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radio-chromatography

the chromatography and electrophoresis of radiolabelled compounds

T.R. Roberts

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The Chromatography and Electrophoresis of Radiolabelled Compounds

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Preface

During the last decade there has been a steady increase in the use of radiotracers in organic and biological chemistry and this has been accompanied by continued advances in the efficiency and sophistication of chromatographic methods. As a result, the chromatographic and electrophoretic separation of radiolabelled compounds, referred to as "radiochromatography", is now carried out in many laboratories throughout the world.

The aim of this book is to describe and discuss the various radiochromatography and radioelectrophoresis methods in a single volume. The historical development of the techniques is outlined and the reasons for choice of a method for any particular application are discussed. This is followed by a more detailed consideration of the practical aspects and examples of applications taken from the recent literature.

I am grateful to many people for their help and support during the preparation of this book, particularly Alan Dutton for help with parts of Chapter 6 on radio-high-performance liquid chromatography and Neill Wright for useful discussions. I am also grateful for the help of Mr. Claudot's excellent team, particularly Rene Green, Janet Churchyard, Janet Charlton and Janine King-Cootes for typing the script, Philip White, Dave Hall and Paul Evans for the artwork and diagrams and Colin Nicholson for some of the photographs. Other photographs were supplied by manufacturers of the instruments.

My thanks also go to my wife, Lynn, for her help and patience and to my children, Christopher, Matthew and Marie for their rather surprising interest in the preparation of the book.

Sittingbourne, 1977 T.R. ROBERTS

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
butyl-PBD	2-(4′-*tert*.-Butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole
cpm	Counts per minute
CI	Chemical ionisation
dpm	Disintegrations per minute
ECD	Electron-capture detector
EI	Electron impact
FID	Flame ionisation detector
GC–MS	Gas chromatography–mass spectrometry
GLC	Gas–liquid chromatography
HPLC	High-performance liquid chromatography
Hyamine 10X	*p*-Diisobutylcresoxethoxethyl dimethylbenzylammonium hydroxide
MS	Mass spectrometry
μCi	Microcurie
nCi	Nanocurie
NMR	Nuclear magnetic resonance
PBD	2-Phenyl-5-(4-biphenylyl)-1,3,4-oxadiazole
PC	Paper chromatography
PM	Photomultiplier
POPOP	1,4-Di-[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-Diphenyloxazole
radio-GLC	Radio-gas–liquid chromatography
radio-HPLC	Radio-high-performance liquid chromatography
radio-PC	Radio-paper chromatography
radio-TLC	Radio-thin-layer chromatography
RNA	Ribonucleic acid
TLC	Thin-layer chromatography
TLE	Thin-layer electrophoresis
Triton X-100	Isooctyl phenoxy-polyethoxy-ethanol

Chapter 1

Introduction

CONTENTS

GENERAL INTRODUCTION

It is traditional when introducing a book on chromatography to describe the early experiments of Tswett [1] and the eventual breakthrough in liquid partition chromatography by Martin and Synge [2] in 1941. At that time, Martin and Synge used a silica gel support coated with water through which a water-immiscible solvent was passed. From then on partition chromatography developed and in 1944 filter paper was used as a chromatographic support by Consden *et al.* [3].

By the mid-1940s the use of radiotracers in biological studies was well established and within a short space of time the separation of radiolabelled compounds by paper chromatography (PC) became of considerable interest. This was the beginning of "radiochromatography". As an example, in 1947 Fink *et al.* [4] reported the use of ^{131}I in metabolic studies with rats in which metabolites were separated by "filter paper partition chromatography" and subsequently detected by autoradiography using X-ray film.

In the following year, Tomarelli and Florey [5] described a simple apparatus for scanning paper chromatogram strips for radioactivity based on the movement of a Geiger counter across the chromatogram. This was the first of many designs of radiochromatogram scanner and the subsequent development of radio-PC is outlined in Chapter 3.

Radiochemical detection methods were modified for use with thin-layer chromatography (TLC) when this technique was discovered and rapidly developed during the 1960s. Advantage was taken of the experience gained with PC and radio-TLC is now very commonly used in chemical and biochemical laboratories generally. The methods used for detection of radioisotopes on TLC plates form the basis of Chapter 4.

Radiotracers are also used during electrophoretic separations and in general the methods used are no different from those used in PC or TLC. The outstanding exception to this is polyacrylamide gel electrophoresis for which specific methods have to be used. With this in mind the aim of Chapter 5 is to describe the ways in which "radio-electrophoresis" differs from radiochromatography.

Most early chromatographic separations were carried out on suitable absorbents in columns but the greater efficiency and resolution of PC, TLC and gas–liquid chromatography (GLC) tended to overshadow column chromatography for some time except for large-scale separations. However, the increasing use of ion exchange and gel filtration as well as the recent rapid advances in high-performance liquid chromatography (HPLC) have re-established column chromatography as a technique of major importance.

This has led to a renewed interest in the detection of radiochemicals in column eluates

and the methods available for this are outlined in Chapter 6 in which particular emphasis is given to radio-HPLC and the considerations necessary when using it.

During the late 1950s the technique of GLC became very well established as a powerful separatory tool (both analytical and preparative) for those compounds with suitable physical properties. Specific element detectors were developed and these added specificity to the high sensitivities that were obtained. Radiochemical separations by GLC have been described in the literature since 1955 [6] and radio-GLC is now very well established. The development, use and application of radio-GLC is described in Chapter 7 but reaction radio-gas chromatography is not considered within the scope of this book.

THE USE OF RADIOISOTOPES IN THE LABORATORY

At one time the use of radioisotopes in the chemical or biological laboratory was regarded as so specialised a technique that it was not in general use. This situation has gradually changed, however, and with the high sensitivity of modern radiochemical detectors, only small amounts of radioisotopes are necessary for tracer experiments. As a result many more laboratories have been upgraded so that weak β-emitters can be used in them and compounds labelled, for example, with ^{3}H or ^{14}C have been invaluable in biochemical tracer experiments and metabolic studies.

The isotopes commonly used together with their energies and half-lives are listed in Table 1.1. Isotopes other than these are not considered within the scope of this book which is concerned primarily with the methods suitable for and problems encountered in the detection of the most commonly used β-emitters after chromatography.

TABLE 1.1

PHYSICAL PROPERTIES OF THE β-EMITTING RADIOISOTOPES COMMONLY USED IN ORGANIC CHEMICAL AND BIOCHEMICAL STUDIES

Isotope	Energy (MeV)	Half-life
^{3}H	0.018	12.5 years
^{14}C	0.15	5500 years
^{32}P	1.70	14.3 days
^{35}S	0.167	87.2 days
^{36}Cl	0.71	4.4×10^{5} years

The detectors used in radiochromatography are described in Chapter 2 but for background information on nuclear physics and the properties of radiations the reader is referred to *Radioisotope Laboratory Techniques* by Faires and Parks [7]. This book also discusses the design of radiochemical laboratories, safety in the radiochemical laboratory and methods of decontamination and disposal of radioactive waste.

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Chapter 2

Radioactivity detectors used in chromatography

CONTENTS

INTRODUCTION

Most texts on radioactivity include descriptions of the methods available for detecting α-, β- and γ-emitting isotopes. These include ionisation chambers, electroscopes, proportional counters, Geiger–Müller counters, scintillation counters, semiconductor detectors, photographic methods and γ-ray spectrometers. However, only a limited number of detection methods are specific and sufficiently sensitive for the detection of weak β-emitters, particularly ^{3}H and ^{14}C.

It is the purpose of this chapter to describe the design of those radioactivity detection methods which have been used in radiochromatography. Several comprehensive texts are available which discuss the design and operation of a wider range of detection methods [1–5] together with background information on the nature of radioactivity.

IONISATION DETECTORS

Proportional counters

Ionisation chambers and proportional counters detect the ionisation caused by radiations emitted from radioactive isotopes. An ionisation chamber comprises two plate electrodes between which there is a potential difference of the order of 100 V. Positively and negative-

ly charged ions formed as a result of the radiation between the electrodes are accelerated towards the electrode of opposite charge before they have time to recombine. Provided that sufficient radioactivity is present the current produced will be a measure of the amount of radioactivity.

Ionisation chambers, although stable and easy to use for general monitoring purposes, particularly for α- and γ-emitters, are not suitable or sensitive enough for the location of weak β-emitters on chromatograms.

If the anode is made from a thin wire instead of a plate and if the potential difference across these electrodes is increased, then the potential gradient in close proximity to the anode wire may increase sufficiently to cause secondary ionisation when accelerated electrons collide with neutral gas molecules. In this situation the secondary ionisation produced is proportional to the initial ionisation caused by the radioactive isotope. Consequently, proportional counters operate in the range of applied voltage where this proportionality occurs (see Fig. 2.1).

Pulses detected with a proportional counter can be recorded with a scaler or, more commonly, a ratemeter can be used to measure the count-rate. However, the size of the pulse produced in a proportional counter is small (relative to that in a Geiger–Müller tube, for example) and a pre-amplifier is usually necessary.

Proportional counters vary considerably in design, but a typical counter would have a cylindrical cathode with a straight wire tensioned along the axis of the cylinder and insulated at either end. Some counters are filled with a suitable gas and sealed and they detect only radioactive particles which pass into the counter.

A more suitable design for radiochromatography is a gas-flow proportional counter since weak β-particles are stopped even by the thinnest windows. In this case a counting gas (such as argon–methane (90:10, w/w)) is passed through the cylinder at a low flow-rate to prevent the access of air into the chamber. Flow-through counters of this design are used for monitoring the effluent of GLC columns for radioactive compounds and this

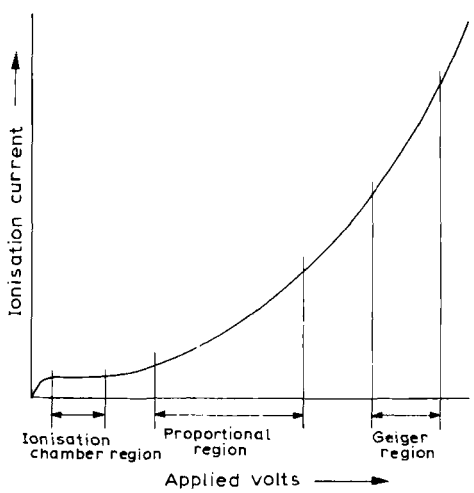


Fig. 2.1. The three regions used for counting.

is discussed fully in Chapter 7. In this case an external γ-ray source can be used initially to establish the operating voltage and other conditions for the counter.

Geiger–Müller counters

Geiger–Müller counters are similar mechanically to proportional counters but they operate with a higher potential difference between the electrodes. The result is that the size of the pulse produced by the presence of ionising radiation is no longer proportional to the number of ions formed initially. In the "Geiger region" (Fig. 2.1) all the discharges produced are nearly equal in magnitude because of an avalanche effect. In fact one of the disadvantages of the Geiger–Müller tube is that the same size of pulse is produced by α- or γ-sources so it is not possible to discriminate between, say, β-particles and background counts from cosmic radiation.

Geiger–Müller counter tubes are filled with a quenching gas in order to prevent a continuous discharge. A typical Geiger–Müller tube contains a gas mixture comprising argon at 80 mm Hg together with ethanol at 10 mm Hg which acts as a quenching agent. The low pressure permits low operating voltages to be used.

One of the commonest designs is shown in Fig. 2.2. The cylindrical cathode is 2–3 cm in diameter and the anode again is in the form of a thin tungsten wire. For detecting solid sources (for example, labelled compounds on chromatograms) the counter has a thin end-window usually made of mica to enable weak β-particles to enter the counter.

Since the pulse produced in a Geiger–Müller tube is large compared with a proportional counter pulse, little external amplification of the pulses produced is necessary. Consequently Geiger–Müller counters are simple to use and they are more stable than proportional counters. However, they are less sensitive, non-specific and currently find less application in radiochromatography. However, some of the earliest TLC radioscanners were based on

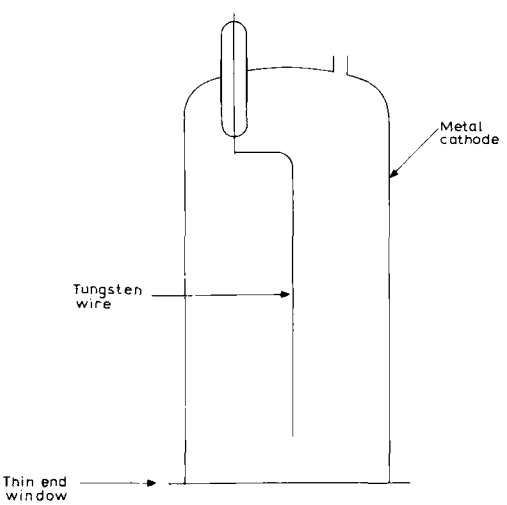


Fig. 2.2. Typical design of a Geiger–Müller counter tube.

the stepwise movement of the chromatogram beneath a Geiger–Müller counter. The Geiger–Müller counter is still used to crudely and quickly locate radioactive sites on chromatograms.

Geiger–Müller counters have also found application in the column chromatography of ^{32}P-labelled samples. The energy of ^{32}P is sufficiently high to be detected efficiently and simple flow cell arrangements can be based on this [6].

Geiger–Müller counters with an end-window are unsuitable for ^{3}H-counting and the sensitivity is low for ^{14}C. Consequently an open-window Geiger counter is used and this is essentially a flow-through counter similar to a proportional counter but operated on the plateau in the Geiger region (see Fig. 2.1).

For TLC and PC, proportional counters form the basis of a number of the automatic radioscanning instruments currently available commercially. In these cases the design of the counter is as shown in Fig. 2.3. A semi-cylindrical cathode is used with the anode wire stretched across the middle. The counter is then closed with a fixed or interchangeable metal plate with either a thin foil-covered slit or an open slit through which β-particles can enter. Once again a low but positive gas flow-rate is used and the detector is held in close proximity to the chromatogram which is to be scanned. A detector taken from a thin-layer plate scanner is illustrated in Fig. 2.4 together with some of the magnetic plates used to close the counter. The development and use of radioscanners for TLC are discussed in Chapter 4.

When scanning paper chromatograms for radioactivity it is possible to scan both sides of the paper using two detectors, and a detector head taken from a Berthold instrument is shown in Fig. 2.5. This gives effectively 4 π detection, and this instrument together with other paper radioscanners is discussed in Chapter 3.

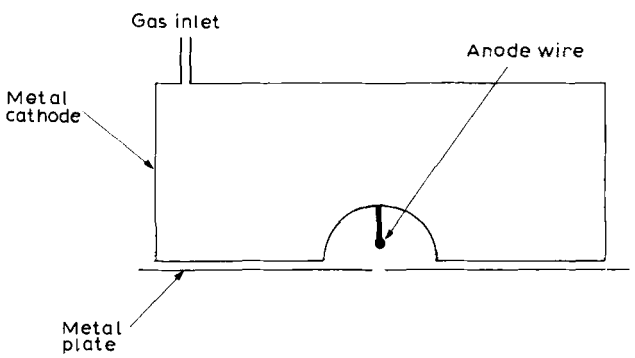


Fig. 2.3. A diagram of the proportional counter design used in radiochromatogram scanners.

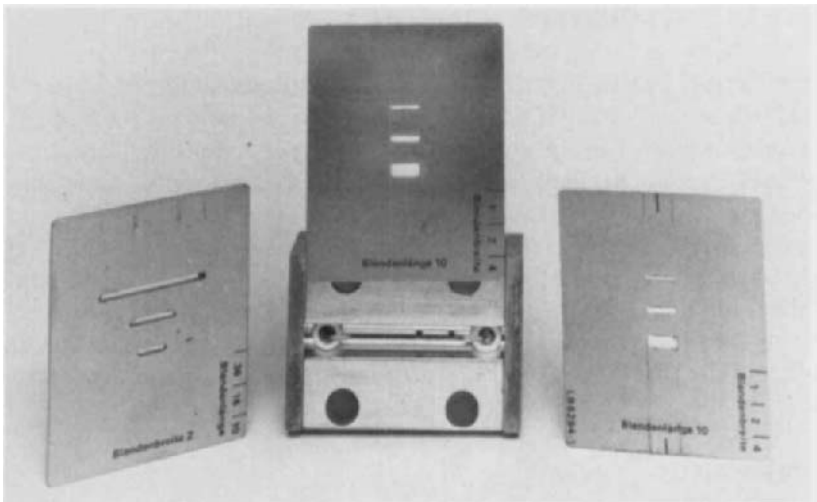


Fig. 2.4. The detector used in the Berthold thin-layer scanner II and some magnetic cover plates with different slits.

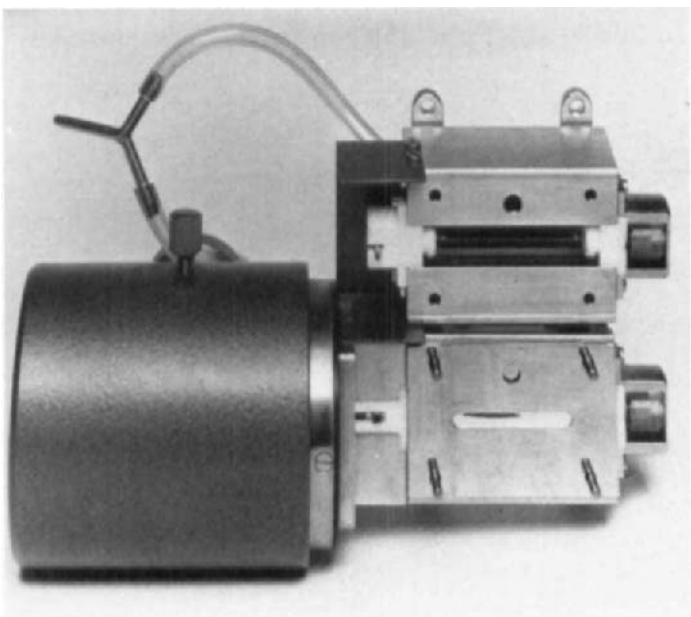


Fig. 2.5. The 4 π detector used with the Berthold thin-layer scanner II for scanning both sides of paper chromatogram strips.

LIQUID SCINTILLATION COUNTERS

There has been a rapid increase in the use of liquid scintillation counting in the last decade. Indeed it is true to say that for the general radioassay of β-emitting isotopes, liquid scintillation counting has essentially superseded the use of proportional counters and Geiger counters except in particular applications such as flow-through counters and contamination monitors.

There are several excellent texts on liquid scintillation counting ranging from relatively simple accounts of the principles [7,8] and practical books [8,9] to basic works written by experts in the field [10,11]. International symposia on liquid scintillation counting have been held by The Chemical Society, London in Great Britain in recent years and the proceedings of these meetings (*e.g.*, ref. 12) provide an up-to-date account of developments of the technique.

The scintillation process

Excitation is caused when ionising radiation passes through a solvent containing a dissolved scintillator. A proportion of the excitation is the result of promoting π electrons from their ground state, S_{0x}, to higher-energy singlet states (see Fig. 2.6). From the S_{0x} state conversion to a lower excited state occurs rapidly and excited solvent molecules can transfer their energy to solute molecules. Energy in the form of fluorescence is then released by the solute to produce the scintillation. This light emission is measured by a photomultiplier tube.

