic modification. Here the published evidence is controversial. Thus, while in some studies no meaningful differences were reported (Padgette *et al*., 1996; Taylor *et al*., 1999), an independent study claimed that GM soya samples had consistently contained significantly fewer isoflavones than the parent cultivars (Lappe *et al*., 1999). In one respect, all authors agreed, i.e. that the isoflavone content of soybean seeds showed considerable variability between sites and was dependent on agronomic conditions. However, Lappe *et al*. (1999) went further and claimed that, while the variability of the GM samples was indeed considerable, conventional soybeans showed less variation in isoflavone content. As the isoflavone content of soybeans might affect human health, there needs to be more awareness of potential health problems due to this variability. While the precise details of the changes in isoflavone content on genetic modification will have to be established in the future, to ensure clinical consistency, the origin and the actual phyto-oestrogen levels in soybean may need to be standardized.

In the study by Padgette *et al*. (1996), no significant differences were found in the levels of antinutrients, such as trypsin inhibitors, lectin and oligosaccharide flatulence factors, between solvent-extracted, toasted or untoasted GM and non-GM soybean seeds. However, the comparisons were made by the same method as for macronutrients and therefore the large range of natural variability excluded the possibility of finding significant differences. Interestingly, in single soybean meal samples of each of the two GTS and parent lines, the trypsin inhibitor (also a major allergen in soybean) content was substantially higher, by almost 30%, in one of the two GTS lines, with a smaller increase in the other. No trypsin inhibitor analyses were performed on the protein isolate or protein concentrate samples originating from the meal samples. Although there were other compositional differences in these processed soybean products, it is difficult to decide from single determinations whether these were significantly different or not.

In conclusion, there is insufficient evidence to date to decide whether the composition of GM and conventional soybeans is equivalent or not. In fact, some data, particularly those for phyto-oestrogens, were

significantly different. Furthermore, because not strictly comparable compositional data were used, the case for equivalence was not properly established. There is therefore an obvious need for further more critical studies.

GM potatoes

Brief references to GM potatoes, particularly those expressing *Bacillus thuringiensis* (Bt) toxin, can be found in non-peer-reviewed book chapters or other articles. In most instances, these contain no data and are therefore of little scientific value. There are two exceptions, one of which is an article on the safety assessment of GM potatoes expressing the soybean glycinin gene (Hashimoto *et al*., 1999a). However, it is not quite clear what the authors wanted to achieve because, at the expression level of glycinin in potatoes of between 12 and 31 mg g^{-1} total soluble protein, no significant improvements in the protein content or amino acid profile could have been expected. Indeed, the results in the paper demonstrated that the total protein content of the GM potatoes appeared to be significantly less than that of the control line and that no improvement in the essential amino acid profile was achieved either. There appeared to be substantial differences in some vitamins between GM and control lines, and the amounts of both solanine and chaconine increased in the GM lines. It is, therefore, not quite clear why it was claimed by the authors that their GM lines were equivalent to the parent line and could be utilized as safely. The other more recent study is a conventional compositional analysis of some macro- and micronutrients of tubers from insect- and virus-resistant potato plants (Rogan *et al*., 2000) performed by methods which currently are accepted by most novel food regulatory bodies. Although these showed some significant differences in a number of tuber constituents, in the absence of toxicological/nutritional animal studies it is difficult to ascertain whether these differences could have any biological effects on humans/ animals, particularly as these conventional analyses could not have revealed the development of any unknown possible toxic/antinutritive components. Additionally, known antinutrients, such as lectins or enzyme inhibitors, were not included in the analysis.

GM rice

GM rice lines expressing the soybean glycinin gene have been developed (Momma *et al*., 1999) by a method similar to that used for GM potatoes. The glycinin expression level was between 40 and 50 mg glycinin g^{-1} total rice protein. The GM rice was claimed to contain 20% more protein, but its moisture content was less than that of the parent line. However, from the paper, it is not quite clear whether the increased protein content was due to the decreased moisture content of the seeds because it was not specified whether the values were expressed for air-dried or fully dried seeds. Thus, most of the arguments in the discussion of whether the higher protein level was due to the positioning effect of gene insertion or metabolic interference will have to await clarification by further work.

GM cotton

Several lines of GM cotton plants have been developed using the gene encoding an insecticidal protein from *B. thuringiensis* subsp. *kurstaki*. These had increased protection against the major lepidopteran insect pests of cotton. As cottonseed is an important source of oil for human consumption, and cottonseed and processed cottonseed meal for animal feed, extensive analytical work has been done to establish whether the GM lines were 'substantially equivalent' to conventional lines (Berberich *et al*., 1996). The levels of protein, fat, carbohydrate, moisture, ash, amino acids and fatty acids in the insect-protected lines were claimed to be comparable with those found in commercial varieties. Moreover, the levels of antinutrients such as gossypol, cyclopropenoid fatty acids and aflatoxin were similar to or less than those in conventional seeds. Thus, the GM varieties

were suggested to be equivalent to conventional seeds and just as nutritious. However, the statistics used by the authors were identical to those used with glyphosate-resistant soya and therefore could be similarly criticized. Although the content of known constituents fell in between the wide range of values of commercial conventional lines, this did not mean that they were compositionally equivalent, particularly as environmental stress could have major and unpredictable effects on antinutrient and toxin levels (Novak and Haslberger, 2000). Thus, without animal experimentation, this approach could not reveal whether any new and unknown toxins/allergens had been created or not.

GM maize

A glyphosate-tolerant (Roundup Ready) maize line GA21 has recently been developed. It was claimed (Sidhu *et al*., 2000) that, except for a few minor differences, which the authors think are unlikely to be of biological significance, the results of compositional analyses of proximate, fibre, amino acid, fatty acid and mineral contents of the grain, and proximate, fibre and mineral contents of forage collected from 16 field sites over two growing seasons showed that control and GM lines were comparable. The comparison was carried out by a method similar to that described for GTS soya (Padgette *et al*., 1996) and this may therefore not be scientifically rigorous enough for the establishment of substantial equivalence.

Nutritional/Toxicological Studies Published in Peer-reviewed Journals

Herbicide-resistant soybean

As part of a safety assessment of GTS, the feeding value, wholesomeness (Hammond *et al*., 1996) and possible toxicity (Harrison *et al*., 1996) of two major GM lines of GTS were compared with those of the parent line. Processed GTS meal was included in the diets of rats, broiler chickens, catfish and dairy cows at the same concentrations as in commercial non-GM soybean rations. Rats and dairy cows were fed these diets for 4, broilers for 6 and catfish for 10 weeks. It was claimed that in rats, catfish and broilers the growth and feed conversion efficiency, in catfish the fillet composition, in broilers the breast muscle and fat pad weights, and in dairy cows milk production and composition, rumen fermentation and digestibilities were similar for both GTS and parental lines. According to the authors, these results confirmed that the GTS and parental lines had similar feeding values.

Rat studies

A critical evaluation of the rat study was hampered by the lack of adequate primary individual data in the paper. Thus, there was no full description of the rat diet. It appears that the total protein content of the diets was adjusted to 247 g kg^{-1} diet to be isonitrogenous with Purina Laboratory Rat Chow by the addition of 24.8 g of GTS and parent soybean meals, respectively (~10% protein), to a base diet. All comparisons were made with rats fed commercial Purina Chow. The protein concentration in these diets was, however, appreciably higher than the usual 10–16% crude protein and exceeded the protein requirements of the rat. This extra protein potentially could have masked any possible transgene product effects, particularly with the raw unprocessed soybean diets in which the GM meals were incorporated only at the level of 50 or 100 g kg^{-1} of the diet. Thus, these meals only replaced 8.5 and 17%, respectively, of the total protein of 247 g kg^{-1} diet. In other words, the GM soybean protein in these meals was diluted by other dietary proteins by 12- and 6-fold, respectively, producing another possible masking effect. The composition of the control Purina Chow diet in the ground raw soybean feeding study was not described. This is important because the identity of the raw control soybeans included in the Purina Chow control diet was not specified.

In the feeding study, four groups of rats (ten males and ten females in each group) singly housed were fed diets containing the

parental line or the GTS lines (40-3-2 or 61-67-1) for 28 days. No individual values (or their ranges) for feed intake or body weight were given. The bar diagrams of the combined body weight of rats at the end of each week of the 4-week experiment were rather uninformative. However, it was observed by the authors that the Purina Chow-fed male rats grew significantly better than the three experimental groups fed toasted soybeans (including the parental line). This was attributed to better commercial processing. However, the bar diagrams also indicated that the growth in the group fed with one of the GTS lines (61-67-1) was probably equal to that of the Purina Chow-fed control and, therefore, by inference, these rats also grew significantly better than the other two experimental lines (the GTS line 40-3-2 and the parental line). This again underlined the importance of giving individual data in papers, without which it is difficult to assess the results. Similarly, there were no individual data for organ weights, such as liver, kidneys and testes. However, it was claimed that the kidney weights of the raw GTS line-fed (and parental control?) male rats were significantly higher than those of the controls, while the testes of the parental line-fed rats were significantly enlarged. According to the authors, as these differences were neither dose related nor only shown by the parental line, they were not caused by genetic modification. Rather curiously, the weights of the stomach and intestines, the main target organ in any nutritional testing, were not recorded. Observations were not recorded on other organs, and no histology appears to have been done on these tissues either. The only tissue which was subjected to microscopy was the pancreas, but the description of the findings was qualitative. Only minimal to mild lesions were found and these were claimed to be common to all groups. However, under these conditions, this was not surprising because no pancreatic hypertrophy was found. This was probably due to the effect of the unusually high dietary protein concentration, which, as the authors pointed out, masked and/or diluted the biological effect of the trypsin inhibitors. This is of particular concern because the trypsin inhibitor content of GTS lines in unprocessed soybean was significantly higher than in the control line (Padgette *et al*., 1996).

It is regrettable that the design of this important rat feeding study had such unfortunate omissions. It is of particular concern that no histology was apparently carried out on gut tissue. Thus, more critical work is needed to decide whether the feeding value of GM and non-GM soybeans is equal or not.

Chicken study

The broiler chicken feeding study's experimental design closely followed that of commercial practice and therefore the results should only be indicative of the commercial feeding and production value of the various soybean lines. As the data were pooled from all birds fed on the same diet, it is not easy to see what, if anything, was the significance of the small differences found in the study, such as the slightly lower body weights, breast and fat pad weights obtained with the GTS lines (particularly with GTS 40-3-2) for the utilization of GM soybean. It would have been better to measure the nutritional performance of individual birds (or small groups) fed on different diets and then compare them after statistical analysis. In the absence of this, we have to rely on the authors' conclusion that the design of the experiment gave the upper limit of differences in weight gain of 3.5% and gain/feed ratio of 2% and that the GTS lines vs. parental line were within this limit. Thus, with this restriction, the feeding value of the GTS lines for broilers was practically equal to that of the parental soybean line.

Catfish experiment

Catfish are excellent and highly sensitive indicators for the feeding value of diets. It was obvious from the results that, similar to the findings with rats, one of the GTS lines, 61-67-1, was superior to the other lines (GTS 40-3-2 and the parental line) in most respects. Thus, fish on GTS line 61-67-1 ate more, had better weight gain and gain/feed ratio and weighed more at the end of the 10-week study than the others, even though the composition of the fillets from these fish was not significantly different. This significant

difference in performance must, therefore, indicate that genetic modification may not be as reproducible as it has been claimed and that the feeding value and metabolic effects of GM and parent lines are not always 'substantially equivalent'.

Study on lactating cows

Milk production and composition and performance data in the lactating cow study showed some significant differences between cows fed diets containing the different lines of soybean, indicating a lack of 'substantial equivalence'. In view of these differences, even though we may not at present know all their biological/nutritional consequences, it may be difficult to maintain the view that the feeding value of the GTS and parent lines is equal, and further work is needed to establish whether the GTS lines are safe or not for humans/animals.

Testing of E. coli *recombinant gene product*

Extensive studies have been carried out to ascertain the safety of the gene product, 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), which renders the soybeans glyphosate resistant (Harrison *et al*., 1996). Unfortunately, there are some flaws in these experiments, the most important of which is that in the acute gavage studies the authors did not use the enzyme isolated from GTS lines but instead that from *Escherichia coli*. Although they were at pains to show that the EPSPS enzyme samples from the two sources were similar in lack of glycosylation, molecular size, reaction with a polyclonal anti-EPSPS antibody and enzyme assays, these methods do not have sufficient power to show unequivocally whether they were identical. The authors themselves pointed out that post-translational modification of the completed polypeptide chains emerging from the ribosomes may be done differently in two such evolutionarily distinct life forms as higher plants and prokaryotic bacteria. Amidation, acetylation and proteolytic processing can have such major effects on the conformation of the protein as to make these gene products behave differently in the digestive system. Thus, the use of the *E. coli* recombinant protein for the acute mice gavage studies may invalidate the authors' conclusion that the gene product from soybean did not have any toxic effects. These studies must be re-done with the gene product isolated from the transgenic plant before the results could be accepted. In any case, in such gavage studies, young, rapidly growing animals must be used to show any distinct effect on growth. As all animal weights were unchanged in the experiment, the test system used could not have detected any effect unless the consequences of the gavaging had been disastrous. Feeding studies with the gene product in young rapidly growing rodents should be the preferred method for the demonstration of the deleterious effects.

The other flaw in the experimental design was the reliance on an *in vitro* simulated gastric/intestinal digestion assay, which was also carried out with the *E. coli* recombinant gene product. To obtain physiologically valid results, it would have been necessary to use the gene product isolated from GM soybean in an *in vivo* assay in the rat (or other suitable animals; see Rubio *et al*., 1994) or a full feeding trial. Thus, it has been shown before that the kidney bean (*Phaseolus vulgaris*) α-amylase inhibitor is fairly stable to proteolytic degradation in the rat gut (Pusztai *et al*., 1995, 1999), but, when its gene was expressed in peas (*Pisum sativum*), it was rapidly digested and inactivated in the rat stomach/small intestine *in vivo* (Pusztai *et al*., 1999). This may have contributed to the safety of GM peas for rats and, by inference, possibly for other monogastric mammals. Thus, *in vitro* digestion assays may have little relevance to the safety of GM food crops.

In a separate feeding study (Teshima *et al*., 2000), the possible harmful effects of toasted glyphosate-resistant GM soybean were investigated at 300 g kg^{-1} inclusion level in the diet of rats and mice. After feeding these animals for 15 weeks, no significant differences in nutritional performance, organ development, histopathology of the thymus, liver, spleen, mesenteric lymph nodes, Peyer's patches and small intestine, and the production of IgE and IgG humoral antibodies between GM and non-GM line diets were

found. However, as rats grew less than 30 g and mice not at all in 15 weeks, the conditions were so unphysiological that no valid conclusions could be drawn from these experiments.

GM maize

In a major commercial-scale broiler chicken feeding study with rations containing transgenic Event 176-derived Bt maize involving 1280 birds (Brake and Vlachos, 1998), it was claimed that no statistically significant differences in survival or bird weights between birds fed diets containing GM maize, Event 176, or an isogenic parent maize line were found. Indeed, birds fed GM maize rations appeared to have significantly better feed conversion ratios and an improved yield of breast muscle. However, the authors cautioned against the conclusion that this enhanced performance could be attributed to the Bt maize *per se*. It is possible that the results might have been due to slight differences in the overall composition of the diets. This is reasonable considering the length of this study and possible problems of consistent diet preparation on a commercial scale. Minor differences in composition such as the slightly lower protein content of the GM maize and fat contents of the diets magnified to the scale of this trial make the results more relevant to commercial than to academic scientific studies.

In a poultry feeding study, it was claimed that the GA21 Roundup Ready maize-based diets gave similar performance data in growth, feed efficiency and fat pad weights to diets containing the parental control line (Sidhu *et al*., 2000). However, this and a similar study carried out in Germany with a maize line expressing PAT-PROTEIN (Flachowsky and Aulrich, 2001) were commercial production experiments and made little contribution to scientific safety assessment.

In a separate study, maize was genetically modified by the transfer of the gene of egg white avidin to make the seed resistant to storage insect pests (Kramer *et al*., 2000). It was also claimed that this GM maize was safe for mice as apparently, when, instead of a balanced diet, they were fed solely on this crop, the mice suffered no ill effects. However, the mice used in the experiment were adults which did not grow at all, and therefore the conclusion that the GM maize was safe is, at best, premature.

GM peas

Diets containing transgenic peas expressing the transgene for insecticidal bean α-amylase inhibitor (~3 g kg^{-1} peas) at two different inclusion levels in the diet, 300 or 650 g kg^{-1}, were subjected to nutritional evaluation with rats in a 10-day feeding trial (Pusztai *et al*., 1999). The nutritional performance of rats fed GM pea diets was compared with those obtained with rats pair-fed iso-proteinic and iso-energetic diets containing parent-line peas and also lactalbumin diets spiked with isolated bean and pea α-amylase inhibitors, respectively. At 300 g kg^{-1}, but not at 650 g kg^{-1} inclusion level, the nutritional value of diets containing transgenic or parent peas was not significantly different. Even at 650 g kg^{-1}, the difference was small, mainly because the transgenically expressed pea recombinant α-amylase inhibitor was quickly (in < 10 min) degraded in the rat digestive tract and therefore its antinutritive effect was abolished. In contrast, spiking the parental line pea diet with the stable bean α-amylase inhibitor reduced its nutritional value (Pusztai *et al*., 1995, 1999).

In this study, unfortunately, no gut histology was done or lymphocyte responsiveness measured, and therefore one had to rely on the evaluation of nutritional parameters that are inherently less sensitive in order to find possible differences in metabolic responses between GM and conventional food components. Although there were significant differences in the development of some organs, mainly the caecum and pancreas, most organ weights were remarkably similar. At the end of the study, cautious optimism was expressed that GM peas could be used in the diets of farm animals, particularly at the low/moderate levels recommended in

commercial practice and if the progress of the animals was monitored carefully. However, this relatively short feeding study with modest objectives cannot at this stage be taken as proof of the safety of GM peas for human consumption. There is a need to carry out further and more specific risk assessment testing procedures, which must be designed and developed with human consumers in mind. It also has to be kept in mind that only one particular line of GM peas was tested in which the endogenous antinutrient levels were selected to be similar to those of the parent peas. In some other GM lines, however, lectin levels could vary, up or down, by a factor of four. Moreover, in some field pea cultivars, such as Laura, the concentration of trypsin inhibitor increased by about 24% and the chymotrypsin by 100%, while the haemagglutinating activity decreased by a factor of four in the GM line compared with its parent (A. Pusztai, unpublished). This strengthens the argument that, in the safety assessment of GM crops, many lines should be included and that, from the results of a single GM line, no blanket approval should be given to other lines developed, even if in the transformation the same vector was used and carried out at the same time.

GM potatoes

There have been four independent studies on different GM potatoes.

Glycinin-expressing potatoes

The safety of transgenic potatoes expressing the soybean glycinin gene was evaluated in a short (4-week) rat feeding study (Hashimoto *et al*., 1999b). With an interesting experimental design, control rats and the experimental groups were fed the same control commercial diet. However, the rats were also daily force-fed by gavage with 2 g of respective potato lines kg^{-1} body weight. The potatoes used were a parental control line and two transformed lines, one with the glycinin gene and another one with a designed glycinin gene (coding for four additional methionines in the gene product), respectively. However, there were a number of problems with this study. Thus, although no difference in growth, feed intake, blood cell count, blood composition and internal organ weights between the groups was found, the uncertainty as to whether the animals were fed with raw or boiled/baked potatoes leaves a question mark over the interpretation of the results.

Bt toxin potatoes

An interesting, mainly histology, study was carried out on the ileum of mice fed with potatoes transformed with a *B. thuringiensis* var. *kurstaki Cry*I toxin gene. As a control, the effect of the toxin itself was also investigated (Fares and El-Sayed, 1998). It was shown that both the delta-endotoxin and, to a lesser extent, the Bt potato caused villus epithelial cell hypertrophy and multinucleation, disrupted microvilli caused mitochondrial degeneration, increased numbers of lysosomes and autophagic vacuoles, and increased activation of crypt Paneth cells. As a result, it was recommended that 'thorough tests of these new types of genetically engineered crops must be made to avoid the risks before marketing'. Unfortunately, some flaws in the experimental design detract from the strength of the conclusions. The most important of these was that, apart from indicating that the gene used in the transformation was the *Cry*I gene from *B. thuringiensis* var. *kurstaki*, there was no description of the Bt potatoes. The gene expression level in the GM potato was not given and it was not clear whether the potatoes in the diet were cooked or raw. Moreover, the amount of the Bt toxin used for supplementing the potatoes within the control potato diet was not specified either. This made it impossible to make a quantitative comparison of the effects on the ileum of the Bt potato with those of the spiked control potato diets. The assumption that the ileum is the most important absorptive part of the rodent small intestine could also be argued against, because 90% of all nutrient absorption in fact occurs in the jejunum. As this was an electron microscopy study, the fixation of the ileal samples was

not done on well-oriented sections but on chopped up fine tissue pieces, and important detail of villus organization was therefore lost. Finally, the delta-endotoxin-induced hyperplastic changes on ileal villi should have been demonstrated by measuring cell proliferation and mitotic rates in ileal (and jejunal) crypts rather than on the villi. However, despite these shortcomings, this study has established once and for all that, in contrast to general belief, exposure of the mouse gut (ileum) to the *CryI* gene product has caused profound hypertrophic and hyperplastic changes in cells of the gut absorptive epithelium and can lead to mucosal sensitization (Vazquez Padron *et al*., 1999, 2000b). This shows up the fallacy of drawing comforting conclusions from *in vitro* simulated gut proteolysis tests. Clearly, concerns about the possible biological consequences of exposure to GM food should be addressed under *in vivo* conditions because, even if an *E. coli* product breaks down *in vitro*, this does not necessarily mean that the same gene product expressed in the transgenic crop should also break down.

GNA GM potatoes

Some of the results of rat feeding studies with GM potatoes expressing the snowdrop (*Galanthus nivalis*) bulb lectin (GNA) gene were similar to the results of Fares and El-Sayed (1998). A part of this work concerning the effect of GNA GM potatoes on the histology of different compartments of the rat gut was published (Ewen and Pusztai, 1999a). Although this peer-reviewed scientific paper was criticized by some, most of the criticisms were unpublished personal opinions. Moreover, most of the published criticisms (e.g. Kuiper *et al*., 1999) were answered adequately (Ewen and Pusztai, 1999b). Some selected results of the nutritional/metabolic studies were, against the wishes of the authors, placed on the website of The Rowett Research Institute (www.rri.sari.ac.uk), where most of the work was done (Bucksburn, Aberdeen, UK). However, so as not to jeopardize their eventual proper publication, these results will only be mentioned briefly.

Young, rapidly growing rats (starting weight of 84 ± 1 g) were strictly pair-fed on iso-proteinic (60 g total protein kg^{-1} diet, most of which was from potatoes) and iso-caloric diets (in contrast to that described in Kuiper *et al*., 2001) supplemented with vitamins and minerals for 10 days. The test diets contained either raw or boiled GM potatoes. The control diets contained the same amount of parental-line potatoes (raw or boiled) alone or supplemented with GNA at the same concentration as expressed in the GM potatoes. A positive control group of rats was also included in the experiment, and these were fed a lactalbumin-based high quality control diet to check for any potential problems in rat behaviour and experimental conditions. As part of the nutritional/metabolic evaluation, samples of stomach, jejunum, ileum, caecum and colon were taken, fixed and stained with haematoxylin and eosin for full quantitative histological evaluation (Figs 16.1, 16.2 and 16.3) or reacted with GNA antibody and subsequently stained using a PAP (peroxidase–antiperoxidase) method to establish whether any GNA was bound to the epithelial surface (Fig. 16.4). By measuring the mucosal thickness of the stomach and the crypt length of the intestines (Ewen and Pusztai, 1999a), it was shown that proliferation in the gastric mucosa was in part caused by GNA, the gene product. However, the growth-promoting stimulus on the small intestine of diets containing GM potatoes leading to crypt enlargement and a part of the stomach enlargement was not a GNA effect. As shown before and confirmed here, there was a slight binding of GNA to the small intestinal epithelium (Fig. 16.4). However, GNA is not a mitotic lectin and therefore it did not induce hyperplastic growth in this tissue (Pusztai *et al*., 1990). Accordingly, the jejunal growth was probably due to some as yet unknown effects of other parts of the genetic construct used for the transformation or the genetic transformation itself. Hyperplasia was shown previously by measuring the increase in crypt length (Ewen and Pusztai, 1999a). However, similar results were obtained by measuring the increase in crypt cell numbers (Table 16.1) and crypt mitotic figures (not fully significant) in the jejunum of GM potato-fed rats (Table 16.2).

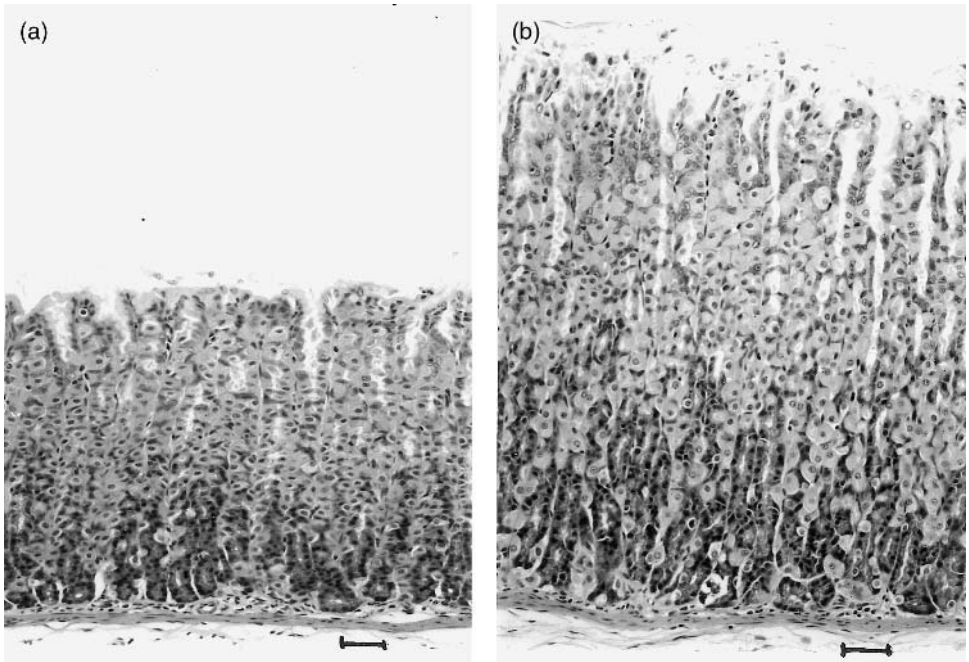

Fig. 16.1. Comparison of the stomach mucosa of rats fed with raw GM potato diet (b) shows marked thickening due to hypertrophy of mucosal cells in comparison with that of rats given the parental line (a) (bar = 100 μm).

The results suggested that it is possible that crypt hyperplasia and an increase in epithelial T lymphocyte infiltration observed with GM potatoes might also happen with other GM plants that had been developed using the same or similar genetic vectors and method of insertion. It is therefore imperative that the effects on the gut structure and metabolism of all GM crops should be investigated thoroughly as part of the regulatory process before their release into the human food chain.

Potatoes expressing cationic peptide chimeras

Desiree and Russet Burbank potatoes expressing N-terminally modified cecropin–melittin cationic peptide chimeras and control line potatoes fed to mice caused severe weight loss. The animals did not grow even after supplementing these potatoes with rodent laboratory chow. According to the authors (Osusky *et al*., 2000), mice fed with tubers from transgenic potatoes were as healthy and vital (*sic*) as those from the control group, and their faecal pellets were comparable. However, the severe weight loss seriously called into question the value of the results of this poorly designed feeding experiment.