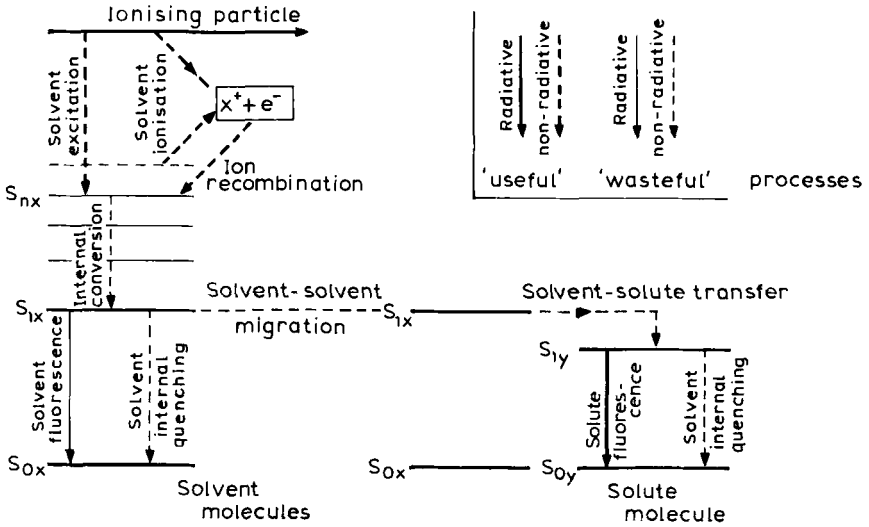


Fig. 2.6. The scintillation process. (Reproduced with permission from Birks [7].)

Solvents and scintillators

Several aromatic solvents are used to make scintillator solutions. Toluene or 1,4-dioxan have been used for many years but toluene-based "cocktails" are preferred as they give high-efficiency counting and toluene is an easier solvent to work with. Dyer [8] has given the requirements shown in Table 2.1 as necessary for a good liquid scintillation solvent.

A wide range of organic scintillators is now available but in practice only two or three are in common use. The best compromise choice is probably butyl-PBD on the basis of solubility and efficiency of energy transfer, except where its alkali lability is a problem. The primary scintillators (Table 2.2) have an emission wavelength close to the response range of most modern photomultiplier tubes but in some cases it is necessary to add a small amount of a secondary scintillator which has the effect of modifying the wavelength of the emitted light. Provided that the correct secondary solute is chosen, the emission from the scintillator can be brought in line with the maximum response range of the photomultiplier tube(s). These have also been referred to as "wavelength shifters". The names and structures of the common secondary scintillators are also shown in Table 2.2. The need for wavelength shifters has declined with the use of modern bialkali photomultiplier tubes, however.

TABLE 2.1

GENERAL REQUIREMENTS FOR A LIQUID SCINTILLATION SOLVENT (AFTER DYER [8])

Available to a reasonable degree of purity
Utility as a solvent for samples to be measured
Ability to efficiently transfer excitation energy to a solute
A low optical density in the appropriate spectral range
Low vapour pressure
Low freezing point
Economic cost

Sample preparation

A wide range of sample types is handled when chromatograms and column eluates are to be analysed by liquid scintillation counting, as illustrated in Table 2.3. A detailed description of recommended sample preparation methods are given in the separate chapters on individual techniques which follow, but some general principles are discussed here.

The simplest liquid scintillation counting "cocktails" are homogenous solutions of the organic scintillator(s) in organic solvent. However, their use is essentially limited to samples in organic solvent (*e.g.*, for example, organic column eluates) and used alone they are not ideal for counting paper sections or thin-layer adsorbents.

The use of scintillator solutions containing detergents such as Triton X-100 (isooctyl phenoxy-polyethoxy-ethanol) permits a much wider range of solvent mixtures, including aqueous solutions, to be counted. A general-purpose scintillator cocktail is made up from butyl-PBD (20 g), Triton X-100 (625 ml) and Analar toluene (made up to 2.5 litres). This can readily accommodate 10 vol.% water with minimal reduction in efficiency.

TABLE 2.2

SOME COMMON PRIMARY AND SECONDARY SCINTILLATORS

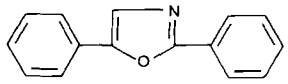
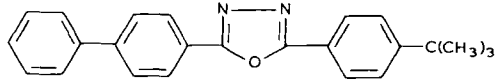
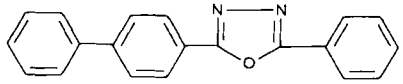
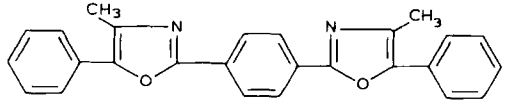
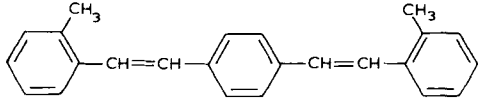
Scintillator	Chemical name	Primary or secondary	Structure	Remarks
PPO	2,5-Diphenyloxazole	Primary		Commonly used
Butyl-PBD	2-(4′-*tert.*-Butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole	Primary		Can be used alone without need for secondary scintillator
PBD	2-Phenyl-5-(4-biphenylyl)-1,3,4-oxadiazole	Primary		Not commonly used
Dimethyl-POPOP	1,4-Di-[2-(4-methyl-5-phenyloxazolyl)]-benzene	Secondary		Usually used with PPO
Bis-MSB	1,4-Di-(2-methylstyryl)-benzene	Secondary		Preferred to dimethyl-POPOP as more quench resistant

TABLE 2.3

TYPES OF SAMPLE ENCOUNTERED IN VARIOUS RADIOCHROMATOGRAPHY METHODS

Method	Sample
Paper chromatography and electrophoresis	Paper sections
Thin-layer chromatography and electrophoresis	Powdered adsorbents
Polyacrylamide gel electrophoresis	Gel slices
Column chromatography	Column eluates ranging from pure organic solvents to aqueous samples

It is possible to suspend TLC adsorbents using a finely divided silica such as Cab-O-Sil in almost any scintillator cocktail, and the merits and pitfalls of this method are discussed in Chapter 4.

A range of solubilisers is available for the digestion of samples such as polyacrylamide gel slices. Hyamine 10X (*p*-diisobutylcresoxethoxethyl dimethylbenzylammonium hydroxide) has been in use for this purpose for a number of years. Although it will solubilise most tissues and gels it causes yellowing of the solution and low-efficiency counting results. The range of solubilisers currently available has been summarised by Dyer [8], and the reader is referred to this reference for specific applications.

Oxidation methods are increasing in popularity with ^{14}C and ^{3}H analyses and overcome many of the problems of sample preparation, chemiluminescence and quenching that can occur with heterogeneous counting systems. The radiolabelled compounds in the chromatogram matrix are oxidised to $^{14}CO_2$ or $^{3}H_2O$ and these are trapped in scintillator solutions, a basic solution (usually containing 2-phenylethylamine) being used to ensure efficient trapping of $^{14}CO_2$. Various oxygen flask and combustion train methods can be used but the most efficient method (and the most expensive in capital terms) is the use of an automatic sample oxidiser. Several models are available commercially (see Chapter 3).

Quenching

It is well known that radiochemical samples and the solvents in which they are dissolved generally cause a lowering in counting efficiency. This effect will vary from sample to sample. The phenomenon is referred to as quenching and there are essentially two causes. One is that coloured materials interfere with the detection of light energy by the photomultiplier tubes and this is called "colour quenching". The more common cause of quenching arises when the solute or solvent molecules associated with the sample added to the scintillation cocktail interfere with the transfer of energy (for example, by absorbing the excitation energy and dissipating it as heat) to the organic scintillator. This is referred to as "chemical quenching" or "impurity quenching". It is common to have both colour and chemical quenching in the same sample.

Quenching occurs primarily with weak β-emitters, including ^{3}H, ^{14}C and ^{35}S and these are the isotopes most frequently handled in bioorganic and biochemical studies. With the higher-energy isotopes such as ^{36}Cl and ^{32}P quenching is not a serious problem and liquid scintillation counting is very straightforward.

It is necessary to measure the efficiency of counting of each sample containing a weak β-emitter since the degree of quenching will vary depending on the amount of solute and solvent added and on the nature and intensity of any colouring. Ways of minimising quenching effects can be found with experience and these include reduction of sample size, choice of solvents which are less likely to cause quenching and removal of colour from samples by bleaching with chlorine gas prior to the addition of scintillator. It will still be necessary, however, to determine the counting efficiency of each sample.

Methods of measuring counting efficiencies have been well documented and the major ones are: (a) internal standardisation; (b) the channels ratio method; and (c) the external standard ratio method.

The advantages and disadvantages of these methods have been discussed elsewhere [8].

Development of liquid scintillation counters

Rapkin [13] has reviewed the developments of instrument design since the early 1950s. Compared with the automatic liquid scintillation counters available today, the first counters were very simple and comprised a single vertically mounted photomultiplier tube housed in lead shielding, and a vial containing the scintillator and sample was placed against the photomultiplier tube. Optical contact was made with a silicone oil. A high-voltage supply was connected to the photomultiplier tube and signals were amplified and in some cases summed with a single-channel analyser. Such instruments operated with low efficiency and high background. The subsequent developments and automation built into liquid scintillation counters are clearly described by Rapkin and the historical aspects will not be discussed here.

It was the development of the coincidence counter that permitted much lower background count-rates to be achieved. A block diagram of a coincidence counter is shown in Fig. 2.7.

Most modern liquid scintillation counters have at least three counting channels which can be set up individually in an energy range which will discriminate between different isotopes or different scintillators. A block diagram of a modern counter (the Intertechnique SL30) is illustrated in Fig. 2.8. Signals from the two photomultiplier tubes are passed to a summation circuit and the resulting combined signal is applied to the amplifier which is connected to three pulse height analysers (the three channels) through a linear gate. The

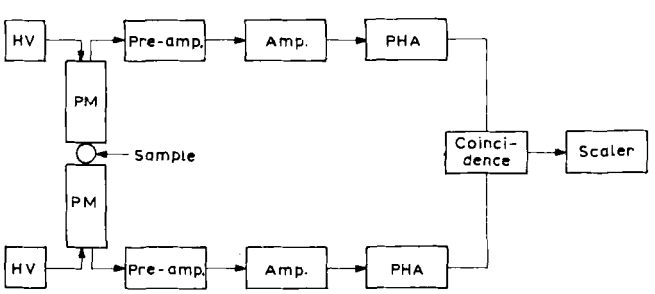


Fig. 2.7. A block diagram of a simple coincidence counter.

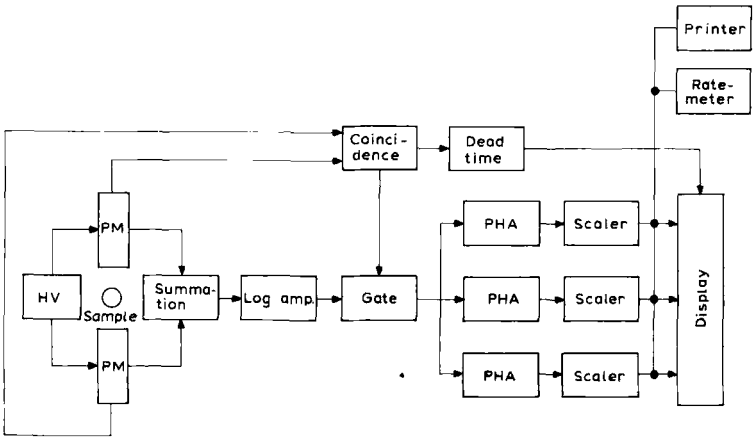


Fig. 2.8. A block diagram of a modern 3-channel liquid scintillation counter, the Intertechnique SL30.

linear gate is only open to coincident signals. The coincidence circuit also controls the clock to make allowances for dead-time losses.

There is currently a wide variety of instruments available from at least ten companies and amongst the more recent additions to the range are the Intertechnique SL 4000 (Fig. 2.9), a bench-top counter from Packard (Fig. 2.10) and the Nuclear-Chicago Mark III. Some counters have built-in computers including the Intertechnique SL 33 but other off-line computer links are also used, depending on the requirements of individual users. Further details of the instruments and suppliers are given in the Appendix.

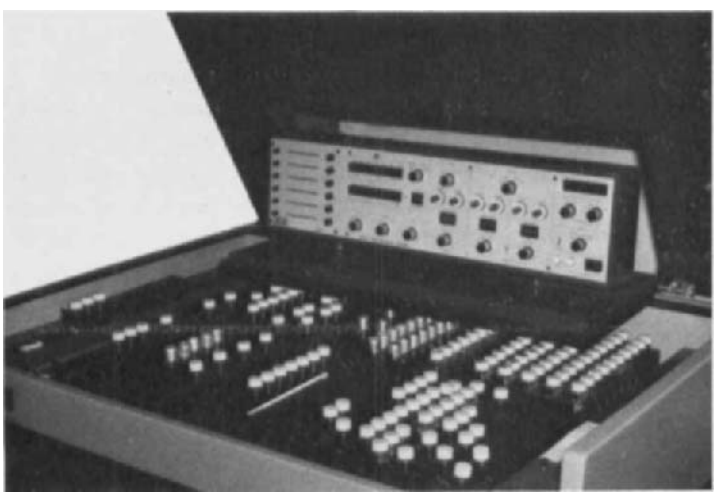


Fig. 2.9. An Intertechnique SL 4000 liquid scintillation counter. (Reproduced with permission from Intertechnique Ltd.)

Fig. 2.10. One of the recent bench-top Packard radiocounters. (Reproduced with permission from Packard Ltd.)

Cerenkov counting

As stated earlier, there are relatively few quenching problems with the isotopes of higher energy, particularly ^{36}Cl and ^{32}P. In fact, when using isotopes such as ^{32}P, sample preparation can be simplified considerably by using the Cerenkov counting method. When β-particles with energies greater than about 300 KeV travel through a medium with a high dielectric constant, a bluish-white (near UV radiation) is emitted and this is referred to as Cerenkov radiation. This can be detected by the modern photomultiplier tubes in use in current instruments with efficiencies of about 25% for ^{32}P and 2.3% for ^{36}Cl [14].

Quenching effects do not occur with Cerenkov counting and no organic scintillator cocktail is required. Consequently, the technique is finding wider application for the determination of ^{32}P and inorganic isotopes of sufficiently high energy.

Solid scintillation counting

The use of solid scintillators began in the late 1950s and the potential of these materials for monitoring radioactivity in solution was soon realised. There are now available plastic scintillators, organic and inorganic scintillators and activated glasses for use in flow cells to measure radioactivity in solution. These have found application in the continuous monitor-

ing of eluates from chromatography columns and the instrumental requirements are similar to those for liquid scintillation counting.

The use of solid scintillators in flow cells has been reviewed by Schram [15] and by McGuinness and Cullen [16]. A detailed discussion, with particular reference to high-performance liquid chromatography, is given in Chapter 6.

AUTORADIOGRAPHY

It is well known that the darkening of photographic emulsions by radioactive elements played an imporatnt part in the discovery of radioactivity towards the end of the 19th century. Since energetic α-particles produce a black line (visible on the film under the microscope), β-particles give rise to a more diffuse darkening and γ-rays produce a uniform darkening of the film, autoradiography in its early days was used as a means of distinguishing between these different types of radiation. It is perhaps surprising that this relatively simple detection method is still widely used today.

In fact, there exists a variety of autoradiographic methods for locating radiolabelled compounds in animal and plant tissue as well as on chromatograms. These include simple "contact autoradiography" in which the sample and film are held in contact during the exposure period, liquid emulsion methods, in which an emulsion or gel is poured over the sample, stripping film techniques and micro-autoradiography using the electron microscope. For detailed descriptions of these methods the reader is referred to the comprehensive coverage given by Rogers [17] and by Baserga and Malamud [18].

The autoradiographic detection method is quite different from those β-particle detection methods which have been described so far. One difference is that a cumulative record of the β-radiation is recorded on the photographic emulsion during the exposure period and when the film is developed a permanent record is obtained. However, the major difference is that the autoradiograph gives a record of the location of the radioactivity in the sample, such as a chromatogram, as well as a measure of the concentration (or intensity) of radiation present in the different parts of the sample. For this reason, the technique is of great value in radiochromatography for the location of the separated compounds on paper and thin-layer chromatograms, and the use of autoradiography for this purpose is discussed in Chapters 3 and 4.

The photographic process

The simplest photographic film used in autoradiography consists of a suspension of silver bromide crystals in a gel. Light and other radiations effect a change in the silver bromide and the film is said to possess a "latent image". When the film is developed, metallic silver is formed only where the interaction between the silver bromide and the radiation occurred, hence the darkening which is observed. Unfortunately, not only radiation from the sample will cause darkening of the film. Mechanical pressure and bending or rough handling of the film and some chemicals will have the same effect and, of course, radiation from other sources (including cosmic radiation) can give rise to a general "background" darkening. The major causes of background are listed in Table 2.4.

Background effects are less of a problem with chromatograms and simple biological

TABLE 2.4

FACTORS CAUSING BACKGROUND DARKENING OF AUTORADIOGRAPHS

Factor	Comment
Exposure to light	Not a serious problem – easily avoided with care
Pressure	Avoid careless handling and bending of X-ray film
Chemical effects	Ensure chromatogram is free of solvent. If necessary use thin protective film
External radiation	Difficult to avoid completely but remove any γ-sources to avoid unnecessary exposure

samples such as whole plants than with microscopic samples in which it can be very difficult to distinguish between external radiation tracks and dark zones due to radioactivity in the sample.

Resolution

The resolution obtained on an autoradiograph will depend on many factors, including the energy of the isotope, the nature and thickness of the film, the length of exposure of the sample to the film and the distance between the sample and the emulsion. Considering a point source of radioactivity, the silver grain density resulting from the radiation will be as shown in Fig. 2.11, and the resolution R has been defined [17] as the distance from the radioactive source at which the grain density is half that directly over the source itself.

It is of interest that better resolution is obtained with the weaker β-emitting isotopes than with the more energetic isotopes since the radiation from the latter will travel greater distances in the film. Consequently, although longer exposure times are necessary with isotopes such as ^{3}H and ^{14}C, better resolution is usually obtained than with, for example, ^{32}P. In contrast, the advantage of liquid emulsion coating, with its unique contact with the sample, is greatest for very weak particles such as those from ^{3}H, when extremely high resolution is obtainable.

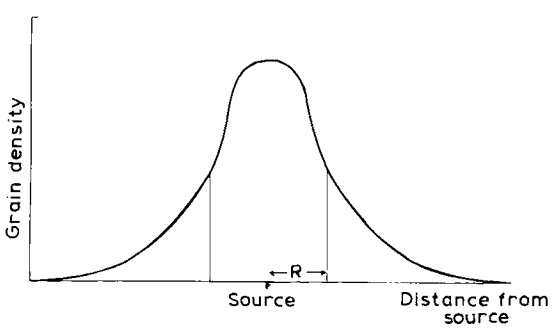


Fig. 2.11. A diagram of the distribution of silver grains around a point source. (Reproduced with permission from Rogers [17].)

THE SPARK CHAMBER

In contrast to the well established technique of autoradiography, spark chambers have been used for the location of radioactivity on chromatograms only since 1965 [19]. One of the earliest designs by Pullan consisted of two parallel arrays of wires (electrodes) placed in a counting gas atmosphere with a potential difference between the electrodes. A TLC plate or paper chromatogram was placed beneath the electrode unit and the β-particles entering the electric field from the chromatogram caused ionisation that led to the formation of a visible spark. A polaroid camera was used to record the sparks produced. In Fig. 2.12 is shown a schematic diagram of the electrode arrangement of the early spark chambers. The wires were arranged perpendicular to each other and were separated by only 2–3 mm. Typically the cathode was made of 24-gauge tinned copper wires stretched over a frame of perspex and the anode was made from 0.1 mm diameter stainless-steel wire.

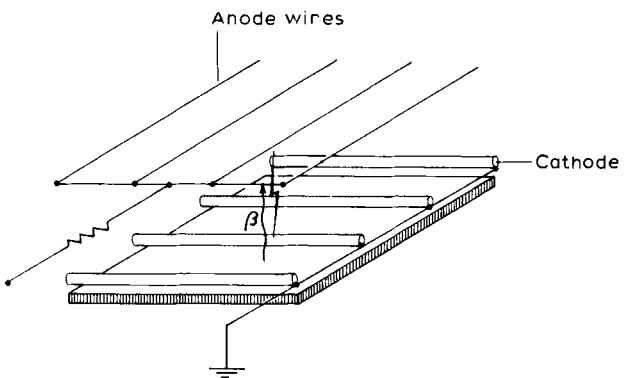


Fig. 2.12. A schematic diagram of the electrode arrangement in a crossed-wire spark chamber. (Reproduced with permission from Pullan [20].)

Spark chambers of this type suffered from several limitations [20] the major one being spurious sparking which gave rise to high backgrounds. The detector was modified with the introduction of a spiral cathode, and it is this design which is incorporated into the commercial instruments described in Chapter 4.

As with autoradiography the location of radiolabelled compounds on the chromatogram is indicated on the resulting photograph (see Fig. 4.10) but essentially the method can only be used qualitatively. Pullan [20] did consider the possibility of using the electrical signals produced in a spark chamber to obtain quantitative data, but the method should really be regarded as a rapid means of locating radioactive zones on chromatograms after which a more precise method of detection should be used. The relative merits of the spark chamber are discussed in more detail in Chapters 3 and 4.

SEMICONDUCTOR DETECTORS

In 1971 Tykva [21] described the use of a semiconductor detector for the detection of β-emitters on chromatograms and he and his co-workers have subsequently described the

use of these detectors in gel electrophoresis [22], PC [23] and TLC [24].

The semiconductor detector is claimed to have high-energy resolution and a low background but is less sensitive than either the autoradiography method or the spark chamber. The detector used by Tykva was a silicon barrier detector which had a relatively small effective area of measurement.

In use with TLC for example (see Fig. 4.7) the chromatogram was placed on a moving table which was moved manually (stepwise) or semi-automatically in 1-mm steps in a measuring chamber which had to be evacuated; therefore the method could not be used to detect volatile compounds. The detector, with an input electrode 1 mm in diameter, was positioned 0.5 mm above the chromatogram. Pulses (measured as a voltage) from the detector are amplified and because the amplitude of the pulse will depend on the energy of the β-emitter, discrimination between say ^{3}H and ^{14}C can be achieved.

Although the use of semiconductor detectors offers a rapid versatile method for a range of isotopes the method has not been widely used or commercialised.

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Chapter 3

Radio-paper chromatography

CONTENTS

INTRODUCTION

Paper chromatography (PC) has been in use since the 1940s as a valuable separatory technique in organic chemistry and biochemistry. Its use reached a peak in the late 1950s and early 1960s at which time it was superseded in many applications by thin-layer chromatography (TLC) which soon became known as a more rapid and efficient chromatographic method.

Between 1950 and 1962–1963, however, there was a very active interest in the use of PC for separation of radiolabelled compounds and numerous publications on radio-PC methods and applications appeared in the literature during that period. Although there is now considerably less interest in PC it continues to be used for those separations which cannot be achieved readily using TLC or other methods.

As a reflection of this situation there are very few commercial radioscanners for determination of radioactivity on paper chromatograms, and some of these consist merely of

attachments to TLC radioscanners. This is the converse of the situation which existed 10–15 years ago.

It is intended in this chapter to trace the development of radio-PC and to assess the advantages and disadvantages of currently available methods.

Current approaches to radio-PC fall into the following categories:

(i) Direct radioscanning of narrow strips or "scanning" of a whole two-dimensional chromatogram using a multi-detector system.

(ii) Autoradiographic methods.

(iii) Techniques based on liquid scintillation counting of sections of the chromatogram with or without prior elution.

(iv) Combustion analysis.

(v) Spark chamber techniques.

Whereas methods (i), (ii) and (v) are non-destructive, (iii) and (iv) are destructive methods although if necessary only a narrow strip of the paper chromatogram can be used for radioassay.

There are relatively few comprehensive reviews of radio-PC [1–3] but that of Pocchiari and Rossi [1] is particularly valuable in that it gives a comprehensive coverage of the literature up to 1961. More recently, Tolgyessy [2] discussed radio-PC in a more general review of radiochromatography and electrophoresis in which the commercial instruments available in 1972 were described. Some early books on PC have chapters on the analysis of radiolabelled compounds (*e.g.* Hais [3]) but the subject has not always been covered in more recent editions.

DEVELOPMENT OF RADIO-PAPER CHROMATOGRAPHY

Radioscanning techniques

A surprisingly large number of devices for the scanning of paper chromatograms have been described in the literature and in their review of quantitative PC Pocchiari and Rossi [1] compiled a comprehensive survey of the literature up to 1961. One of the first radio-chromatogram scanners described was that of Tomarelli and Florey [4] as early as 1948. Their instrument was simply a sliding base plate with the chromatogram placed on it which was moved manually at regular intervals across a Geiger counter. Many more complex set-ups have been built subsequently, and since the literature prior to 1961 has been reviewed and a full description of scanning devices available up to that time has been given, a discussion of the developments made since 1961 will be given here.

McWeeny and Burton [5] built a radioscanner from commercially available components including a modified Panax lead castle, ratemeter and a recording milliammeter.

Two thin-windowed Geiger–Müller tubes were housed in the lead castle with a 0.6 cm gap between the windows. A 2.5 cm wide strip of chromatography paper was then fed from a reel through the gap between the detectors and the resolution could be varied by adjusting the collimator slits. When used for ^{35}S less than 0.2 nCi was detectable with a background of 10.5 cpm although the counting efficiency was not as good as that of a gas-flow counter.

Conway and Lethco [6] modified an end-window counter usually used for radiocounting of samples on planchets for use in PC. This "adaptor" could be used manually (stepwise) or automatically if a ratemeter and recorder were available. The aim was to produce a low-cost attachment for an end-window counter but the approach has not been widely used.

Shipotofsky [7] has discussed the limitations of some of the paper scanners which were built prior to 1964 in that many instruments were intricate and difficult to construct without machining facilities. He described a simple, low-cost scanner which was in use in his own laboratory, and this is shown schematically in Fig. 3.1. It was based on a pair of thin-window Geiger–Müller tubes for ^{14}C counting and open-window tubes for detecting ^{3}H. The detectors were mounted face to face, to achieve effectively 4 π counting, and the chromatogram was again passed through metal plates with collimator slits. The microswitch beneath the scanner was positioned so that it was triggered by a weight fixed to the end of the chromatogram. This simple device operated with a background count-rate of 25 cpm which could be reduced to about 14 cpm with additional lead shielding.

In 1964 Dobbs [8] reported a study of the errors and causes of poor reproducibility encountered when scanners based on windowless gas-flow counters were used for detecting weak β-emitters on paper chromatograms. A number of scanners incorporating windowless counters have been described [9–11], and the instrument used by Dobbs is shown schematically in Fig. 3.2. In this instrument the paper chromatogram was cut into strips and fixed to a plate which passed through the counting chamber. The scanning speed could be varied by a rack and pinion drive mechanism and the slit width of the metal plate covering the open window could also be varied. The detector was flushed with a stream of argon–methane (90:10, v/v) quenching gas.

Using this scanner Dobbs studied the effects of gas flow-rate, the build up of static charge on the paper and loss of compounds by volatilisation on the reproducibility of repeated scans. He also discussed the problem of uneven penetration of radiochemicals through the paper, which leads to different results when opposite sides of the paper are scanned. This problem is discussed in more detail later in this chapter (see p. 32).

Using the different approach of impregnating a developed paper chromatogram in a liquid scintillator [12], Sprott [13] built an instrument in which chromatograms treated in this way could be scanned continuously. The aim was to enhance the sensitivity of

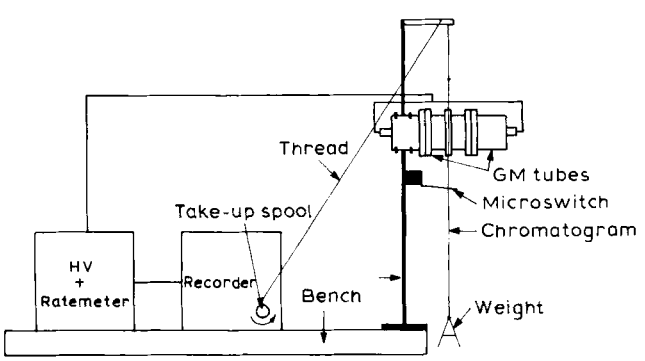

Fig. 3.1. A schematic diagram of Shipotofsky's scanner. (Reproduced with permission from Shipotofsky [7].)

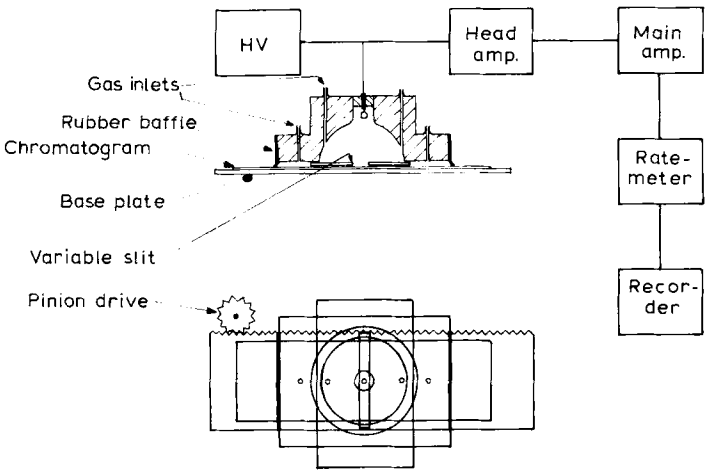

Fig. 3.2. A diagram of a radioscanner built in 1964 by Dobbs [8]. (Reproduced with permission.)

detection and at the same time overcome the inconvenience of liquid scintillation counting of individual sections of the chromatogram. The chromatograms were cut into 2.5 cm wide strips which were impregnated with a liquid scintillator and fixed onto an aluminium drum which fitted into a lead shield. As the drum rotated, the paper strip passed over a silica prism light pipe, and the light pulses produced passed through the silica to a photomultiplier tube which was in turn connected to a ratemeter and recorder. The response of the instrument was linear down to approximately 0.1 nCi of ^{14}C but the background count-rate was rather high at 72–90 cpm.

Although, as Sprott claims, this sort of instrument can be made up simply from redundant electronic components such as obsolete ratemeters which have been replaced with more sensitive units, it is unlikely to have a wide appeal in laboratories where a lot of PC is carried out.

Pocchiari and Rossi [1] pointed out an important limitation of most radioscanners in that they could only scan narrow strips of paper, so that a two-dimensional chromatogram had to be cut up into strips. Unless an autoradiograph of the chromatogram had already been obtained it was difficult to avoid cutting through radioactive zones, thus making the interpretation of the resulting scan difficult. It can also be a time-consuming and cumbersome method of locating the radioactive sites. To overcome these problems several two-dimensional scanners were developed and alternative methods of locating radioactive zones on two-dimensional chromatograms were described. Chain and co-workers [14] built an automatic two-dimensional scanner as early as 1956 and improvements to the basic design have been made over the years [15–17]. A full description of a computerised version appeared in 1970 [17]. In this model the radiochromatogram was scanned by two Tracerlab gas-flow counters run on argon–isobutane (98:2) gas held on either side of the paper. The detectors were moved discontinuously across the chromatogram in discrete steps and the radioactivity at sites on the paper was monitored for a pre-set time period before the detectors moved on to the next position. The position, radioactivity count and counting time for each unit area are then recorded on paper tape.

An assembly of up to ten scanners could be used simultaneously by means of a time-sharing arrangement. Although this very complex system has been in use for many years for studying the metabolic fate of radiolabelled compounds it is probably unique and must have absorbed a great deal of development time. The output is similar to that of an autoradiograph although data are presented quantitatively and the results are obtained more quickly. However, the most accurate quantitative results and the highest resolution will be obtained if a large number of small areas are radiocounted for long periods, and if this is done the time advantage over autoradiography could be lost.

Perkins and Tyrrell [18] built a scanner for two-dimensional chromatograms which produced facsimiles of chromatograms on electrosensitive paper, rather like an autoradiograph. Chromatograms of up to 52 cm square were fastened around a drum and a sheet of electrosensitive paper (such as "Teledeltos" type L-48) was fastened around a second drum. A stylus of nichrome wire relayed the signal from a thin-windowed Geiger–Müller tube to the paper. As little as 35 cpm of ^{14}C above background could be detected and there was a considerable time saving compared with autoradiography. It has been pointed out, however [19], that autoradiographs may be obtained simultaneously on a large number of paper chromatograms so that a single such scanner requiring approximately 2–3 days to complete a single two-dimensional scan can be of only limited interest.

Moses and Lonberg-Holm [19] used a rather different approach to radio-PC. Individual pieces of the chromatogram which contained radioactivity were cut out and mounted between two thin strips of mylar film and this was fed from a spool between two closely arranged detectors where each spot is held for a predetermined time interval for radioassay. A diagram of the counter arrangement is shown in Fig. 3.3. Once the mylar tape had been prepared the apparatus operated automatically and a tape with 1250 spots to be counted was fed through the machine with minimal attention over an 8-day period. The major drawback of this approach is that it is necessary to make an autoradiograph of the chromatogram so that the positions of the radioactive compounds are known before they are cut out for mounting on the tape.

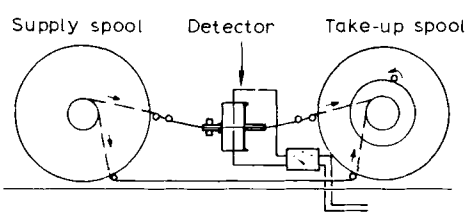

Fig. 3.3. The counter arrangement used in a scanner built by Moses and Lonberg-Holm [19]. (Reproduced with permission.)

Autoradiography

Autoradiography, which is a very popular procedure with TLC plates (see Chapter 4), seems to be used less extensively as a means of locating radioactive compounds on paper chromatograms. Although most weak β-emitters can be detected using autoradiography, since the energy of the β-radiation from ^{3}H is low ($E_{max.}$ = 18.5 KeV; $E_{av.}$ = 5.7 KeV) and

its range in the adsorbent layer and in air is so short, exposure times in excess of 2 weeks may be necessary.

The sensitivity of detection of ^{3}H on paper has been enhanced by soaking or impregnating the chromatogram with a liquid scintillator, and the resulting light emission is detected more readily by the X-ray film. This has been described as "fluorography" or "scintillation autography". There is no doubt that fluorography permits a photograph of the chromatogram to be obtained within a reasonable exposure time; but soaking the chromatogram with a scintillator solution can introduce severe problems not the least of which is extraction of compounds from the paper into solution. Consequently, Wilson's method [20] of leaving the chromatogram and film in a tank of terphenyl solution in toluene for several days was not widely used.

Markman [21] overcame this problem to some extent by spraying the chromatogram with a liquid scintillator solution (Ilford G5) after which it was dried and exposed to the film, the whole operation being carried out in a dark room.

Tykva and Pavlu [22] carried out a study of some of the factors affecting blackening of films during fluorography of ^{3}H in which quantitative measurements were made. They found that the degree of darkening of the X-ray film was lower the thicker the chromatography paper used. The degree of darkening was also dependent on the concentration of organic scintillator (PPO) in the paper.

A fuller description of the literature on the basic technique of autoradiography is given in Chapter 4 and the effects of paper thickness and drying methods on autoradiography of paper chromatograms are discussed later in this chapter (see p. 35).

Liquid scintillation counting of sections of paper chromatograms

As an alternative to radioscanning or autoradiography a representative portion of a paper chromatogram can be cut into small sections which are added to liquid scintillator in a counting vial. The amount of radioactivity present can then be determined by liquid scintillation counting. This is particularly useful for ^{3}H-determinations (particularly for compounds soluble in the scintillator) and for counting low concentrations of ^{14}C and other isotopes of similar energy since a counting time can be chosen to give good counting statistics, depending on the amount of radioactivity present.

The problem encountered with scanning and autoradiography of uneven distribution of different radioactive compounds through the paper is overcome by the liquid scintillation counting method but it is replaced by another variable, namely the different degree with which compounds of different polarities are desorbed from the paper into the organic solvent in the vial. Furthermore, the size and orientation of the paper sections in the vial can give rise to considerable variations in results. These problems have been discussed by several authors [23–26] with experience of the technique, and ways of overcoming the problems have been suggested [27,28]. A more detailed discussion of the practice of liquid scintillation counting of paper chromatograms is given in a later section on p. 36.

Elution analysis, in which radiolabelled compounds are eluted from the paper with a suitable solvent followed by radioassay of the solution, is also used but it is of less interest than with TLC. A more common procedure is to radioscan the chromatogram to locate the labelled compounds after which each radioactive zone is eluted separately for quantification by liquid scintillation counting.

Hariharan *et al.* [29] constructed a multi-detector scanner for the rapid scanning of paper chromatograms after which compounds were recovered from the paper using the simple arrangement shown in Fig. 3.4. This method was used to isolate unstable products from wet chromatograms in the shortest possible time. A wet paper strip was placed inside the plastic tube which had a small hole at the bottom. The plastic tube was placed inside a centrifuge tube which was spun on a low-speed bench centrifuge for one minute. This permitted more than 85% of the radiolabelled material to be eluted from the paper; much of the remaining material was eluted by adding water or buffer solution to the paper and repeating the procedure.

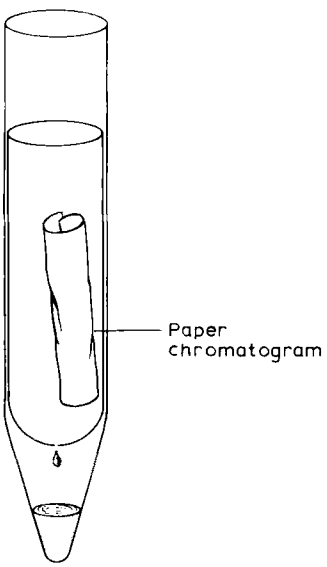

Fig. 3.4. Arrangement for rapid elution of compounds from paper strips. (Reproduced with permission from Hariharan *et al.* [29].)

Spark chamber techniques

Radiochromatogram spark chambers [30] have been introduced relatively recently (the late 1960s) and several commercial models are now available for the rapid location of radiolabelled compounds on chromatograms. All of these instruments are designed with TLC in mind and they accommodate 20 X 20 cm plates. However, small paper chromatograms may also be examined by the technique. For instance, the Birchover instrument (Fig. 4.12) will take paper sheets but the detector area is limited to 22 X 24 cm.

As it is more relevant to radio-TLC, the development of spark chamber techniques is covered in Chapter 4.