GM tomatoes

Finally, an important study will have to be described even though it was not published in a peer-reviewed journal, but the ideas and experiments described had some influence on the development of GM regulation (Noteborn *et al*., 1995). Thus, a new laboratory GM tomato line was developed using the *B. thuringiensis* crystal protein CRYIA(b) gene but, instead of the cauliflower mosaic virus 35 S promoter (CaMV 35 S), which is used in practically all first-generation GM crops, a potentially safer plant promoter was used. Although with this the expression level of the Bt toxin was only about 1/20th of that found with CaMV 35 S, this might be improved upon in future. In contrast with most other studies with GM crops, there was a

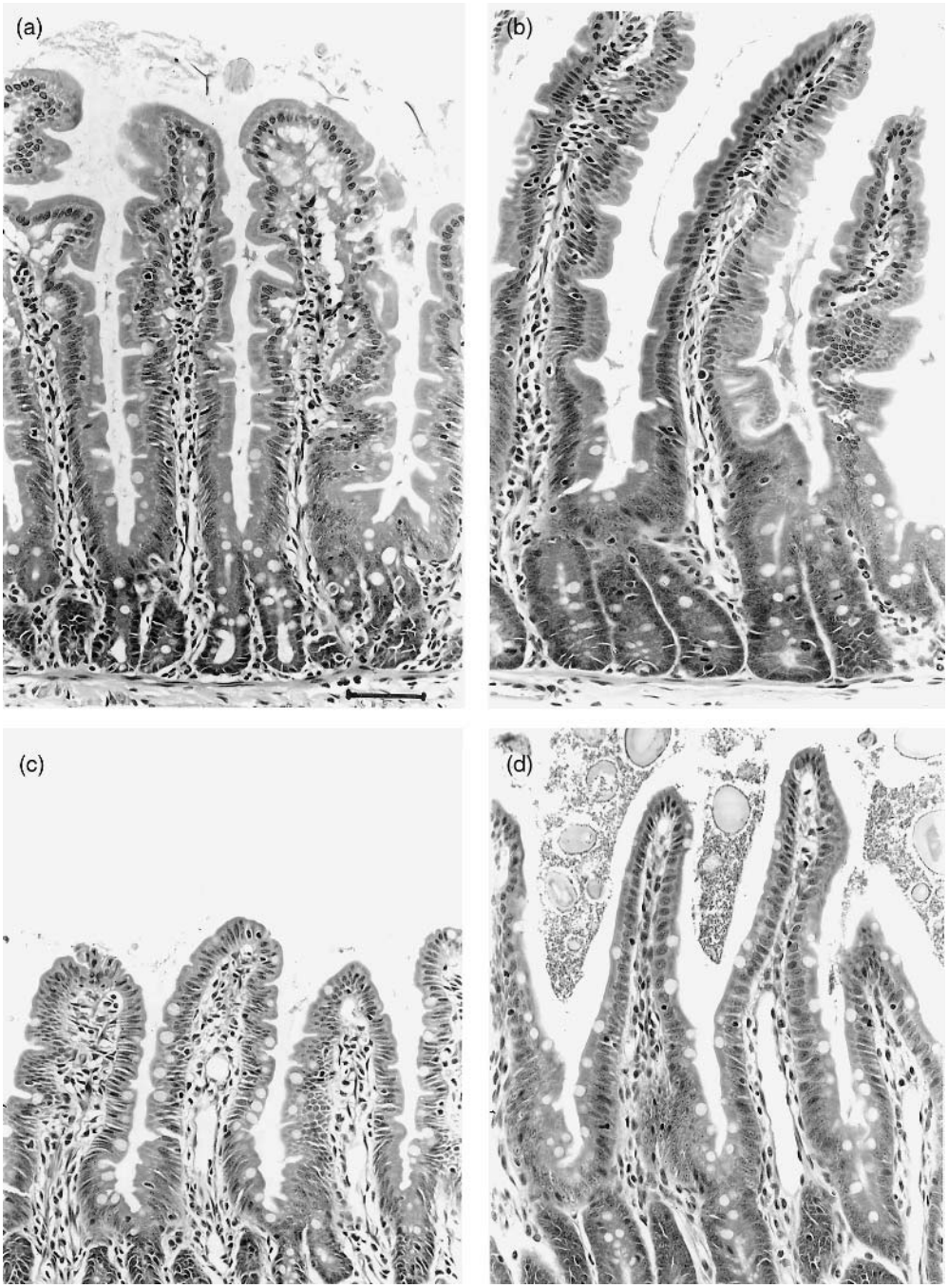

Fig. 16.2. Histology of the jejunum and ileum of rats fed raw GM or parent potato diets. Jejunal crypt length and cells exhibit marked enlargement after feeding rats GM potato diets for 10 days (b) in comparison with those of rats given parental line potato diets (a). The villus length is similar in both, but intraepithelial lymphocyte cell counts appear to be increased on the GM potato diet. In the ileum, both crypts and villi of rats on GM potato diets are elongated (d) in comparison with parent potato-fed rats (c) (bar = 100 µm).

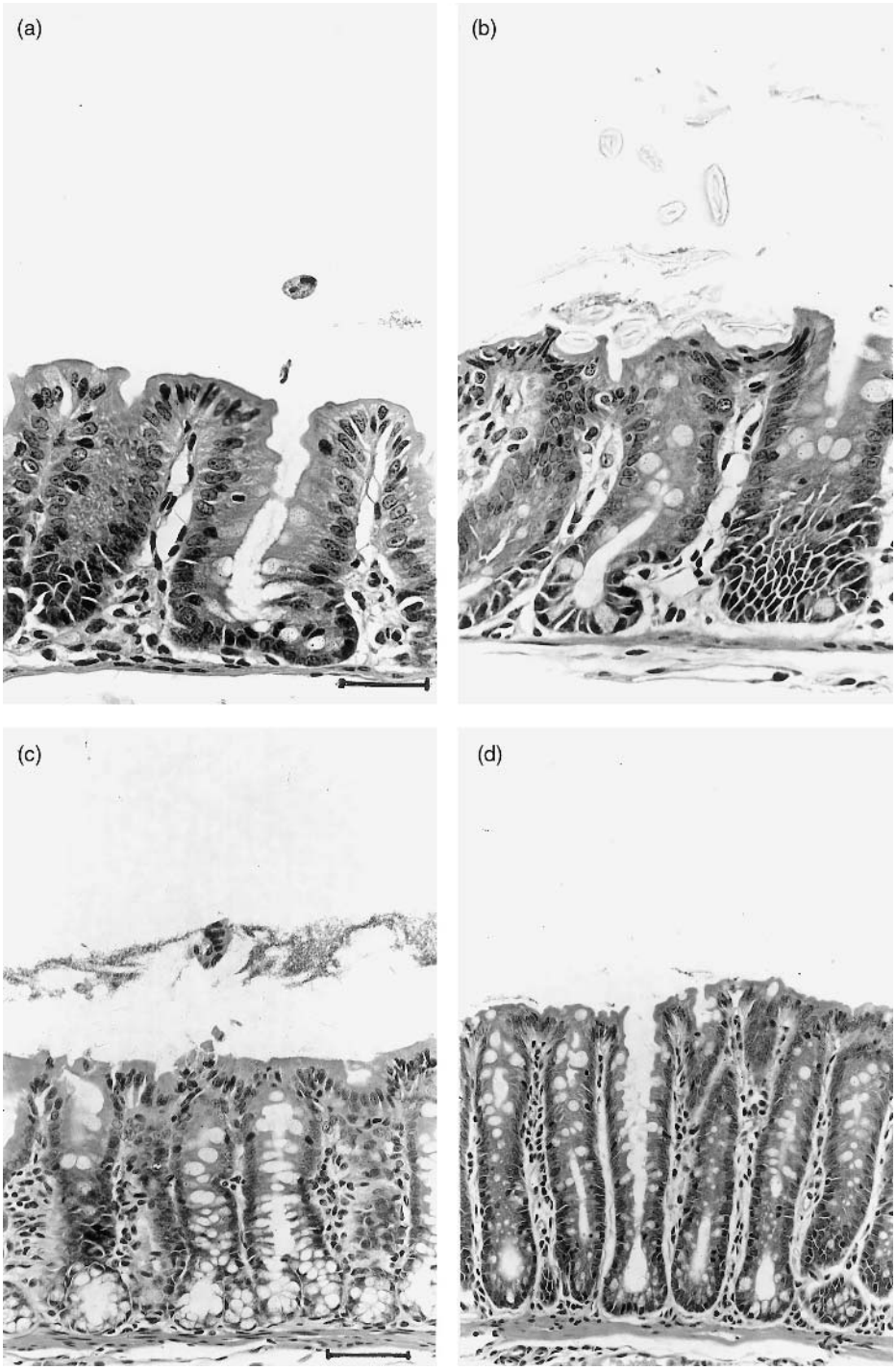

Fig. 16.3. The mucosa of the caecum demonstrates little change. Differences between GM-fed (b) and parent line potato-fed rats (a) are slight, while the colonic mucosa is moderately thickened in GM-fed rats (d) compared with that of rats given the parental line (c) (bar = 50 μm).

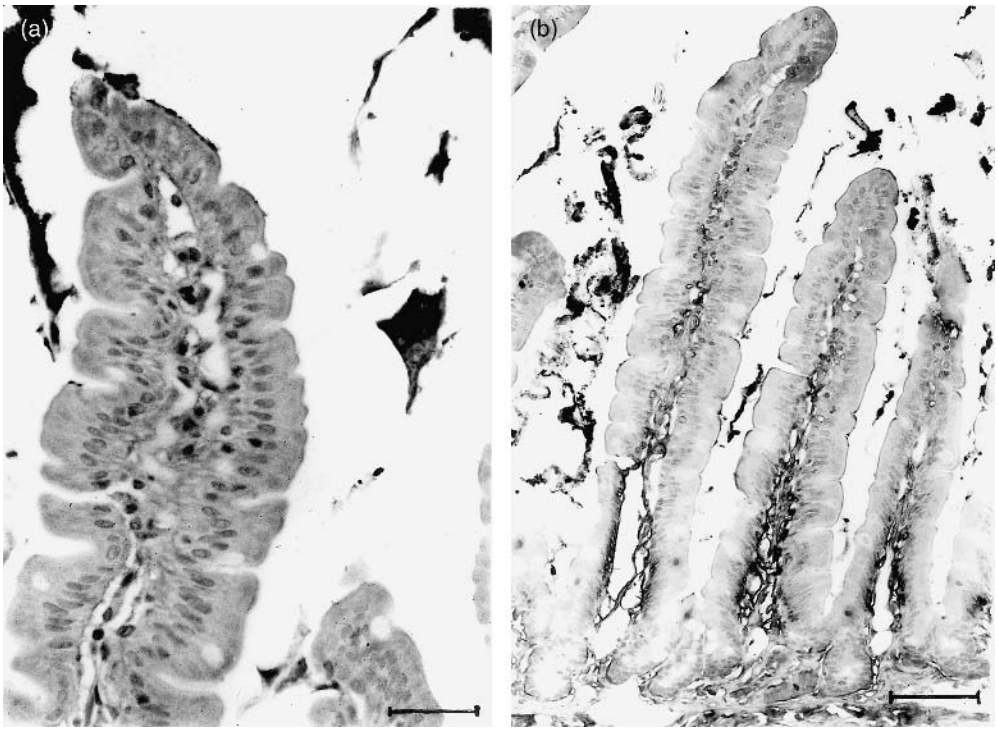

Fig. 16.4. Immunocytochemistry of the jejunum of rats fed raw GM potato diets for 10 days (a) shows moderate binding of GNA to villus tips (bar = 50 μm). Similar binding of GNA to jejunal villus tips is found in rats given parent potato diet supplemented with GNA in amounts equivalent to that expressed in the GM potato (b) (bar = 100 μm). Sections were first treated with anti-GNA rabbit antibody (diluted 1/100), followed by visualization with PAP. Note the strong antibody reactivity of feed particles in the sections.

Table 16.1. Number of crypt cells in the jejunum of rats fed various potato diets.[a]

Diet	Parent	Parent vs. parent + GNA (*P*)	Parent + GNA	Parent vs. transgenic (*P*)	Transgenic	Parent + GNA vs. transgenic (*P*)
Raw	15.9 (0.5)	0.037	17.0 (0.7)	0.000	20.3 (0.8)	0.006
Boiled	17.8 (1.1)	0.466	18.2 (0.2)	0.749	18.2 (1.2)	0.769
Raw vs. boiled (*P*)	0.006		0.003		0.003	

[a]The number of nuclei were counted sequentially on well-oriented haematoxylin and eosin paraffin sections (4 μm). Values represent means (SD) for six rats per treatment; ten crypts per rat were counted. Differences between treatments are significant when $P < 0.05$ (Student's *t* test). The effect of boiling ($P = 0.759$) is not significant, while that of GNA added or as transgene product ($P = 0.019$) and the effect of transformation ($P = 0.000$) are highly significant. The interactions between GNA and cooking ($P = 0.043$) and between transformation and cooking ($P = 0.018$) are also significant (multivariant analysis with Tukey's test).

commendable attempt to measure the binding of the gene product to the rat gut surface *in vivo* rather than using spurious arguments as to why the gene product should not bind. Although no *in vivo* binding was found, this should not detract from the significance of this initiative because, due to the lack of availability of sufficient quantities of Bt toxin

Table 16.2. Mitotic numbers per 100 crypts in the jejunum of rats fed potato diets.[a]

Diet	Parent	Parent + GNA	Transgenic
Raw	48	49	75
Boiled	57	56	57

[a]Mitotic cells were expressed per 100 crypts.

isolated from GM tomatoes, an *E. coli* recombinant and potentially less stable form of the gene product was used in the experiment, and its possible degradation in the gut may have accounted for the lack of binding. However, Bt toxin was shown by immunocytochemistry to bind to gut sections, including the caecum and colon, from humans and rhesus monkeys *in vitro*. Unfortunately, their short-term toxicity testing in mice (and rabbits) and the *in vitro* simulated proteolysis assays were also carried out with the *E. coli* recombinant gene product and therefore their conclusions of finding no toxic effects may not be valid. Commendably, the authors carried out a 91-day feeding study with rats using freeze-dried GM vs. parent line tomatoes, which were included at a 10% level in the diets, but no differences in food intake or body and organ weights were found. However, because the Bt toxin expression level in the tomatoes was low, the daily intake of the gene product(s) by the rats was also low. Moreover, as the daily input of tomato proteins was only about 5–6% of the total dietary protein intake of the rats, it was somewhat optimistic to expect any significant changes in these nutritional parameters. To have any reasonable chances to show up small differences in the nutritional value of GM vs. parent line crops, it would have been important to use as high a protein concentration as possible such as that in the 110-day GM potato feeding study carried out at The Rowett Institute, in which the GM protein in the diet was diluted only twofold by other dietary proteins, and this allowed the significant differences in the growth rates of rats fed on baked GM potato diets vs. parent potato diets to show up. In fact, to equalize the growth rates of the rats on the GM potatoes to that of the controls, the GM diet had to be supplemented with an extra 12 g lactalbumin kg^{-1} diet, and this extra protein gave a quantitative measure of the difference of the nutritional value between GM and non-GM potatoes. Even at these similar growth rates, the weights of some of the rats' vital organs, such as the gut and particularly the small intestine, the liver and kidneys, were still significantly different.

There were other omissions in the Bt tomato study, the most important of which was that no Bt toxin survival was measured in the gut lumen and no gut histology was done to see if there was any Bt toxin binding to or possible structural changes in the gut epithelium or whether lymphocyte infiltration occurred. This omission is particularly important because later studies showed that the similar Bt toxin Cry1Ac could bind to gut epithelial cells in mice (Vazquez Padron *et al*., 2000a,b) and induce mucosal antigenic sensitization (Vazquez Padron *et al*., 1999, 2000a,b). The allergenic potential of Bt tomatoes was not investigated either. However, despite some of its shortcomings, this study showed many novel and commendable features, which, after some improvements, may, hopefully, be incorporated into the general GM food testing procedures.

Allergenicity

One of the major health concerns with GM food is its potential to increase allergies in the human population through the food chain. The possibility of fatal anaphylaxis in sensitized individuals after their unwitting exposure to allergenic proteins in unlabelled GM foodstuffs is a real danger. When a gene is transferred from a source of known allergenic potential, the assessment of the allergenicity of the GM crop is relatively straightforward. This can be done using *in vitro* tests with sera from individuals sensitized to the allergen from the original source. Similarly, it is relatively easy to assess the effect of genetic engineering on endogenous allergens in crops with some evidence of allergenicity. With tests such as the radioallergosorbent test (RAST), RAST inhibition

and immunoblotting, the allergenic potential of the GM crop is easily measured. There are now several examples for these, such as the demonstration of the allergenicity of the Brazil nut 2S seed storage protein in transgenic soybean (Nordlee *et al*., 1996) or the codfish allergy in potatoes genetically engineered with cod protein genes to make the potatoes tolerate cold storage (Bindslev-Jensen and Poulsen, 1997). The claim that in glyphosate-tolerant soybean the introduction of the herbicide resistance gene does not affect the allergenicity of the soy endogenous allergens is also a good example (Burks and Fuchs, 1995). Having shown in a surveillance programme of farm workers before and after exposure to *B. thuringiensis* pesticide sprays that some developed skin sensitization and IgE antibodies to the Bt spore extract and that two of them had a positive skin-prick test, it may now be possible to test for the allergenicity of Bt toxins engineered into various crops (Bernstein *et al*., 1999). This is all the more important because the Cry1Ac toxin has now been shown to be a potent oral immunogen and adjuvant (Vazquez Padron *et al*., 1999, 2000a,b).

It is much more difficult to assess the allergenicity of GM foods when the gene is transferred from a plant whose allergenic potential is unknown. Moreover, it is also possible that, as a result of the gene transfer or vector insertion, a new allergen is developed or the expression level of a minor allergen is increased in the GM crop. The gene product can also have an allergenic adjuvant effect on a food component previously of low allergenic potential, or some component in the GM food may have an adjuvant effect on the allergenicity of the transgene product. Unfortunately, while there are good animal models for nutritional/toxicological testing, no satisfactory animal models have been developed so far for allergenicity testing (Helm and Burks, 2000). For the time being, only indirect methods are available for the assessment of the allergenic potential of GM foods derived from sources of unknown allergenicity. There are a number of recommended approaches to be followed. A useful preliminary step is to establish if there are any sequence homologies in the transgenic protein to any of the about 200 known allergens. If there are, *in vitro* tests for IgE reactivity need to be performed. It is thought that the peptide length in the transgenic protein which is optimally needed for binding B-cell epitopes requires the presence of at least eight contiguous identical or similar amino acids. However, the amino acids in the allergenic epitopes are rarely contiguous. Moreover, the absence of a positive reaction in *in vitro* testing does not guarantee that the transferred protein is not an allergen. In a decision-tree type of indirect approach, the next step is to consider the molecular size, glycosylation, stability, solubility and isoelectric point of the transgenic protein and compare them with those of known allergens (O'Neil *et al*., 1998). Unfortunately, in most studies to date, the all-important stability of the transgenic protein to gut proteolysis is established in an *in vitro* simulated gastric/intestinal system (Astwood *et al*., 1996; Metcalfe *et al*., 1996), and this is fundamentally flawed. The results, therefore, are at best misleading and at worst erroneous. Reliance on the concept that most allergens are abundant proteins is probably also misleading because, for example, Gad c1, the major allergen in codfish, is not a predominant protein (Bindslev-Jensen and Poulsen, 1997).

When the gene responsible for the allergenicity of a crop is known, its cloning and sequencing open the way for its reduction by antisense RNA strategy. Thus, in rice, the low molecular weight α-amylase/trypsin inhibitors are major allergens. A part of the genomic sequence encoding this protein in an antisense direction was constructed between the promoter of the rice allergen gene and its waxy terminator, and this was introduced into rice protoplasts. The allergenicity of the regenerated plants was significantly less than that of parental wild-type rice (Nakamura and Matsuda, 1996).

In conclusion, allergenicity testing appears to be one of the Achilles heels of GM food safety. It is clear that, if and when it is known that the protein gene is derived from a source with a history of allergenicity, there is a reasonable certainty that the GM crop will be allergenic. Unfortunately, the reverse is not true: the use of a gene from something that is not allergenic will not guarantee that the GM

crop will not possess allergenicity. In the absence of new and reliable methods for allergenicity testing, particularly the lack of good animal models, at present it is almost impossible to establish definitely whether a new GM crop is allergenic or not in advance of its release into the human/animal food/feed chain.

Conclusions

One has to agree with the opinion expressed in *Science* (Domingo, 2000) that there are many opinions but very few data on the potential health risks of GM foods, even though research to exclude such risks should have been carried out before the GM crops were introduced into the food chain. Our present database is therefore woefully inadequate. This is clearly seen from a closer scrutiny of the reference lists of recent reviews which contain only a handful of toxicological/nutritional and immune studies of GM food crops published in peer-reviewed science journals (Ruibal-Mendieta and Lints, 1998; Betz *et al*., 2000; Kuiper *et al*., 2001; Pusztai, 2001). Moreover, the scientific quality of even what is published is, in most instances, not up to the standards that ought to be expected. In this review, data published in peer-reviewed and some non-peer-reviewed journals have been examined in detail. However, as our future is claimed to be dependent on the success or failure of the promise of genetic modification delivering GM foods which will be wholesome, plentiful and, most importantly, safe for us all, the emphasis was on strict but fair criticism.

From the results, the conclusion seems inescapable that the present crude method of genetic modification has not delivered GM crops that are predictably safe and wholesome. The promise of a superior second generation of GM crops is still in the future. It is possible that some of the first generation of GM crops may superficially satisfy some commercial end points, such as their use in broiler chicken production. However, we need to consider that these GM feed ration-fed animals eventually will be consumed by humans, and there is absolutely nothing known about the potential hazards (if any) for human health of this indirect exposure to GM food. Furthermore, the examples in the papers highlighted some differences even between such crude things as macronutrient composition of GM and conventional lines. It is argued by some that these differences have little biological meaning. However, it was clear that most GM and parental line crops would arguably fall short of the definition of 'substantial equivalence'. This crude, poorly defined and unscientific concept outlived its possible previous usefulness. There is an urgent need to come up with novel scientific methodologies to probe into the compositional, nutritional/toxicological and metabolic differences between GM and conventional crops if we want to put this technology on a proper scientific foundation and also to allay the fears of the general public. We need more science and not less. For proper safety assessment, our first concern ought to be to establish on a case-by-case basis the impact of components of GM foods on the digestive system, its structure and metabolism, because the way our body will respond to GM foods will be predetermined at this level. According to the Royal Society (1999), we need 'to refine the experimental design of the research done to date'. New ideas were also advocated in the *Lancet* debate (Ewen and Pusztai, 1999b; Kuiper *et al*., 1999) and at the OECD Conference in Edinburgh in February 2000.

Recommendations

Main tasks and methods for safety assessment of GM crops

1. For compositional analysis and comparison, the parent and transformed lines must be grown under identical conditions, treated and harvested the same way. In addition to proteins, starch, lipids, etc. of the parent and GM lines, their contents of bioactive components should also be compared by novel methods (proteomics, fingerprinting, etc.).

2. The stability to degradation by acid or pepsin or other proteases/hydrolases of GM

products, foreign DNA, including the gene construct, promoter, antibiotic resistance marker gene, etc., has to be established in the stomach and intestines of model animals *in vivo*. With GM lectins, including Bt toxin, the presence/absence of their epithelial binding should also be demonstrated by immunohistology.

3. The biological, immunological, hormonal properties and allergenicity of GM products must be established with the GM product isolated from the GM crop, and not with recombinants from *E. coli*, as these two may have substantially different properties.

4. As GM food is unlikely to be highly poisonous, 'toxicity' is an unhelpful concept and difficult to assay. In contrast, nutritional studies in which GM crop-based diets are fed to young growing animals should reveal their possible harmful effects on metabolism, organ development, immune/endocrine systems and gut flora, which together determine the safety of the GM crop and the development of the young into healthy adults.

5. For animal testing iso-proteinic and iso-energetic diets need to be formulated in which most of the dietary protein is derived from the GM crop. The composition of the control diets should be the same as the GM diet but containing the parent line with or without supplementation with the isolated gene product at the same level as expressed in the GM line. Groups of animals (five or more per group), of similar weight, should be pair-fed in short- and long-term experiments. Urine and faecal samples should be collected for the determination of net protein utilization (NPU), nitrogen balance and feed utilization ratios. Blood samples should be taken before, during and at the end of the experiments for immune studies (i.e. lymphocyte proliferation assay, Elispot), hormone assays (insulin, cholecystokinin, etc.) and for the determination of other blood constituents. The animals are to be weighed daily and any abnormalities observed. After killing the animals, their bodies should be dissected, the gut rinsed and its contents saved for further studies (enzymes, GM products, DNA). Sections should be taken for histology, and the wet and dry weights of organs recorded and analysed.

Evaluation

With suitable statistical analyses (ANOVA, multiple comparisons and/or multivariate analysis), the significance of differences, if any, in the parameters should be established.

- If differences between animals fed GM and parent line diets indicate that the genetic modification must have had a significant effect on utilization and nutritional value, the GM crop cannot be accepted for inclusion in the human/animal diet.
- If, similarly to the GM diet, the parent line diet spiked with the gene product shows differences, the use of this gene in GM food/feed is not acceptable.
- If negative effects are not observed with the parent line diet containing the isolated gene product, it is likely that the harm is caused by the use of the particular construct or by an unwanted or unforeseen effect of the gene insertion on the genome.

Animal testing is but a first step and not a substitute for human studies. If there is no indication of harm to the animals, the results will have to be validated with human volunteers in clinical double-blind, placebo-controlled drug-type tests. Such studies may have to go on for considerable lengths of time. It must also be kept in mind that any potential harm with GM food may be most acute in the young, elderly and sick, particularly those suffering from HIV, hepatitis or other viral diseases. Many people suffer from allergies and other disorders of the gastrointestinal tract, and for these the consumption of GM food may have unforeseen consequences and some of these may be irreversible. Thus, for these, the clear labelling of GM food must be made mandatory.

There is a compelling need to develop further the concepts of biological testing, particularly for potential long-term effects. Since the GM potato work with male rats showed abnormalities in the development of their sexual organs, it is imperative that similar experiments should be done with female rats to be followed by studies of the effects on

reproductive performance of rats (or other animals) reared and maintained on GM vs. non-GM diets for several generations.

If there is a general willingness to fund research along these or similar lines and the regulators accept the concept of biological/toxicological testing transparently and inclusively, the methods are available for the work to start. Following this route, publishing the results and consulting the public will ensure that a technology which promised safe and plentiful food will deliver it for us all, and we are confident that if people see that everything has been done to establish its safety they will accept it willingly.

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17 Radionuclides in Foods: the Post-Chernobyl Evidence

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Introduction

The Chernobyl accident in April 1986, the worst nuclear accident in history, had far-ranging and long-term implications for the safety of the food chain. The explosion and subsequent fire at the nuclear power station in Ukraine spread volatile radioactive elements (e.g. radioiodine, ^{131}I, and radiocaesium, ^{137}Cs) over large areas of the former Soviet Union (fSU) and parts of Western Europe. The pattern of deposition of radioactivity (Fig. 17.1) was complex; in Western Europe, the highest deposition of radiocaesium isotopes occurred in areas where rainfall intercepted the radioactive plume as it dispersed. Less volatile elements such as isotopes of strontium and plutonium were deposited principally within 30 km of the reactor, in the form of small particles of radioactive fuel ('hot particles').