CHOICE OF METHOD FOR RADIO-PAPER CHROMATOGRAPHY

It is apparent from the foregoing survey that there exists a variety of techniques for the

radioassay of paper chromatograms and the technique chosen in any laboratory will depend on the following major factors:

(i) The isotope or isotopes to be detected.

(ii) The amounts of radioactivity present (*i.e.*, sensitivity).

(iii) The complexity of the mixture to be separated (*i.e.*, the resolution required).

(iv) The speed with which results need to be obtained.

(v) The finances available for purchase of equipment taking into account the existing radiochemical equipment.

(vi) The availability of radiolabelled sample and the need to recover material from the chromatogram.

(vii) The need for qualitative or quantitative data.

These factors also need to be considered when planning radio-TLC and they are discussed at some length in Chapter 4 (p. 59). However, a brief discussion of those factors which are peculiar to radio-PC is given here.

Tritiated compounds on paper chromatograms may be located by scanning with an open-window detector but the efficiency is very low (*ca.* 1–2%). Autoradiography or some form of fluorography can also be used if time is available but liquid scintillation counting of small sections of the chromatogram either directly or after combustion is the most sensitive technique. If direct counting is used then desorption of the tritiated compounds from the paper may be necessary.

With ^{14}C and other isotopes of similar energy radioscanning, autoradiography or liquid scintillation counting methods can all be used and the particular method chosen will depend on the other factors listed above. The use of PC in dual-label work is rather limited, but in this case a liquid scintillation counting method or selective masking in autoradiography would be preferred.

In general, the resolution obtained on a paper chromatogram is not as good as can be achieved with TLC and there is consequently less interest in autoradiography with one-dimensional chromatograms. The resolution obtained by strip-scanning is good and in laboratories which have access to a paper scanner such as the Packard Model 7201 or Berthold LB280 this is generally the preferred method. It is a non-destructive method and if necessary several chromatograms can be wound onto the spool for automatic feeding through the 4 π detector. Furthermore most thicknesses of paper can be scanned. When considering costs, commercial chromatogram scanners are relatively expensive. The Packard Model 7201 and Berthold Model LB280 are the only radioscanners currently on the market in Europe primarily for use with paper chromatograms (although the Packard instrument will also accomodate 5 cm wide TLC plates). Several TLC radioscanners, including models made by Panax Instruments and by Berthold, have attachments for use with paper and these are more commonly found since most laboratories use both radio-TLC and radio-PC.

Where a liquid scintillation counter is.available, counting of paper sections becomes an attractive proposition for quantitative work. The method is only partially destructive if only a narrow pilot strip of the chromatogram is taken but it can become very laborious and time consuming if a large number of chromatograms is involved.

With the fully automatic sample oxidisers now available, combustion of paper sections prior to scintillation counting is gaining popularity. If anything, it is more time consuming

than direct liquid scintillation counting since the operator needs to feed paper sections into the sample oxidiser at regular intervals. However, it can overcome problems of quenching that can arise when paper sections are added directly to liquid scintillant in the vial. The advantages and disadvantages of these methods are discussed in more detail on p. 39.

The complexities involved when two-dimensional chromatograms are run have already been referred to. Although scanners designed specifically for two-dimensional chromatograms have been described and are in use in some laboratories, no commercial instrument is available and this does not appear to be a widely used approach. It is more common either to obtain an autoradiograph, or possibly a spark discharge photograph, and this is often followed up by liquid scintillation counting of the located spots. A review of the recent literature has shown that two-dimensional PC is far less common now than it used to be and it is certainly less common than is TLC.

RADIO-PAPER CHROMATOGRAPHY TECHNIQUES IN PRACTICE

Radioscanning

Design and use of the instruments

Although a great variety of PC scanners has been described in the literature there are now only three to four commercially available instruments. Of these the Packard Model 7201 (Fig. 3.5) is perhaps most suited for PC and the Berthold LB280 is less well known in Great Britain. The Packard scanner can accommodate strips of paper between 1.25 cm and 5 cm wide and thicker papers such as Whatman No. 3 MM can also be scanned. The paper strips are wound onto reels and the paper is pulled from the reel through the gap between two windowless Geiger gas-flow counters arranged in 4 π configuration. There is a choice of four slit widths on the detector heads, ranging from 2.5 to 10 mm and clearly the resolution obtained will depend on the slit width used. The narrower the slit width the lower the peak amplitude will be. There are 16 scanning speeds (0.02 cm/min up to 200 cm/h) and a choice of 8 time constant settings (0.1–300 sec) so that a slit width, scanning speed and time-constant suitable for each chromatogram can be chosen.

The detectors are housed in specially designed lead shields which together with an anti-coincidence circuit connecting both detectors reduce the background to less than 10 cpm.

In use a number of strips from one or more chromatograms can be wound onto the same spool and in order to align the recorder chart with the chromatograms after the run each strip can be coded with a hole-punch. There is also an event marker linked to an automatic switch which indicates the positions of recorder and paper strip at the end of the run.

For scanning of TLC plates the lower of the two detectors can be removed and a TLC adaptor fitted. This is of somewhat limited interest, though, because only 5-cm plates can be scanned.

The Berthold and Panax radioscanners can be used for scanning paper chromatograms using attachments such as that shown in Fig. 3.6 for the Panax instrument. These simply

Fig. 3.5. A Packard Model 7201 radiochromatogram scanner. (Reproduced with permission from Packard Ltd.)

involve the use of a 4 π detector to scan both sides of the paper. Alternatively the paper chromatogram could be taped onto the platen of a TLC scanner and one side of it scanned with the 2 π detector. This is, of course, less sensitive than the 4 π mode but overcomes the inconvenience of interchanging the detectors for TLC and PC. Care must be taken, however, in interpreting quantitative data obtained when only one side of the paper is scanned because of the possibility of uneven distribution of compounds throughout the paper (see p. 35). If necessary both sides of the paper can be scanned in turn and the results compared.

Optimum radioscanning conditions

The variables that need to be considered when radioscanning chromatograms are primarily the slit width, the scanning speed, the time-constant of the ratemeter and the distance between the detector and chromatogram. With commercial paper scanners the latter can be fixed and need not be considered further. Johnson [31] has discussed the

other variables in quantitative terms. The best sensitivity and resolution is obtained when the slit width is half the distance between the peaks. Clearly, high sensitivity can be obtained with a wide slit but resolution suffers and the peak shape is not a true representation of the distribution of radioactivity on the chromatogram.

Regarding time-constant, Johnson developed the following relationship as a working rule for determining the minimum measuring range for use with a given time-constant:

$$\text{Minimum range (cpm full scale)} = \frac{1000\sqrt{\text{background count-rate}}}{\sqrt{\text{time-constant}}}$$

If too short a time-constant is used the fluctuations observed may produce an unacceptable scan from which peak areas cannot readily be measured. Alternatively, if the time-constant chosen is too long for the scanning speed, the resulting peak height will be lower and the peak shape may be a poor representation of the actual distribution of radioactivity. Consequently, a compromise must be made and a time-constant should be chosen which is long enough to reduce fluctuations to an acceptable level without having a serious effect on peak shape.

The choice of time-constant will also depend on the scanning speed chosen (the lower the scan speed, the longer the time-constant) and if low levels of radioactivity are present, a low scanning speed should be used.

Quantitation

Using a radioscanner, quantitation of results can be done by peak area measurements, or with an integrator incorporated into the scanner ratemeter. Alternatively an external

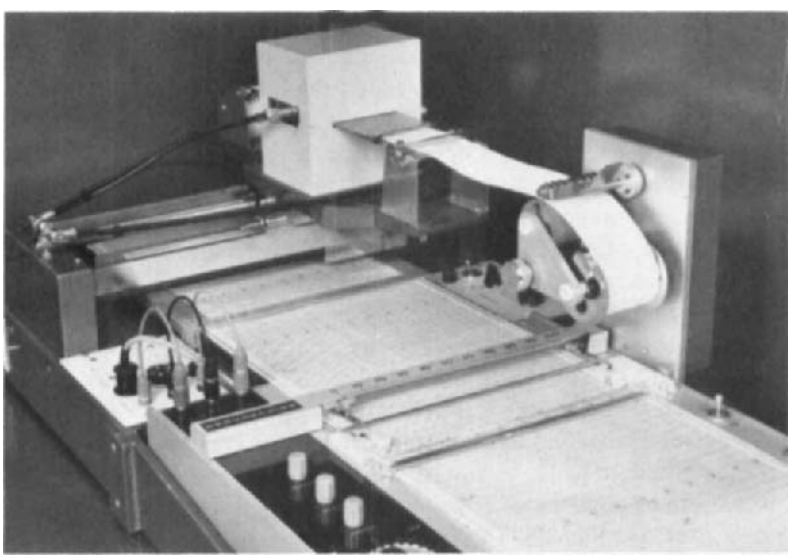

Fig. 3.6. The attachment used for scanning paper strips on a Panax radiochromatogram scanner. (Reproduced with permission from Panax Equipment Ltd.)

integrator with digital output, such as the ESI Nuclear 5680 printing autoscaler, can be attached to a scanner.

For most work, peak area measurements are satisfactory although it is often necessary to rescan a chromatogram several times using different ratemeter ranges to ensure that all the peaks are on scale. A digital output integrator overcomes this problem and is a more convenient facility for quantitative work.

Radio-paper chromatography technique with special reference to radioscanning

It is important with radio-PC to use good chromatography practice as outlined in several PC tests, one of the most recent being *Chromatography and Electrophoretic Techniques, Vol. 1* (4th edition) [32]. With reference to the technique of radio-PC itself, it has received surprisingly little coverage in this and other texts although the use of the spark chamber is discussed by Smith and Mitchell [30].

Experience has shown that there are several important considerations which should be made when chromatograms are to be radioscanned after development in solvent. For instance, when chromatographing extracts of biological samples the solution is usually applied to the paper as a wide band (since the scanner detector is up to 3 cm wide). The width of the band applied will also depend on whether spots of non-labelled reference compounds also need to be applied.

Solutions should preferably be applied to the paper and allowed to air-dry but if the extract or solution to be analysed is aqueous then it is often necessary to apply aliquots as a band and dry them with a hot-air blower. When one aliquot is dry then another is added to the same area. This procedure should be used with caution, however, as some compounds in the mixture may be thermally unstable.

When the chromatogram has been run it should be removed from the developing tank and hung up to dry in as even an air current as possible. This is particularly important when thicker papers are used as not only can labelled compounds move short distances across the paper during drying but they can also migrate from one surface to the other. For example, if a wet chromatogram is placed flat on a sheet of glass or a "benchkote" the solvent will evaporate unevenly from the upper surface and radioactive zones, particularly those of high R_F in the solvent, will tend to migrate to the upper surface. If the chromatogram is then scanned on both sides with a 2 π detector as described earlier the resulting scans could be quite different and neither would be an accurate quantitative representation of the chromatography achieved. This is overcome to a large extent by using a scanner with a 4 π detector but it is still recommended that uneven drying should be avoided since some compounds will tend to evaporate from the paper more than others. This was exemplified by Dobbs [8] who showed that on standing, benzoic acid was lost from a paper chromatogram more readily than was stearic acid (see Fig. 3.7). This variable evaporation and migration to the surface can also be a problem with autoradiography and it is discussed on p. 35.

The differences that can be obtained with scanning both sides of the paper as opposed to 4 π scanning are illustrated in Fig. 3.8. This is a chromatogram on Whatman No. 3 MM paper of an extract of barley plants treated with a ^{14}C-labelled sample of the wild oat herbicide flamprop-isopropyl. This extract was an aqueous solution and contained several

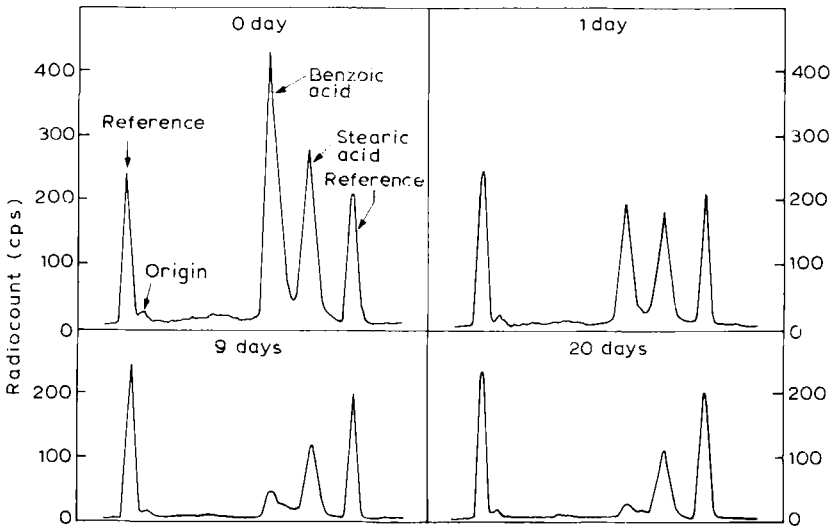

Fig. 3.7. The variation of scans obtained by exposing a chromatogram to different drying conditions. (Reproduced with permission from Dobbs [8].)

polar metabolites which were thought to be conjugated with plant carbohydrates. PC was carried out in an attempt to separate and clean-up the individual conjugates prior to a more detailed examination. From Figs. 3.8a and 3.8b it is clear that there are differences in the radioscans of opposite sides of the paper. The small amount of material with lower R_F value on the upper surface did not penetrate through the paper, but the two components with higher R_F values did. Even so, the relative peak areas of the compounds with higher and lower R_F values are different when measured separately for both sides. (A:B = 48:52 for the top-side and 40:60 for the underside.) Similar effects have been observed by many workers over the years including Dobbs [8].

In Fig. 3.9a is shown a 4 π scan of the same chromatogram obtained on a Packard radioscanner and the rate of peaks A:B is 38:62. It is interesting to note that when a shorter time-constant was used (Fig. 3.9b) although the noise level increased, the presence of a third peak between A and B was clearer than in the other scans.

Autoradiography

Whereas the preferred method for radioassay of one-dimensional chromatograms is radioscanning, autoradiography is more commonly used for two-dimensional chromatograms. Dark room requirements and exposure times are discussed in Chapter 4 (p. 68) and there are several early reports of the technique with reference to PC [3,34].

Practical considerations

There are fewer problems of handling with paper chromatograms than with TLC plates

the surface of which can be scraped or damaged when X-ray film is placed against them. As with TLC, any means of maintaining the film and paper in contact in the dark and without disturbance can be used. A cassette or simple press is suitable or the dark paper and envelope which hold the film could be used. Ideally some means of aligning the exposed film and the paper after development should be used. For example, spots of a radioactive standard can be put in corners of the chromatogram prior to exposure to the film

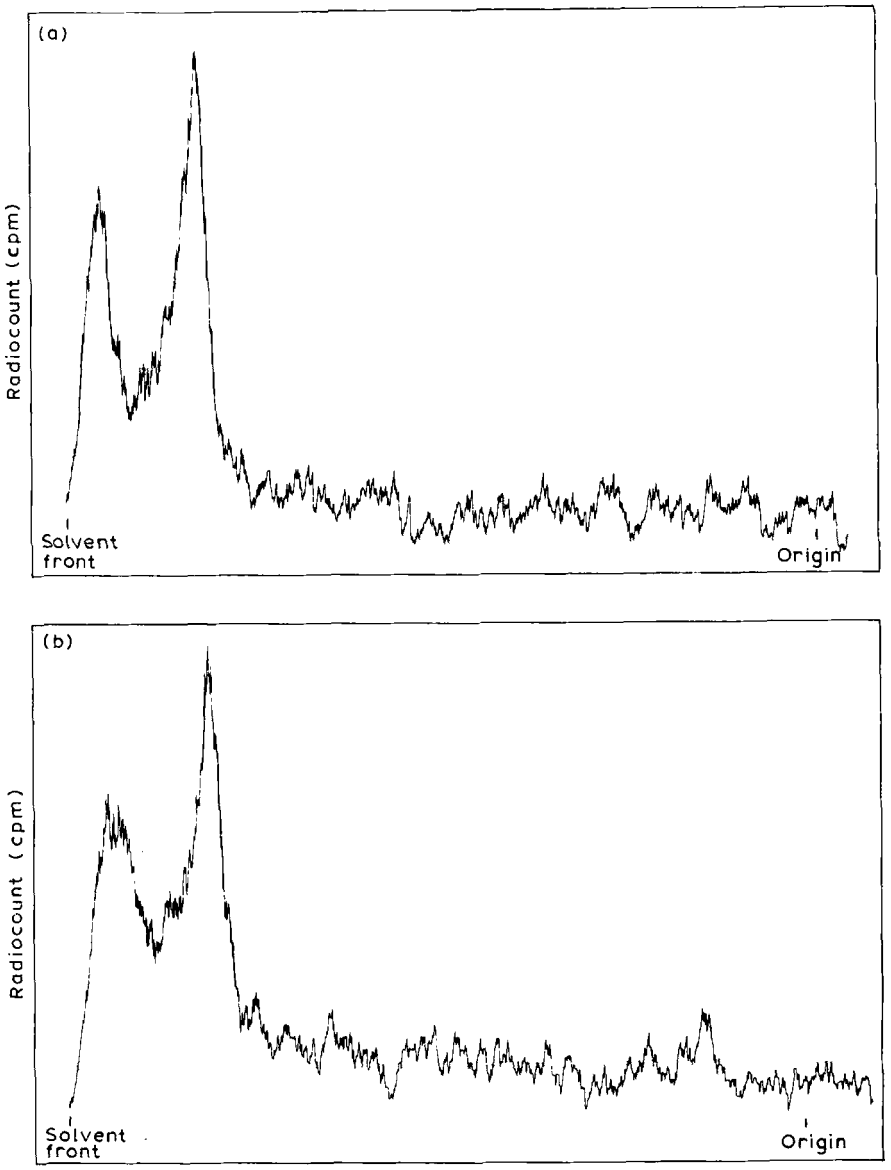

Fig. 3.8. The separate scanning of both sides of a paper chromatogram on Whatman 3MM paper using a 2 π detector.

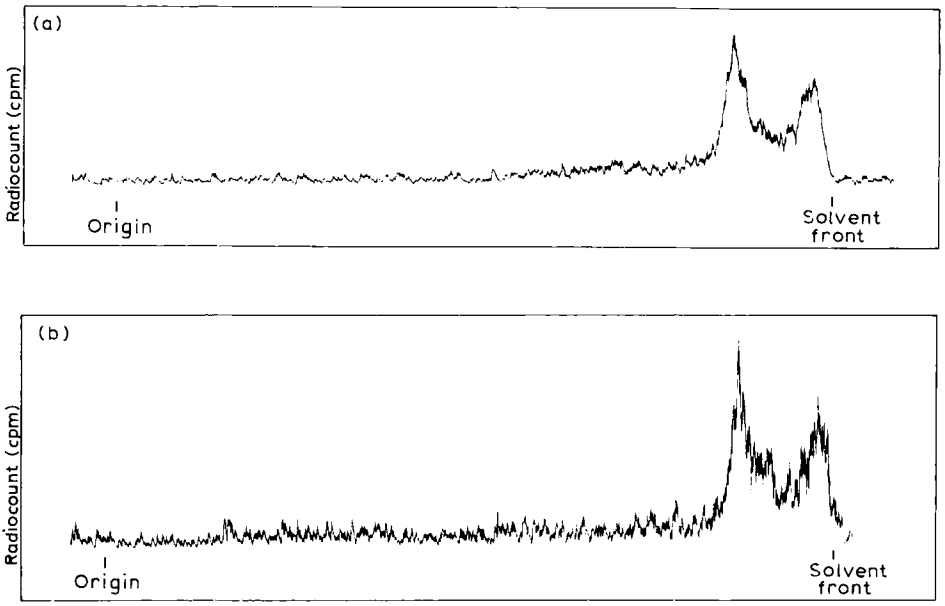

Fig. 3.9. Radioscans of the paper chromatogram shown in Fig. 3.8 obtained using a Packard Model 7201 radiochromatogram scanner. A shorter time-constant was used in scan (b) than in (a).

in such a way that the two can be aligned correctly after exposure and development.