Radiologically important isotopes and half-lives

The importance of different radioactive elements varies with time after a nuclear accident because of differences in their physical half-lives. The radioactive half-life is defined as the time taken for one half of a given amount of a radioactive element to decay radioactively. For example, 2.6×10^{17} becquerels (Bq) of ^{131}I were emitted from Chernobyl. ^{131}I has a physical half-life of 8.05 days (Table 17.1) so, after 8.05 days (one half-life), 1.3×10^{17} Bq of ^{131}I remained in the environment and, after 32.2 days (four half-lives), this was reduced to 0.1625×10^{17} Bq ($½ \times ½ \times ½ \times ½ = 1/16$ of the original amount).

Initially, the radiation dose to humans was primarily from ^{131}I (with contributions from other short-lived isotopes) in the first few weeks after the accident. Over months–decades after the accident, longer lived isotopes, primarily ^{137}Cs with contributions from ^{134}Cs and ^{90}Sr, formed the major part of the dose. Over hundreds to thousands of years, Pu isotopes (with ingrowth of ^{241}Am) will form the major part of the dose as the ^{137}Cs decays away (Fig. 17.2). In this chapter, we will concentrate primarily on the mid- to long-term consequences, and therefore on ^{137}Cs, which has been the focus of the majority of scientific and regulatory attention following the Chernobyl accident.

Summary of contamination of the food chain

Initial concerns over safety of the food chain were due primarily to short-lived ^{131}I in

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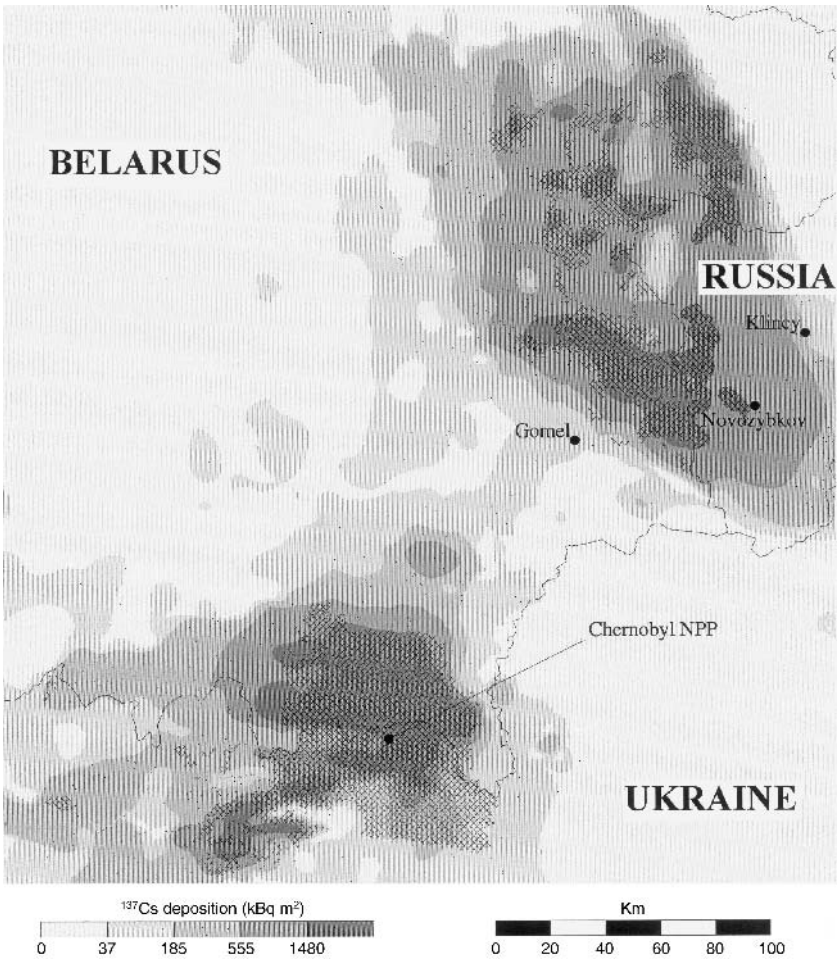

Fig. 17.1. Extent of lands within the former Soviet Union in 1999 contaminated as a consequence of the Chernobyl accident. Cross-hatched areas denote areas of abandoned land. ^{137}Cs deposition is interpolated from contours presented by de Cort *et al*. (1998).

milk and fresh vegetables (Tsyb *et al*., 1996; Drozdovitch *et al*., 1997). Radioiodine is accumulated in the thyroid, a problem exacerbated by low natural levels of (stable) iodine in the diet of the affected regions of the fSU. Iodine deficiency was relatively common in these regions, so accumulation of the radioactive form was believed to have been extremely high. The primary, well-documented, health effect arising from the Chernobyl accident has been the development of thyroid cancers by children in the fSU, largely as a consequence of ^{131}I ingestion via contaminated milk (e.g. Demidchik *et al*., 1996). At the present time, statistically significant increases in the incidence of other radiation-induced cancers have not been observed as a result of the accident (UNSCEAR, 2000).

Following the first few weeks after the accident, physical decay had reduced activity concentrations of ^{131}I (and many other short-half-life isotopes) to insignificant levels, as shown in Fig. 17.2. In the medium term after the accident, ^{137}Cs made up the major component of radiation doses to humans. Significant contributions to dose were made by the shorter half-life ^{134}Cs and also by ^{90}Sr. The γ

Table 17.1. Physical half-lives and amounts released of some radiologically important radionuclides from Chernobyl (adapted from IAEA, 1991). Radionuclides of primary radiological concern (over different time scales) after the accident are in bold.

Radionuclide	Physical half-life	Percentage of reactor core released (%)	Amount released (Bq)
^{89}Sr	53 days	4.0	8.0×10^{16}
^{90}Sr	**28.8 years**	**4.0**	**8.0×10^{15}**
^{95}Zr	65.5 days	3.2	1.4×10^{17}
^{103}Ru	39.5 days	2.9	1.2×10^{17}
^{106}Ru	368 days	2.9	5.8×10^{16}
^{131}I	**8.05 days**	**20**	**2.6×10^{17}**
^{134}Cs	**2.1 years**	**10**	**1.9×10^{16}**
^{137}Cs	**30.2 years**	**13**	**3.8×10^{16}**
^{140}Ba	12.8 days	5.6	1.6×10^{17}
^{144}Ce	284 days	2.8	9.0×10^{16}
^{238}Pu	87.7 years	3	3.0×10^{13}
^{239}Pu	2.4×10^4 years	3	2.6×10^{13}
^{240}Pu	6.5×10^3 years	3	3.6×10^{13}
^{241}Pu	**14.4 years**	**3**	**5.1×10^{15}**
^{242}Cm	164 days	3	7.8×10^{14}

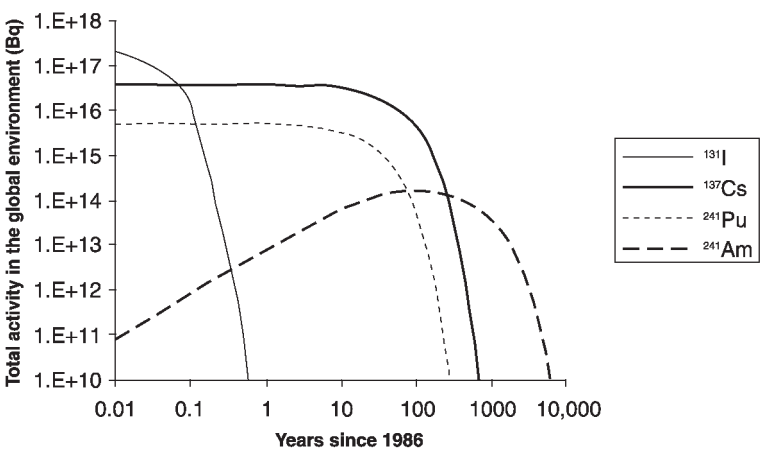

Fig. 17.2. Change in amounts of some released radionuclides over time after Chernobyl due to radioactive decay. The increase in ^{241}Am activity concentration is due to ingrowth from ^{241}Pu.

radiation (see Table 17.2 for a description of α, β and γ radiation) emitted by radiocaesium presents a potential hazard from deposits on the ground (external dose) as well as from ingestion of contaminated foodstuffs (internal dose). ^{90}Sr is a β emitter and presents little external irradiation risk, with the exception of direct contact between contaminated materials and the skin.

Within the most affected areas of the fSU, approximately 150,000 people were evacuated from land with ^{137}Cs deposition greater than 1480 kBq m^{-2}, to reduce the potential risk from external and internal exposure by radiocaesium. The 1480 kBq m^{-2} evacuation level was set in order to ensure that a person's lifetime radiation dose from Chernobyl radiocaesium was less than 350 millisieverts (mSv). For comparison, the average lifetime dose from all radiation sources (the majority being natural background radiation and from medical uses of radiation) to people in Europe is approximately 300 mSv (from data in de Cort *et al.*, 1998), although there is

Table 17.2. Characteristics of some radioactive emissions.

Radiation type	Description	Stopped by:	Approximate relative biological effectiveness[a]
α particle	Helium nucleus	Air or outer layers of skin	20
β particle	Electron	A few millimetres of aluminium	1
γ ray	Electromagnetic wave	A few centimetres of lead	1

[a]Relative biological effectiveness is used to convert radiation energy absorbed by the body to radiation dose: for a given absorbed energy, α radiation is estimated to be 20 times more damaging than β or γ radiation.

considerable variation in this value because of varying environmental factors. The present extent of evacuated areas within the fSU is shown in Fig. 17.1 (Beresford and Wright, 1999). While there are no plans to reuse much of this area in the foreseeable future, people are 'illegally' living within many of the areas (including the 30 km exclusion zone surrounding the Chernobyl nuclear power plant) and producing their own food.

Contamination of the food chain spread much wider than the evacuated areas, affecting many areas of Belarus, Ukraine and European Russia. Foodstuffs were contaminated in some areas of relatively low ^{137}Cs deposition as a result of high accumulation from certain soil types. For instance, Beresford and Wright (1999) report that, in areas of soddy podzolic soils, a ^{137}Cs deposition in the range of 140–500 kBq m^{-2} will result in an annual ingested dose of 1 mSv. In areas of peaty soil this level of dose is reached at ^{137}Cs depositions as low as 7–50 kBq m^{-2} (see Fig. 17.1 for deposition within the fSU). Parts of Western Europe were also affected, with advice not to consume fresh vegetables being given in, for instance, parts of Italy and Germany. Most affected were the Scandinavian countries, where activity concentrations in reindeer, goat milk, sheep, game animals and freshwater fish were above intervention levels. As with sheep in some upland areas of the UK, some parts of Scandinavia are still subject to restrictions (Howard *et al*., 2001).

Previous nuclear accidents

The Chernobyl accident was on a much greater scale than previous accidental releases of radioactivity to the environment. The largest nuclear accident prior to Chernobyl was the explosion in 1957 of a high-level waste tank at the Mayak plutonium production and reprocessing facility in Siberia. Releases of a mixture of radionuclides, including long-lived ^{90}Sr (physical half-life 28 years) resulted in evacuation and removal from agricultural production of an area of approximately 1000 km^2. By 1997, 82% of this land had been reclaimed (Joint Norwegian–Russian Expert Group, 1997).

Following the 1957 fire at the Windscale nuclear reactor in the UK, a ban on the consumption of milk because of high ^{131}I activity concentrations was implemented over an area extending to a maximum of 518 km^2 (Jackson and Jones, 1991). It is probable that, at present day intervention levels, temporary precautionary bans on foodstuffs, including meat and milk, would also have been implemented as a consequence of radiocaesium contamination. This may also be the case for some food products in some areas as a consequence of fallout from the atmospheric nuclear weapons testing era (predominantly 1952–1962). The accident at Three Mile Island in the USA did not result in significant contamination of the environment and food chain; the highest activity concentration in a food product determined in a sample of goat milk was only 1.5 Bq l^{-1} of ^{131}I, collected 2 km from the site (Katherine, 1984).

Studies Prior to the Chernobyl Accident

Prior to Chernobyl, much was already known about the movement of radionuclides (radiocaesium and radiostrontium in particular) in

the environment. Studies had been carried out on the environmental and food chain transfer of global fallout from the above-ground nuclear weapons tests of the 1950s and 1960s. Laboratory studies had shown that the sorption of ^{137}Cs by soils and sediments is dominated by specific sorption to certain sites on illitic clay minerals (Jacobs and Tamura, 1960). This sorption was known to have a slow kinetic component, in which ^{137}Cs is transferred to less available sites in the mineral lattice (Evans *et al*., 1983), a process often referred to as 'fixation'. Caesium competes for binding sites on the clay mineral lattice with similarly sized K^+ and NH_4^+ ions. Thus, a common measure of the 'availability' or 'exchangeability' of radiocaesium sorption to soils and sediments is to carry out an extraction with ammonium acetate or ammonium chloride solution. Coughtrey and Thorne (1983) report measurements by Evans and Dekker (1966) that a large proportion (~ 85%) of radiocaesium in soils was in fixed form, with only 15% exchangeable with a molar solution of ammonium acetate, though this fraction varied considerably with soil type. Environmental studies of global fallout from nuclear weapons testing showed that transfers of radiocaesium to milk were much higher in the Faroe Islands than in Denmark. This was attributed to greater availability of ^{137}Cs in the organic soils of the Faroe Islands than in more mineral soils in Denmark (Aarkrog, 1979).

Environmental variability in ^{137}Cs binding to soil (and consequent uptake by plants) was therefore linked to the clay mineral (specifically illitic clays) content of soils. A second important influence on ^{137}Cs transfers through the food chain was shown to be the potassium content of soils (and, in aquatic systems, the potassium content of lake or river water). Because of its chemical similarity to potassium, an important nutrient, caesium is accumulated in biota through the same mechanisms as potassium. Thus, by a dilution effect, in aquatic systems, concentration factors of ^{137}Cs in fish were known to be inversely related to the K^+ concentration of the surrounding water (Fleishman, 1973; Blaylock, 1982). In terrestrial systems, addition of fertilizer containing K^+ was shown to decrease ^{137}Cs uptake by plants in some soils, though the effectiveness of application was reduced by the potential for K^+ ions to de-sorb ^{137}Cs from the soil binding sites (Coughtrey and Thorne, 1983).

For a given activity concentration of radiocaesium in soil (Bq kg^{-1}), therefore, it was known that bioaccumulation factors of radiocaesium could be very high, but this accumulation was extremely variable 'showing a range covering four orders of magnitude' (Coughtrey and Thorne, 1983). As discussed above, uptake was known to be influenced by a number of environmental factors, but was expected to be highest in soils of low nutrient status (low K^+) and low clay mineral content. These effects were illustrated by data collected by the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) on the transfers of radiocaesium from nuclear weapons test fallout to humans. Whole-body radiocaesium activity concentrations were approximately two orders of magnitude greater in people living in 'marginal' arctic and subarctic environments with nutrient-poor soils (e.g. Finnish reindeer herders, populations in northern Russia and Alaska) than in populations whose diet originated predominantly from intensive agriculture (UNSCEAR, 1977). This higher whole-body radiocaesium activity concentration was largely the result of the consumption of foods with especially high radiocaesium transfers (e.g. reindeer meat).

Radiostrontium exists in solution in cationic form (as Sr^{2+}), and its chemical and biological behaviour is similar to that of calcium. In most circumstances, the environmental transfer of radiostrontium was shown to be determined by the Ca^{2+} status of a system. In contrast to radiocaesium, radiostrontium is believed to be predominantly sorbed reversibly to ion-exchange sites in soils (e.g. Tikhomirov and Sanzharova, 1978, quoted in Coughtrey and Thorne, 1983). Transfers to biota are less variable than for ^{137}Cs, and bioaccumulation factors are often significantly lower. Uptake by plants is related to the Ca concentration, with the Sr/Ca ratio in plants being approximately equal to that in soil (Tikhomirov and Sanzharova, 1978, quoted in Coughtrey and Thorne, 1983). In aquatic

environments, ^{90}Sr accumulation in fish is in inverse proportion to the calcium concentration of the water (Blaylock, 1982). Similarly, ^{90}Sr absorption in the animal and subsequent transfer to animal-derived food products was shown to be inversely proportional to dietary calcium intake, with Sr/Ca ratios in tissues and milk being lower than those in the diet (e.g. the ratio of Sr/Ca in milk to Sr/Ca in the diet is ~0.1 for mammals) (Comar *et al*., 1966; Beresford *et al*., 1998). An important feature of transfers of Sr to animals and humans is its incorporation into bone, and hence its long retention time in the body.

Radioactivity in Foodstuffs after Chernobyl

Reference levels of radioactivity in foodstuffs

A reference level is a level of radioactivity in a foodstuff at which some action must be taken by regulatory authorities. Exceeding the reference level can result in a ban or limit on consumption of the product, but, where appropriate, may simply indicate the need for further investigation. Reference levels vary between countries and according to circumstance. In part, reference levels are set such that the overall ingested dose resulting from consumption of foodstuffs at this level is below a certain limit. For example, they may be higher in the event of a short-term, temporary increase in levels of radioactivity in food than in the case of long-term contamination. They may also vary according to the number of foodstuffs contaminated: if a number of foodstuffs, forming an important part of the diet, are being considered, the reference level may be lower than that for the case in which a single, less important product is considered.

An example of a reference level used in the European Community (EC) (EURATOM Council Regulations Nos 3958/87, 994/89, 2218/89 and 770/90) is the maximum permitted level (MPL), as shown in Table 17.3. Reference levels are usually determined by calculating the mean activity concentration in foodstuffs which, assuming consumption over a 1-year period, would lead to a radiation dose deemed to present a negligible risk. The dose is determined by the radioactivity in a product and its consumption rate; thus, MPLs may be affected by the rate of consumption of a foodstuff. For example, minor foodstuffs such as herbs and spices have higher MPLs (by a factor of 10) than the values shown in Table 17.3, which assume high consumption rates and apply to major foodstuffs such as dairy produce, potatoes and beef. The MPLs in force in the EC assume that the average activity concentration of a person's total diet is a fraction (10%) of the contamination of certain individual foodstuffs. This assumption is considered to be appropriate since the total diet, particularly in Western Europe, is a combination of foodstuffs of different types originating from very disperse areas.

The MPLs for ^{137}Cs activity concentrations in foodstuffs in the fSU countries are shown in Table 17.4. These are generally lower than the EC levels, partly because there are

Table 17.3. Agreed maximum permitted levels of radionuclides in foods in place in the European Community.

Radioactivity in food in Bq kg^{-1} or Bq l^{-1}	Baby foods	Dairy produce	Other foods (except minor foodstuffs)	Liquid foodstuffs
Isotopes of strontium, notably ^{90}Sr	75	125	750	125
Isotopes of iodine, notably ^{131}I	150	500	2000	500
α-emitting isotopes of Pu and trans-Pu elements, e.g. ^{239}Pu, ^{241}Am	1	20	80	20
All other nuclides of half-life > 10 days, notably ^{134}Cs, ^{137}Cs	400	1000	1250	1000

multiple exposure pathways in the Chernobyl affected areas and diets tend to be much more localized in the fSU countries than in the EC. In addition, limits in the fSU countries have generally decreased with time after the accident. In the first year after the accident, for pragmatic reasons, limits were higher than at present. It has been argued that current limits in the fSU are too restrictive, leading to implementation of food bans and expensive remedial measures where actual radiation risks are low.

Iodine-131

Ingestion of milk and leafy vegetables was believed to have formed the major pathway for radioiodine dose to the thyroid following the Chernobyl accident (IAEA, 1991). The short half-life of ^{131}I (8.05 days) means that there are few available measurements in foodstuffs. In the UK, the maximum reported level of ^{131}I in cow milk was 371 Bq l^{-1} (measured in Cumbria on 4 May 1986), although higher levels were recorded in goat (1040 Bq l^{-1} measured in Cumbria on 9 May 1986) and sheep milk (2095 Bq l^{-1} measured in Surrey 4 May 1996). The maximum permitted level of ^{131}I in dairy produce in the UK is 500 Bq kg^{-1} (data from UK Ministry of Agriculture, Fisheries and Food monitoring). Kryshev (1994) presents measurements of ^{131}I in fish in the Kiev reservoir shortly after Chernobyl. Activity concentrations in fish flesh declined rapidly by physical decay from around 6000 Bq kg^{-1} on 1 May 1986 to 50 Bq kg^{-1} on 20 June 1986.

Caesium-134, 137

The transfer of radionuclides to foodstuffs is commonly estimated using an aggregated transfer factor (T_{ag}, m^2 kg^{-1}):

$$T_{ag} = \frac{\text{Concentration of radionuclide in foodstuff, Bq kg}^{-1}}{\text{Density of deposited radioactivity, Bq m}^{-2}} \quad \text{m}^2\ \text{kg}^{-1}$$

Since the transfer of radionuclides to foodstuffs is dependent on a number of factors (including transfer through the soil, soil composition and chemistry, time after fallout), the T_{ag} is clearly a simplified concept. It is, however, a valuable tool for estimating activity concentrations in foodstuffs from maps of contamination density. In addition, some models (e.g. Yatsalo *et al*., 1997) can improve estimates of the T_{ag} by accounting for changes over time and using soil-specific T_{ag} values. More complex models utilize soil property data, such as percentage clay, potassium content and percentage organic matter, to estimate radiocaesium transfer to food crops and grass (Crout *et al*., 1999).

As could have been predicted from work carried out before the Chernobyl accident, foodstuffs from intensively managed systems

Table 17.4. Intervention limits for the ^{137}Cs activity concentration (Bq kg^{-1}) in foodstuffs within Belarus, Russia and the Ukraine as in place in 1999.

	Belarus	Russia	Ukraine
Fresh milk	111	370	370
Butter	185	370	
Cheese		370	
Beef	600	740	740
Pork	370	740	740
Chicken	370	740	740
Bread	74	370	370
Potatoes	100	370	590
Other vegetables	100	370	590
Cereals and legumes	100	370	
Cultivated berries	100	370	
Other fresh fruit	100	370	590
Baby food	37	185	185

(in the fSU, these took the form of collective farms) had lower radiocaesium concentrations than those arising from semi-natural and natural systems. In recent years, intervention limits (Table 17.4) have generally not been exceeded in intensively produced foodstuffs (i.e. the output of collective farms) (Firsakova *et al*., 1996). In Belarus, for example, approximately 525 kt of milk and 21 kt of meat were above the intervention limit during 1986: by 1994, this had dropped to 12.4 and 0.003 kt, respectively (Firsakova *et al*., 1996). Reductions in the amounts of animal-derived foodstuffs exceeding intervention limits have occurred through natural declines in ^{137}Cs bioavailability (see below) as well as by the use of soil-based countermeasures. In the case of meat, reductions were brought about mainly by the selective feeding of uncontaminated fodder or transportation of animals to less contaminated areas during the final fattening stage prior to slaughter.

The Chernobyl accident highlighted the potential importance of foods not derived from intensive agriculture in the transfer of radioactivity to people. The rural populations within the fSU produce, or gather, much of their own diet. Many families own a dairy cow and keep pigs and poultry. Vegetables and fruit are produced in the garden or on nearby land allocated to villagers by the collective. There is a common tradition of collecting edible fungi and berries from forests (often termed forest gifts) and, in some areas, fish from lakes or rivers. Therefore, intensive agricultural products are often of little importance to the diet. Radiologically, this local food source is important as the transfer of radiocaesium to wild foods (such as fungi, ericaceous berries and some species of freshwater fish) is often much greater than that to other foodstuffs. In addition, effective ecological half-lives (T_{eff}, the time taken for the amount of radioactivity in a foodstuff to reduce by one half by physical decay and environmental processes (see below)) are often much longer than in agricultural systems. Also, privately owned cows often graze poor quality pastures, forest clearings or meadows along watercourses. The soils in such pastures allow a comparatively high root uptake of radiocaesium. Consequently, a greater proportion of privately produced milk has ^{137}Cs activity concentrations in excess of national intervention limits than milk produced on collective farms. Within Belarus, there were 73 villages in which privately produced milk had ^{137}Cs activity concentrations in excess of 111 Bq kg^{-1} between 1995 and 1998 (Beresford and Wright, 1999). (Since the villages represent measurement centres, it is likely that the total number of villages producing milk in excess of 111 Bq kg^{-1} was greater than 73.) ^{90}Sr activity concentrations in the milk of privately owned cattle also exceed intervention limits within Belarus (Rolevich *et al*., 1996). The contribution of different foodstuffs to radiocaesium in the diet is illustrated in Table 17.5, using data from a village in the Ukraine (Beresford and Wright, 1999).

In rural settlements surveyed within Belarus, Russia and the Ukraine, 40–75% of interviewees consumed wild fungi, 60–70% forest berries and 20–40% fish from local lakes (Strand *et al*., 1996). Shutov *et al*. (1996) estimated that fungi and berries could contribute up to 60–70% of dietary ^{137}Cs intake of those adults within Russia. Indeed, within the rural population in the affected parts of Russia, a mean increase in the whole-body radiocaesium activity of 60–70% in autumn as a result of fungi consumption has been noted (Skuterud *et al*., 1997). Similar correlations were reported between the consumption of both forest berries and freshwater fish caught in local lakes and ^{137}Cs whole-body measurements in two Russian settlements (Strand *et al*., 1996). Table 17.6 illustrates the differences in ^{137}Cs T_{ag}s between different foodstuffs and environments.

INFLUENCE OF STABLE POTASSIUM ON ^{137}CS UPTAKE BY THE FOOD CHAIN Post-Chernobyl studies confirmed previous work which had shown the influence of potassium on radiocaesium uptake by the food chain. Uptake of radiocaesium by plants is inversely related to the potassium concentration of the soil interstitial water. One of the most commonly used countermeasures to reduce ^{137}Cs in foodstuffs in the fSU has been to increase rates of fertilizer application with potassium based-fertilizers. However, the current poor

Table 17.5. Example of consumption rates of different foodstuffs and the contribution of each foodstuff to the daily ^{137}Cs intake, as determined during June/July 1997 in Milyach, Ukraine.[a] Results are presented as median values, and the range in intake rates is indicated in italics.

Foodstuff	^{137}Cs activity concentration Bq kg^{-1}	Consumption rate kg day^{-1} fresh weight	^{137}Cs intake rate Bq day^{-1}
Milk	87	1.00	87 *22–900*
Meat	58	0.10	5.8 *1.8–22*
Potatoes	27	0.70	19 *6.5–45*
Other vegetables	23	0.20	4.5 *1.3–18*
Fungi	215	0.02	43 *0–759*
Berries	456	0.009	4.1 *0–14*
Fish	55	0.02	1.1 *0.3–15*
Bread	22	0.50	11 *7.0–28*
Total daily intake	79	2.72	215 *52–1390*

[a]Data from Beresford and Wright (1999).

Table 17.6. ^{137}Cs transfer factors of different foodstuffs. Measured T_{ag} values typically vary by several orders of magnitude and the values given here are for illustrative purposes only. The higher contamination density is illustrative of land in the 0.55–1.5 MBq m^{-2} contaminated zone in the fSU, the lower is illustrative of the most contaminated areas in Western Europe.

		Predicted ^{137}Cs in foodstuff at different contamination densities	
Foodstuff	Transfer factor (T_{ag}) m^2 kg^{-1}	2×10^4 Bq m^{-2}	1×10^6 Bq m^{-2}
Milk, Dubrovitsa, Ukraine 1994/95[a]	1.5×10^{-4} Gley soil 3.7×10^{-3} Peat soil	3 Bq kg^{-1} 74 Bq kg^{-1}	150 Bq kg^{-1} 3,700 Bq kg^{-1}
Sheep meat, Norway, 1993[b]	42.7×10^{-3}	854 Bq kg^{-1}	42,700 Bq kg^{-1}
Cow meat, recommended[c]	6×10^{-3}	5.1 Bq kg^{-1}	256 Bq kg^{-1}
Wild mushroom *Suillus luteus*, 1994, Belarus[d]	41.7×10^{-3}	834 Bq kg^{-1}	41,700 Bq kg^{-1}
Berries, *Vaccinium myrtillus*, 1989–1994, Belarus[d]	7.7×10^{-3}	154 Bq kg^{-1}	7,700 Bq kg^{-1}
Predatory fish, range of lakes, 1993–1997[e]	1.0×10^{-3} Low transfer 50×10^{-3} High transfer	20 Bq kg^{-1} 1,000 Bq kg^{-1}	500 Bq kg^{-1} 50,000 Bq kg^{-1}

[a]Howard *et al.* (1996); [b]Dahlgaard (1994); [c]IAEA (1994); [d]Kenigsberg *et al.* (1996); [e]Smith *et al.* (2000a).

economic climate has led to a reduction in the rates of fertilizer use within contaminated areas. Alexakhin *et al*. (1996) report that the K_2O fertilizer application rate in the Bryansk oblast of Russia decreased from 81 kg ha^{-1} in 1991 to 18 kg ha^{-1} in 1993, and that there was a consequent threefold increase in the transfer of ^{137}Cs to cereals and potatoes.