It has been shown by several workers that, rather surprisingly, there is little loss in sensitivity when thicker papers are used for chromatography. At first this was thought to be due solely to the formation of smaller, more compact zones on thicker papers [35] but it has also been shown to result from migration of radioactive compounds to the surface during drying of the chromatogram, a problem which has already been referred to (p. 35). Duncombe [36] compared the effect of drying wet chromatograms with a hot-air blower on one side with natural air-drying in the laboratory. Predictably migration to one surface occurred on forced drying whereas even migration to the surface occurred on paper dried equally on both sides in still air.

The interesting point is that migration still occurs when a chromatogram is dried naturally and the degree of migration of spots to the surface will depend on the R_F value. Duncombe's results which are shown in Table 3.1 have confirmed this. Consequently, it is difficult to interpret autoradiography results from paper chromatograms quantitatively (using a densitometer) and some other method of quantification, either combustion or direct liquid scintillation counting of the spots located by the X-ray film is recommended.

Details of how to develop X-ray film have been given on p. 69.

Tritium detection

As conventional autoradiography of tritiated compounds on paper chromatograms requires very long exposure times, fluorographic methods are preferred since with

TABLE 3.1

EFFECT OF UNEQUAL DRYING OF EACH SIDE OF A PAPER CHROMATOGRAM ON THE AMOUNT OF ^{14}C AT THE SURFACE (REPRODUCED FROM DUNCOMBE [36], WITH PERMISSION)

Labelled compound	Solvent*	Paper (Whatman No.)	R_F value	Ratio $\frac{\text{counts on heated side}}{\text{counts on other side}}$
Glucose	1	3MM	0.05	1.31
Glucose	2	3MM	0.21	1.75
Glucose	3	4	0.25	1.46
Glucose	4	3MM	0.42	1.67
Glucose	5	3MM	0.80	2.37
Glucose	6	4	0.88	3.87
Glucose	7	3MM	0.91	3.49
Citric acid	8	3MM	0.02	1.18
Citric acid	2	3MM	0.47	1.79
Succinic acid	8	3MM	0.44	1.23
Succinic acid	2	3MM	0.82	3.05

*Solvents: (1) acetone–water (92:8); (2) *sec.*-butanol–acetic acid–water (12:3:5); (3) *n*-butanol; (4) acetone–water (60:40); (5) acetone–water (45:55); (6) water; (7) acetone–water (35:65); (8) phenol–water (4:1).

fluorography a 10-fold improvement in the limit of detection of ^{3}H can be obtained [37]. Several workers have used liquid emulsions which were either sprayed onto the chromatogram [21] or used to dip the paper in before exposure to the X-ray film [22,38]. Undoubtedly better results are obtained when the paper is sprayed with scintillator and there are several commercially available products which are suitable for this. For example Nuclear Enterprises NE216 liquid scintillator has a high counting efficiency for ^{3}H.

Liquid scintillation counting of sections of paper chromatograms

Direct counting methods

There are fewer handling problems in the preparation of strips of paper chromatograms for liquid scintillation radioassay than with TLC plates. A representative strip of the paper is simply cut up into pieces of equal size and the pieces are added separately to individual counting vials, after which a suitable liquid scintillation cocktail is added.

As indicated earlier, the problem of orientation of the paper in the vial, which can give variable results, needs to be considered. It has been generally concluded by several workers that the position of the paper in the vial is not important for ^{14}C work but it can give serious variations in results when ^{3}H is being counted. The problem can readily be overcome by cutting the paper into pieces small enough to allow them to rest on the bottom of the vial. Furthermore, the smaller the pieces the better the resolution of the "scan" (a convenient size for this is 1 cm^2). In this way reproducible results should be obtained.

A more serious problem with scintillation counting of sections is the variable desorption of radiolabelled compounds from the paper into organic solution referred to earlier

in this chapter. For example, Wang and Jones [23] obtained counting efficiencies for ^{14}C of 85% for compounds soluble in the scintillator cocktail compared with only 35% for insoluble compounds. The problem is serious for ^{3}H and efficiencies as low as 1–2% can be expected for tritiated compounds which remain adsorbed onto the paper. The situation is particularly bad for low-level work. Cayen and Anastassiadis [28] found that higher efficiencies could be obtained if the paper sections were soaked with a small volume of methanol, to elute the compounds, before addition of a PPO–POPOP–toluene scintillator solution. The amount of methanol used (0.2 ml for each 10 ml scintillator) had no serious quenching effect. Some results taken from the original paper are given in Table 3.2.

An alternative method used in our own laboratory is to add 1 ml of water to each vial containing the paper sections and, after 30 minutes, to add 15 ml of a scintillator cocktail containing detergent, namely toluene–Triton X-100–butyl-PBD. This has the advantage that the scintillator solution can accept 1 ml of water without a reduction in overall counting efficiency and many polar materials from biological samples which are chromatographed on paper may not be completely dissolved in a small volume of methanol. Some results using this method are given in Table 3.3. A standard volume (10 μl) of an extract containing radiolabelled compounds which were insoluble in toluene was pipetted onto 1-cm^2 pieces of chromatography paper and dried with a cold-air blower before transfer to plastic counting vials. In one case only the toluene–Triton X-100–butyl-PBD solution was added while in another case 1-ml volumes of water were added to the vial and left to stand for 30 minutes after which the scintillator solution was added. The experiment was carried out using both Whatman No. 1 and 3 MM papers and all samples were prepared and radiocounted in triplicate. The amount of radioactivity in a 10-μl aliquot of the extract was determined separately.

It is clear from the results that a considerable improvement in counting efficiency can be obtained by the addition of water and better results were obtained with water than with methanol. Furthermore, when counting paper sections containing compounds of different solubilities in toluene and water, this method should ensure that all compounds end up in solution before they are radiocounted.

TABLE 3.2

EFFECT ON COUNTING EFFICIENCY OF SOAKING CHROMATOGRAPHY PAPER SECTIONS IN METHANOL BEFORE ADDITION OF SCINTILLATOR SOLUTION (AFTER CAYEN AND ANASTASSIADIS [28], WITH PERMISSION)

Compound	Chromatography paper (Whatman)	Sample	Average cpm
Coumestrol-^{3}H	None	Standard	129,500
	No. 42	Without methanol	64,700
	3MM	Without methanol	56,800
	No. 42	With 2% methanol	102,600
	3MM	With 2% methanol	99,200
Biochanin A-^{14}C	None	Standard	8,210
	No. 42	Without methanol	6,780
	3MM	Without methanol	6,490
	No. 42	With 2% methanol	7,730
	3MM	With 2% methanol	7,540

TABLE 3.3

EFFECT ON COUNTING EFFICIENCY OF SOAKING CHROMATOGRAPHY PAPER SECTIONS CONTAINING TOLUENE-INSOLUBLE ^{14}C-LABELLED MATERIAL

Each sample contained 860 dpm.

Chromatography paper (Whatman) and treatment	Average cpm	Counting efficiency (%)
No. 1 only	402	46.7
No. 1 + 1 ml water	746	86.7
3MM only	412	47.9
3MM + 1 ml water	703	81.7
3MM + 0.5 ml methanol	653	75.9

Elution analysis

Elution of sections of a paper chromatogram and liquid scintillation counting of the eluate is rarely used as a radioassay method, mainly because it is laborious and time consuming. It is preferable to carry out the elution in the vial as described in the previous section.

In preparative PC only a strip is taken for radioscanning, liquid scintillation counting or combustion analysis and the radioactive zones are located and eluted from the remainder of the chromatogram. This can be done by cutting up the zone to be eluted and soaking it

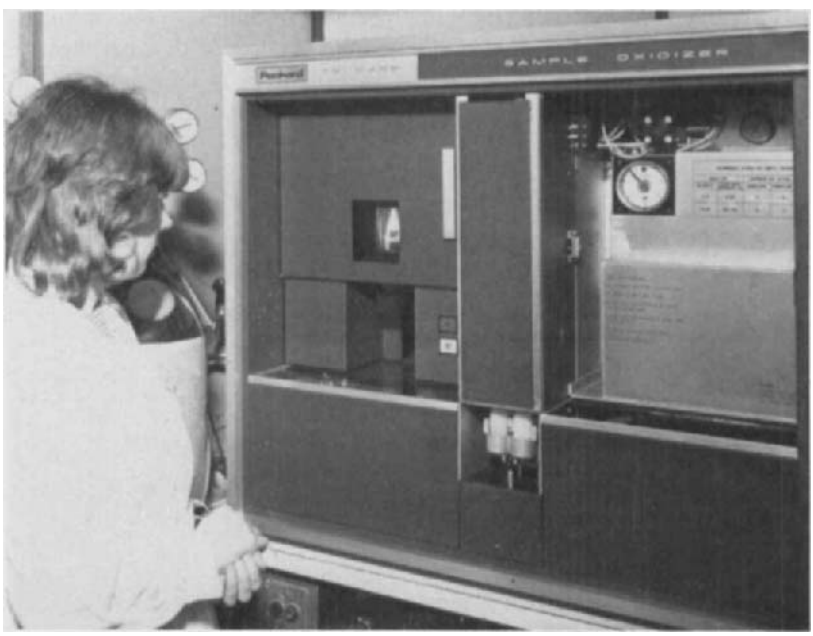

Fig. 3.10. The Packard Model 306 sample oxidiser.

in a suitable solvent followed by filtration through sintered glass. Alternatively the components may be washed off by eluting the chromatograms with solvent by downward development and collecting the eluate in a beaker as it leaves the paper.

Combustion analysis

Radioassay of paper chromatogram sections by combustion analysis has been increasing in popularity since automatic sample oxidisers such as the Packard Model 306 (Fig. 3.10) and Intertechnique Oxymat (Fig. 3.11) have become available. The method is limited to compounds containing ^{14}C (which is oxidised to $^{14}CO_2$) and ^{3}H (which is oxidised to $^{3}H_2O$).

The procedure followed is first to check the recovery of a standard through the oxidation cycle, preferably using a solution of the extract or compound chromatographed. For example, a known volume of standard solution is pipetted onto cellulose contained in a small paper cup which is placed in the wire holder and combusted. Several replicate analyses are preferable, after which the paper sections placed in combustion cups are oxidised in turn. The recovery of ^{14}C or ^{3}H should be checked again both during and after the analysis to ensure that it has been essentially constant during the run. The amount of $^{14}CO_2$ or $^{3}H_2O$ trapped in scintillator solution is then determined.

In the author's experience, recoveries of 85–90% can be obtained using ^{14}C and these

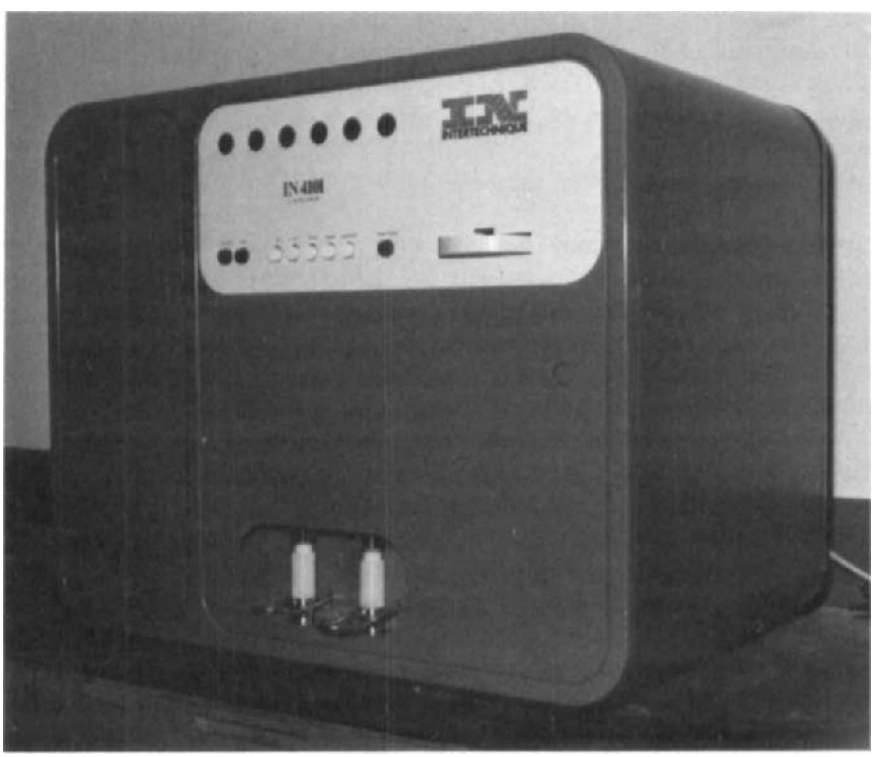

Fig. 3.11. The Intertechnique Oxymat sample oxidiser. (Reproduced with permission from Intertechnique Ltd.)

are similar to those obtained by the direct liquid scintillation counting method recommended on p. 36. As the combustion method is more time consuming it is likely to be used less frequently than liquid scintillation counting. However, it can be very useful if problems of colour quenching are encountered when the direct method is used.

Spark chamber techniques

Radiochromatogram spark chambers are straightforward to operate and quick to use and they are gaining in popularity as a very rapid qualitative method of locating labelled compounds on chromatograms. A detailed description of the technique in practice is given on p. 71 and an example of a spark discharge photograph obtained on a paper chromatogram is shown by Smith and Mitchell [30]. The exposure time was only 10 minutes and although the result is not as clear as an autoradiograph, the time-saving is considerable.

APPLICATIONS OF RADIO-PAPER CHROMATOGRAPHY

Radio-PC is used far less now than it was in the 1960s and for many applications it has been superseded by more rapid and more efficient techniques, particularly radio-TLC and column separations. In spite of this, radio-PC is superior to TLC in certain situations, particularly in the chromatography of more polar compounds, which are less readily analysed by TLC. Furthermore, preparative PC gives separations very similar to those obtained under analytical conditions. This is not always true for TLC, especially with ready-made TLC plates with thick layers. These and other advantages are apparent from the following section in which some applications of radio-PC are discussed.

Pesticide and drug metabolism studies

In the past, PC was used extensively in studies of the metabolism of radiolabelled pesticides and drugs and despite the decline in its use it is still valuable as a complementary method to radio-TLC. Whereas simple, relatively non-polar metabolites are readily chromatographed on silica-gel TLC plates (see Chapter 4, p. 80) this is not always true for metabolites which are bound or conjugated to naturally occurring molecules such as sugars, amino acids or peptides for which PC can be more suitable.

For instance, Hutson and co-workers in their work on the metabolism of the insecticide endrin in the rabbit [39] and rat [40] separated the sulphate ester and glucuronide of 12-hydroxyendrin (R_F values 0.70 and 0.10, respectively) from each other and from several "free" metabolites, including 12-hydroxyendrin (R_F value 0.95), using Whatman No. 1 or No. 3 paper developed in *n*-butanol–ammonia (s.g. 0.880)–water (86:1:13, v/v) [39]. Radioactive metabolites of [^{14}C]endrin were located by radioscanning in a Packard Model 7201 instrument.

Radio-PC is also suitable for larger-scale preparative separations of metabolites [39] and for the separation of labelled metabolites from extraneous interfering material which had been co-extracted from the plant or animal tissue. Hodgson *et al.* [41] found that whereas the herbicide diphenamid and its metabolites could not be separated from free sugars and naturally occurring plant glycosides by TLC, this separation could be achieved

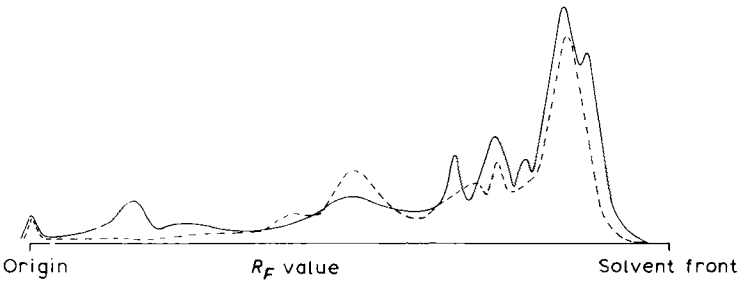

Fig. 3.12. PC of a methanol extract of faeces from rats exposed to [^{14}C-ring] herbicide (——) or [^{14}C-ethyl] herbicide (– – –). (Reproduced with permission from Crayford and Hutson [42].)

using descending PC. This permitted pure samples of metabolites conjugated with sugar to be isolated for further examination by hydrolysis and enzyme assay of the amount of sugar present in the molecule.

Crayford and Hutson [42] claimed that PC can be the most suitable method for analysis of faecal extracts and Fig. 3.12 shows methanol extracts of faeces from rats which had been fed [^{14}C]-2-chloro-4-(ethylamino)-6-(1-cyano-1-methyl-ethylamino)-*s*-triazine (labelled at the ring or at the ethyl group). The two traces are of metabolites formed when the herbicide, labelled separately in two different positions, was administered to the rat.

In the author's laboratory radio-PC is used to separate mixtures of polar metabolites from different sources to determine whether the same products occur in each case. For example, polar products formed when the herbicide benzoylprop-ethyl (the structure of which is based on 3,4-dichloroaniline) was added to soil, were chromatographed with extracts of soil treated with 3,4-dichloroaniline itself [43]. In this way an indication was obtained as to whether the polar metabolites of benzoylprop-ethyl in soil were formed as a result of its initial degradation to 3,4-dichloroaniline.

Waring [44] used radio-PC in a study of the metabolism of the fungicide Vitavax in the rat and the rabbit.

Similar examples to these may also be found in the literature on drug metabolism [45–53]. Wang *et al.* [45] determined the radiochemical purity of a 5-nitrofuran-based drug and they also analysed urine of rats treated with the ^{14}C-labelled drug by PC. Smith and Griffiths [46] studied the metabolism of phenacetin, paracetamol and related compounds in the rat. Urine samples were again chromatographed on paper and areas on the chromatogram located by either UV absorption or autoradiography were cut out for liquid scintillation counting.

Chapman and Marcroft [49] used radio-PC in a study of the metabolism of [^{14}C]-amphetamine in the horse in relation to the known effect of the drug on the performance of man and horses in athletic events. Aitio [53] looked into the more general question of glucuronide conjugation in rat and guinea-pig using radio-PC and autoradiography.

The technique is also used for other metabolism studies, and Burke *et al.* [54] used autoradiography and radioscanning to locate metabolic products of ^{35}S-labelled anionic detergents on paper chromatograms.

Biochemical studies

PC is frequently used in radiotracer studies of metabolic pathways especially when

amino acids or carbohydrates are being separated. Firmin and Gray [55] used radio-PC to separate metabolic products of acetonitrile in bacteria. Descending chromatography was used in a range of solvents and the dried chromatograms were cut up for liquid scintillation counting. Alternatively compounds were eluted from the paper with water and the resulting aqueous solution was radioassayed by liquid scintillation counting in the usual way.

Griffiths and Tudball [56] have used PC in their recent work on the fate of cystathionine in rat brain using both ^{35}S- and ^{14}C-labelled products. The developed chromatograms were dried and sprayed with ninhydrin to visualise amino acids and radioactive zones were detected by autoradiography for periods of up to 1 month. Liquid scintillation counting of paper strips was used to quantify the results.

Wang and Yang [57] were able to separate ATP and ADP from each other and from inorganic phosphate using Whatman No. 1 paper impregnated with polyethyleneimine. Liquid scintillation counting of paper sections was used to locate the ^{32}P-labelled products and a typical result is shown in Fig. 3.13.

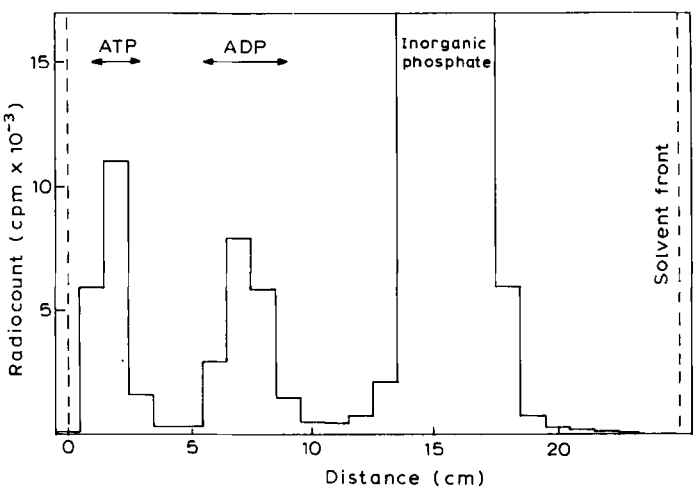

Fig. 3.13. A radiochromatogram of ^{32}P-labelled products, obtained by liquid scintillation counting of sections. (Reproduced with permission from Wang and Yang [57].)

Thwaits *et al.* [58] working on the enzymic acetylation of histones used the less common technique of combustion analysis. Two narrow strips were cut from each chromatogram, one for spraying with ninhydrin reagent and the other for cutting into sections for combustion in a sample oxidiser. A chromatogram reproduced from the original paper is shown in Fig. 3.14.

Radiosynthesis

Reference has already been made to the use of radio-PC for preparative-scale separations of labelled compounds in the presence of biological material. Although PC is seldom used as a means of checking radiochemical purities it is often used for purification of a

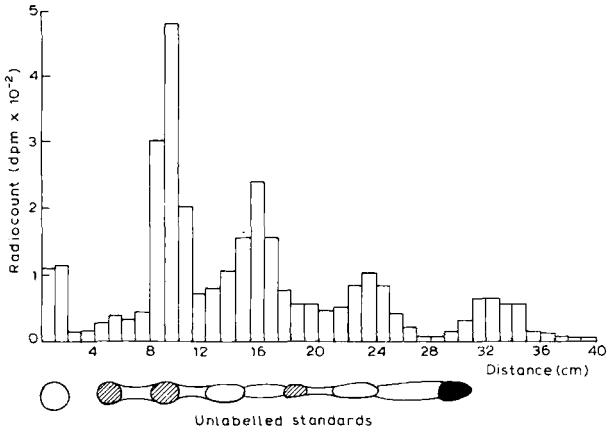

Fig. 3.14. A radiochromatogram obtained by combustion of paper sections. (Reproduced with permission from Thwaits *et al.* [58].)

synthesised compound on a preparative or semi-preparative scale. Falecki [59] separated 10–100-mg amounts of synthesised [^{14}C]choline, [^{14}C]glutamic acid, [^{14}C]indoleacetic acid and other compounds on Whatman No. 3 paper and obtained products with radiochemical purities of 98–99%.

In general, however, column chromatography methods are preferred for relatively large-scale preparations and separations of products from radiochemical syntheses but PC can be more suitable for separations on the milligram scale especially when conditions and suitable solvent systems are known from analytical work.

Sheppard and co-workers at The Radiochemical Centre, Amersham have used PC to study the radiation-induced self-decomposition of ^{3}H-labelled nucleotides and related compounds of high specific activity [60]. PC was apparently more suitable than TLC for the separation of, for example, [^{3}H]thymidine from some of its more polar self-decomposition products.

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Chapter 4

Radio-thin-layer chromatography

CONTENTS

INTRODUCTION

Since the technique of thin-layer chromatography (TLC) came into general use in the early 1960s considerable progress has been made in the quantitative radioassay of TLC plates. Initially, most approaches to radio-TLC were merely extensions or modifications of techniques which had been used in paper chromatography (PC), but as the full potential of

TLC was realised, instruments were designed specifically for monitoring radioactivity on thin layers.

The reason for this was the physical difference between the paper chromatogram, which could be handled relatively easily and could be cut into strips or pieces for radioassay, and the rigid TLC plate with its more fragile layer of adsorbent.

The techniques used for detecting β-emitting isotopes on thin-layer chromatograms may be conveniently divided into the following categories:

(i) scanning with a radioactivity detector;
(ii) autoradiographic methods;
(iii) spark chamber techniques;
(iv) zonal analysis and elution techniques based on liquid scintillation counting;
(v) combustion analysis.

Techniques falling into categories (i)–(iii) monitor radioactive areas on the intact plate whereas with (iv) and (v) the adsorbent on the chromatogram is divided up and the labelled material cannot readily be recovered for further examination. With the exception of the more recently introduced spark chamber techniques, all of the approaches described above were first used for PC. However, particularly in the case of scanning, new instruments have been designed to scan rigid plates on one side only compared with paper scanners, which use detectors with 4 π geometry to scan both sides of the paper simultaneously.

The state of the art of radio-TLC has been reviewed at intervals since 1962 including those by Mangold [1, 2] (1962, 1969), Snyder [3, 4] (1967, 1972), Figge *et al.* [5] (1970) and Prydz [6] (1973).

DEVELOPMENT OF RADIO-THIN-LAYER CHROMATOGRAPHY

Radioscanning techniques

Mention has already been made of the possible use of paper chromatogram scanners for the radioassay of TLC plates provided that the plates are of a suitable size. For example, Rosenberg and Bolgar [7] described the use of a Tracerlab SC 55 instrument for scanning 20 cm x 4.6 cm TLC plates. Larger plates could not be scanned unless they were cut into 4.6-cm strips. Although the efficiency for ^{14}C was only around 2%, the method was used to determine radiochemical purities.

An alternative way of utilising a paper chromatogram scanner was to stabilise the silica gel layer and remove it from the glass plate. One way of doing this [8] was to spray the developed TLC plate with "Neatan" (a commercial aqueous dispersion of polyvinyl propionate). When the Neatan was dry, transparent tape was placed on the surface and the layer was soaked in water and peeled off. It was then necessary to spray the *underside* of the chromatogram with "Neatan" in order to stabilise the whole layer, which could then be cut into strips and fed into a paper scanner. The sensitivity for ^{14}C on layers prepared in this way was found to be almost as good as for paper but the technique was of only limited interest since it was fraught with handling problems. Other examples of related procedures can be found in the early literature.

The widespread use of TLC soon led to the development of detectors and scanners de-

signed specially for radio-TLC. In 1962, Schulze and Wenzel [9] described a proportional gas-flow counter with a flat aperture plate as shown in Fig. 4.1. Methane was used as quenching gas. For scanning, the TLC plate was placed on a flat carriage which was moved along at a distance of 1–3 mm below the detector. In this way, soft β-emitters including ^{3}H could be detected. Typical efficiencies were around 40% for ^{14}C and 0.3% for ^{3}H with TLC layers of 0.25 mm thickness. This detector formed the basis of the Desaga scanner which was first available commercially in 1963. This scanner (Fig. 4.2) was evaluated by Wilde [10] and comprised a platform which could be moved at one of a range of speeds beneath a fixed proportional gas-flow detector and preamplifier, a ratemeter with power supply and a recorder with chart speeds identical to those of the scanner deck. This allowed direct alignment of the recorder trace and the chromatogram. The instrument proved to be sensitive and to give good resolution and reproducibility.

The features of the Desaga scanner were also described by Berthold [11], including its use for scanning two-dimensional chromatograms and an alternative form of data presentation, namely the dot printer. This produced a dot on pressure-sensitive paper with every pulse (or with a pre-selected number of pulses) to give an output similar to that of an autoradiograph. Paper chromatograms could also be scanned, using a 4 π detector fitted instead of the 2 π head for TLC plates. Improvements to this basic instrument have been made over

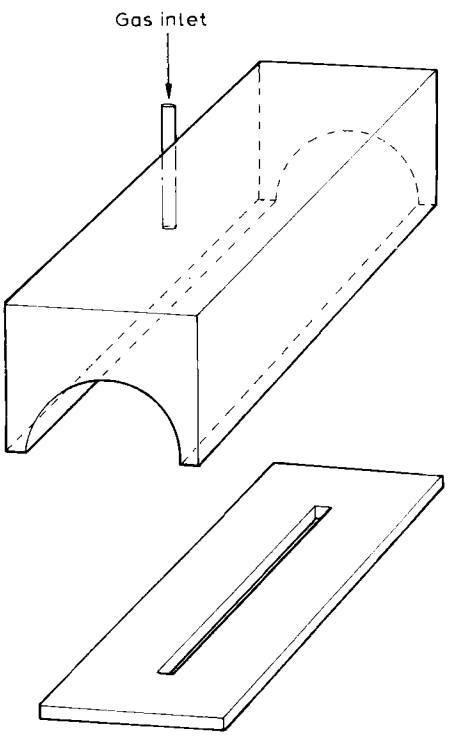

Fig. 4.1. The proportional gas-flow counter described by Schulze and Wenzel [9]. (Reproduced with permission.)

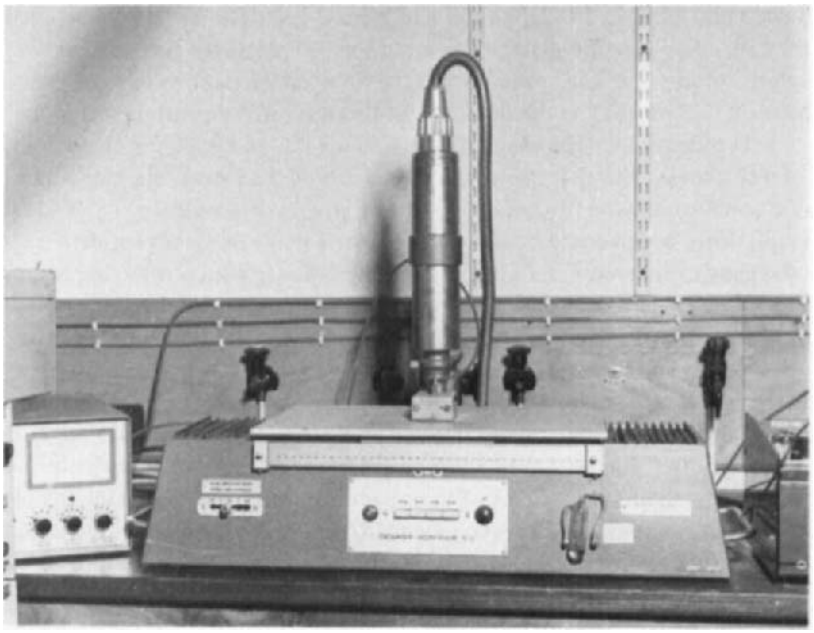

Fig. 4.2. An early Desaga thin-layer radioscanner.

the years and the radioscanners currently available from Berthold/Desaga are described later in this chapter.

Several reports have appeared in the literature of TLC scanners which have been built up from detectors, ratemeters and other components. Moye [12] used an Ekco Geiger–Müller counter mounted on a platform with a continuous belt drive and a synchronous motor, which had to be changed when different scanning speeds were required. Although the detector used here had a considerably lower efficiency than the proportional counter of Schulze and Wenzel, it was suitable for use with isotopes of higher energy than ^{3}H and the scanner was successfully used in kinetic studies [13]. West *et al.* [14] built a TLC scanner from a Nuclear Chicago C 100B Actigraph strip feeder which moved the TLC plate under a gas-flow counter and ratemeter which was used to drive a recorder synchronously with the feeder. The instrument was used for the TLC analysis of thyroid hormones.

In 1967, Ravenhill and James [15] published a detailed paper on their scanner which was based on a proportional counter design which had proved successful in radio-gas–liquid chromatography. This was claimed to be the first TLC scanner with automatic count integration. The proportional counter was modified with a slit of 1 cm x 1.0–2.5 mm at its centre and this was mounted above the TLC plate. Argon–carbon dioxide (95:5, v/v) counting gas was passed into the detector at both ends to ensure that it emerged through the slit. The design of the whole apparatus is shown schematically in Fig. 4.3. The counting efficiency for ^{14}C was found to be 37%. Full details of the scanner and circuit diagrams are given in the original paper [15] together with examples of scans made in the differential and integral modes.

A quite different approach to radio-TLC based on solid scintillation detection was used by Melo and Prydz [16] who were interested in the monitoring of both ^{14}C and ^{3}H individually on the same thin-layer chromatogram. Silica gel plates were sprayed with a saturated solution of anthracene in benzene and, when the benzene had evaporated, a layer of anthracene crystals remained. The plate was then scanned with a photomultiplier tube and the signals were detected and passed through an amplifier to discriminators which distinguished between ^{14}C and ^{3}H pulses. The equipment used is shown in Fig. 4.4. In fact, if only a single discriminator and ratemeter were used the plate could be scanned first with settings for ^{14}C and the scan could then be repeated with the discriminator set to collect ^{3}H pulses. In practice there was little overlap between the two counting channels.

Another novel scanner [17] was built in the same laboratory and the detection method used this time was an electron multiplier. This was a rather complex instrument which needed to be operated in a vacuum of 5×10^{-4} torr and it was housed in a cylindrical vacuum chamber with a volume of about 75 l. The detection system is shown diagrammatical-

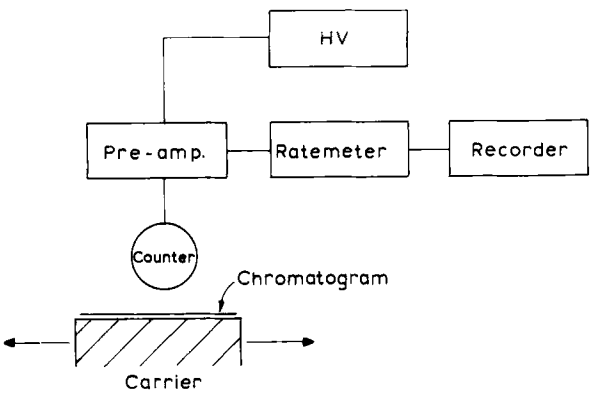

Fig. 4.3. A schematic diagram of the scanner described by Ravenhill and James [15]. (Reproduced with permission.)

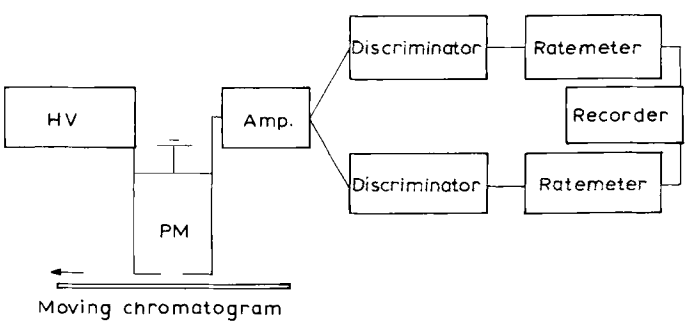

Fig. 4.4. A schematic diagram of the scanner designed by Melo and Prydz [16]. (Reproduced with permission.)

ly in Fig. 4.5. It was hoped that, as the electron multiplier was operated under vacuum, it would detect ^{3}H with high efficiency since the β-particles emitted from ^{3}H travel only a few millimetres in air. In practice the detection limit was found to be 5 μCi for ^{14}C in a single spot (compared with approximately 0.1 μCi for the Berthold scanner) so the system is of limited interest to the chemist or biochemist working with low levels of radioactivity.

A new version of the Berthold (Desaga) radioscanner was introduced in 1968–1969 and Wenzel and Hoffman [18] published data obtained with it in 1971. This modified instrument was a two-dimensional scanner which could again be used with a recorder or dot printer output. The proportional counter, which was again run on methane or argon–methane (90 : 10, v/v) was closed with one of a set of magnetic plates with slits ranging from 1 to 4 mm width. Plates with slit lengths of 1 cm or 3 cm were available and the slits were either left open or they were covered with very thin foil. Detection limits were determined [18] using standards and were found to be around 200 dpm for ^{14}C and 3000 dpm for ^{3}H. Radioactivity on two-dimensional chromatograms could be located by scanning after each development and using a recorder in the manner shown in Fig. 4.6. Alternatively a dot printer could be used after the chromatogram had been eluted in both directions. A more detailed description of this scanner is given in a later section (p. 64).

Another method for the determination of two isotopes simultaneously on the same TLC plate was reported by Tykva and Votruba [19] who used a semiconductor detector [20]

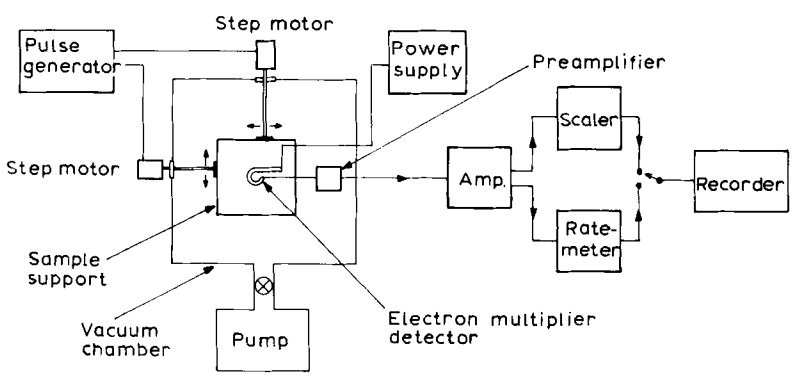

Fig. 4.5. A schematic diagram of the detection system used by Prydz *et al.* [17] incorporating an electron multiplier. (Reproduced with permission.)

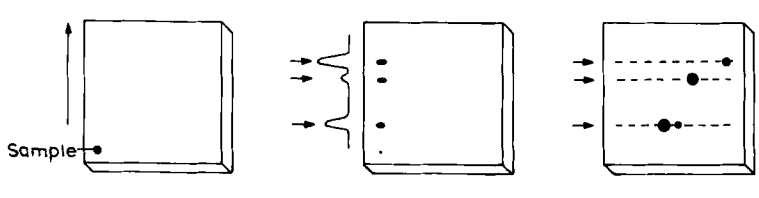

Fig. 4.6. A method of scanning two-dimensional chromatograms. The chromatogram is scanned after the first elution and again after the second elution along tracks known to contain radioactivity.