In agreement with studies of weapons test fallout, inverse relationships were observed between the concentration factor (CF) of ^{137}Cs in fish and the K^+ concentration of lake waters (Smith *et al*., 2000a). Thus, it was found that fish in lakes in agricultural areas (where potassium concentrations tend to be higher as a result of runoff of fertilizers) tend to be significantly less contaminated than those in semi-natural areas, which have lower potassium concentrations. Post-Chernobyl research has demonstrated, however, that dietary potassium has little influence on the transfer of radiocaesium to mammals.

Strontium-90

Though information on ^{90}Sr contamination from Chernobyl is relatively sparse, results indicate much lower activity concentrations in foodstuffs than for ^{137}Cs (IAEA, 1991). The data indicate $^{137}Cs/^{90}Sr$ ratios in foodstuffs of between 10/1 and 100/1, as a result of relatively lower releases of ^{90}Sr and lower accumulation by biota. For example, milk samples from the Ovruch region with 1068 Bq l^{-1} ^{137}Cs contained only 5 Bq l^{-1} ^{90}Sr (IAEA, 1991). In the Chernobyl cooling pond, ^{90}Sr activity concentrations were around 2 kBq kg^{-1} in fish during 1986, compared with around 100 kBq kg^{-1} for ^{137}Cs (Kryshev, 1994). It is likely that $^{137}Cs/^{90}Sr$ ratios will tend to be lower closer to Chernobyl than in samples from areas further away since less volatile ^{90}Sr was associated with fuel particles deposited mainly within the 30 km zone. Weathering of such 'hot particles' has increased in ^{90}Sr in vegetation over time after the accident in some parts of the exclusion zone (Kashparov *et al*., 1999).

Time-dependent Transfer of Radiocaesium to Foodstuffs

During the years after Chernobyl, the bioavailability and environmental mobility of radiocaesium declined markedly, resulting in large changes in contamination of foodstuffs, vegetation and surface waters. Laboratory studies on the sorption of radiocaesium by soils and sediments quantified the selective binding of Cs to specific sorption sites ('frayed edge sites', FES) on illitic clay minerals (Cremers *et al*., 1988). On these sorption sites, radiocaesium is available for ion exchange with ions that have a similar hydrated radius, specifically potassium and ammonium. Over time, however, radiocaesium slowly diffuses into the illite lattice (Comans and Hockley, 1992) becoming unavailable for direct ion exchange, a process commonly known as 'fixation'.

During the weeks to months after a fallout event, radiocaesium activity concentrations in both vegetation and surface waters are determined by short-term processes. Activity concentrations in plants are determined by the interception and washoff rates of the initial fallout, as well as uptake by the roots (e.g. Fesenko *et al*., 1997). Similarly, in rivers and lakes, activity concentrations are high initially as a result of direct deposition to the water surface and rapid runoff of ^{137}Cs before it is sorbed by catchment soils (Monte, 1995; Smith *et al*., 1997). Activity concentrations then decline over a period of weeks to months as a result of reduced runoff from catchments and, for lakes, loss of ^{137}Cs through the outflow and deposition to bottom sediments.

On long time scales (years), the processes which determine radiocaesium transfers to and from many different ecosystem components (e.g. between plants and animals) are fast in comparison with the slow decline in radiocaesium availability in soil (Fig. 17.3). Thus, the change in radiocaesium activity concentration in the main environmental compartments should be controlled by slow changes in its soil–soil-solution partitioning. To test this hypothesis, Smith *et al*. (1999) analysed many long-term field studies of temporal changes in radiocaesium in three different ecosystem components: vegetation, surface waters (dissolved phase) and milk following the Chernobyl accident (examples shown in Fig. 17.4(a)).

Long-term rates of change in the radionuclide content of vegetation and biota are described by assuming an exponential decline in radioactivity concentration, C:

$$C = C(0)e^{-\lambda t}$$

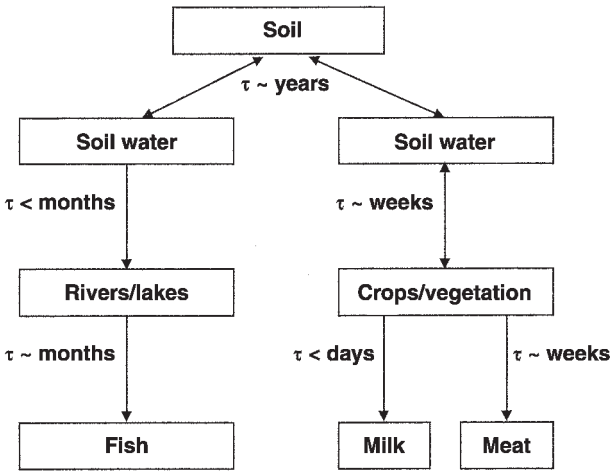

Fig. 17.3. Schematic diagram indicating time scales, τ, of release of radiocaesium from soils to terrestrial and aquatic ecosystems during the years after a fallout event (adapted from Smith *et al.*, 1999). The time scale of 'fixation' in soils is significantly longer than rates of retention and release of radioactivity in the other parts of the ecosystem. Thus, in the long term, changes in the soil–soil-water partitioning control changes in radioactivity concentration in surface waters, vegetation, etc.

Values of λ include a physical decay component (^{137}Cs decay constant: 0.023 year^{-1}). The rate of decline, λ (year^{-1}), is often quoted as an effective ecological half-life (the time taken for the amount of radioactivity in a foodstuff to decline by one half), T_{eff} (years), where $T_{eff} = \ln(2)/\lambda$.

Figure 17.4(b) shows a histogram of measured T_{eff} values in the three different ecosystem components, vegetation, milk and water. Combining results for all three ecosystem components gives a mean T_{eff} = 1.7 years, with 91% of all measurements falling within the range 1–4 years.

Clearly, there is natural variation in T_{eff} values, which may be linked to soil characteristics. However, to our knowledge, there is currently no systematic way of predicting such variation. Nevertheless, the range in rates of decline observed (T_{eff} = 1–4 years) is relatively small: a value of T_{eff} = 2 years will adequately describe most cases.

However, as shown in Fig. 17.4(c), recent data (Jonsson *et al.*, 1999; Smith *et al.*, 2000b) show a two-component exponential decline ($C = C_1e^{-k_1t} + C_2e^{-k_2t}$) in ^{137}Cs activity concentrations in surface water, terrestrial vegetation and fish. The observations show that the effective ecological half-life in young fish, water and terrestrial vegetation has increased from 1–4 years during the first 5 years after Chernobyl to 6–30 years in recent years. From the observed persisting mobility of radiocaesium in the environment, and particularly the increase of T_{eff} towards the physical decay rate of ^{137}Cs ($t_{½}$ = 30.2 years), it was concluded (Smith *et al.*, 2000b) that the sorption–desorption process of radiocaesium in soils and sediments is tending towards a reversible steady state.

Predicting future ^{137}Cs contamination

The continuing mobility of ^{137}Cs in the environment means that foodstuffs will remain contaminated for much longer than was expected initially. In the UK, restrictions on the sale and slaughter of sheep are currently in place on 389 upland farms (~232,000 sheep), on which some sheep have ^{137}Cs activity concentrations above the UK limit for the entry of meat into the food chain (1000 Bq kg^{-1}). During studies conducted on three restricted farms between 1991 and 1993, the maximum ^{137}Cs level in sheep meat was 1870 Bq kg^{-1}. Assuming that this is typical of restricted farms within the UK, and using the rates of long-term decline estimated above,

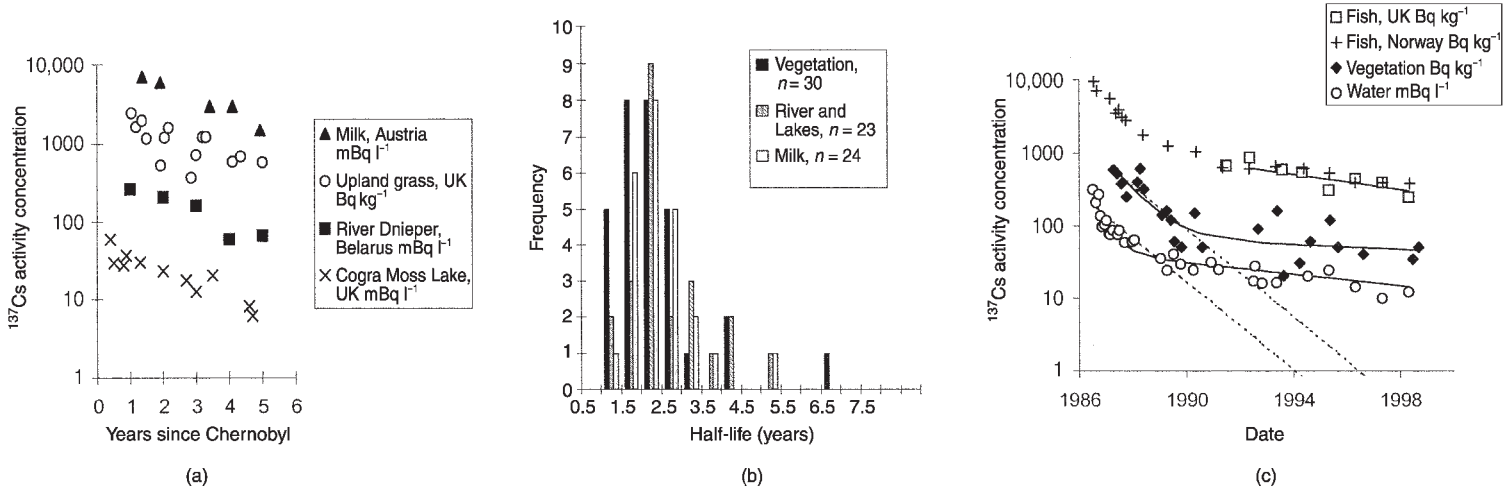

Fig. 17.4. (a) Examples of changes in ^{137}Cs activity concentration in different ecosystem components after Chernobyl (Smith *et al.*, 1999). (b) Frequency distribution of effective ecological half-lives in different ecological components during the first 5 years after Chernobyl (Smith *et al.*, 1999). (c) Long-term changes in ^{137}Cs in brown trout, Norway (Jonsson *et al.*, 1999), perch, terrestrial vegetation and water, UK (Smith *et al.*, 2000b). The decline in ^{137}Cs in immature fish, water and vegetation during the first 5 years has a T_{eff} of 1–4 years as a result of 'fixation'. The dotted lines demonstrate a hypothetical continuation of irreversible fixation. Fits of the two exponential models to the data, indicating reversible 'fixation', are shown as solid lines. Long-term declines in the UK and Norwegian systems are similar (T_{eff} 6–30 years).

restrictions may need to remain in place on some farms for a total of 30 years after the Chernobyl accident; similar predictions for the continuing need for restrictions have also recently been made by Nisbet and Woodman (1999). The predicted time period for restrictions is more than 100 times longer than was expected initially. In some areas of the fSU, consumption of forest berries, fungi and fish (current ^{137}Cs content 10–100 kBq kg^{-1}), which contributes significantly to people's radiation exposure, will need to be restricted for at least a further 50 years (Beresford *et al*., 2001).

Risk Assessment

A full description of the methods of radiation risk assessment is beyond the scope of this chapter. Radiation risk assessment is a complex subject, and the following is only a brief outline of the principles of assessment of risks from ingestion of radioactivity in foodstuffs.

The amount of radiation absorbed in the body is measured in grays (Gy), where one gray equals one joule of radiation energy absorbed per kilogram. This absorbed energy can be estimated quite precisely using models for the transfer of ionizing radiation through tissues. The damage that the absorbed radiation does to people is much more difficult to quantify, particularly at the relatively low doses associated with environmental contamination. There are, however, a number of epidemiological studies relating absorbed dose to risk of adverse health effects (primarily cancer risk).

Because different types of radiation damage cells in different ways, radiation risk is measured in dose equivalents, sieverts (Sv), rather than in total energy absorbed. For β and γ radiation, 1 Gy of absorbed energy results in a dose equivalent of approximately 1 Sv. For the (potentially) more damaging α particles, 1 Gy of absorbed energy results in a dose equivalent of approximately 20 Sv (see Table 17.2). The dose equivalent (Sv) is estimated from a concentration of radioactivity in a foodstuff (in becquerels) using an ingestion dose coefficient (Sv Bq^{-1}). Ingestion dose coefficients are calculated from models to estimate the absorbed radiation energy by the body from ingestion of radioactivity (ICRP, 1990). Such models account for the energy and type of emitted particle, the extent of absorption of the radionuclide by the body, and its distribution and retention time in the body. Dose coefficients are determined for different ages (e.g. infants, children and adults), their values generally decreasing with age.

Using epidemiological studies, primarily of survivors of the Hiroshima and Nagasaki atomic bombs, radiation protection agencies have estimated the lifetime fatal cancer risk to people from exposure to ionizing radiation. Current risk estimates (ICRP, 1991) predict a 5% lifetime risk of fatal cancer per sievert of dose to each individual of a population. This means that, if a population is exposed to radiation leading to a dose equivalent of 1 Sv to each person, these risk estimates predict a 5% increase in the number of fatal cancers in that population. These cancers do not occur immediately, but may arise some time after exposure. Since dose rates to most individuals after Chernobyl were generally much lower than 1 Sv (so predicted excess cancer risk is significantly less than 5% for most exposed populations), increases in fatal cancers can be expected to be difficult to detect against an existing cancer mortality rate of around 20% in populations in industrialized countries.

The fatal cancer risk from consuming 'contaminated' foodstuffs is calculated by estimating the total amount of ingested radioactivity. For example, if lamb of ^{137}Cs activity concentration 1000 Bq kg^{-1} is consumed at the UK average adult consumption rate of 8 kg over 1 year (NRPB, 1996), the total activity of ^{137}Cs ingested is 8000 Bq during the year. The dose arising from this radioactivity is calculated by multiplying the total becquerels ingested by the ingestion dose coefficient. For ^{137}Cs in, for example, adults, the ingestion dose coefficient is 1.3×10^{-8} Sv Bq^{-1} (ICRP, 1990), which results in an estimated dose of 1.44×10^{-4} Sv for an intake of 8000 Bq year^{-1}. According to current risk estimates (5% per sievert for an exposed population, for chronic, low dose radiation), this is approximately

equivalent to a probability of fatal cancer of 7.2×10^{-6} or 1 in 140,000.

In such radiation risk assessments, it is current practice to assume, as we have done above, that even very low dose radiation carries with it an associated cancer risk, though this is a matter of some debate within the radiation protection community. Epidemiological studies have not shown clear evidence of increased cancer risk at very low doses and low dose rates (~100 mSv or less). The large-scale studies required to show the very small increase in cancers expected have shown no evidence of an increase, or have proved inconclusive, largely because of the many confounding factors associated with such large-scale studies. Indeed, some studies of populations exposed to varying levels of natural radioactivity have observed a decrease in cancer rates with increased exposure to radiation. Thus the risks from low doses (and low dose rates) of radiation (less than ~100 mSv) could perhaps best be described as theoretical, but, for radiation protection purposes, it is conservatively assumed that every exposure to ionizing radiation carries a potential risk.

Risk Management: Countermeasures

Because many of the radionuclides released by Chernobyl were short lived, radiation doses were greatest in the few days after the accident. Thus the most effective measure to reduce radiation doses was the rapid evacuation of the population from the immediate area. Long-term bans on consumption of contaminated produce were also put in place, and these were believed to have reduced ingestion dose rates substantially. Such bans, however, are not always adhered to because of the difficulty of obtaining good, radioactively 'clean' produce in the affected areas of the fSU. Thus, in many areas, consumption of privately produced milk and meat above intervention levels and of 'wild' foods (mushrooms, berries, freshwater fish) still contribute significantly to radiation doses.

Radiation doses from ^{131}I were reduced by bans on consumption of milk in Russia, but to a lesser extent in Belarus. After the 1957 accident at Windscale, a ban on the consumption of milk was implemented, which averted between 55 and 75% of the potential maximum ^{131}I dose to a child's thyroid (Jackson and Jones, 1991) in the area covered by the ban.

After Chernobyl, distribution of iodine tablets was not widespread and could have significantly reduced doses to the thyroid. However, a World Health Organization/European Commission working group concluded that, for adults over 40 years old living well away from the site of an accident, controls on food would be a more appropriate measure to reduce radioiodine ingestion rates and hence doses to the thyroid (Rubery and Smales, 1990). This is because there are members of the population who are sensitive to large intakes of stable iodine, so the potential risks of stable iodine prophylaxis can be greater than the benefit of reduced radioiodine burdens.

Restrictions on foodstuffs were also implemented in Western Europe, as a result of ^{137}Cs contamination of the food chain. These included controls on the sale and slaughter of more than 4 million sheep on 8914 holdings in the UK. Restrictions were also put in place in parts of Scandinavia because of high radiocaesium activity concentrations in many food products including goat milk, reindeer, sheep meat and game animals. Consumption of freshwater fish declined by up to 50% in the more contaminated areas of Sweden, and the sale of freshwater fish to the general public was prohibited in these areas (Brittain *et al*., 1991).

A requirement to respond to the Chernobyl accident provided a considerable impetus to developing effective countermeasures (Howard and Desmet, 1993). Reductions in the amounts of animal-derived foodstuffs exceeding intervention limits have been brought about by natural declines in ^{137}Cs bioavailability (see above) as well as by the use of soil-based countermeasures such as deep ploughing (to reduce activity concentrations in the surface soil layers) or addition of potassium fertilizers. In the case of meat, reductions were also brought about by the selective feeding of uncontaminated

fodder or transportation of animals to less contaminated areas during the final fattening stage prior to slaughter. Radiocaesium in milk and meat can also be reduced by adding chemicals which bind radiocaesium (hexacyanoferrates or 'Prussian blue') to the diet of grazing animals. The hexacyanoferrate reacts with consumed radiocaesium in the intestine to form a complex that is eliminated in the dung instead of passing into the animal's bloodstream. In Western Europe, much of the long-term problem after the Chernobyl accident has been connected with free-ranging or wild animals. To cope with this, delivery systems which do not need daily access to animals were developed in which the hexacyanoferrate binder is incorporated into different matrixes, including rumen-dwelling boli and salt licks (Hove, 1993).

Though many countermeasures have been used to reduce radioactivity after Chernobyl, there has also been an increased awareness that it is not only the effectiveness of a countermeasure that has to be considered but also issues of feasibility, cost effectiveness, side effects and public acceptance (Voigt *et al*., 2000).

Conclusions

Chernobyl was one of the worst industrial accidents in history, causing potential risks to the safety of the food chain on a continental scale. Thousands of square kilometres of land in Belarus, Russia and Ukraine still cannot be used for agricultural production and have been defined as areas of strict radiation control. Populations of whole towns and villages have been relocated, creating hundreds of thousands of 'Chernobyl refugees'.

Scientific studies on Chernobyl radioactivity in the food chain have established which areas and foodstuffs are most affected, as well as identifying many of the environmental factors which determine food chain contamination. It is now known that the contamination of foodstuffs (particluarly 'wild foods') by ^{137}Cs will remain a problem for 50 years or more in the affected regions of the fSU. In spite of the extensive environmental contamination, however, current radiation doses to the Chernobyl-affected populations are low. Indeed, the social and economic consequences of the accident may well outweigh the direct health effects of the radiation. In addition to the social and economic consequences of evacuating large areas of land, a major problem in dealing with the long-term effects of the accident is the public's overestimation of radiation risk. An International Atomic Energy Authority (IAEA) study found the psychological effects of Chernobyl to be 'wholly disproportionate to the biological significance of the radiation' (IAEA, 1991). Populations in the contaminated areas have been shown to have higher levels of stress, worse perceived health and greater use of medical facilities (e.g. number of doctor visits) than similar unaffected populations. The challenge for scientists and decision makers now is to determine how best to manage the contaminated areas in the decades to come. A key element of this management strategy is an information and education programme for the people who have to live with Chernobyl's radioactive legacy.

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18 Radionuclides in Foods: American Perspectives

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Introduction

The US Food and Drug Administration (FDA) has the responsibility for the wholesomeness of the food supply in the USA. In 1961, the FDA initiated a programme for monitoring the radionuclides in foods in the teenage diet (FDA, 1963; Laug *et al*., 1963). This was in response to the above-ground weapons testing. At the same time, the US Public Health Service (PHS) instituted a nationwide pasteurized milk network (PMN); in cooperation with the FDA (Roecklin *et al*., 1970), milk samples were collected from at least one of the largest cities in each state.

These samples were forwarded to one of three PHS laboratories for analysis. The radionuclides of interest were ^{131}I, ^{137}Cs, ^{140}La, ^{90}Sr and ^{89}Sr. Various food products were collected as they were harvested. The PHS also instituted a food programme (Roecklin *et al*., 1970) called the Institutional Diet Program (IDP) for collecting samples from various institutions of teenage diets, similar to the FDA programme, except that in the PHS study the total diet sample was homogenized, rather than analysing each component. With the signing of the US–Soviet above-ground weapons testing ban treaty, these programmes were changed.

The FDA had stopped sampling and analysing the foods in the teenage diet in 1969 to avoid duplication. In 1973, the FDA decided to resume its programme, due to a governmental reorganization. Two of the PHS laboratories had been transferred to the Environmental Protection Agency (EPA) and were no longer analysing for radioactivity in food (Anderson and Nelson, 1962). The PMN was put on a standby and EPA in 1973 cancelled the IDP.This new FDA programme included the teenage and infant diet sample and was responsible for analysing radioactivity in food (Food and Drug Administration, 1973). This Teenage and Infant Diet Program was also a part of its Total Diet Program Study, which analyses for various other components. The radionuclides in the food programme also included imported foods that had domestic status. In addition, the FDA would be able maintain a capability for analysing radionuclides in food samples in the event of a release from a nuclear accident. Such incidents occurred, the first in 1979, when there was an accident at the Three Mile Island Nuclear Power Plant near Harrisburg, Pennsylvania. Later, in 1986, there was an

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accident at the Chernobyl nuclear power plant near Kiev in the Ukraine, USSR. (see Smith and Beresford, Chapter 17, this volume).

Nature of Radionuclides of Interest

The primary radionuclides found in foods and milk were the short-lived fission products ^{89}Sr, ^{131}I, ^{140}Ba and ^{140}La. In addition, there were several longer-lived fission products such as ^{137}Cs and ^{90}Sr. ^{134}Cs was found in products from the Chernobyl incident. The food products contained other short-lived and longer-lived fission products such as ^{65}Zn, ^{95}Zr, ^{95}Nb, ^{103}Ru, ^{106}Ru, ^{140}Ce and ^{141}Ce. These radionuclides were not found in milk. The milk and milk products that were produced from the cow contained only the former, as the cow's digestive system discriminated against the other radionuclides.

Background – nature of radionuclides

The modern epoch in physics may be said to have begun with the discovery of X-rays by Roentgen in 1895. This was followed by the discovery of radioactivity by Becquerel in 1896. These two discoveries led to other developments in the understanding of nuclear structure, reactions and the various types of particles involved. All these investigations finally culminated in the discovery of 'fission', which resulted in the development of atomic energy, in both peace and war.

Atomic structure

All matter is made up of elements. The smallest part of an element is the atom. The size of a hydrogen atom, which is the smallest, is about 1.5×10^{-8} cm while its weight is about 1.67×10^{-24} g. In comparison, a uranium atom would weigh about 4.0×10^{-22} g. The atom itself consists of a central nucleus surrounded by a cloud of electrons ranging in number from one for hydrogen to 92 for uranium. These electrons are said to move in orbits around the central nucleus. The size of the nucleus is considerably smaller than that of the atom, the size of the hydrogen nucleus being 1.4×10^{-13} cm. The nucleus itself consists of protons, which carry a unit positive charge 'e' ($e = 1.6 \times 10^{-17}$ coulombs), and neutrons, which carry no charge. The mass of the neutron is only slightly greater than that of the proton. The electron carries a unit negative charge and has a mass approximately 1/1840 of that of a proton or a neutron. Hence, most of the mass of the atom is carried in the nucleus. It is also known that most of the energy of the atom is also stored in the nucleus.

Elements and isotopes

All the elements of the periodic table are made up of protons and neutrons in the nucleus and electrons orbiting around it. The total charge carried by the nucleus is equal to the number of protons in it. This number is called the 'atomic number' and is characteristic of each element. Since the atom as a whole is electrically neutral, there will be as many electrons around the nucleus as there are protons in it. The number of protons plus neutrons gives the 'mass number' of the atom. Thus, the mass number of carbon, $^{12}_{6}C$, is 12 and the atomic number is 6. If two nuclei have the same number of protons in the nucleus, but different number of neutrons, they are called isotopes. For example, $^{7}_{4}Be$ and $^{10}_{4}Be$ are isotopes of beryllium having the same number of protons and the number of neutrons being three and six, respectively. Isotopes have the same chemical properties, which depend on the number of electrons in the orbits around the nucleus, but different physical properties, which depend on the central nucleus.

Radioactivity

As mentioned before, it was noted early in this century that certain substances are radioactive, i.e. they spontaneously emit different types of radiations and transform into other elements depending on the emitted radiations. This process, which was first noted in

the heavy elements such as uranium, radium, etc., was found to obey an exponential decay law, i.e. the amount of radiation emitted decreases with time in such a manner that the period for reduction to half the initial value is constant. This period is called the 'half-life' and is characteristic of the concerned isotope. The various types of radiations emitted in radioactive decay are described in subsequent sections.

α *Radiation*

This is emitted generally by the heavy elements such as uranium, thorium, radium, etc. They are identical to the nucleus of the helium atom, carrying two protons and two neutrons. Hence, their mass is about four times that of the nucleus of the hydrogen atom (one proton only) and the charge is +2e, i.e. twice that of the hydrogen nucleus. The α radiation is usually highly energetic, having energies in the range of 4–9 million electron volts (1 MeV = 1.602×10^{-6} ergs). In view of their heavy mass and charge, α particles are highly ionizing and are easily absorbed. Hence, they can travel only a few centimetres or so in air. They cannot penetrate beyond the skin and are harmful only when they enter the body organs either by inhalation or by ingestion of contaminated food.

β *Radiation*

This type of radiation consists of particles identical to electrons. They are emitted in radioactive decay with various energies ranging from nearly zero to a maximum energy characteristic of the decaying radioactive isotope. Hence, their energy spectrum differs from that of α rays, which have discrete energies. The β rays can travel a few feet in air but cannot penetrate much beyond the depth of the skin of a person. Hence, like the α rays, they are harmful only when they are inside the body. From outside, they can cause only skin burns. β Rays are absorbed in an approximately exponential manner, i.e. they have a characteristic thickness in the particular manner that will reduce the radiation intensity to half the initial value. This value depends on the atomic number of the material, its density and the maximum energy of the β particles.