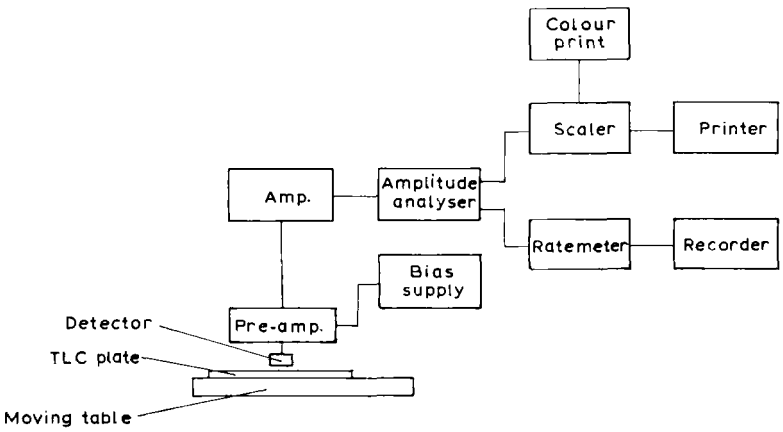

Fig. 4.7. A schematic diagram of a scanner incorporating a semiconductor detector. (Reproduced with permission from Tykva and Votruba [19].)

incorporated into the scanner shown diagramatically in Fig. 4.7. Pulses from the detector were amplified and were selected by a pulse height analyser, and if two isotopes such as ^{14}C and ^{35}S were to be measured then a dual-channel analyser was used with suitable discriminator settings. The scanner deck could be operated semi-manually or it could be programmed by a computer to move in fixed steps of between 1 and 7 mm. In the latter case, the radioactivity present at each position was measured and the result was printed out as a series of coloured dots which represented different levels of radioactivity.

For measurements on a single isotope this semiconductographic method would not appear to offer any advantage over scanners based on gas-flow counters, but for more complex dual-isotope work it obviously has potential.

Autoradiography

The use of autoradiography in PC has already been discussed in Chapter 3 and this is perhaps the most widely used method of locating labelled compounds on TLC plates. Its popularity stems from the fact that it is easy to use, it does not require complex equipment and as a consequence it is very inexpensive in capital terms compared with scanning techniques although the cost of materials needs to be considered. Nevertheless, in order to do quantitative work it is necessary to have access to either a densitometer or an alternative quantitative radiochemical instrument such as a liquid scintillation counter. The latter approach has been found to be more commonly used.

All weak β-emitters can be detected using the autoradiographic technique and it is easier to monitor tritiated compounds on TLC plates than on paper chromatograms. However, exposure times in excess of two weeks are usually necessary for ^{3}H. Furthermore, no intermediate protecting layer can be used (*e.g.* Mylar film). Long exposure times are not as serious a drawback with other isotopes as is generally believed and with ^{14}C-, ^{35}S- and ^{36}Cl-labelled compounds exposure times of 1 or 2 days give acceptable results as long as quantities in excess of approximately 10 nCi are available.

The basic technique has been described in several books and reviews including those of Mangold [2], Rogers [21], Fischer [22] and of Fischer and Werner [23] and a discussion of autoradiography appears in a later section of this chapter (p. 68). There are relatively few detailed publications on the autoradiography of the higher-energy isotopes but there has been considerable interest in methods of improving the sensitivity of detection for ^{3}H. A useful method of overcoming the self-absorption problem of ^{3}H is to impregnate the adsorbent layer with a scintillator thus giving rise to an enhanced darkening of the photographic emulsion. This has been described as "fluorography" or "scintillation autography" [24, 25]. Luthi and Waser [26] described a fluorographic technique in which anthracene was powdered in a ball mill with silica gel and TLC plates were prepared from this mixture. The sensitivity of detection was also markedly improved at low temperature. For example, a ^{3}H fluorograph was twice as intense (as determined by densitometry) when the exposure was made at −30°C than at +4°C and as much as 30 times as intense at −70°C. Anthracene is particularly suitable for fluorography because of its low solubility in many common TLC elution solvents and its low chemical reactivity as well as its high fluorescence efficiency.

In 1970 Randerath [27] reviewed the state of the art with regard to ^{3}H detection by autoradiography and pointed out the surprising variation in opinion as to which was the optimum method. For example, contrary to the results of Luthi and Waser, Chamberlain *et al.* [28] found no advantage in fluorography over conventional autoradiography unless the TLC powder was impregnated with an X-ray emulsion. This was of only limited interest since the radiolabelled compounds could not be recovered from layers of adsorbent impregnated in this way. In fact various sensitivities for the detection of ^{3}H have been re-

TABLE 4.1

EFFECT OF TREATMENT OF CHROMATOGRAM AND TEMPERATURE OF EXPOSURE ON SENSITIVITY OF ^{3}H-FLUOROGRAPHY (AFTER RANDERATH [27])

Exposure time 24 h.

Treatment*	Temperature (°C)	Film	Sensitivity (nCi/cm^2)
None	+25	Royal blue	120–150
	−78.5	Royal blue	120–150
	+25	No-screen	400–600
	+25	Tri-X pan	120–150
	+25	Royal-X pan	120–150
2% PPO	−78.5	Royal blue	25–35
7% PPO	−78.5	Royal blue	4–6
	−78.5	RP/S X-omat	5–6
	−78.5	GAF-X	5–8
	+25	Royal blue	80–100
	−78.5	No-screen	50–80
	−78.5	Tri-X pan	30–40
	−78.5	Royal-X pan	10–20

*A solution of PPO in diethyl ether was poured over the chromatogram (35–40 μl/cm^2).

ported ranging from 25 to 300 nCi/cm^2/day [28, 29]. For this reason Randerath carried out a comparative study between autoradiography and fluorography using various amounts of scintillator, and exposures were made at two temperatures. It was found that, although the sensitivity of detection of ^{14}C was more or less independent of the method used, in the case of ^{3}H the limit of detection varied considerably with the amount of scintillator incorporated and with the temperature. Some of Randerath's results [27] for ^{3}H are shown in Table 4.1.

This detailed study clearly demonstrated the high sensitivity of fluorography when working with ^{3}H and it also confirmed the enhancement in intensity at low temperature. At the same time it also showed that for isotopes of higher energy fluorography offered no advantage.

Meeks *et al.* [30] found similar results when they evaluated a commercially available intensifier. The product permitted a 100-fold increase in sensitivity to be achieved for ^{3}H when used according to the manufacturers recommendations, but again there was no improvement for ^{14}C.

Spark chamber techniques

A rapid method of locating radiolabelled compounds on chromatograms has been introduced more recently and this involves a quite different detection procedure from those described so far. Reference has already been made (p. 40) to the "spark chamber", which was first used for radio-TLC by Pullan and co-workers [31, 32] in 1968 and the use of radiochromatogram spark chambers has recently been reviewed [33]. In its simplest form the detector comprised two parallel electrodes, consisting of arrays of parallel wires, between which an electric field could be produced. The whole system was maintained in an atmosphere of a suitable gas such as argon–methane (90 : 10, v/v). When placed above a TLC plate, a β-particle from a compound on the plate would give rise to a visible spark. Since the spark appeared at the same location as the β-particle, a photograph (taken with a polaroid camera) of the light produced gave a record of the location and relative intensities of the spots, similar in appearance to an autoradiograph. A diagram of the early spark chamber is shown in Fig. 4.8 and examples of a spark chamber record of a one-dimensional chromatogram are shown in Fig. 4.9. Although the result is not so clear and precise as the corresponding autoradiographs it must be remembered that the spark chamber exposure time scale is only minutes compared with hours or days for the autoradiograph.

An inconvenient feature of the early model, which was available from Birchover Instruments, was that the film obtained was smaller than the TLC plate and, unlike an autoradiograph, it could not be compared directly with the chromatogram. This has been overcome in more recent instruments by the use of overhead projection of the print image directly back onto the original chromatogram. The first instruments made by Pullan and co-workers [31, 32] contained a crossed-wire arrangement but a coiled (spiral) cathode was found to be more stable (Fig. 4.10). A modification of the earlier models was described by Smith *et al.* [34] and examples of its use were cited. Instruments currently available include the Birchover 450 Radiochromatogram Spark Chamber (Fig. 4.11), the Panax Beta-graph (Fig. 4.12) and the Berthold Beta-Camera (Fig. 4.13).

The Birchover is available as a standard model (No. 450B) or with optional ultraviolet or

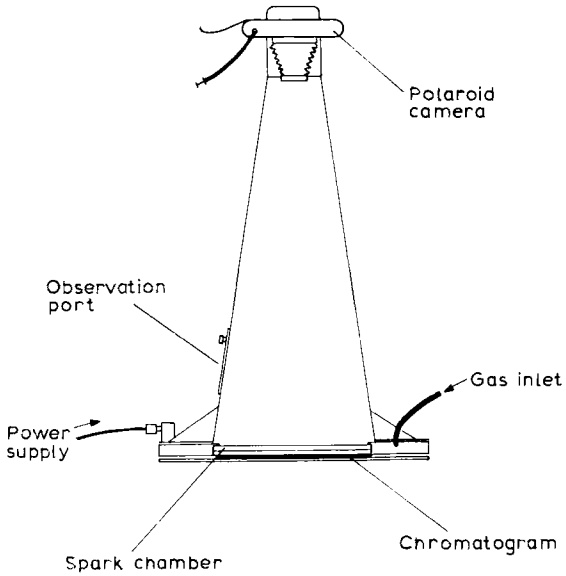

Fig. 4.8. A diagram of an early version of the radiochromatogram spark chamber. (Reproduced with permission from Pullan [32].)

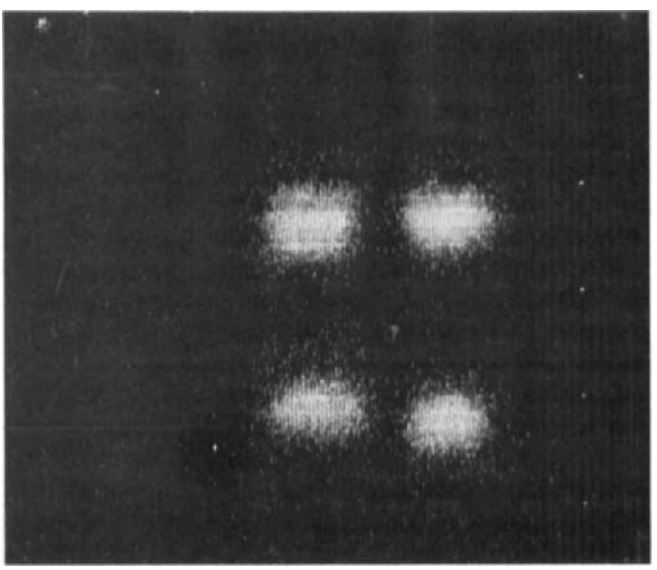

Fig. 4.9. A typical spark-discharge photograph obtained on a Berthold Beta-Camera.

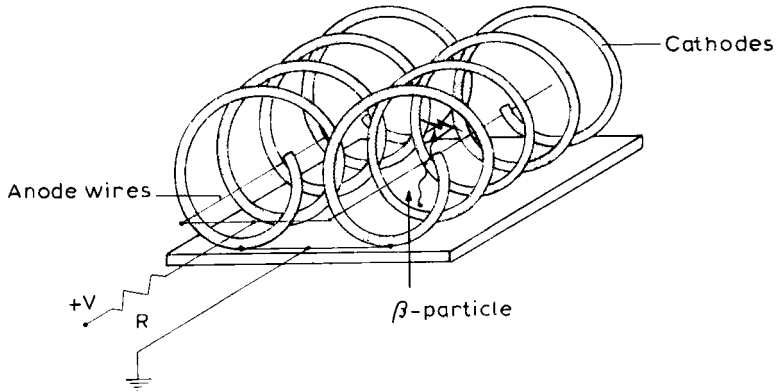

Fig. 4.10. A diagram of the spiral cathodes used in current radiochromatograph spark chambers showing the anode wires running along the axes of the spirals.

Fig. 4.11. The Birchover 450 Radiochromatogram Spark Chamber. (Reproduced with permission from Birchover Instruments Ltd.)

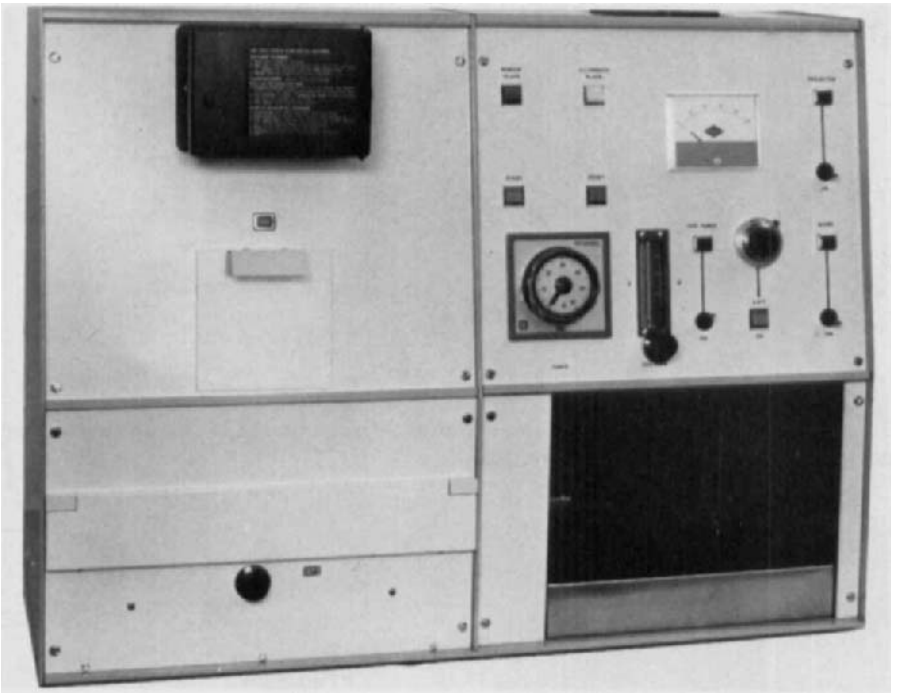

Fig. 4.12. The Panax Beta-Graph. (Reproduced with permission from Panax Equipment Ltd.)

natural illumination for the comparison of radioactive zones with unlabelled compounds present on the chromatogram as references. An alternative CU5 polaroid camera and suitable film are then used to obtain a permanent record of the chromatogram under the appropriate light conditions. Details of the procedure are given on p. 71.

The Panax Beta-graph is a similar instrument but it incorporates an oscillating spiral cathode which was designed to overcome the problem of background "hot-spots". However, this results in only marginal improvements in resolution and has limitations (see ref. 33).

The Berthold Beta-camera claims to have a resolution of 3 mm for ^{3}H and 5 mm for ^{14}C, which is similar to that of the Panax and Birchover instruments. However, the design of the grid in the Berthold instrument is such that the rather disturbing effect of the grid pattern which can be observed is minimised.

Zonal analysis and elution techniques

Many variations on the basic procedure of scraping off small areas of adsorbent from TLC plates (so-called "zonal analysis") are possible and this technique can be used in any laboratory with access to a liquid scintillation counter. The adsorbent can either be transferred directly to a counting vial to which a scintillator solution is added or alternatively the labelled compound(s) may be eluted from the TLC powder prior to radioassay of the eluate by liquid scintillation counting. The use of both methods is described on p. 72

but consideration is given here to the development of methods aimed at mechanising and increasing the precision of what can be a tedious and time-consuming process.

A major effort was made in this area during the 1960s by Snyder and co-workers. Initially, Snyder and Stephens [35] (among others) used the simple procedure of dividing the area on a TLC plate between the origin and the solvent front into 5 or 6 zones which were carefully removed with a spatula and transferred into separate counting vials. A toluene–PPO–POPOP scintillator solution containing 4% (w/v) of a thixotropic gelling agent was added and the radioactivity in each vial was determined. This was used to examine ^{14}C- and ^{3}H-labelled lipids on TLC plates and highlights a major advantage in that more than one isotope can be measured simultaneously. Furthermore, for ^{3}H and for low concentrations of other, more energetic isotopes, liquid scintillation counting is the most sensitive means of detection. Consequently, zonal analysis methods are both sensitive and versatile although they suffer from the drawbacks that they are destructive, tedious and subject to serious mechanical losses.

In 1964 Snyder [36] described a manual "zonal scraper" for the rapid and quantitative transfer of areas of adsorbent into vials. The scraper was designed for use only with 2 cm wide glass plates so chromatography was carried out on glass plates of size 20 x 2 x 0.4 cm coated with silica gel. It was possible to scrape off areas of 1, 2 or 5 mm x 2 cm and an example of a typical scan is shown in Fig. 4.14.

This manual instrument was the prototype of a fully automatic instrument described by

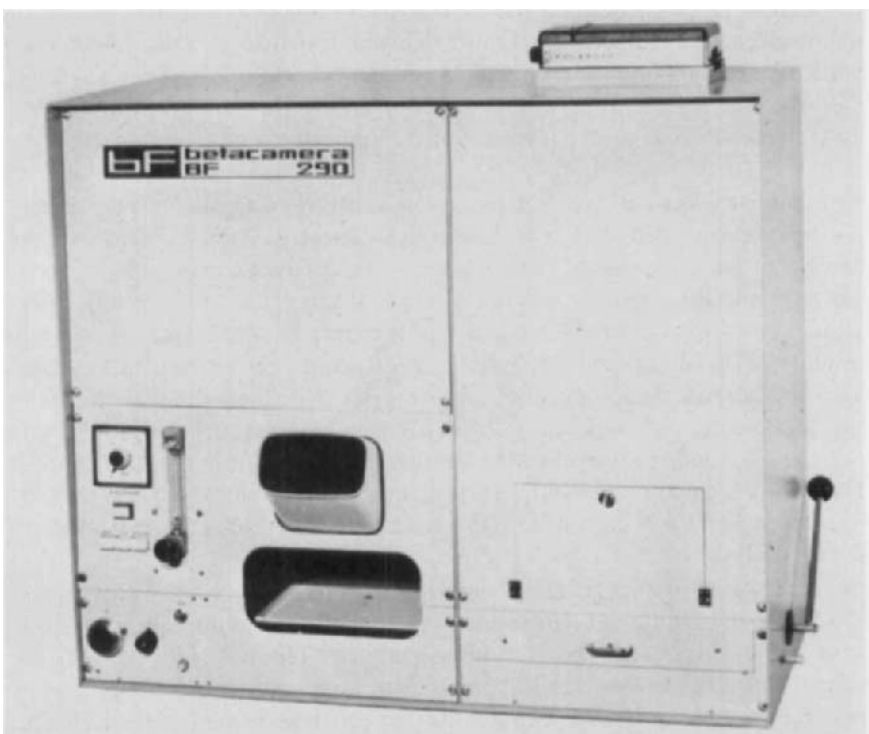

Fig. 4.13. The Berthold Beta-Camera. (Reproduced with permission from Intertechnique Ltd.)