γ *Radiation*

Gamma radiation is electromagnetic radiation similar to light but of much higher energy. The wavelength of γ rays is much shorter than that of visible light. The energy of the electromagnetic radiation or photon is given as $W = h\nu$, where h is the Planck's constant, ν (nu) is the frequency, equal to $c\lambda^{-1}$, c being the speed of light and λ (lambda) the wavelength of the radiation. γ Rays are emitted in radioactive decay along with α or β radiations. Like α rays, γ rays have discrete energies. They are absorbed exponentially in materials but, in view of their great penetrating power, only thick blocks of concrete, lead or other high-density materials can reduce their intensity to a small value. The γ rays can deliver a whole-body dose from either outside or inside the body due to their high penetrating power. The element does not change due to γ radiation, but will change depending on the accompanying α or β radiation.

Distribution of Radionuclides in the Food Chain

Man's use of nuclear energy involves the production of artificial radionuclides, which present a potential public health problem with regard to the contamination of the terrestrial food chain. The release of these radionuclides has not introduced to our environment a new pollutant, but rather has increased an old pollutant in terms of radiation exposure. The problem then is a matter of evaluating the extent of man-made radioactivity in the environment and its subsequent health significance. At present, fallout from past nuclear weapons tests is the most widespread source of artificial radionuclide contamination in the terrestrial environment. With limited nuclear testing, other sources such as nuclear reactor operations, nuclear fuel reprocessing, waste disposal, and medical and industrial uses of radioisotopes will be receiving more

emphasis as significant sources of environmental contamination.

The food chain

The pathways which radionuclides follow in moving from their origin to man constitute the food chain. Radioactive materials are removed from the atmosphere by meteorological processes, primarily precipitation. In general, the most serious food chain contamination problem arises from direct deposition of radioactive materials on animal feed crops or on food crops directly consumed by man. Following this initial deposition, various processes may remove the radioactive materials, such as being washed off by rain or blown off by wind. The extent of this removal is a function of many physical and biological parameters. Man's intake of radioactive material may occur from contaminated food crops, from contaminated meat and meat products and from contaminated milk or milk products. The inhalation route (atmosphere directly to man) may be important under special circumstances and is not discussed here. The relative importance of the various pathways of intake will depend on many factors, among which are the physical half-lives of the radionuclides, the rate and route by which they pass through the food chain and the dietary habits of the population. The immediate and generally most significant pathway is pasture–cow–milk–man for the more significant radionuclides up to approximately 100 days following deposition (one time event). Plant losses are such that, after this time period, adsorption by plants is the most significant pathway for the longer-lived radionuclides. The final step in the food chain (uptake by man) primarily depends on the chemical characteristics of the radionuclide and the metabolism of the concentrating organ.

As an example of radionuclide behaviour in the terrestrial food chain, the pathway of the fission product ^{90}Sr may be cited. It has been shown that the grazing animal population readily transfers this nuclide to man from atmospheric nuclear testing by deposition on the earth's surface, with subsequent plant uptake and transference to milk.

In considering the relationships between the food chain and any particular radionuclide, it becomes necessary first to consider the source of the contaminant. The source should be considered as it relates to the physical and chemical state of the radionuclide, since these properties relate to the degree of movement in the food chain. Fission products from an atmospheric burst would not be expected to exist in the same physical and chemical state as those from a reactor incident, fuel reprocessing plant or waste treatment facility. Even among the latter operations, a wide variety of physical and chemical states of the fission products would be expected

Studies of the movement and effects of radionuclides in the food chain are of value because they:

- make possible the prediction of the relationships between the kinds of radioactivity in the various steps within the food chain and the resulting levels in the human population;
- provide a means for evaluating ways by which the levels in the human population may be minimized;
- provide background information for setting up environmental sampling programmes and for interpreting the data obtained;
- make available animal data, which may be useful for estimating behaviour of radionuclides in humans;
- make possible predictions of the possible effects of environmental contamination, especially in the grazing animal population.

Radioactive materials from atmospheric releases are deposited on the earth's surface by precipitation or direct deposition. Although the discussion of possible fallout patterns is beyond the scope of this chapter, it should be stated that fallout distribution depends on many parameters. For nuclear weapons testing, these include meteorological conditions, fission yield, type of explosion (i.e. ground, water or air) and geographical location. In the case of a nuclear incident, such as an air release from a nuclear reactor, the factors of

major concern would be the existing fission product inventory, the micrometeorological conditions and the nature of the surrounding terrain.

Contamination of animals and animal products

Certain radionuclides are readily transferred to the human population via domestic grazing animals, which are effective collectors of contamination from various vegetative forms (Eisenbud, 1973). There are many factors which affect the degree to which animals are contaminated. The most important include:

- pasture type used for grazing;
- extent of barn feeding (purchased and stored), miscellaneous feeding practices – age of food and supplemental feeding use of purchased feed, etc.;
- water source; and
- animal housing – degree of sheltering animals from surface contaminants.

Because of the many variables involved, the degree of animal and animal product contamination may be quite variable, even from apparently similar sources.

Of particular interest in this pathway of the food chain are the relationships that exist between the quantities of radionuclides ingested by the animal and the subsequent quantities which are deposited in the tissues and secretions that serve as human food. To study these relationships requires knowledge of the metabolic characteristics of the animal and the particular radionuclide. Only those radionuclides which enter the food chain at a significant rate and quantity will be of importance to man. These radionuclides must also possess characteristics that allow for their continued movement through the chain.

Radionuclide Metabolism

Classically, metabolism refers to the biological processes whereby complex cellular elements are synthesized.

General considerations involved in radionuclide metabolism

For practical purposes, only the gastrointestinal mode of entry of nuclides into the compartments of a biological system is important (Thompson, 1960; National Academy of Sciences–National Research Council, 1961). In special situations, the pulmonary and skin routes may be important in permitting assimilation of the nuclides. The intravenous route of entry is artificial and only important as an experimental tool. However, it must be noted that it simulates the situation once a nuclide is absorbed into the bloodstream. This method to some extent by-passes the uncertainties involved in a study with natural routes of assimilation.

The gastrointestinal system is probably the most important route of entry for soluble forms of nuclides. Insoluble forms will be dependent on the degree of solubility and remain in the intestine, with this organ receiving the bulk of the radiation exposure. α Emitters will dissipate about 1% of their energy in the tissues of the gastrointestinal tract. A much greater percentage of the energy from β emitters will be absorbed and dissipated within the gastrointestinal tract. Insoluble γ emitters will effectively radiate the intestine; however, the rest of the body will also be exposed. Pertinent examples of the more important fission products are given in (Table 18.1).

Metabolic classification of radionuclides

No completely satisfactory classification of nuclides is possible. One approach is to group the nuclides according to their positions in the periodic table. It would be expected that nuclides of the same group would behave similarly because of their similar chemical properties. The contamination of the terrestrial food chain from the various uses of nuclear energy constitutes a potential health problem to man. To cope with this problem requires a consideration of the physical, chemical and biological characteristics of the radio-contaminants and the terrestrial

Table 18.1. Fission products of biological importance.[a,b]

Chemical character	Isotopes (pairs) important on account of abundance and half-life	Gastrointestinal uptake from GI tract to		
		Total body	Critical organ	
Halogens	^{131}I, ^{133}I, ^{135}I	1.0	0.3	Thyroid
Oxygenated anions	^{132}Te, ^{132}I	0.25	–	–
Alkali metals	^{137}Cs, ^{137}Ba	1.0	0.4	Muscle
Alkaline earths	^{89}Sr, ^{90}Sr, ^{90}Y	0.3	0.2	Bone
	^{140}Ba, ^{140}La	0.05	0.04	Bone
Rare earths	^{91}Y, ^{95}Zr, ^{95}Nb	10^{-4}	3×10^{-5}	Bone, liver
	^{141}Ce, ^{144}Ce, ^{144}Pr			
	^{143}Pr, ^{147}Nd, ^{147}Pm			
Noble metals	^{103}Ru, ^{106}Ru, ^{106}Rh	0.03	4×10^{-6}	Bone, liver

[a]Food and Agriculture Organization (1960) and Interlaboratory Technical Advisory Committee (1965).
[b]Isotope pairs are classed according to the chemical and biological characteristics of the parent.

food chain. The methods of fission product deposition along with the mechanisms of soil, plant and animal contamination need to be fully understood. With the above considerations in mind, the particular radionuclides of health importance to man in the terrestrial environmental can be identified and their metabolic relationships studied. By sampling the appropriate media, a comparison of the quantities of these artificially produced fission products with the naturally occurring radionuclides can be made, and then an assessment of the degree of environmental contamination can be obtained.

Milk and milk products contain naturally occurring chemicals such as sodium, calcium, strontium and potassium. The latter chemical has a natural occurring radionuclide ^{40}K. Its abundance is 0.118% in nature. The half-life is 1.26×10^9 years. It decays by β emission (89%) and electron capture (11%). It also decays by γ rays (11%), with an energy of 1.460 MeV. This is readily detectable in milk. The metabolism of the cow is such that the long-lived radionuclides found in milk are ^{137}Cs, ^{134}Cs (notably from Chernobyl) and ^{90}Sr. Short-lived radionuclides include ^{131}I, ^{140}Ba, ^{140}La and ^{89}Sr. The latter radionuclides are usually found in fresh fission products. Table 18.2 lists these radionuclides by emission decay, half-life and energy. Food products may contain these radionuclides and other fission products. These are listed in Table 18.3.

Metabolism of radiation in man and animals

Many radionuclides are produced by man's use of nuclear energy. Of these, only a limited number are important as sources of internal radiation to the human body. These specific radio-contaminants can reach man by way of the terrestrial food chain. This discussion has a twofold purpose: (i) to describe the characteristics that determine the environmental significance of a radionuclide; and (ii) to present a detailed description of the food chain behaviour of several of these radionuclides of environmental significance.

Characteristics of radionuclides of environmental importance

In order for a radionuclide to be a significant environmental contaminant, it must possess certain characteristics. These characteristics must be such that they allow the nuclide to move from its point of origin through the food chain, and still remain a possible health hazard to man. In general, the radionuclides of environmental significance are those man uses which are readily taken up by plants and animals. They are either isotopes of elements important in metabolism or closely similar to them. For example, the alkali metal ^{137}Cs is metabolically similar to potassium. It is readily absorbed and circulates freely throughout the body, irradiating all tissues. The fission product ^{131}I, an isotope of the

Table 18.2. Characteristics of the more important fission products of food chain significance.[a]

Radioisotope[b]	^{235}U (%)	^{238}U (%)	Type of radiation	Physical half-life	GI absorption (%)
^{90}Sr/^{90}Y	5.1	3.2	β	28 years	30
^{137}Cs	6.2	6.2	β, γ	29 years	100
^{147}Pm	2.9		β	2.6 years	0.01
^{144}Ce	5.0	4.5	β, γ	285 days	0.01
^{106}Ru/^{106}Rh	0.5	2.7	β, γ	1.0 years	0.03
^{95}Zr	6.3	5.7	β, γ	65 days	0.01
^{89}Sr	4.6	2.0	β	51 days	30
^{103}Ru	3.4		β, γ	39.7 days	0.03
^{95}Nb	6.3		β, γ	35 days	0.01
^{141}Ce	6.0		β, γ	33 days	0.01
^{140}Ba/^{140}La	6.1	5.7	β, γ	12.8 days	5
^{131}T	3.1		β, γ	8.04 days	100

[a]Food and Agriculture Organization (1960) and Katcoff (1958, 1960).
[b]Isotope pairs are classed according to the chemical and biological characteristics of the parent.

Table 18.3. Fission products important due to fission abundance and half-life.[a,b]

Chemical character	Isotope	Fission abundance (%)	Half-life
Halogens	^{131}I, ^{133}I, ^{135}I	3.1, 6.3, 6.0	8.1 days, 22 h, 6.7 h
Oxygenated anions	^{132}Te, ^{132}I	4.0	7.8, 2.4 h
Alkali metals	^{137}Cs, ^{137}Ba	6.2	37 years, 2.6 months
Alkaline earths	^{89}Sr, ^{90}Sr, ^{90}Y	4.6, 5.1	51 days, 26 years, 61 h
	^{140}Ba, ^{140}La		12.8 days, 40 h
Rare earths	^{91}Y, ^{95}Zr, ^{95}Nb	–[c], 6.3	57 days, 65 days, 35 days
	^{141}Ce, ^{144}Ce, ^{144}Pr	6.0, 5.0	33 days, 290 days, 17.5 months
	^{143}Pr, ^{147}Nd, ^{147}Pm	–[c], 2.9, 2.7	13.7 days, 11.6 days, 3.7 years
Noble metals	^{103}Rh, ^{106}Ru, ^{106}Rh	3.4, 0.5	40 days, 1.0 year, 30 s

Atom yield > 0.03%; half-life > 10 h.
[a]Food and Agriculture Organization (1960) and Katcoff (1960).
[b]Fission abundance values are approximately the same for ^{233}U, ^{235}U and ^{239}Pu.
[c]Data lacking on abundance.

essential element iodine, concentrates in the thyroid gland. ^{90}Sr and ^{226}Ra are alkaline earths similar to calcium and follow it to the bone. ^{14}C and ^{3}H, isotopes of two very essential elements, are distributed throughout all living tissues. The metabolic processes of all plants and animals are similar; radionuclides which concentrate in animal tissues are usually those that pass most readily through the food chain.

SPECIFIC CHARACTERISTICS The relative importance of individual radionuclides depends on many factors. Among the most important factors, the following can be cited: the magnitude of the hazard from radionuclide deposition in the body is dependent on the type and energy of radiation. For the deposition of the same activity of a radionuclide in a given organ, the hazard from the type of radiation would be $\alpha > \beta > \gamma$. The hazard from the given emitter would also increase with increasing energy of emission.

CHARACTERISTICS BASED ON PHYSICAL, CHEMICAL AND BIOLOGICAL PROPERTIES An arbitrary separation of the above-listed characteristics into physical and biochemical categories can be made for discussion purposes. Developments in this area are concerned with the retention of the liberated radon. Recent investigation indicates that, in long-standing cases

of radium poisoning, an average of 70% of the radons produced are exhaled.

1. *Chemical properties*. As examples, elements of group VII, fluorine, chlorine, bromine, iodine and astatine, have strikingly different modes of metabolism. Fluorine is deposited in bone; chlorine and bromine are fairly equally extensive within the extracellular fluid space; and iodine is concentrated in the thyroid gland. Astatine is also localized in the thyroid gland.

2. *Physical properties*. The fission process gives rise to a mixture of radionuclides with a wide range of half-lives. Each of these nuclides is produced in a certain proportion (abundance), which is dependent on the fission materials and the energy of the fissioning neutrons. The abundance of the various nuclides produced has been found to be approximately the same for the different fissionable materials. Table 18.1 presents the most important fission products, based on fission abundance and half-life, which are of immediate concern in environmental contamination. High fission abundance and a moderate to long half-life when considering parent–daughter relationships characterize these nuclides. Many of these radioisotopes can subsequently be eliminated from food chain consideration because they are not present significantly long enough to be a long-term health hazard. The more important nuclides will be those which are formed in high abundance, with moderate to long half-lives and which are isotopes of, or chemically similar to, essential elements.

3. *Biochemical properties*. The chemical and biological properties of the various radionuclides greatly affect their ability to move through the food chain. Table 18.2 presents the fission products of biological importance grouped according to similar chemical characteristics, and shows the relative uptake by the total body and critical organ from the gastrointestinal tract. From Table 18.2, it is seen that the important fission products are those which comprise the rare earths, the zirconium–niobium isotopes, the noble metals, particularly ruthenium and rhodium, the isotopes of iodine, the alkali metal caesium and the alkaline earths, especially strontium and barium. Examining the fractional uptake from the gut of these various groups of isotopes biologically can give some indication of their relative importance. This leaves the alkaline earths strontium and barium, the alkali metal caesium and the iodine isotopes as nuclides of primary importance. Depending on the particular situation, these nuclides will assume a greater or lesser degree of importance in the food chain. Additional biological factors which aid in accessing the potential health hazard from the particular nuclide include: (i) the quantity deposited and the residence time of the nuclide in the critical organ; and (ii) the essentialness or indispensability of the critical organ to the organism.

Specific radionuclides

The fission products which enter the environment from fallout or from various nuclear facilities include more than 30 radioactive isotopes. From the above discussion, it is evident that all of these radionuclides are not equally harmful to the human population. Intensive study of fission product behaviour in the food chain has revealed that ^{89}Sr, ^{90}Sr, ^{140}Ba, ^{131}I and ^{137}Cs are the radionuclides of major concern. ^{90}Sr and ^{137}Cs are radionuclides of long physical half-life and are considered long-term hazards. ^{89}Sr, ^{140}Ba and ^{131}I, due to their shorter physical half-lives are only short-term hazards. This discussion will deal primarily with the environmental behaviour of ^{90}Sr, ^{131}I and ^{137}Cs.

^{89}Sr and ^{90}Sr

The radionuclides of major importance in the alkaline earth series are ^{89}Sr, ^{90}Sr and ^{140}Ba. Like calcium, these alkaline earth radionuclides are deposited in large amounts in the skeleton (Food and Agriculture Organization, 1960, 1964; Comar *et al*., 1961; Comar, 1963; Russell, 1963; Comar and Bronner, 1964; Kahn *et al*., 1965). All three radionuclides are produced in relatively large abundance during nuclear fission (see Table 18.2) and assume a greater or lesser degree of importance in food chain contamination depending

on the time period considered after the contaminating event. ^{89}Sr and ^{90}Sr behave the same chemically so that the food chain behaviour of one also applies to the other. As an example of the behaviour of a particular radionuclide in the food chain, the movement of ^{90}Sr in the pathway from atmosphere to soil to plant to animal to milk to man can be cited. As a given quantity of ^{90}Sr moves through this pathway, the various environmental components, including man, tend to discriminate or reduce the quantity, which is finally available for bone deposition in man. The ^{90}Sr is deposited on the soil and effectively diluted with soil constituents before being assimilated by the plant. The grazing animal receives the contaminant through ingestion of various vegetative forms and reduces the quantity of ^{90}Sr excreted into the milk by inherent discriminatory processes. The remaining ^{90}Sr is then available for deposition in man.

Radioiodine

In nuclear fission, a number of radioisotopes of iodine are formed. Among the most prominent are ^{131}I (half-life 8.1 days), ^{132}I (half-life 2.2 h) and ^{133}I (half-life 21 h). In fresh fission products, the shorter-lived radioiodine isotopes initially will make the major exposure contribution because of their greater abundance. However, in older fission products (of the order of a few days), the shorter-lived iodine isotopes will have decayed, and ^{131}I will be the radionuclide of major concern. Thus, the study of the environmental behaviour of radioiodine has been concentrated on ^{131}I, which has a relatively long half-life compared with the other iodine isotopes. Because of the relatively short half-life ^{131}I, the soil pathway is not important. Since ^{131}I is only of major concern for relatively short periods of time following the deposition of fresh fission products, it is only necessary to consider dietary foods that reach man shortly after contamination. Milk is the primary example of such a food. Experimental studies and dietary surveys have indicated that milk is the only food product that contributes a significant amount of ^{131}I to the human diet (Comar and Bronner, 1964; Food and Agriculture Organization, 1964). Thus the most important pathway for ^{131}I is atmosphere–plants–animals–milk–man. It is possible that the pathway atmosphere–plants–man could result in ingestion of significant amounts of ^{131}I from unwashed fruits and vegetables that have been exposed to surface contamination. However, exposure by this route would most probably be significant only on a local or individual basis (Table 18.4).

Table 18.4. Relative biological availability of ^{131}I to individuals.[a]

Age	Thyroid weight (g)	% Uptake in thyroid	% Uptake g^{-1} of thyroid	Fresh milk consumption (l day^{-1})	(% ^{131}I g^{-1} thyroid) × (l milk day^{-1})
Fetus					0
12 weeks		0	0		0.1–1
12–15 weeks			0.1–1	1	1–5
15–32 weeks			1–5	1	
Person					
0–6 months	2	30	15	0–0.5	0–7.5
6–12 months	2	30	15	0.5	7.5
1–2 years	2–5	30	12	0.5	6
2–5 years	3–5	30	10–6	0.5	5–3
5–10 years	5–10	30	6–3	0.7	4–2
10–15 years	10–15	30	3–2	0.7	2–1
15–20 years	15–20	30	2–1.5	0.6	1
20–30 years	20	30	1.5	0.3	0.4
> 30 years	20	30	1.5	0.2	0.3

[a]Comar *et al.* (1961).

Radiocaesium

In contrast to ^{90}Sr, ^{137}Cs does not readily follow the soil uptake route (Food and Agriculture Organization, 1964). This nuclide is fixed in forms largely unavailable to plants as a result of entrapment in the lattice structure of certain clays. Plant contamination, therefore, occurs primarily by direct deposition. Since ^{137}Cs is capable of concentrating in soft tissues (e.g. muscle), the step from animal to animal products (other than dairy products) assumes importance for this radionuclide. A long-term genetic dose is thus possible from ingestion of foods contaminated with ^{137}Cs. Since a significant quantity of ^{137}Cs is excreted into animal's milk, the pathway atmosphere–plants–animals–milk–man is most important for diets containing average amounts of milk. In general, the biological significance of ^{137}Cs is somewhat less than that of ^{90}Sr because of its shorter effective half-life in the body.

Natural radioactivity

There are a number of naturally occurring radionuclides in the biosphere. They enter and are transferred through the food chain to varying extents. A major portion of the work with natural occurring radionuclides has been undertaken in order to establish baseline levels in the environment. Such levels have been used for comparison in assessing the degree of contamination of the environment with artificial radioactivity.

The most important naturally occurring radionuclides belong to the uranium, thorium and actinium series. The actinium series is the least abundant of the three. These natural series are composed of a number of the heavier elements of varying half-lives that exhibit extremely complex decay schemes. Studies of the transfer of these radionuclides to man via the food chain involves measuring the activity levels in plants which are consumed directly by man, and plants such as grasses which form the principal food of animals, which in turn become the principal food of man. Dietary surveys indicate that there is a wide range of natural activities in vegetation and there appears to be no simple correlation with the activities found in the soil, which have a much smaller range. Also, there is not much information available concerning the discrimination factors for soil–food and man–food processes. It appears that plants and animals absorb the majority of these radionuclides to only a very small extent when compared with the most pertinent artificial radioisotopes. Few quantitative data are available on absorption phenomena. The greatest attention has been paid to radium since this element appears to be the principal one absorbed by plants. However, dietary surveys have indicated that the occurrence of this element in the majority of foods is well below that of man-made ^{90}Sr.

The following naturally occurring radionuclides are present in a singular form in the environment: rubidium, lanthanum, samarium and ruthenium. Little information is available on plant and animal absorption of these natural radionuclides. They are known to be present in the environment at considerably lower concentrations than the most abundant naturally occurring radioisotope, ^{40}K. Consideration of several of their characteristics such as their chemical nature, the behaviour of their man-made counterparts and their essentialness to plants would tend to indicate that these radionuclides are little absorbed by plants. The remaining fission products, activation products and naturally occurring radionuclides that are found in the terrestrial environment are of lesser hazard to man as a result of their limited movement in the food chain. However, depending on special circumstances some of these radionuclides may assume a greater importance than normally expected.

Monitoring

Surveillance

The Total Diet Program was reinitiated in 1973 (Food and Drug Administration, 1973). This programme was necessary to:

- monitor the foodstuffs in the USA. It was part of an overall programme to ensure

that the food products were safe and wholesome; and

- maintain a capability in the event that: (i) above-ground testing was initiated; (ii) leaks from around nuclear facilities occurred; (iii) a nuclear accident occurred; and (iv) there was increased use by medical facilities and increased commercial radioactive materials.

The data collected for this programme can be found in the reports that have been generated in the literature (Simpson *et al*., 1974, 1977, 1981; Tanner and Baratta, 1980; Strobe *et al*., 1985; Cunningham *et al*., 1989, 1994; Caper and Cunningham, 2000). The data have shown that the radioactive content of the food grown and consumed in the USA meets the requirements of the Federal Radiation Council (FRC) as promulgated in 1961 (Table 18.5). These results are also below the requirements of newer regulations issued in 1998 (US Department of Health and Human Services, 1998).

This capability was soon to be put to use. In 1979, an incident occurred at the reactor at Three Mile Island (Unit II) in Pennsylvania, resulting in some releases of radioactive gases. However, the containment vessel was not breached. This resulted in the sampling and analyses of thousands of samples during the first month. These were mainly milk and produce samples from the farms and dairies in the immediate area. Also included were foodstuffs processed and manufactured from that area. Sampling from that area continued for another year at a less vigorous pace. The only radionuclide found was ^{131}I, and the concentrations were in range II of the FRC's radiation protection guidelines (FRC, 1961). Intake in this range calls for active surveillance and routine control. The limits for range II are 0.37–3.7 Bq l^{-1} (Table 18.5). This radionuclide (^{131}I) was only detected during the first week. The limit of detection for this method at the 2 sigma level is ± 0.37 Bq l^{-1}. The ^{90}Sr content in all the samples tested was similar to its content due to the worldwide fallout. No ^{89}Sr was detected, which confirmed this.

The second occasion that required extensive sampling and analyses was when an incident occurred at the Chernobyl nuclear power plant in the Ukraine, of the then USSR. The radionuclides from the incident at Chernobyl did not increase the dietary intake of foods grown and processed in the USA. However, the foods imported into the USA did contain 'fallout'. In the early period, the major radionuclide of concern was iodine. Several mushroom samples and soft cheese samples were detained as they exceed the US Department of Agriculture (USDA)–FDA levels of concern (FDA, 1986) (Table 18.6). Other samples, particularly spices and herbs, contained high amounts of fission products. Later, samples of cheese, apple juice and pasta

Table 18.5. Radiation protection guides (RPGs) and derived concentration action ranges (Bq kg^{-1})[a] for selected radionuclides for the general population.[b]

Radionuclide	Target	RPG dose (mSv $year^{-1}$)	I[c]	II	III
^{131}I	Thyroid	5	0–0.37	0.37–3.7	3.7–37
^{137}Cs[d]	Whole body	1.7	0–54	54–540	540–5,400
^{90}Sr	Bone	5	0–0.74	0.74–7.4	7.4–74
Tritium (^{3}H)[d]	Whole body	1.7	0–7,400	7,400–74,000	74,000–740,000

[a]Derived concentrations were calculated on the basis of an average contaminated food intake of 1 kg day^{-1} (includes water and other beverages).
[b]Federal Radiation Council (1965).
[c]Range I requires no specific action; range II requires surveillance and routine control of upward trends towards range III; range III requires surveillance and controls to reduce exposure to range II (1); the range II–range III transition corresponds to the RPG dose 1 Bq = approximately 27 pCi.
[d]^{137}Cs and tritium were not considered by the FRC. The ranges were derived by using the radionuclide concentrations in water, tabulated by the National Council on Radiation Protection for occupational exposure, × 1/30 to apply to the general population.

Table 18.6. Post-Chernobyl 'action levels' applied by the US FDA–USDA as of December 1986 for radionuclides in imported foods.[a]

Radionuclide	Bq kg^{-1}
^{131}I Infant foods	56
^{134}Cs[b] Other foods	300
^{137}Cs All foods	370

[a]Food and Drug Administration (1986).
[b]Refers to the combination of both radionuclides – ^{134}Cs and ^{137}Cs – as they appear in Table 18.7.

were detained due to their exceeding the levels of concern (Cunningham *et al.*, 1989, 1994).

Other concerns have been raised over the years, and the FDA has been called upon to participate in them. One of these concerns was the 'dumping' of low-level radioactive waste in various harbours. The dumping had ceased in 1970. Samples were collected in 1980–1982, in cooperation with the EPA. Samples collected at that time were analysed for the various radionuclides such as ^{137}Cs and ^{90}Sr. No activity above background was detected. Later, there was concern that $^{239/240}$Pu was one of the radionuclides 'dumped' in these areas. Again, extensive sampling in cooperation with the EPA was conducted. Results of this study showed that the concentrations were below background levels or what would be expected from the $^{239/240}$Pu fallout from the previous above-ground testing in the 1960s (Baratta, 1995).

Methodology

The methodology used in the analyses of these radionuclides has been developed and tested previously by the standard setting societies, such as the AOAC International (2000) *Official Methods* and the APHA–AWWA–WEF (1998) *Standard Methods*, or have been published in the open literature (Baratta and Reavy, 1969; Baratta, 1992, 1994, 1998). The FDA has maintained a quality assurance programme, using the EPA (now discontinued) for its quality control samples. The results of this programme showed that the analytical data produced are within the expected acceptability for control (Baratta, 1993).

Toxicity and Effects

Amount deposited and retained in the critical organ

The most hazardous irradiation situation results from radionuclides that are concentrated by essential organs of the body in relatively large quantities and are retained in these organs for long periods of time. The deposition of ^{90}Sr, a bone-seeker of long biological half-life, is an example of such a situation.

Type and energy of radiation emitted

The magnitude of the hazard from radionuclide deposition in the body is dependent on the type and energy of radiation. For the deposition of the same activity of a radionuclide in a given organ, the hazard from the type of radiation would be $\alpha > \beta > \gamma$. The hazard from the given emitter would also increase with increasing energy of emission.

Physical properties

The fission process gives rise to a mixture of radionuclides with a wide range of half-lives. Each of these nuclides is produced in a certain proportion (abundance), which is dependent on the fissioning material and the energy of the fissioning neutrons. The abundance of the various nuclides produced has been found to be approximately the same for the different fissionable materials. Table 18.3 presents the most important fission products based on fission abundance and half-life, which are of immediate concern in environmental contamination. One fission product of biological importance is ^{90}Sr. It has been estimated that all detonations by the USA, the UK and the USSR until the latter part of 1958 produced approximately 7,000,000 curies of this radionuclide.

Risk Assessments

Philosophy of radiation protection

The setting and execution of guidelines for radiation protection are based on an underlying philosophy in which two factors are of prime importance. First is the assumption that radiation effects follow a linear or non-threshold dose–response relationship. There is convincing evidence, particularly in so far as the genetic effects of radiation are concerned, that there exists a non-threshold phenomenon. Although positive proof is lacking thus far, it has been deemed prudent to adopt this more conservative hypothesis in setting protection standards for large numbers of people. According to the non-threshold concept, there is no radiation dose so small that it does not involve some degree of risk. The non-threshold relationship, therefore, implies that there is no radiation protection guideline, no matter how low, which can ensure *absolute* safety to every individual in a large population receiving the guideline dosage. However, since the magnitude of the risk is proportional to the dose received, untoward effects would become manifest at very low dose levels only if extremely large numbers of exposed individuals were observed.

The radiation protection guide (RPG) (FRC, 1965) may be defined as the radiation dose which should not be exceeded without careful consideration of the reasons for so doing. In light of the non-threshold phenomenon, every effort should be made to encourage the maintenance of radiation exposures as far below the guide as practicable. Methods of estimating guides are experiments, which have contributed greatly to the study of the effects of radiation. From this combined knowledge and from an understanding of the relative biological damage produced by various types of radiation, protection guides for whole-body exposure and for various organs have been recommended. These guides, of course, represent doses far below those at which any effects have thus far been observed.

Basis for radiation protection guides

Establishment of 'safe' levels of a long-term radiation dose requires knowledge of the cause–effect relationship between radiation dose and biological damage. Such damage may appear many years after initial exposure and is usually indistinguishable from the normal diseases and impairments of man. Information accumulated on this subject is, therefore, difficult to evaluate and is often controversial. Nevertheless, observations involving man and animal life have resulted in the accumulation of significant data.

Factors influencing radioactivity concentration guides

The radioactivity concentration guide (RCG) is the concentration of radioactivity in the environment that is determined to result in whole-body or organ doses equal to the RPG. In calculating RCG values for a given radionuclide, the following factors must be taken into consideration.

INITIAL BODY UPTAKE Large fractions of some elements are absorbed when taken into the body. In the case of certain other elements, only small fractions are absorbed in passage through the gastrointestinal tract. Therefore, the greater retention would increase the hazard from the first group as compared with the second, other factors being equal.

When radionuclides are inhaled, unless information specific to the radionuclide is available, it is assumed, in the case of soluble compounds, that 25% is retained in the lower respiratory tract. From here, the nuclides move into the bloodstream and a portion of each is deposited in its critical tissue within a few days. Approximately 50% is held in the upper respiratory tract and swallowed. In the case of insoluble compounds, it is assumed that 12% is retained in the lower respiratory tract, which is usually taken as a critical organ. The remainder is eliminated by exhalation and swallowing.

FRACTION RETAINED IN THE BODY The rate of elimination from the blood and tissues of the body varies considerably for different elements or compounds. The time required for one-half of the original quantity of radioactive material to be removed from the body by biological processes is called the biological half-life.

Some materials in the bloodstream are eliminated rapidly from the body, whereas large fractions of others remain in the body organs. For example, radium, plutonium and strontium are deposited in the bone where the rate of turnover is very slow, i.e. the biological half-life is many years. Radioisotopes of these elements are much more hazardous than those of carbon, sodium, sulphur and those that have biological half-lives of a few days or weeks. The principal biological methods of elimination of radionuclides from the body are the urine, faeces, exhalation and perspiration. Usually, elimination is much more rapid before the radionuclide is translocated from the body to a more permanent area, such as the bone. This time is usually from a few days to a few weeks. After the initial period, the elimination rate becomes more nearly exponential, and the application of the term 'biological half-life' has more meaning.

Regulatory Issues

The US FRC issued its Report No. 2 that set up the RPGs in September 1961 (FRC, 1961). These guides were set up to ensure that the public in the USA would be protected from the radioactive 'fallout' (Table 18.5). During the 'fallout' from Chernobyl, imported foods were regulated using a guide approved by both the USDA and the FDA. These were known as the USDA–FDA 'levels of concern' (Food and Drug Administration, 1986) (Table 18.6). They were primarily for ^{131}I, ^{137}Cs and ^{134}Cs. In 1998, the FDA published in the US Federal Register new guides: *Accidental Radioactive Contamination of Human Food and Animal Feeds: Recommendations for State and Local Agencies* (US Department of Health and Human Services, FDA, CDHR, 1998). This replaces the previous US Federal Radiation Council Report No. 2. (FRC, 1961, 1965). The USDA–FDA 'levels of concern', became the 'derived intervention levels' or 'DILs'. The values remain the same. Table 18.7 shows the new levels recommended in the *Accidental Radioactive Contamination of Human Food and Animal Feeds: Recommendation for State and Local Agencies* (US Department of Health and Human Services, FDA, CDHR, 1998).

The previous guidelines (FRC, 1965) were predicated upon the more or less constant release of low level radioactivity into the environment from the routine uses of radiation, and assumes continuous radionuclide intake by the population. Control for the population is based on the source of the release. There are cases, however, in which the contamination of the environment might be accidental or unforeseen, producing contamination that is transient and not likely to recur; these might include, for example, reactor incidents that result in relatively high but temporary local radioactivity levels. In cases of this kind, the 'contaminating event' would not occur on a regular basis, and control of the population might base protective action upon limiting or changing the uptake of certain contaminated foods. However, the impact of such measures

Table 18.7. Recommended derived intervention level (DIL)[a] or criterion for each radionuclide group.[b]

Radionuclide group	Bq kg^{-1}
^{90}Sr	160
^{131}I	170
$^{134}Cs + {}^{137}Cs$	1200
$^{238}Pu + {}^{239}Pu + {}^{241}Am$	2
$^{103}Ru + {}^{106}Ru$	[C3/6800 + C6/450] < 1[c]

[a]The DIL for each radionuclide group (except for $^{103}Ru + {}^{106}Ru$) is applied independently. Applicable to foods as prepared for consumption (for dried or concentrated food products, such as powdered milk or concentrated juices, adjust by a factor appropriate to reconstituted product). For spices, which are consumed in small quantities, use a dilution factor of 10.

[b]US Department of Health and Human Services (1998).

[c]Due to the large differences in DILs for ^{103}Ru and ^{106}Ru, their individual concentrations are divided by their respective DILs and then summed. The sum must be less than 1. The C3 and C6 must be the concentrations at the time of measurement.

upon the community which they are designed to benefit requires careful consideration by responsible authorities to ensure that the benefit of the action taken is not outweighed by its other effects. To deal with this kind of situation, the FRC, in its Report No. 5, introduced the protective action guide (PAG) concept. The PAG is defined as the projected absorbed dose to individuals in the general population that would warrant protective action following a contaminating event. It is assumed that the corresponding projected dose to a suitable sample of the exposed population would be one-third of the PAG. These guidelines have, thus far, been established for ^{131}I, ^{90}Sr and ^{137}Cs. It should be noted that a decision to take action to limit a community's intake of an important basic food item containing radionuclides involves balancing of the health benefits to be attained against undesirable features of the protective action, such as disruption of dietary habits and nutritional needs. It follows, then, that control actions could be employed at dose levels above or below the PAG, depending upon the degree of total impact that the action has upon the community.

Based on current information, RPGs for radiation doses have been recommended. These levels are subject to modification as more knowledge is gained. In view of their status and the possibility that any radiation dose may be damaging, it is well to recall the definition of the RPG as that radiation dose which should not be exceeded without careful consideration of the reasons for doing so. Every effort should be made to encourage the maintenance of radiation doses as far below the RPG as practical. Table 18.5 summarizes the previous recommendations of the FRC. Table 18.6 gives examples of the post-Chernobyl 'action levels' as applied by various countries. Table 18.7 is an example of the present recommended DIL or criterion for each radionuclide group (US Department of Health and Human Services, FDA, CDHR, 1998).

Conclusions

The US FDA has been monitoring radionuclides in foods since 1961. During this period, it has found that the food supply in the USA has met the criteria as set by the FRC (FRC, 1961) and the levels of concern (Food and Drug Administration, 1986) following the incident at the Chernobyl reactor. The incident at Three Mile Island showed that what little activity that was released was well within the FRC guidelines. The results of this monitoring have been reported in the literature as have other data concerning possible contamination (Simpson *et al*., 1974, 1977, 1981; Tanner and Baratta, 1980; Strobe, *et al*., 1985; Cunningham *et al*., 1989, 1994; Baratta, 1992; Caper and Cunningham, 2000). The foods imported after the Chernobyl incident that exceeded the levels of concern were detained. The results of the seafood tested from the former low level dump sites were found to be near or at background levels (Baratta, 1995).

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19 Widespread and Continuing Concerns over Food Safety

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Introduction

Even the most perfunctory perusal of this book will lead to the conclusion that, notwithstanding the best efforts of established and new government agencies, food contamination is likely to be a continuing issue for the foreseeable future. Despite measures implemented in many countries, the general consensus is that there is very little scope for complacency. In the short term, every nation will be faced with events that undermine public confidence in a wide range of food items. The recent past has provided us with an unremitting catalogue of food scares arising from decades of pollution, careless deregulation and under-funding of services in monitoring, research and education. The problems associated with negligence on this scale will not disappear immediately.

Detailed examination of this book will highlight on-going and widespread contamination of the principal foods that make up our diets. A number of deleterious constituents are intrinsic to particular foods, for example allergens in cereals and nuts. Other contaminants occur as a result of environmental pollution, while others arise from poor standards of hygiene. Microbial contamination has emerged as a particularly intractable problem both in terms of range of foods affected and in the diversity of causal organisms. *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes* are of particular concern in food safety. Theoretically, the risks associated with microbial contaminants should be relatively easy to minimize, but in practice this is proving to be more difficult. The spectre of antibiotic resistance has also emerged from recent studies. Thus, Walsh *et al*. (2001) demonstrated that the overall incidence of antibiotic resistance in *Listeria* species is low. However, they noted a continuing pattern of the emergence of strains of *Listeria* species isolated from foods that are resistant to one or more of the common antibiotics used to treat listeriosis in humans. Resistance of *Campylobacter* species and *E. coli* O157 to antimicrobial agents has also been reported (Aquino *et al*., 2002; Schroeder *et al*., 2002). This chapter is designed to highlight those foods that present a risk now and are likely to do so in the foreseeable future. Food legislation will also be considered from a holistic point of view, with the aim of suggesting further statutory controls over pollution and specific contaminants. The chapter includes a review of recent alerts and cases of contravention of legislation, and ends with a summary of future research priorities in key areas of

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food safety. The research programmes suggested are not comprehensive by any means and the reader is referred to individual chapters within this book and to specialist documents published by government epartments and food agencies (e.g. Ministry of Agriculture, Fisheries and Food, 1992a,b,c; Food Standards Agency, 2000a,b, 2001a,b,c,d,e,f,g,h), particularly in Europe and North America.

Statistics

Recent statistics reinforce widespread and continuing disquiet over food safety. This section is not comprehensive due to limitations of space. Nevertheless, the scale of the problem is encapsulated in the findings of Mead *et al*. (1999). They estimated that each year 76 million cases of food poisoning occur in the USA, with 325,000 individuals requiring hospitalization and 5000 fatalities. Nearly 2.4 million cases were caused by *Campylobacter* species and 1.4 million cases by non-typhoidal serovars of *Salmonella*. In May 2000, 1485 cases of *Campylobacter* poisoning were recorded in the UK, coinciding with the barbecue season. In the 1996 outbreak of *E. coli* O157 contamination in Scotland, 496 cases were recorded, including 18 deaths. This level of mortality was the second highest in the world for an epidemic caused by *E. coli* O157. In Japan, around 1000 outbreaks of food poisoning involving 30,000–35,000 individuals have been reported to occur annually (Suzuki *et al*., 2002). In Canada and the USA, major outbreaks of illness have been linked with the consumption of vegetables contaminated with *L. monocytogenes* (cited by Li *et al*., 2002). Kingsley and Richards (2001) stated that 1.4 million cases of hepatitis A virus (HAV)-mediated illness occur each year on a worldwide scale. Later in this chapter, reference will be made to a number of food alerts and to successful actions directed at individuals and small retail businesses. Many of the food alerts were associated with undeclared potential allergens in bread and cakes. Thus the full spectrum of staple foods has been linked with specific contaminants and human disorders. It is, therefore, appropriate to review the risks presented by particular foods in different countries.

Cereals and Nuts

The principal contaminants of cereals, bread and nuts are presented in Table 19.1. Cereals and nuts constitute a particular risk to consumers in Africa, Asia and possibly South America by virtue of mycotoxin contamination of these foods. The aflatoxins and fumonisins have been linked with the incidence of specific types of cancer (D'Mello, Chapter 4, this volume). The export of such

Table 19.1. Principal contaminants/toxicants of cereals, bread and nuts.

Food	Contaminant/toxicant	Comments/status	Further information
Cereals			
Maize	Aflatoxins; fumonisins	On-going in Africa and Asia	Chapter 4
Wheat	Allergens (gliadins)	Coeliacs at risk	D'Mello (1991)
Wheat	Deoxynivalenol	Canada and the USA; subject to advisory directives	Chapter 4
Bread	Pesticide residues above 'reporting limits'	In decline	Ministry of Agriculture, Fisheries and Food (1992a)
Nuts			
Peanuts	Aflatoxins	On-going in producer countries	Chapter 4
	Allergens	Precipitating life-threatening and other conditions in susceptible individuals; requires constant vigilance	Sampson and Burks (1996)
Pistachio	Aflatoxins	On-going in producer countries; currently under surveillance	Chapter 4

foods also presents potential risks to consumers in the developed world, to the extent that legislation is in place or proposed for these mycotoxins. In the UK, a recent survey found aflatoxin B_1 levels above regulatory limits in 13% of nuts and nut products (Food Standards Agency, 2002). In addition, certain individuals are at risk from allergens occurring in wheat and peanuts. Allergens in peanuts can cause life-threatening conditions (Spencer and Berman, Chapter 1, this volume) in susceptible people. Avoidance of peanuts is rendered more difficult by their ubiquitous distribution in manufactured foods, including biscuits, cakes, sauces and complete meals.

In a survey by the Ministry of Agriculture, Fisheries and Food (1992a), samples of bread were found to contain pesticides at or above the reporting limits (RLs). However, incidence declined from 32% in 1988 to 22% in 1990. In addition, the pattern of residues had changed, with less malathion and more primiphos-methyl.

Vegetables and Fruit

The contaminants of vegetables and fruit listed in Table 19.2 include nitrates, pesticides, arsenic and *L. monocytogenes*. The levels of nitrate in food and water have elicited considerable concern among medical practitioners and specialists (Eichholzer and Gutzwiller, Chapter 10, this volume). In some samples, nitrate concentrations in lettuce, celery and beetroot may exceed 1000 mg kg^{-1}. However, in a report by the UK Ministry of Agriculture, Fisheries and Food (1992b), it was concluded that only vegetarians might be at risk and that surveillance of their nitrate intakes should continue. Nevertheless, it is prudent to control nitrate exposure in the general population by reducing nitrogenous pollution.

Pesticide residues (Cabras, Chapter 5, this volume) in certain vegetables and fruit continue to be a problem in many countries. Recent surveillance results in the UK indicate a distinct lack of progress in reducing levels of pesticide residues in imported yams (Table 19.2). The levels were in excess of legal maximum residue limits (MRLs). However, the levels of contamination were deemed not to be a risk to consumers; MRLs are not safety standards. In the UK, 69% of lettuce samples recently were reported to contain pesticide residues. In three samples, residues exceeded the legal MRLs, while two samples contained non-approved pesticide residues. Celery, grapes, oranges and apples have all been found to contain multiple residues of pesticides (Table 19.2). Regarding grapes, the Pesticide Residue Committee in the UK recently reported that 67% of samples contained pesticides, 29% being contaminated with multiple residues. As indicated later in this chapter, specific food alerts have been issued on human health risks presented by pesticides in vegetables and fruit.

Table 19.2. Principal contaminants in fresh vegetables and fruit.

Vegetables/fruit	Contaminant	Data source
Lettuce, celery and beetroot	Nitrates	Ministry of Agriculture, Fisheries and Food (1992b)
Lettuce	Pesticide residues in excess of maximum residue limits (MRLs) in UK-grown and imported samples	Food Standards Agency (2001a)
Lettuce (also celery and tomatoes)	*Listeria monocytogenes*	Li *et al.* (2002)
Celery	Multiple residues of pesticides	Food Standards Agency (2000b)
Spinach	Arsenic	Munoz *et al.* (2002)
Yams	Pesticide residues in excess of MRLs in yams imported into the UK	Food Standards Agency (2001e)
Grapes, oranges and apples	Multiple residues of pesticides	Food Standards Agency (2000b, 2001c)

Li *et al*. (2002) cited two outbreaks of listeriosis, one in Canada and the other in the USA, that were both linked with contamination of vegetables with *L. monocytogenes*. The use of farmyard manure has raised questions about the microbiological safety of vegetables, particularly those grown under organic conditions. The potential for the transfer of bacteria from contaminated manure and irrigation water to vegetables is no longer a theoretical issue in the light of evidence presented by Solomon *et al*. (2002). Sagoo *et al*. (2001) conducted a microbiological examination of ready-to-eat organic vegetables obtained from retail outlets in the UK. They demonstrated the absence of *L. monocytogenes*, *Salmonella*, *Campylobacter* and *E. coli* O157 in their samples, and implied that overall agricultural, hygiene, harvesting and production practices were satisfactory. The situation in many other countries where organic farming systems have been adopted needs to be assessed individually. However, it is reassuring that McGee *et al*. (2001) were able to demonstrate that, although *E. coli* O157 can survive in cattle slurry for an extended period of time, a substantial decline in numbers of the pathogen occurs during storage. Assuming the same effect is true for other pathogens, the application of animal wastes in organic farming may not be a major factor affecting the safety of vegetables, as is sometimes implied. However, a watching brief should be maintained, as the use of microbiologically contaminated animal wastes is associated with some risks.

In parts of Chile, geological factors mean that consumers are exposed to above-average levels of arsenic. Concentrations in soil and aquifers of up to 1099 mg kg^{-1} and 11 mg l^{-1}, respectively, have been reported (Munoz *et al*., 2002). Not unexpectedly, vegetables and fruit grown in these areas can become contaminated with arsenic. In the study of Munoz *et al*. (2002), highest levels (up to 0.6 mg kg^{-1} fresh weight (FW)) were recorded for spinach. The level allowed by Chilean legislation is 1 mg kg^{-1}, but other countries, for example Poland, require levels to be considerably lower (0.2 mg kg^{-1}).

Despite the foregoing, the value of vegetables and fruit as part of a balanced diet must always be stressed. Vegetables and fruit are an important source of nutrients and fibre. Significantly, secondary compounds present particularly in leafy vegetables may offer protection against certain types of cancers and asthma.

Seafood

Estuarine and marine pollution has become a major environmental issue with consequences for the safety of seafood. In addition to the compounds listed in Table 19.3, there are many reports of increased levels of contaminants arising from authorized discharges from radiochemical plants and other sources around the world (Smith and Beresford, Chapter 17, and Baratta, Chapter 18, this volume). For example, recent studies have highlighted the unexpectedly high levels of tritium in fish and shellfish from Cardiff bay and the River Taff. It has been suggested that these elevated concentrations have not compromised food safety even for local seafood consumers (Food Standards Agency, 2001g). Nevertheless, a watching brief must be maintained particularly around major nuclear establishments in Europe and America.

Despite the Minamata episode of the 1960s in Japan, mercury contamination of seafood continues to present multiple risks to consumers on a wider geographical scale. Thus, Dickman *et al*. (1998) observed a link between mercury in fish and subfertility in Hong Kong men. In the Faroe Islands, mercury exposure occurs through consumption of whale meat. Children with pre-natal exposure to this element have been observed to display cognitive deficits (Grandjean *et al*., 1997).

Major concerns continue to arise from microbial sources in seafood (Table 19.3; see also Gago Martínez and Lawrence, Chapter 3, this volume). Contamination of shellfish with HAV is a significant risk to seafood consumers in Europe, the USA and China. Coastal waters in the USA are classified as 'approved', 'conditional', 'restricted' or 'prohibited' for shellfish harvesting, depending on levels of pollution. Similarly, in England and Wales, a revised classification of bivalve mollusc harvesting

Table 19.3. Principal contaminants in seafood.

Food	Contaminant	Comments/status	Further information
Fish	Mercury	Contamination continues despite the Minamata episode of the 1950s	Dickman *et al*. (1998)
	Arsenic	May be affected by cooking temperatures	Devesa *et al*. (2001)
	PCBs	In farmed salmon	Food Standards Agency (2001b,h)
	Dioxins	In farmed salmon	Food Standards Agency (2001b,h)
	Vibrio parahaemolyticus	Serotype O3:K6 associated with recent outbreaks	Hara-Kudo *et al*. (2001)
Shellfish	Diarrhoetic shellfish poisons	Specific compounds identified	Chapter 3
	Hepatitis A virus (HAV)	Approximately 1.4 million cases of HAV-induced illness occur globally	Kingsley and Richards (2001); Seymour and Appleton (2001)
	E. coli	Revised harvesting classification issued by Food Standards Agency	Food Standards Agency (2001f)

PCBs, polychlorinated biphenyls.

has been introduced in response to *E. coli* contamination (Food Standards Agency, 2001f). Three categories have been introduced: in Class A areas, molluscs may be harvested for direct human consumption, whereas, in Class B and Class C areas, shellfish are classified as fit for human consumption only after certain relaying procedures have been followed.

Vibrio parahaemolyticus (serotype O3:K6) recently has been associated with foodborne illness in South-east Asia. This pathogen spread to Taiwan, Laos, Japan, Thailand, Korea and even the USA between 1997 and 1998. Hara-Kudo *et al*. (2001) detected the bacteria in a variety of seafoods, particularly clams and mackerel.

Meat

The safety of meat has been brought into sharp focus in the aftermath of the bovine spongiform encephalopathy (BSE) crisis in Europe and specifically in the UK. It would, therefore, be easy to assume that the BSE agent has been a major determinant of meat safety. Such a view would be quite erroneous as the major contaminants have been, and continue to be, of bacterial origin (Table 19.4; see also Johnson, Chapter 2, this volume). It is reassuring, therefore, to note that the Food Standards Agency survey recently indicated that the incidence of *Salmonella* contamination in retail chickens in the UK dropped to the lowest level ever recorded. Further information is available at the Food Standards Agency website (www.foodstandards.gov.uk). In addition, a study of a variety of retail meats obtained from the greater Washington DC area of the USA (Zhao *et al*., 2001) showed that only 3% of samples were contaminated with *Salmonella*. Despite the foregoing, it should be noted that other bacterial contaminants continue to represent a major public health hazard, with worldwide impact. Thus, in the UK, there is still extensive contamination of poultry meat with *Campylobacter*. In the greater Washington, DC area of the USA, 71 and 14%, respectively, of retail chicken and turkey samples obtained for the study by Zhao *et al*. (2001) were contaminated with *Campylobacter*, and 91% of stores visited had *Campylobacter*-contaminated chicken. The incidence of *E. coli* in that study was 39% for chicken, 12% for turkey, 16% for pork and 19% for beef. Furthermore, other studies point to the presence of both *Salmonella* and *Campylobacter* on the external and internal surfaces of material used as packaging of retail chickens (Harrison *et al*., 2001).

According to Bolton *et al*. (2002), *E. coli* O157:H7 contamination of beefburgers is

Table 19.4. Principal contaminants of meat, eggs and dairy products.

Food	Contaminant	Comments/status	Further information
Meat			
Beef	BSE agent	In decline, but incidence of vCJD in humans set to rise in the medium term	Chapter 14
Beef/lamb	*E. coli*	On-going	Chapter 2
Beef/lamb	Veterinary residues	On-going on a worldwide scale	Chapter 13
Lamb	Radionuclides	On-going on a regional scale	Chapters 17 and 18
Poultry	*Salmonella*	On-going on a worldwide scale	Chapter 2
	Campylobacter	On-going on a worldwide scale	Chapters 2 and 13
	Undisclosed	Illegal diversion of unfit poultry meat into the food chain in the UK; possibly worldwide	Food Standards Agency (2001f)
Pork	*Salmonella*	On-going worldwide; new tracing methods in development	Giovannacci *et al*. (2001)
	Ochratoxin A	On-going on a regional scale	Chapter 4
Beef, lamb, poultry and pork	Dioxins	Associated with a specific contamination incident in Belgium	Food Safety Information Bulletin (1999)
Eggs	*Salmonella*	On-going on a worldwide scale	Chapter 2
Milk	*Listeria monocytogenes*	On-going on a worldwide scale	Chapter 2
	Mycobacterium avium subspecies *paratuberculosis*	Possible link with Crohn's disease in humans	Food Standards Agency (2001d)
	Dioxins	On-going on a regional scale	Chapter 7; Ministry of Agriculture, Fisheries and Food (1992c)
	Aflatoxins	On-going on a regional scale	Chapter 4
	Radionuclides	On-going on a regional scale	Chapters 17 and 18; Ministry of Agriculture, Fisheries and Food (1994)
Cheese	*Listeria monocytogenes*	On-going on a worldwide scale	Chapter 2

vCJD, variant Creutzfeldt–Jakob disease.

significant, accounting for 50% of outbreaks associated with this pathogen in the USA during the period 1982–1994. Their studies, moreover, indicated that the current methods available for controlling the pathogen during production of beefburgers are ineffective.

Eggs

The recent loss of confidence in food safety can, arguably, be traced to the '*Salmonella*-in-eggs crisis' in the UK (Table 19.4). Stringent controls over breeding and laying flocks together with the introduction of

vaccination against *Salmonella enteritidis* have now resulted in reduced contamination of eggs in the UK and elsewhere. This decline has correlated with reduced incidence of human salmonellosis. However, in a second report on *Salmonella* in eggs, the Advisory Committee on the Microbiological Safety of Food (2001) warns against complacency since there may be other *Salmonella* serotypes capable of infecting hens and thus contaminating eggs. Furthermore, that Committee made a number of specific recommendations on future policy. For example, it was suggested that 'use by' dates would elicit greater health benefits to consumers than 'sell by' or 'use before' dates. This recommendation is based on the conclusion that safety with respect to *Salmonella*-contaminated eggs decreases markedly some 21–28 days after lay due to yolk membrane disintegration. Implementation of this recommendation would, however, require permission via revised EU regulations.

Milk and Dairy Products

Milk and dairy products are subject to contamination with a wide variety of organisms (Johnson, Chapter 2, this volume) and other substances (Table 19.4). For example, *L. monocytogenes* is a regular contaminant of dairy foods (Kalmokoff *et al*., 2001), and the organism is capable of adhering to many materials in food-processing plants, including dairies (Beresford *et al*., 2001). The recent observation that *Mycobacterium avium* subspecies *paratuberculosis* can survive pasteurization of milk has renewed interest in a possible link between the bacterium and Crohn's disease in humans. However, various food agencies stress that people should continue to consume milk and other dairy products. Analyses of farm-gate milk in the UK between 1988 and 1989 showed low levels of aflatoxin M_1 contamination, but more than 50% of milk samples in Tanzania were found to contain the mycotoxin (D'Mello and Macdonald, 1998).

Levels of dioxins (Fiedler, Chapter 7, this volume) in UK milk supplies were observed to be higher in samples obtained from farms near to urban and industrial sites than in those from farms in rural areas (Ministry of Agriculture, Fisheries and Food, 1992c). The UK national milk supply has also been affected by contamination with radionuclides arising from the testing of nuclear weapons and from the Chernobyl discharge in 1986 (Ministry of Agriculture, Fisheries and Food, 1994).

Supplements and Additives

Dietary supplements and additives are widely used for a variety of purposes (Simon and Ishiwata, Chapter 11, this volume). Controversy over the use of these compounds continues even with permitted products. Thus, although aspartame has been approved in the UK since 1983, the Food and Advisory Committee has advised the Food Standards Agency to review recently published evidence to allay persistent consumer apprehension about the safety of this artificial sweetener. There currently is considerable debate about the safety of foods for children, with at least one press report suggesting the labelling of some items with a health warning on the basis of additions of dyes, artificial flavourings and flavour enhancers. Similarly, vitamin C, at levels corresponding to 200 mg day^{-1}, has been linked to cellular damage and cancer under *in vitro* conditions. While the human health implications of relatively high doses have still to be elucidated, current advice is that all vitamin C requirements can be satisfied with a balanced diet containing five portions of fruit and vegetables per day.

The addition of salt and sugar to manufactured foods currently is under scrutiny in many of the developed countries following medical and dental advice. Reduction of salt intake is perceived as a vital step towards control of hypertension and cardiac disease. It is reassuring to observe that in the UK salt levels in bread have declined by 21% since 1998 (Food Standards Agency, 2001h). There is, however, an urgent need to reduce levels of salt and indeed sugar in snacks and confectionery.

Other Food Products

A list of contaminants in miscellaneous food items is presented in Table 19.5. This list is not an exhaustive compilation but is indicative of recent concerns in the UK. Food safety agencies elsewhere should be able to provide comparable information more attuned to local food safety issues. In the cases of mycotoxin contamination of coffee, spices and dried vine fruits, remedial action currently is being implemented. Petterson *et al*. (1991) commented on the conflicting evidence concerning the pharmacological and physiological effects of caffeine and discounted the potential teratogenic effects in humans on the basis of the large doses required to elicit such responses. However, current advice (Food Standards Agency, 2001g) is that pregnant women should restrict their caffeine intake to 300 mg day^{-1} as a prudent measure to avoid negative effects such as low birth weights and spontaneous abortion. Another group at risk are cigarette smokers, who tend to consume more caffeine than non-smokers and metabolize the alkaloid at a faster rate.

The UK Food Standards Agency suggests that consumers may continue to eat Chinese foods despite the occurrence of cancer-causing chloropropanols in soy sauce samples stocked by specialist retail outlets. The compounds investigated included 1,3-dichloropropanol (1,3-DCP) and 3-monochloropropane-1,2-diol (3-MCPD). The final advice was based on results indicating that soy sauce products sold in major retail chains were safe in this respect. Levels of bisphenol A (Petersen, Chapter 12, this volume) in some canned vegetables were also deemed not to pose significant risks to human health.

In Finland, the National Food Agency has provided specific advice on contaminants in honey, olive oil pomace, *Hypericum*, raspberries and imported noodles. In the USA, allergy alerts have been issued by the Food and Drugs Administration over undeclared ingredients in cookies, peanut butter bars and low-fat milk products. Further details of these and other food alerts are provided in a later section.

Food Allergy

Allergic responses to certain foods (Spencer and Berman, Chapter 1, this volume) have been well documented both in anecdotal accounts and in scientific investigations (Sampson and Burks, 1996). A true allergic response reflects a hypersensitivity to food or particular components that evokes an immunological reaction in a subject. When a food elicits a response that is not clearly linked with an immune reaction, an alternative term, 'food intolerance', is invoked to characterize the resulting adverse effects. Lactose intolerance is one example of such a syndrome. In addition, many individuals may react adversely to the various proteins in milk. The proteins of eggs have also been implicated in allergic reactions, with children being particularly susceptible.

Table 19.5. Contaminants in miscellaneous food products available in Britain.

Food products	Contaminant	Data source
Soy sauce	Chloropropanols	Food Standards Agency (2001d)
Spices	Aflatoxins and ochratoxin A (OTA)	Chapter 4
Canned vegetables	Bisphenol A	Food Standards Agency website[a]
Olive pomace oil	Benzo(*a*)pyrene	Food Standards Agency website[a]
Turkish helva (halva)	*Salmonella*	Food Standards Agency (2001e)
Dried vine fruits	OTA	Chapter 4
Coffee	OTA; caffeine	Chapter 4; Food Standards Agency (2001g)

[a]www.food.gov.uk

Effects of food allergens range from mild reversible symptoms to life-threatening anaphylaxis in sensitive individuals. Adverse reactions to peanuts exemplify the full spectrum of such effects, requiring total avoidance by individuals displaying the anaphylactic symptoms. Fruit and vegetables also contain allergens, often mediated in cross-reactions with pollen components. Thus, a dual and complex allergy may be identified in this type of syndrome (Vieths, 1997).

Special labelling of foods is required to protect consumers, particularly those susceptible to peanut allergies. The warning 'may contain nuts' is a common feature of all types of manufactured foods. Consumers sensitive to other food components are required to check the declared list of ingredients. A later section in this chapter gives examples of particular batches of foods that have been withdrawn from sale following allergy alerts.

Processing and Packaging

Processing and packaging are critical components of any strategy to ensure that foods are safe for the consumer. Regrettably, unless stringent methods are adopted, processing and packaging are attended with potential risks. In addition, hygiene conditions and practices in the slaughterhouse will have a significant impact on the safety of meat. Thus, contamination of broiler meat with *Campylobacter* may occur at slaughter through contact with excreta (Heuer *et al*., 2001). The efficacy of removal of specified risk material, as required by law, will determine the safety of beef and lamb.

Examples of industrial processing techniques include:

- cooking
- pasteurization
- freezing
- dehydration
- curing
- use of preservatives
- irradiation
- packaging

Contamination of foods during processing occurs on a disturbing scale and with regular frequency. For example, *L. monocytogenes* is often isolated not only from dairy and other food products but also from food-processing plants (Kalmokoff *et al*., 2001). Taormina and Benchat (2002) commented on the survival of *L. monocytogenes* in commercial food-processing equipment cleaning solutions. They advised that elimination of this organism could be achieved by the use of alkaline cleaning solutions and appropriate sanitizers. Chasseignaux *et al*. (2001) isolated *L. monocytogenes* from raw meat and associated products in poultry- and pork-processing plants in France.

Thermal methods

Adequate cooking is an obvious and effective method of overcoming the potential effects of bacterial contamination, and new technologies are being developed for this purpose (Robey *et al*., 2001). However, improper storage and handling subsequently can and often does negate the effects of cooking. The classical example is the storage of cooked meat under uncooked meat in commercial and domestic refrigerators. In the case of green coffee (Table 19.5), roasting conditions during processing determine the efficacy of reduction of ochratoxin A. Van der Stegen *et al*. (2001) commented on variable reductions ranging from 69 to 96% in different studies.

It is maintained that thermal procedures are not efficient for the complete inactivation of shellfish toxins and that alternative technologies need to be developed. Gonzalez *et al*. (2002) reported the basis of a new method involving the use of supercritical fluids. The thermostability of staphylococcal enterotoxin A has also been reported. Suzuki *et al*. (2002) suggest alternative strategies for food hygiene management based on the use of an electrolysed anodic solution.

Baking of bread may have differential effects on allergenic potential. Simonato *et al*. (2001) showed that some wheat allergens, for

example the 16 kDa protein, can be denatured after baking, but others can be made more resistant to digestive processes. Consequently, they may interact with the gut mucosa in an immunologically active mode. These findings also imply that current diagnostic tests for identifying wheat allergies may be flawed.

Seafood contaminated with certain species of arsenic may constitute potentially greater risks to human health after cooking. Thus, Devesa *et al*. (2001) concluded that arsenobetaine in seafood may undergo thermal conversion to more toxic species.

Food-processing methods such as grilling and smoking may induce formation of polyaromatic hydrocarbons (PAHs), as described by Guillén and Sopelana in Chapter 8 (this volume). Smoke from cooking oils may also cause adverse effects by virtue of the production of PAHs. Chen and Chen (2001) summarized the evidence for these effects and compared the mutagenic potential of smokes from different cooking oils. It is salutary to recall that cooking meat under grills and on barbecues is attended by additional risks in that harmful bacteria may persist in uncooked portions or through inadvertent contact with raw meat.

Irradiation

Irradiation is now permissible for the processing of certain foods intended for direct human consumption since this method is effective against common food spoilage microbes (WHO, 1994). However, organoleptic changes undermine its efficacy for particular food items unless microbial loads are low, as demonstrated by studies with saffron (*Crocus sativus* L.; Zareena *et al*., 2001).

Freezing

Freezing has long been used as a method of food preservation, and storage of foods at –20°C in household appliances is now common practice. However, it is assumed that freezing kills all microbial pathogens. The work of Moorhead and Dykes (2002) dispels that notion with respect to *Campylobacter*, which is able to survive both the freezing process and subsequent frozen storage. The authors conclude that current methods of frozen meat preservation do not add a significant margin of safety as regards this pathogen.

Curing

N-Nitroso compounds (NOCs; Eichholzer and Gutzwiller, Chapter 10, this volume) have been implicated in the aetiology of gastric cancer in humans. Exposure results from the intake of foods cured with nitrate and nitrite and by the smoking and chewing of tobacco products. Processed foods that may be relevant in this respect are frankfurters, dried salted fish and certain soy sauces. Haorah *et al*. (2001) reported that 90% of NOCs and 75% of NOC precursors in frankfurters were not present in fresh meat. It was suggested that most of the precursors arose during processing or storage, while most NOCs were generated by reaction with the added nitrite. As regards other forms of curing, it should be noted that cold smoked fish products are likely to be contaminated with *L. monocytogenes*, a pathogen that appears to survive this type of processing. In a survey conducted by the Finnish National Food Agency, 15–20% of vacuum-packed cold smoked fish products were contaminated with this pathogen during 1997–1998, although low levels were found in 2000.

Effects of Environmental Pollution

Enhanced levels of environmental pollution have undermined food safety; this is self-evident and indeed has formed a major theme in this book. The association between nuclear incidents and food radioactivity is well documented and constitutes the basis of two chapters in this book (Smith and Beresford, Chapter 17, and Baratta, Chapter 18). The importance of this link has been acknowledged in a document entitled

Radioactivity in Food and the Environment, 2000 (Centre for Environment, Fisheries and Aquaculture Science, 2001). The report was issued on behalf of the Food Standards Agency and the Scottish Environment Protection Agency in the UK. Perusal of Chapters 17 and 18 will show the global extent to which basic food supplies have been, and continue to be, contaminated with radionuclides. Levels in meat and seafood have been highlighted despite the claim that contamination has not compromised human health. Nevertheless, the authors of Chapters 17 and 18 will agree that monitoring of seafood and agricultural produce must continue, particularly around major nuclear establishments in Europe, North America and Japan.

Of equal concern are the human health implications of estuarine and coastal pollution caused by discharges of sewage. Bacterial and viral contaminants arising from such sources accumulate in shellfish and constitute a serious health hazard to consumers (Table 19.3).

Consumer concern has also emerged over persistent organic pollutants such as polychlorinated biphenyls (PCBs) and dioxins (Arnold and Feeley, Chapter 6, and Fiedler, Chapter 7, this volume). Highest concentrations occur in fatty foods such as meat and oily fish. In the UK, dietary exposure has fallen by 75% since the 1980s, with concomitant reductions in health risks associated with these compounds. It has been stated that, at the levels occurring in UK foods, PCBs and dioxins are unlikely to be associated with adverse effects even in young children (Food Standards Agency, 2001h). The perception elsewhere appears to be quite different. Thus, Patandin *et al*. (1999) concluded that maternal body burden of PCBs should be reduced as *in utero* exposure may affect subsequent cognitive performance of pre-school children.

Of the inorganic elements, mercury continues to present a risk worldwide (Jorhem, Chapter 9, this volume), particularly with respect to contamination of seafood (Table 19.3). The detrimental human health effects of mercury may be compounded by exposure to other environmental contaminants including PCBs and organochlorine pesticides (Grandjean *et al*., 1997).

Water quality

The quality and safety of water used for direct consumption or for irrigation of food crops are very much determined by prevailing levels of pollution. The levels of lead and nitrates have been a source of concern for many years. In addition, the occurrence of pathogenic enteric microorganisms derived from sewage pollution in water is an emerging issue. Outbreaks of enteric virus illness in humans have been associated with water on many occasions arising from pipeline failures, pollution of wells and contamination of municipal supplies with sewage (Wyn-Jones and Sellwood, 2001).

Food Labelling

It is axiomatic that food labelling is an important way to keep consumers informed of exactly what they are eating. Indeed, labelling regulations exist in the EU for foods destined for the ultimate consumer or for delivery to catering establishments (see following section and Joint Food Safety and Standards Group, 1998).

In the case of hypersensitive individuals, accurate labelling is an imperative component of their efforts to avoid food allergies. Consequently, the use of terms such as 'spices', 'colourings' and 'flavourings' is not helpful to such individuals, and food manufacturers and retailers must be encouraged to provide precise details if inadvertent intake of allergens is to be avoided. Equally, the appearance of terms such as 'may contain nuts' on the labels of a large number of packaged foods considerably reduces choice for hypersensitive individuals.

Although labelling of manufactured and packaged foods is regulated and actively encouraged by the major food agencies around the world, attention currently is being focused on misleading labels. It is argued that terms such as 'farmhouse', 'country style', 'fresh' and 'natural' have no place on food labels. Such terms are not defined by law and if used without due care can baffle and mislead the consumer.

Certainly such terms have no place in any strategy designed to uphold food safety. It is clear that more stringent enforcement of food labelling guidelines and regulations is needed.

Legislation

General

Elaborate directives and statutory instruments exist to underpin food legislation in the EU. Extensive regulations have also been developed in other countries including, for example, Canada and the USA. It is not intended to review all such legislation here. In any event, relevant legislation pertaining to other countries will be found in various chapters in this book. The following account should illustrate that within the UK in particular and the EU in general there is now a framework that could well serve as a model for other countries where food legislation is still rudimentary or non-existent. As will be seen shortly, comprehensive legislation was required to reduce BSE contamination of beef supplies in Britain. It is tempting to suggest that a similar network of regulations in the UK and elsewhere might be required to bring about more satisfactory control of bacterial contaminants. The incidence of *Listeria, E. coli* and *Campylobacter* in meat and dairy products continues unabated and on a worldwide scale (Table 19.4). Any legislation in this respect, however, will depend upon achieving global consensus on issues such as sampling, methodology, monitoring and enforcement.

Food labelling

Current EU directives are incorporated into legislation of Member States. In the UK, the Food Labelling Regulations 1996 implement these directives. The main provisions of the Regulations are that foods intended for direct consumption or for delivery to catering establishments must be labelled with specific details as follows.

- Name of the food.
- List of ingredients.
- Appropriate durability indication, e.g. 'best before' or 'use by' dates. The latter term applies to foods which in the microbiological context would be considered as being highly perishable and, in consequence, after a short period of time, likely to constitute an immediate health hazard.
- Any special conditions for storage or use.
- Name or business name and an address or registered office of the manufacturer or packer or of a seller established within the EU.

In certain circumstances, additional regulations apply, e.g. origin of the food, if failure to give such information might mislead consumers, and instructions for use if, in the absence of such information, consumers might encounter difficulties in making appropriate use of the food.

Contaminants

Legislation is now in place for the major food contaminants, but there are wide international differences in the scope and detail of the regulations. These discrepancies explain the occurrence of unregulated and banned pesticides in foods imported into the EU. In a large number of developing countries, food safety legislation is virtually non-existent and, furthermore, commands low priority in government business. As pointed out by D'Mello in Chapter 4 (this volume), some 13 countries have no regulations for mycotoxins in foods and, for about 50 countries, mostly in Africa, no data on levels of these contaminants are available. For all countries, a particular concern is the lack of statutory or advisory regulations for control of fumonisins in foods (Chapter 4).

For particular foods, there may be consumer safety implications following export of unregulated commodities to countries with more comprehensive legislation. A good example is the use of banned substances such as specified pesticides or hormone growth promoters in agriculture when the end products are intended for export to Europe

and North America where stringent food safety regulations are in place.

Relevant food legislation issues are addressed in detail in several chapters of this book. However, it is instructive to illustrate the range of legislation at the disposal of food safety agencies in the UK and in other parts of the EU. These regulations should provide a useful framework for those countries where food safety legislation is still in its infancy. The list in Table 19.6 is not designed to be exhaustive but rather indicative of the wide array of general and specific provisions in existing and new legislation. Many of the specific regulations have come into force in the post-BSE era. The information in Table 19.6 has been adapted from a most useful book by Jukes (1993). However, this list has been updated to include new legislation published by the Food Standards Agency (FSA) in its website (see Table 19.6). It will be seen that, in addition to the general provisions of the Food Safety Act (1990), more specific regulations exist, particularly for meat and eggs. In addition, there are specific regulations for processing and packaging, labelling, additives and named contaminants. One cannot help but enquire why, in the face of such

Table 19.6. A summary of food legislation in the UK and EU: a selection of provisions, specific regulations, orders and amendments for particular categories relevant to food safety.[a]

Category	Legislation
General	Food Safety Act (1990)
	Definition of food
	Rendering food injurious to health
	Selling food not complying with food safety requirements
	Inspection and seizure of suspected food
	Improvement notices
	Prohibition orders
	False description
Hygiene and health	Food Safety (General Food Hygiene) Regulations 1995
	Food Safety (Temperature Control) Regulations 1995
	Fresh Meat (Hygiene and Inspection) Regulations 1992
	Fresh Meat (Beef Control) (No. 2) (Amendment) (England) Regulations 2000
	Meat Products (Hygiene) Regulations 1994
	Minced Meat and Meat Preparations (Hygiene) Regulations 1995
	Poultry Meat (Hygiene) Regulations 1976
	Poultry Meat, Farmed Game Bird Meat and Rabbit Meat (Hygiene and Inspection) (Amendment) (England) Regulations 2001
	Ungraded Eggs (Hygiene) Regulations 1990
	Egg Products Regulations 1993
	Eggs (Marketing Standards) Regulations 1995
	Salmonella in poultry: Processed Animal Protein Order 1989; Poultry Breeding Flocks and Hatcheries Order 1993; Zoonoses Order 1989
	Dairy Products (Hygiene) Regulations 1995
	Specified Risk Material (Amendment) 2000: implementation of EU Decision 2000/418/EC
	Food Safety (Fishery Products) Regulations 1992
	Food Safety (Live Bivalve Molluscs and Other Shellfish) Regulations1990
	Imported Food Regulations 1997
Medical foods	Medical Foods (England) Regulations 2000
Additives	Antioxidants in Food Regulations 1978
	Colours in Food Regulations 1995
	Emulsifiers and Stabilizers in Food Regulations 1989
	Flavourings in Food Regulations 1992
	Mineral Hydrocarbons in Food Regulations 1966

continued

Table 19.6. *Continued.*

Category	Legislation
Contaminants	Aflatoxins in Nuts, Nut Products, Dried Figs and Dried Fig Products Regulations 1992
	Arsenic in Food Regulations 1959
	Animals and Animal Products (Examination for Residues and Maximum Residue Limits) Regulations 1997
	Lead in Food Regulations 1979
	Pesticides (Maximum Residue Levels in Food) Regulations 1988
	Tin in Food Regulations 1992
Processing and packaging	Quick-frozen Foodstuffs Regulations 1990
	Food (Control of Irradiation) Regulations 1990
	Food Irradiation Provisions (England) Regulations 2000
	Materials and Articles in Contact with Food Regulations 1987
	Plastic Materials and Articles in Contact with Food Regulations 1992
	Plastic Materials and Articles in Contact with Food (Amendment) (England) Regulations 2000
Labelling	Food Labelling Regulations 1996: implement EU directive relating to the labelling, presentation and advertising of foodstuffs for sale to the ultimate consumer
	Food Additives Labelling Regulations 1992
Food protection in an emergency	Food and Environment Protection Act 1985

[a]Compiled from Jukes (1993); Joint Food Safety and Standards Group (1998); Food Safety Agency website (www.foodstandards.gov.uk/regulations.htm).

comprehensive legislation, food safety continues to be a major source of concern in the perception of European consumers.

Food Safety Act 1990 (Great Britain)

The main provisions of this Act came into force on 1 January 1991. The Act relates to Great Britain and provides the basic framework for all its food legislation (Table 19.7).

Key provisions

The key provisions of the Food Safety Act 1990 are divided into four parts.

1. *Part I: preliminary*. This part contains a definition of 'food' and other basic terms used in the Act such as 'food business', 'food premises' and 'food source'. This part also sets out presumptions applying to food and food ingredients. For example, food commonly used for human consumption found in certain food premises is presumed to be intended for sale.

2. *Part II: main provisions*. This part describes the offence of rendering food injurious to health and defines the offence of selling or possessing for sale food that does not comply with food safety requirements. This is food that has been rendered harmful to health, which is unfit for human consumption or is so contaminated as to be unfit for human consumption. General enforcement provisions confer enforcement officers with powers to inspect food and to seize and detain food suspected of not complying with food safety requirements. Other sections of the Act provide for improvement notices to be issued following infringements of food hygiene or food-processing regulations. The provision of specific prohibition orders allows courts, authorized officers and Ministers to take appropriate action when food safety is at risk.

3. *Part III: administration and enforcement*. This part describes who may enter premises to enforce the Act and explains what they can do while on the premises. A section makes it an offence to obstruct a person enforcing the Act. Time limits for prosecutions and penalties and modes of trial are also set out in this part.

Table 19.7. A selection of the statutory instruments implementing the Food Safety Act 1990 in Great Britain.[a]

Instruments	Provisions
The Food Safety Act 1990 (Commencement (C40) No. 1) Order 1990	New powers for Ministers to use in emergencies
The Food (Sampling and Qualifications) Regulations 1990	Procedures to be followed by enforcement officers when taking samples for analysis or microbiological examination. Qualification requirements for Public Analysts and Food Examiners
The Food Safety Act 1990 (Consequential Modifications) (England and Wales) Order 1990	Continuation of milk and dairies legislation by amendment of previous regulations
Detention of Food (Prescribed Forms) Regulations 1990	Prescription of forms of notice which may be issued for the detention of food under the 1990 Act
Food Safety (Improvement and Prohibition Prescribed Forms) Regulations 1991	Forms of notice which may be issued in connection with improvement notices, prohibition orders or emergency prohibition notices or orders under the respective Sections of the 1990 Act
The Food Premises (Registration) Regulations 1991	Registration of food premises (including vehicles and other movable structures) by food authorities
The Food Safety Act 1990 (Commencement No. 3) Order 1992	Brings into force Section 59(4) of the Act which repeals previous legislation about the registration of food handlers and food businesses
The Food Safety Act 1990 (Consequential Modifications) (Local Enactments) Order 1992	Repeal or revocation of certain parts of local law relating to registration of food premises. These provisions are no longer necessary as The Food Premises (Registration) Regulations 1991 have been implemented
The Food Premises (Registrations) (Amendment) Regulations 1993	Exemption of childminders caring for no more than six children from the requirement to register their premises as a food business
The Food Premises (Registrations) (Amendment) Regulations 1997	Exemption for people who prepare food at home for sale in WI Country Markets Ltd from the requirement to register their premises as a food business

[a]Compiled from the Joint Food Safety and Standards Group (1998).

4. *Part IV: miscellaneous and supplemental*. Sections in this part provide for the Act to apply to the Crown and to Crown premises subject to special arrangements and certain exemptions. Another section amends the Water Act 1989 to extend its controls on the quality of water used for domestic purposes to cover water used in food production.

Statutory instruments

A number of statutory instruments implementing the Food Safety Act 1990 have come into force. The Joint Food Safety and Standards Group (1998) lists these in some detail (Table 19.7). Some of the instruments relate to England and Wales while others apply to the whole of Great Britain. The repeal or revocation of outdated legislation is provided in certain instruments.

Included in this list are The Food (Sampling and Qualifications) Regulations 1990, which set out the procedures to be followed by enforcement officers when obtaining samples for analysis or microbiological examination. The Food Premises (Registration) Regulations 1991 provide for the registration of food premises by food authorities. In addition, the instruments provide emergency powers for government Ministers, and set out qualification requirements for Public Analysts and Food Examiners. The emergency provisions were invoked recently in connection with dioxin contamination of Belgian foods (Fiedler, Chapter 7, this volume). The Food (Animals and Animal Products from Belgium)

(Emergency Control) (England and Wales) (No. 2) Order 1999 came into force on 18 August 1999.

Codes of practice for local authorities

Codes of practice issued under Section 40 of the Food Safety Act 1990 are not legislation but documents issued by Ministers for the guidance of food authorities, and the provisions can be enforced by direction and court order. The Joint Food Safety and Standards Group (1998) listed some 20 codes of practice for local authorities in Great Britain. A selection of these is presented in Table 19.8.

BSE control

Stringent regulations are enforced in Europe to control BSE contamination of meat (Hunter, Chapter 14, this volume). The statutory instruments are detailed in Table 19.9 to illustrate the diverse and formidable array of legislation required to contend with a single food poisoning issue. There is growing optimism that the BSE legislation has had the desired effect in terms of reduced prion protein contamination of UK beef. These regulations also provide an insight into the protracted time scale required for the statutory control of BSE in British herds.

In Britain, compliance with these and other meat hygiene regulations at abattoirs and cutting plants is the responsibility of the Meat Hygiene Service (MHS). Cattle and sheep carcasses are examined for specified risk material (SRM), which includes the intestines and additionally, in cattle over 6 months of age, head, thymus, spleen and spinal cord. Any SRM is removed at abattoirs and cutting plants, stained and disposed of under the supervision of MHS technicians. Disposal involves sealing the SRM in impervious containers before being transported to renderers where it is destroyed. However, as pointed out in Chapter 14, it is more difficult to remove spinal cord from sheep and goats compared with cattle. The BSE control legislation includes the 'over 30 month' rule, which means that cattle over this age are excluded from the human food chain. Another feature of BSE legislation forbids the use of mammalian meat and bone meal as animal feed. It is not yet possible to quantify the role of mechanically recovered meat in human foods on the spread of variant Creutzfeldt–Jakob disease (vCJD). The use of such material has been prohibited since 1995. Of particular concern to those involved in food safety is the continued use of meat and bone meal as a component of animal feeds in many non-EU countries.

Compliance and enforcement

Statutory regulations are not meaningful unless they are complied with and enforced by the relevant authorities. In the UK, much emphasis has been placed on enforcement

Table 19.8. A selection of codes of practice for local authorities in Great Britain.[a]

Responsibility for enforcement of the Food Safety Act 1990
Inspection, detention and seizure of suspect food
The use of improvement notices
Prohibition procedures
Food hygiene inspections
Enforcement of temperature control requirements of food hygiene regulations
Enforcement of the Food Premises (Registration) Regulations
Quick-frozen foodstuffs: enforcement of temperature monitoring and temperature measurement
Enforcement of the Food Safety (Fishery Products) Regulations 1992
Enforcement of the Meat Products (Hygiene) Regulations 1994
Enforcement of the Dairy Products (Hygiene) Regulations 1995
Exchange of information between Member States of the EU on routine food control matters

[a]Compiled from the Joint Food Safety and Standards Group (1998).

of the BSE legislation. However, BSE control failures have been reported in the UK. For example, for the period January to October 2001, 20 cases were recorded of beef imports being contaminated with traces of spinal cord. Some of these cases related to imports from The Netherlands. Exports of beef and related products are subject to stringent control under the provision of the Bovines and Bovine Products (Trade) Regulations of 1999; compliance is monitored by regular checks at ports. A number of alleged SRM offences have been considered for possible prosecution.

As previously indicated, the spread of microbial contamination of foods should, in

Table 19.9. BSE legislation as enforced in Great Britain. The following list is a selection of the statutory instruments that make direct and indirect provisions for food safety. Indirect provisions relate to animal feed and fertilizer legislation.[a]

Instrument	Details
Zoonoses Order 1988, under the Animal Health Act 1981	Order designated BSE as a zoonosis, enabling powers under the Act to be used to reduce any human health risk from BSE
Bovine Spongiform Encephalopathy (Miscellaneous Amendments) Order 1994, under the Animal Health Act 1981	Order banned the use of mammalian protein in ruminant feedingstuffs
Fertilisers (Mammalian Meat and Bone Meal) Regulations 1996, under the Agriculture Act 1970	Regulations prohibited the use of meat and bone meal as or in fertilizer applied to agricultural land
Fresh Meat (Beef Controls) No. 2 Regulations 1996, under the Food Safety Act 1990	Prohibited the sale for human consumption of meat from any bovine animal slaughtered after 28 March 1996 in which, at the time of slaughter, there were more than two permanent incisors erupted, unless there was documentary evidence to prove that the animal was no more than 2 years and 6 months old
Beef Bones Regulations 1997, under the Food Safety Act 1990	Required all beef from animals aged over 6 months to be deboned before sale to consumers; prohibited these bones from being sold for human consumption or to be used in the preparation of food
Specified Risk Material Order 1997, under the Animal Health Act 1981	Introduced controls on the import of specified risk material (SRM) and certain food products and feedingstuffs containing SRM
Specified Risk Material Regulations 1997, under the Food Safety Act 1990	Extended controls on the handling and permitted use of SRM from cattle, sheep and goats; extended existing controls on heads of sheep and goat SRM to include the removal of spleen of all sheep and goats and the spinal cord and tonsils of those over 12 months old or with one or more permanent incisors erupted through the gum
Specified Risk Material (Amendment) Regulations 1997, under the Food Safety Act 1990	Amended the Specified Risk Material Regulations 1997 to clarify that the requirement for removal of spinal cord from sheep and goat carcasses aged over 12 months does not apply to carcasses of animals slaughtered before 1 January 1998
Bovines and Bovine Products (Trade) Regulations 1998	Implemented fully in Great Britain (GB) the requirements of EU Commission Decision 98/256/EC, which replaced previous Decision on emergency measures to protect against BSE; continued to prohibit the dispatch from GB to third countries and Member States of bovine animals and embryos and meat and other products derived from bovine animals slaughtered in GB

[a]Compiled from the Joint Food Safety and Standards Group (1998).

theory at least, be relatively easy to minimize providing basic hygiene measures are adopted at abbatoirs, cutting plants and catering establishments. In addition, there are stringent regulations pertaining to cattle identification, bovine and bovine products (trade), animal by-products, SRM and tuberculosis in the UK and elsewhere in the EU. Notwithstanding, there are many actions, both successful and pending, which have been taken by the Food Standards Agency and other authorities in the UK. Similar actions are being pursued by food agencies in other countries. In the UK, most actions have related to individual farmers, small slaughterhouses and retail outlets (Table 19.10). The infringements relate to quite basic deficiencies in good practice. At the heart of the matter are questions such as communication of relevant legislation to trade personnel and training of staff. One is also bound to enquire whether more inspections might lead to an increased number of actions.

In the USA, the Food and Drug Administration (FDA) publish updated lists of 'Recall and Safety Alerts' (Tables 19.11 and 19.12). Recalls are actions taken by food businesses to withdraw products from the market. Recalls may be conducted voluntarily by the business, if, for example, internal audits demonstrate the need for such action. In addition, recalls may be at FDA request or by FDA order under statutory enforcement. Three classes of recall are defined and implemented by the FDA (Table 19.11). Class I food recalls for the 60 days to 6 December 2001 are listed in Table 19.12. In addition, individual states may issue separate recalls and alerts relevant to local food issues (Table 19.12). Food alerts published by the National Food Agency in Finland are listed in Table 19.13. All actions and alerts summarized in Tables 19.10–19.13 confirm parallel research and surveillance studies (Chasseignaux *et al*., 2001; Harrison *et al*., 2001; Zhao *et al*., 2001) showing that microbial contamination of foods continues to be a worldwide health issue. In the UK, recent recalls have included 'customer return notices' for tinned hot dog sausages. No reasons were given for these recalls, which were issued to affirm the manufacturer's 'commitment to the highest standards of food safety and quality'.

Food alerts may also be highly focused, concentrating on a particular group of contaminants, for example pesticides (Cabras, Chapter 5, this volume) or veterinary residues (Paige and Tollefson, Chapter 13, this volume). Table 19.14 contains recent alerts for pesticide contamination of foods in the EU. In many cases, levels were deemed to present no unacceptable risk to consumers. In a few instances, however, levels were considered to exceed acute reference doses for both adults and children. In February 2002, an alert was issued by the EU concerning the presence of veterinary residues and other substances in meat, fish and shellfish from China, leading to suspension of imports. This action was taken after an inspection visit to China in November 2001.

Monitoring and Surveillance

The need for continued and increased monitoring of food for contaminants is a theme that appears in all chapters of this book. It is axiomatic that a sound monitoring and surveillance service will help to restore consumer confidence in food safety. Thus, although human salmonellosis in the UK and elsewhere has declined as a consequence of vaccination of commercial flocks against *S. enteritidis*, surveillance must continue since there may be other *Salmonella* serotypes capable of infecting hens and thus contaminating eggs. At the local level, it is worthy of note that *Salmonella* was linked with deaths and infection of a number of patients at a Glasgow hospital in January 2002.

Recent actions in the UK have demonstrated the value of monitoring and surveillance in that a number of food items and products have been identified as sources of potentially harmful contaminants (Tables 19.1–19.5). High-profile court cases have resulted in convictions for illegal diversion of unfit poultry meat into the food chain (Table 19.4). An action plan has been proposed to include the introduction of staining

'high-risk' poultry meat destined for pet food and development of a code of practice on the handling and disposal of animal by-products by the meat trade.

Current surveillance indicates unavoidable, widespread and continuing mycotoxin contamination of basic plant products, with global implications for human health (D'Mello, Chapter 4, this volume). For example, concentrations of aflatoxins in maize and peanut kernels regularly exceed safety threshold limits, and monitoring must be given high priority, particularly in the developing countries of Africa and Asia. Furthermore, monitoring must continue in countries importing such commodities.

Table 19.10. Reasons for and outcomes of recent actions/statements by various authorities in the UK against individuals and companies or their directors.[a]

Body	Reasons for action/statement	Outcome
Food Standards Agency (FSA)	Failure to ensure that pig carcasses were refrigerated at or below 7°C as required by the Fresh Meat (Hygiene and Inspection) Regulations 1995	Fine plus payment of legal costs
Department for Environment, Food and Rural Affairs (DEFRA)	Export of minced meat derived from bovine animals intended for human consumption produced in unapproved premises in the UK: an offence under the Minced Meat and Meat Preparations (Hygiene) Regulations 1995	Fine plus payment of legal costs
FSA	Failure to establish a staff training programme and to comply with requirement to cleanse and disinfect equipment	Imprisonment
FSA	Use of exposed wooden pallets in a meat preparation area, in breach of the Fresh Meat (Hygiene and Inspection) Regulations 1995	Fine plus payment of legal costs
FSA	Failure to ensure that cleaning products were used in such a manner as not to affect the fitness of any fresh meat	Fine plus payment of legal costs
FSA	Beef from a calf whose mother contracted BSE illegally entered human food chain; statement made in January 2002	No action taken; risk that meat contained BSE assumed to be low
Ministry of Agriculture, Fisheries and Food (MAFF)[b]	Cutting fresh poultry at unlicensed premises in breach of the Poultry Meat, Farmed Game Bird Meat and Rabbit (Hygiene and Inspection) Regulations 1995. Also breaking an order banning the proprietor from managing a food business. Ban imposed under the Food Safety Act 1990	Imprisonment
MAFF	Failure to comply with Improvement Notices served under the Food Safety Act 1990	Voluntary surrender and subsequent revocation of licence for premises
MAFF	Failure to stain specified risk material (SRM) as required by the Specified Risk Material Regulations 1997	Community service
Meat Hygiene Service	Failure of cutting premises to operate within the Fresh Meat (Hygiene and Inspection) Regulations 1995 (as amended)	Licence revoked
Local authority	Failure to store SRM in an impervious container as required by the Specified Risk Material Regulations 1997	Fine plus payment of legal costs

[a]Compiled from UK Meat Hygiene Enforcement Reports and BSE Enforcement Bulletins; exact issue numbers and other details not disclosed here in the interest of confidentiality.
[b]Now DEFRA.

Table 19.11. Classification of recalls issued by the Food and Drug Administration (FDA) in the USA.[a]

Classification	Definition
Class I recall	Circumstances in which there is a reasonable probability that the use of or exposure to a violative product will cause serious adverse health effects or death
Class II recall	Circumstances in which the use of or exposure to a violative product may cause temporary or medically reversible adverse health effects or in which the probability of serious adverse health effects is remote
Class III recall	Situation in which the use of or exposure to a violative product is not likely to cause harm to consumers
Market withdrawal	This occurs when a product represents a minor violation that would not be subject to FDA legal action

[a]Compiled from the FDA website: www.fda.gov/opacom/7alerts.html

Table 19.12. Safety alerts issued in 2001 by the Food and Drug Administration (FDA) of the USA[a] and by the state of Alaska. The FDA list is mainly Class I recalls relating to food safety.

Date of issue or preparation of product	Alerts
FDA	
6 December	Crystallized ginger: undeclared sulphites
6 December	Allergy alert due to undeclared peanuts and eggs in soft cookies
4 December	Allergy alert following undeclared dairy ingredient in cinnamon biscuits
30 November	Allergy alert following undeclared peanut butter in cake
30 November	Allergy alert following undeclared eggs in raisin cake
27 November	Allergy alert following undeclared pasteurized egg yolks in milk product
31 October	Allergy alert following undeclared milk in assorted chocolates, assorted chocolate-covered nuts and peanut butter bars
26 October	Allergy alert following undeclared egg in bread
26 October	Allergy alert following undeclared sulphur dioxide ingredients in cake mix
15 October	Allergy alert following undeclared whey in margarine
15 October	Allergy alert following undeclared walnuts in cake
Alaska	
19–20 October	Cheese voluntarily recalled due to potential contamination with *Listeria monocytogenes*
2 August	Turkey ham products recalled due to possible contamination with *L. monocytogenes*
30 July to 3 August	Chicken products recalled due to possible under-processing
30 July to 5 September	Turkey products incorrectly labelled
Not specified	Chicken product recalled due to potential contamination with *L. monocytogenes*

[a]Compiled from following websites for, respectively, the FDA and Division of Environmental Health of the State of Alaska: www.fda.gov/opacom/7alerts.html and www.state.ak.us/dec/deh/sanitat/sanalert.htm

Food safety agencies

The food safety agencies in Europe and North America play a major role in monitoring and surveillance of food for contaminants. It is right that this book should contain chapters written by several authors emanating from these agencies. Thus, the FDA of the USA, the Food Directorate, Health Canada, the Swedish National Food Administration and the National Food Agency of Denmark are all represented in this book. In the UK, the FSA was recently set up in response to growing concerns over BSE contamination of beef and

Table 19.13. Recent food safety alerts in Finland.[a]

Food or supplement	Reasons/status
Honey	*Clostridium botulinum* spores; honey not recommended for children under 1 year of age; a multiplex PCR assay for detection of the pathogen has just been published by Finnish authors (Lindstrom *et al.*, 2001)
Olive oil and pomace olive oil (imported)	Unacceptably high concentrations of benzo(*a*)pyrene, above Finnish limit of 2 μg kg^{-1}
Hypericum (St John's wort)	Sales ban on *Hypericum* preparations as foodstuffs due to adverse interactions with several important medicines; reclassified as herbal remedies regulated under the Medicines Act of Finland
Raspberries (imported)	Calicivirus contamination from water
Noodles (imported)	Excessive concentrations of chloropropanol (3-MCPD)
Butter	*Listeria monocytogenes* isolated from both butter and patients; 25 cases of listeriosis recorded in outbreak
Fish products	*L. monocytogenes* occurred in 15–20% of vacuum-packed cold smoked products during 1997–1998; low levels of contamination in 2000

[a]Compiled from the Finnish National Food Agency website: www.elintarvikevirasto.fi/english/tiedotteet/tiedotteet/press0900.html
PCR, polymerase chain reaction; 3-MCPD, 3-monochloropropane-1,2-diol.

Table 19.14. European Union food alerts relating to pesticide contamination. A summary of selected statements issued during 2001.[a] The UK Pesticides Safety Directorate attached comments[b–e] to specific alerts.

Food	Origin	Pesticides
Kiwi fruit	Greece	Methidathion
Grapefruit	Turkey	Chlorpyrifos; parathion-methyl[b]
Grapes (table)	Greece	Fenpropathrin, fludioxonil, penconazole, pyrimethanil, pirimifos-methyl
	India	Triazophos[c]
Apples	France	Dicofol, chlorpyrifos, parathion-methyl and dimethoate
Oranges	Greece	Parathion-methyl[d]
Raisins	Spain	Fenthion
Mushrooms (shiitake)	China	Formaldehyde[e]
Chilli peppers	Thailand	Methamidophos
Mint	Egypt	Chlorpyrifos, chlorpyrifos-methyl and malathion
Chillies (red, kibbled) and chilli powder (hot, red)	India	Ethion, triazophos, cypermethrin and chlorpyrifos
Curry powder	India	Cypermethrin, fenvalerate and phosphamidon
Tea	China	Pirimicarb, fenvalerate
Tea (jasmine)	China	Buprofezin, fenpropathrin and DDT
Milk (curdled, buffalo)	Romania	Organochlorine pesticides

[a]Compiled from www.pesticides.gov.uk/citizen/residues/other/other_residues.htm
[b]'Possibly in excess of acute reference dose and chronic dietary parameters for toddlers.'
[c]'Exposures above the acute reference dose for both adults and toddlers.'
[d]'Possibly in excess of acute reference dose and chronic dietary parameters for toddlers and infants.'
[e]'An unacceptable risk to consumers.'
DDT, dichlorodiphenyltrichloroethane.

the incidence of vCJD in humans. It remains to be seen whether the FSA will be proactive rather than merely reactive to the issues of the day. It will need to take initiatives over such topics as the safety of genetically modified (GM) foods (Gasson, Chapter 15, and

Pusztai *et al*., Chapter 16, this volume), attributes of organic foods and the rising tide of food-related allergy and intolerance in the UK. In the developing countries of Africa, Asia and South America, where there is an overwhelming need for monitoring and surveillance, it is regrettable that no agencies exist to uphold food safety on behalf of consumers.

Research and Development

The message in this book overwhelmingly underlines the need for further research in virtually every aspect of food safety. Indeed, the need for scientific research was a primary stimulus in the production of this book. Research proposals will be found in virtually every chapter in the book. Neither is there any shortage of research recommendations from expert groups. Table 19.15 contains a selection of proposals from just three Working Groups in the UK. There are recommendations for other contaminants and other countries. All Working Groups recognize that financial resources are not infinite and that the burden of allocation of funds, rightly, is the responsibility of the research councils. Any implementation will depend upon current research programmes and competing priorities within other scientific disciplines.

With regard to bacterial and viral contaminants of food, concerted research should focus on virulence factors in pathogenic strains. Work in this area has already begun on certain aspects of virulence in *Salmonella* (Cano *et al*., 2002), but there is a need for similar investigations on *Campylobacter, E. coli* O157:H7 and HAV. Similarly, the concept that enterococcal cells communicate with each other to coordinate toxin production (Dunny, 2002) is worth investigating in other microbes of relevance in food safety. Investigations of cell–cell signalling mechanisms should

Table 19.15. Examples of Working Group recommendations for research on three regular contaminants of foods.

Contaminant	Recommendations
E. coli O157[a]	Development of DNA-based methods to identify accurately the different strains of the organism
	Asymptomatic excretion of the organism in animals and humans
	Bacterial load of individual animals and humans
	Interactions with other enteric organisms, e.g. *Campylobacter* species
Salmonella[b]	Growth of *Salmonella* species in egg contents
	Contamination of eggs in egg-packing plants
	Virulence and pathogenicity
	Detection and differentiation of strains
	Contamination of the farm environment as a source of *Salmonella*
	Egg washing and subsequent growth of *Salmonella*
	Consumer behaviour
Radioactivity[c]	Evaluation of the significance of potential sources of radionuclide contamination in the food chain
	Pathways in routine surveillance
	More sensitive and efficient methodologies for measurement of radionuclides in food
	Improved methods of data handling and processing

[a]Pennington Group (1997); this report followed an investigation into the *E. coli* outbreak of 1996 in Central Scotland.
[b]Advisory Committee on the Microbiological Safety of Food (2001); this report focused on *Salmonella* in eggs.
[c]Centre for Environment, Fisheries and Aquaculture Science (2001); this report was compiled on behalf of the UK Food Standards Agency and the Scottish Environment Protection Agency.

provide insights into pathogenicity and treatment strategies. The application of molecular techniques in the elucidation of virulence factors and pathogenicity is likely to be crucial. There is an undisputed need for the development of appropriate tools for monitoring food contaminants. Polymerase chain reaction (PCR) methods are likely to form the basis of several diagnostic kits for the detection and identification of food pathogens (Lindstrom *et al*., 2001), but there is scope for much wider application of such techniques for other food contaminants.

It is unlikely that conventional toxicological assays will be adequate to evaluate fully the attributes and safety of GM (Chapters 15 and 16) and organic foods. Innovative methodologies and technologies will be required to discern even the most subtle effects of these and other foods on, for example, immunocompetence, cancer initiation and promotion, brain function and behavioural responses. Consumer concern over the safety of GM foods continues unabated, and the observations of Quist and Chapela (2001) indicating that transgenic DNA has introgressed into traditional maize landraces in Mexico is likely to fuel additional debate among environmentalists.

Levels of individual pesticides in common foods are generally below the MRLs and RLs. However, there is at present no way to predict the human health implications of combinations of different pesticides and other environmental contaminants occurring in our food. The extent to which these contaminants may induce additive or synergistic effects will require a level of innovation that does not exist at the present time.

Issues associated with BSE contamination of meat, in all its facets, will continue to demand research inputs for many years to come. One particular conundrum requiring immediate resolution is whether sheep in the national UK flock are carriers of BSE. Scientists still cannot give an unequivocal answer to this issue although they maintain that the risk is 'theoretical' in the absence of confirmed cases of BSE in the national flock. While this is essentially a monitoring issue, a research programme will be needed if the results confirm the presence of BSE in sheep or if the findings are inconclusive. In any case, the problem cannot be resolved without the development of a screening procedure capable of distinguishing between BSE and scrapie.

With regard to allergens in nuts (Spencer and Berman, Chapter 1, this volume), the nature of the protein causing life-threatening conditions in certain individuals needs to be characterized fully in terms of the main effects as well as cross-reactivity with other food components and with other factors. Food allergies and intolerance have been on the increase in recent years with respect to the incidence and severity of reactions, and the reasons underlying both aspects need to be elucidated.

There undoubtedly is an urgent need for radical thinking and development of innovative technologies in all aspects of food safety. Suzuki *et al*. (2002) reported the potential application of electrolysed anodic solutions in the inactivation of staphylococcal enterotoxin A. Silva *et al*. (2002) suggested the potential of bacteriocins as food preservatives, while Gonzalez *et al*. (2002) described the use of supercritical fluids to inactivate shellfish toxins. Much more work is required to develop these and other technologies and to examine their potential for use with a wide range of food matrices.

Consumer Advice

The purpose of this book is to provide a scientific discourse on all matters relating to food safety. However, in the interest of completeness, some basic consumer advice might be appropriate here. The various food agencies around the developed world provide a continuous stream of advice in the form of nutritional information, 'best practice' and alerts. However, it is clear that the message is not reaching butchers, caterers and consumers, as demonstrated by the significant increases in food poisoning incidents. The media have an important role to play in this respect in the various food programmes, but safety in the handling of particular foods, such as uncooked meats, is rarely given any attention; indeed, 'best practice' guidelines

often are blatantly contravened in popular television broadcasts.

Bacterial and viral contaminants

Basic hygiene guidelines must be implemented if the spread of food poisoning from bacterial and viral sources is to be avoided. The following protocols should be noted.

- Hands should be scrubbed and washed after visiting washrooms and before handling food; this procedure should also be followed after handling uncooked meat and seafoods.
- Cooking utensils, knives and cutting boards should be washed thoroughly before and after use.
- Catering staff should not handle food if they have recently contracted specified enteric illnesses, particularly if they are involved in distributions to vulnerable groups in day-care centres for young children and in nursing homes and hospitals for the elderly.
- Raw meats and seafoods should be kept apart from other foods; in refrigerators, uncooked meat and seafood should be placed on the bottom shelf.
- Frozen meat should be thawed thoroughly before cooking. The appropriate temperature and duration of cooking should also be ensured.

Fungal and plant toxins

For these toxins, the scope for consumer advice is limited due to the diversity and stability of compounds.

- Consumers should discard visibly mouldy foods; nuts should only be purchased from reputable sources, such as supermarkets, that implement high standards of quality control.
- Individuals with specific allergies should avoid peanuts and other nuts or their derivatives in food.
- Raw kidney beans and the like should be soaked in water overnight before boiling vigorously for at least 20 min; use of a slow cooker is not recommended for this purpose.
- Green potatoes should always be discarded to reduce glycoalkaloid intake.
- Pregnant women should limit their caffeine intake to less than four average cups of coffee, or six average cups of tea, or eight cans of regular cola drink per day.

Environmental chemicals

Consumers are restricted in terms of the protocols that they might adopt to reduce intake of the major chemical pollutants. However, the following points may be noted.

- Vegetables and fruit should be washed thoroughly before cooking or eating to reduce intake of pesticides.
- For the same reason, root vegetables should, where possible, be peeled to remove potential contaminants such as organophosphate pesticide residues in the epidermal layer.
- Fish from contaminated lakes and rivers should not be eaten.

Nutritional attributes

Providing basic protocols are adopted, particularly with respect to personal hygiene and adequate preparation of foods, vegetables, fruit, poultry meat and seafood should form a valuable part of a balanced diet. The low fat attributes of chicken, turkey and fish are well recognized. Significant benefits also result from the consumption of fatty fish containing particular fatty acids that may help in the prevention of heart disease. In addition to providing fibre, vegetables may play an important role in the prevention of certain cancers. Both vegetables and fruit are excellent sources of vitamins and are recommended in place of the equivalent pure supplements. It is therefore an issue of considerable concern that in the developed countries much of this advice is ignored.

Food and Cancer

Different foods have been implicated in both the induction and prevention of cancer. Foods contaminated with *N*-nitrosoamines, PCBs and dioxins have all been associated with the precipitation of cancer, and the epidemiological evidence seems convincing. However, momentum is gathering over the benefits of various foods in the prevention of cancer. The importance of this role may be gauged by the publication of four papers in a single recent issue of the *Journal of Nutrition* (Table 19.16). Tomatoes, green leafy vegetables, certain mushrooms and soya and whey proteins are all attributed with compounds that offer protection. It should be emphasized that the list in Table 19.16 is not comprehensive and other putative anticancer agents are thought to occur in vegetables. These include lycopene, phyto-oestrogens, glucosinolates and *S*-methylcysteine sulphoxide among many others (see Kinghorn and Kennelly, 1997).

Action Points

Unless remedial action is implemented urgently and on an international scale, consumers can look forward to a period of sustained food safety scares over many years. There are few quick-fix options. The following list is not exhaustive but should help in kick-starting the process of food safety improvements.

- Improved standards of hygiene must be implemented and monitored regularly in all cutting plants, butchers, catering establishments, hospitals and homes for the elderly.
- Steps must be taken to reduce the microbial risks associated with poultry and seafood since, in unadulterated forms, these foods can constitute a valuable component of reduced-fat diets.
- It is necessary to undertake a detailed review of the justification for and need for pesticides and fertilizers in agriculture and horticulture.
- Although levels of individual pesticides are generally below the MRLs and RLs, there is at present no way to predict the human health implications of the combined effects of several pesticides and other environmental contaminants occurring in our food. The extent to which these contaminants may induce additive or synergistic effects will require the development of novel and more sophisticated methodologies as conventional assays have failed to provide satisfactory clues.
- The use of additives in foods and beverages must be reduced to the very minimum. In this regard, a culture change may be necessary among food technologists and manufacturers as consumers begin to demand that food ought to be a source of nutrients (and, of course, pleasure) and not a vehicle for administration of chemicals and other man-made molecules.
- The use of antibiotics, banned drugs and other compounds in animal production must cease and concerted action must be taken on a worldwide basis.

Table 19.16. Vegetables and cancer prevention as exemplified in four papers published in a single issue of the *Journal of Nutrition*.

Vegetables	Cancer types	Putative anti-cancer agents	Reference
Cruciferous vegetables	Oestrogen-related, e.g. cervical cancer	Indole-3-carbinol	Chen *et al.* (2001)
Leafy green vegetables and edible brown algae	Prostate cancer	Neoxanthin and fucoxanthin	Kotake-Nara *et al.* (2001)
White button mushrooms	Oestrogen-related, e.g. breast cancer	Various phytochemicals inhibiting aromatase activity	Grube *et al.* (2001)
Soya	Liver and breast cancer	Proteins (and phyto-oestrogens)	Rowlands *et al.* (2001)

- Improved standards of hygiene must be implemented on livestock farms. Much still needs to be accomplished in this respect even in developed countries. Antibiotics and probiotics should not be used as substitutes for good hygiene.
- The use of catering waste and animal by-products as livestock feed must not be permitted.
- The use of meat and bone meal as animal feed in non-EU countries should be discontinued as soon as possible.
- Steps must be taken to reduce environmental pollution, particularly in agriculture.
- All governments must invest in education and training, research and monitoring services. In the long run, it would be cheaper to underpin these services than to pay compensation to consumers. Care of consumers affected by food poisoning and loss of productivity in the workplace caused by such illness would add to the financial burden.
- Efforts must be redoubled to ensure that those involved in monitoring have the appropriate tools. In this respect, the development of diagnostics must be given the highest priority.
- All the above action points should be accompanied by measures to depoliticize food safety issues. Politicians patently have failed to allay consumer fears in all the recent food scares in Europe, and there is little reason to believe that they have learned from past mistakes. In this book, we have been at pains to emphasize that food safety issues can only be addressed by underpinning food policy with appropriate scientific processes and sound legislation.

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