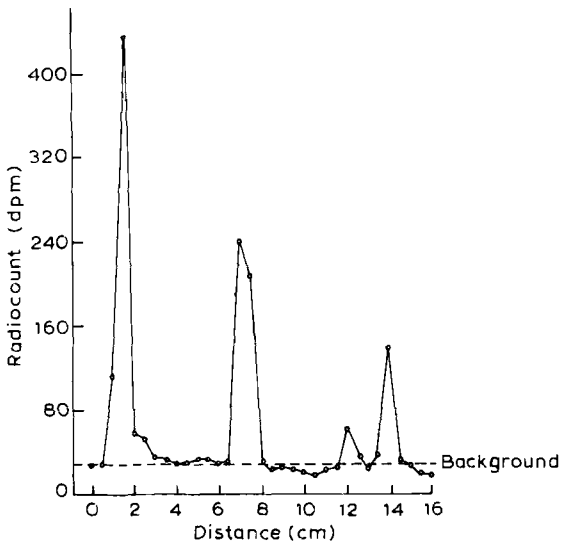

Fig. 4.14. A zonal scan of a TLC plate with only small amounts of radioactivity present. (Reproduced with permission from Snyder [26].)

Snyder and Kimble [37] in 1965. Again only 2-cm plates could be used and 1-, 2- or 5-mm steps could be taken. The scraper incorporated a circular tray from a Packard 4000 Series liquid scintillation counter and the tray held 24 counting vials. The adsorbent was scraped directly into the vials, scintillator solution was added and the tray could be transferred directly to the counter. A commercial version of the automatic scraper is available from Analabs Inc.

An important consideration with zonal scanning is that when adsorbents containing weak β-emitters are combined directly with scintillator cocktails there is a need for quench correction. One of the main sources of error arises when different compounds are desorbed at different rates into the organic scintillator solution. If this occurs then the efficiency of counting could vary from vial to vial and it should therefore be determined for each sample so that absolute values (dpm) can be calculated. A thixotropic gel can be used to suspend the particles of adsorbent throughout the scintillator (see p. 73) and although this gives better results it does not overcome the problem completely. A better way of avoiding the problem of variable solubility is to elute the labelled compounds from the adsorbent prior to liquid scintillation counting [38–40] taking care to use a solvent which is known to elute the compound to $R_F > 0.8$ on TLC. This has been referred to as "elution analysis" or "zonal elution analysis".

Another approach with silica gel plates was to actually dissolve the silica using hydrofluoric acid before the addition of scintillator [41]. This claimed to increase the counting efficiency of ^{14}C to 87% and of ^{3}H to 32% using a toluene–Triton X-100–PPO–POPOP cocktail. Apart from the obvious handling problems of hydrofluoric acid, it may also give rise to chemiluminescence problems with scintillation counting. In view of the high counting efficiencies obtainable with modern organic scintillators, this procedure must now be obsolescent.

Stripping film techniques were referred to in connection with radioscanning methods and they may also be used in zonal analysis. Several stripping agents are available commercially (including Neatan and Collodion). Redgwell *et al.* [42] used a stripping mixture comprising cellulose acetate (7 g), diethylene glycol (3 g), camphor (2 g), 1-propanol (25 ml) and acetone (75 ml). This was found to be suitable for removing cellulose and cellulose/silica layers from glass plates.

After the stripping solution had been applied and allowed to dry, individual spots could be cut out with a scalpel or razor blade and placed in a counting vial. The film of stripping agent becomes transparent when the scintillator solution is added and good counting efficiencies (*i.e.* 70% for ^{14}C) can be obtained. Alternatively the whole thin layer can be removed from the plate intact before it is cut into sections.

Combustion analysis

Combustion of sections of adsorbent taken from TLC plates is gaining in popularity with the availability of more automatic sample oxidisers although it is not as frequently used as with PC. This is undoubtedly due to the greater difficulty in handling and transferring aliquots of TLC adsorbent from the plate to a combustion boat without mechanical losses. Examples of its use are given later in this chapter.

CHOICE OF METHOD FOR RADIO-THIN-LAYER CHROMATOGRAPHY

It is apparent from the foregoing survey that there exists a variety of techniques for the radioassay of TLC plates and the method or methods chosen in any laboratory will depend on the following main factors:

(i) the isotope or isotopes to be detected;

(ii) the levels of radioactivity present (*i.e.* sensitivity);

(iii) the complexity of the mixture to be separated (*i.e.* the resolution required);

(iv) the finances available for the purchase of equipment, taking into account the existing radiochemical equipment available;

(v) the speed with which results need to be obtained;

(vi) the availability of radiolabelled material and the need to recover material from the chromatogram; and

(vii) the use of safe-handling methods.

Range of isotopes

Tritium, because of the low energy with which it emits β-particles, is detected with least efficiency and it therefore warrants special consideration. Since the most efficient method of detecting ^{3}H is liquid scintillation counting, zonal analysis techniques are particularly valuable with this isotope. Tritium can also be detected readily using fluorographic methods and both zonal analysis and fluorography should give a good record of the resolution obtained on a chromatogram. Most radioscanners detect ^{3}H with lower efficiency and are not as well suited to low-level work with it unless larger amounts are present than in ^{14}C work.

All of the methods described are applicable to weak β-emitters other than ^{3}H. The most commonly used isotope, ^{14}C, and isotopes of energy greater than this may be detected by any technique although more time is required for autoradiography than for any other method. When handling the higher-energy isotopes such as ^{32}P or ^{35}S, care must be taken to avoid too long an exposure on autoradiography or with the spark chamber. This can result in a blurred, diffused picture due to the longer distance travelled (greater penetration) of the β-particle in these cases.

When working with two isotopes, methods of determining more than one isotope on the same TLC plate are more limited. The most effective and quantitative way to monitor two isotopes is zonal scanning using a liquid scintillation counter set up for dual isotope measurement. Good discrimination between pairs of isotopes of different energies can be obtained with most modern instruments. Alternatively selective masking can be used with autoradiography to discriminate between ^{3}H and another isotope.

As an alternative, if one of the two isotopes is ^{3}H then the total radioactivity on the plate can first be determined by radioscanning with an open window after which the plate can be re-scanned with a foil-covered window, which will allow the detection of only the higher-energy isotope. This procedure is rather less precise than zonal scanning and can only be used semi-quantitatively.

Finally, the solid scintillation counting method of Melo and Prydz [16] may also be used for dual isotope work although in view of its lower sensitivity it is not likely to be as widely applicable in biochemical studies as are the other methods described.

Sensitivity

Since liquid scintillation counting is the most sensitive detection method for weak β-emitters any radiochromatography method based on it (*i.e.* zonal scanning) will be very sensitive. Autoradiographic methods are in general somewhat more sensitive than scanning techniques although for low levels of ^{3}H and ^{14}C long exposure times are necessary in order to obtain maximum sensitivity. For most biochemical and organic-chemical applications radioscanning is perfectly adequate with respect to sensitivity and only in a few instances is it necessary to seek a more sensitive procedure.

The approximate limits of detection of ^{14}C by various techniques are shown in Table 4.2. Clearly, based on sensitivity alone, there is not a major difference between all of these techniques although for low-level work the ten-fold increase in sensitivity of liquid scintillation and radioscanning can be very useful.

TABLE 4.2

LIMITS OF DETECTION OF VARIOUS RADIO-TLC TECHNIQUES

Technique	Limit of detection (nCi)
Liquid scintillation counting	0.01
Autoradiography	0.05
Spark chamber	0.05
Radioscanning	0.1–0.2

Resolution

TLC is capable of resolving complex mixtures of compounds and in such cases it is important to use detection methods which give an accurate display of the resolution obtained. Perhaps the most valuable feature of autoradiography methods is that they produce a pictorial display of the resolution obtained on a chromatogram. From this it can readily be seen if good chromatography has been achieved or if the chromatogram has been poorly developed.

Examples are given in Fig. 4.15 which shows a radioscan and an autoradiograph of the same TLC plate on which two closely related compounds were separated, (a) with good chromatography and (b) with poor elution and separation.

The resolution obtained with radiochromatograph spark chambers is definitely inferior to that of corresponding autoradiograph (see Fig. 4.16). In spite of this, the spark chamber will give a quick indication of whether a separation has been achieved in which case this can be followed up by radioscanning or by autoradiography. In this way a spark chamber can be a valuable addition to the radiochemical laboratory although they are relatively expensive.

High resolution can be obtained using zonal analysis as long as narrow bands are taken. The difficulty in practice is that either some semi-automatic scraping device is required or very careful and precise technique is needed on the part of the operator to ensure that quantitative transfer of the adsorbent to the scintillation counting vials is achieved.

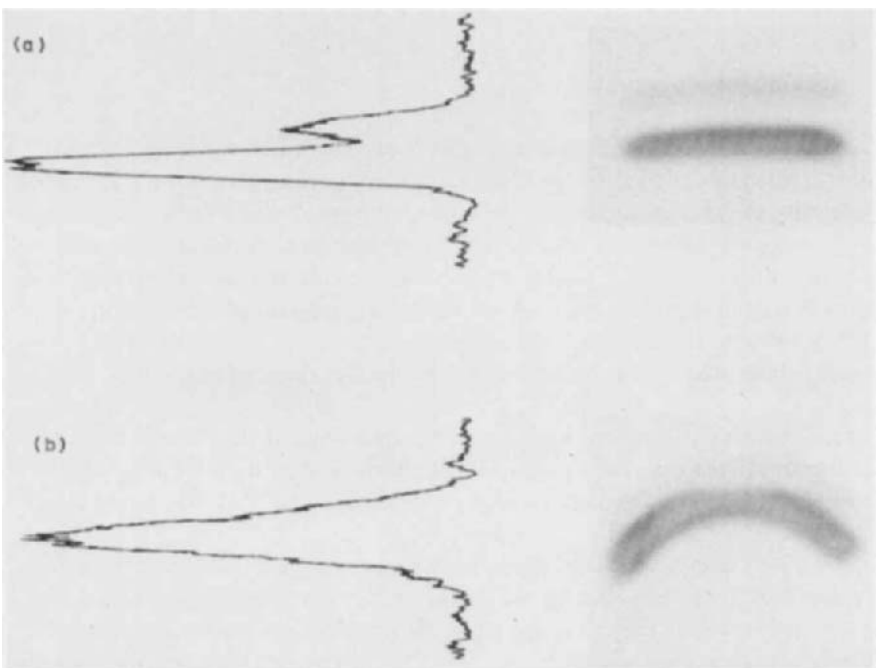

Fig. 4.15. A comparison of autoradiographs (right) and radioscans (left) of a TLC plate in which (a) good chromatography was achieved and (b) poor elution and separation was obtained.

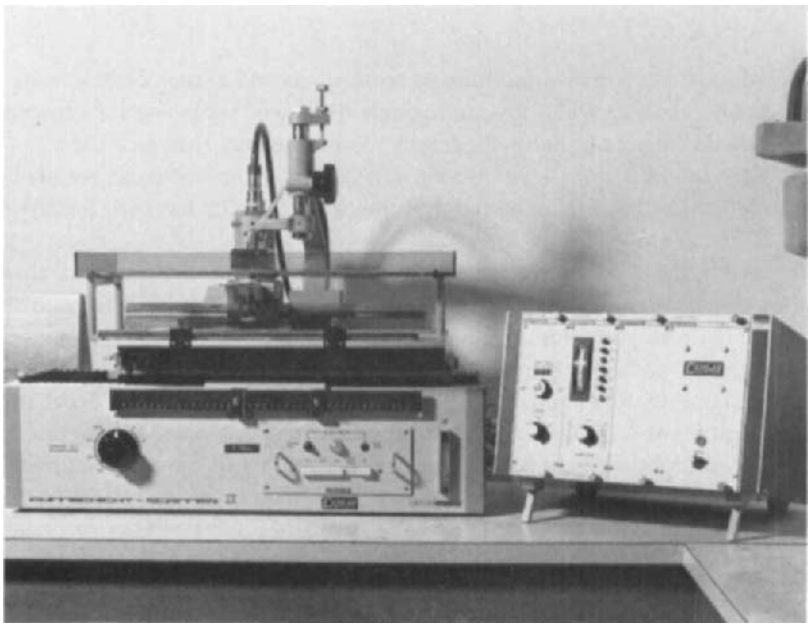

Fig. 4.16. A recent Berthold radioscanner.

Cost and availability of equipment

The finances available for radiochromatography equipment will vary from laboratory to laboratory and when considering the purchase of new equipment, the choice will depend on the availability of other radiochemical detection equipment. Radioscanners and spark chambers cost between £2000 and £4500 (1977 Great Britain prices) and are therefore expensive relative to the cost of the modest materials needed for autoradiography. A densitometer is the only capital expenditure to be considered for autoradiography and it is only required for quantitation of the actual autoradiograph.

Scrapers for zonal analysis are available but in most cases the scraping of TLC plates is done by hand.

Since most laboratories handling weak β-isotopes are equipped with one or more scintillation counters or at least have access to one it is unlikely that an instrument would be bought solely for radiochromatography analysis unless, of course, this were being done on a very large scale.

When equipping a completely new laboratory with basic apparatus for radiochemical work including TLC, first consideration should be given to the purchase of a liquid scintillation counter and materials for autoradiography. This will permit qualitative and quantitative work to be got under way. Consideration can then be given to the purchase of a radioscanner and/or a spark chamber the choice depending on the nature of the work done and on personal preference (since the costs of both are similar).

Speed of analysis

The urgency with which radio-TLC data are required can have an important bearing on the choice of method used. When rapid results are required, at first sight the spark chamber might appear to be the most obvious choice. However, a more quantitative radioassay of the chromatogram will almost certainly be required. For an initial quick result, though, the spark chambers should certainly be considered.

For most work with one-dimensional chromatograms a radioscan may be obtained in less than an hour although longer scanning times would be necessary for plates with only small amounts ($< 1-2$ nCi) of radioactivity present.

The slowest technique is undoubtedly autoradiography although the actual operator time involved is minimal. However, if chromatograms are prepared in duplicate, one can be left in contact with X-ray film while the other is examined by a more rapid technique.

Zonal analysis methods can be very time consuming, especially in laboratories where radio-TLC is extensively used. The same is true for combustion analysis since even with fully automatic oxidisers it is necessary for the operator to prepare combustion boats containing individual bands from the chromatogram and attend to the oxidiser. There is an additional subsequent delay with both methods while the liquid scintillation counting is carried out.

Recovery of radiolabelled materials

As indicated at the beginning of this chapter, some radio-TLC techniques are destructive and labelled compounds cannot easily be recovered, whereas others are non-destructive. Zonal and elution analysis and combustion analysis are destructive and cannot be used when radioactive materials for chromatography are very limited. In many cases, though, it is possible to take a narrow pilot band of the chromatogram for radioassay and only part of the sample is lost. In those situations where materials cannot be wasted, however, it is advisable to use either radioscanning or autoradiography.

Safe-handling

With the introduction of the Health and Safety at Work Act in Great Britain, the relative hazards represented by different radiochromatography techniques need consideration. It is now recognised that the handling of silica gel powders in the open laboratory is undesirable since it can expose the operator to some risk (albeit small compared with major industrial handling situations) of silicosis. For this reason, any method of radioassaying silica TLC plates which involves scraping the powder from the plate (*i.e.* zonal analysis, elution analysis or combustion) is less favoured from the safety viewpoint.

Conclusions

From the above discussion it is clear that no single radio-TLC method is ideal in every respect. For example, high sensitivity and good resolution may be obtained on an autoradiograph but it could take weeks to obtain the result. Similarly, most TLC scanners are

easy and quick to use but they do not achieve such good resolution as does autoradiography. For these reasons it is of value to have more than one technique available and very useful results can be obtained using one of the following combinations:

(a) radioscanning followed by autoradiography (or simultaneously on two identical plates);

(b) spark chamber followed by radioscanning;

(c) radioscanning followed by zonal analysis or combustion; or

(d) in the case of ^{3}H, autoradiography or fluorography followed by zonal analysis or combustion.

RADIO-THIN-LAYER CHROMATOGRAPHY TECHNIQUES IN PRACTICE

Radioscanning

Design and use of the instruments

Of the commercial radioscanners listed in the Appendix one of the most widely used instruments is the Berthold/Desaga scanner. Modifications in design have been made since the scanner was first introduced in 1963 and indeed the latest model (Fig. 4.16) is quite different in appearance to the first. Nevertheless, all versions are similar in principle and comprise a transport mechanism for the TLC plate, a detector, a pre-amplifier, a ratemeter and a suitable output (usually a chart recorder).

The TLC plate to be scanned is placed on the platen beneath the proportional counter head, which is fixed in position. A range of scanning speeds are available and a suitable one is selected prior to scanning. During the scan the platen is moved at a constant speed immediately beneath the detector. On the model illustrated (Fig. 4.16) preset scan speeds ranging from 12 to 30,000 mm/hour are available. Reference has already been made (p. 50) to the way in which two-dimensional scans can be made by first scanning in one direction until a limit switch is triggered. This moves the platen a preset distance perpendicularly after which the plate is scanned along the new track in the opposite direction. This facility is valuable not only for scanning TLC plates which have been eluted in two directions but also for scanning several tracks on a one-dimensional plate.

The instrument may also be set up to scan to and fro over a single track. This can be of interest when working with a short-lived isotope or, more significantly, when checking for loss of volatile radiolabelled compounds from TLC plates. It also gives a check on the reproducibility of the scan.

The gas-flow detector (see Fig. 4.17) is designed as a versatile unit and the detector head is closed with a magnetic metal plate with slits of width 1, 2 or 4 mm. An open slit is used for ^{3}H monitoring but thin foil-covered windows are available for other isotopes. Before use, the characteristics of the proportional counter should be checked and the detector is operated in the proportional region using either methane or, better, a mixture of 90% argon and 10% methane. The latter mixture can be obtained in cylinders from gas suppliers and is preferred since it permits the detector to be operated at a lower voltage (1750–1800 V) than with methane alone (2700–3000 V). Moreover, it is safer to use since it is nearly non-flammable.

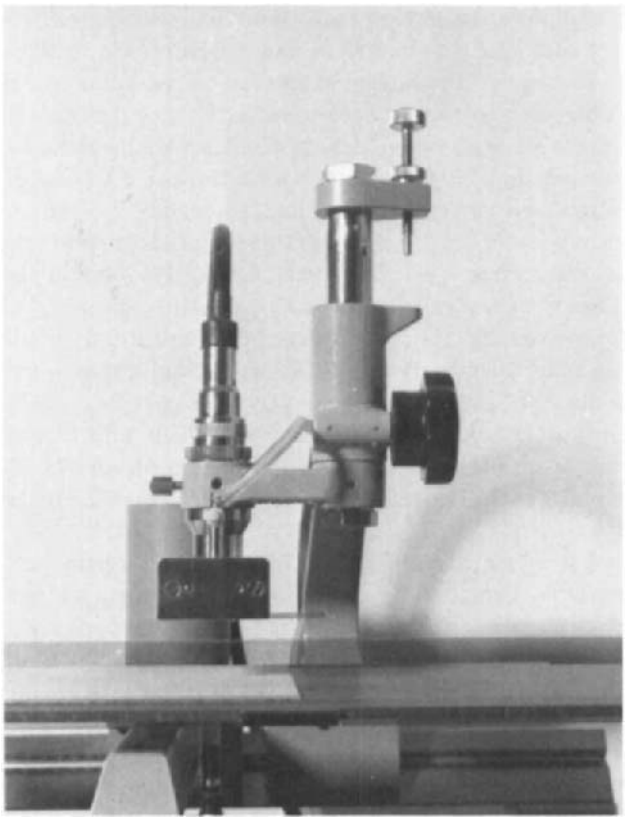

Fig. 4.17. The gas-flow detector on the Berthold radioscanner.

The ratemeter has sensitivity ranges from 1 to 30,000 cps and a separate channel is used for integration. The time-constant determines the length of time over which a signal is averaged, and time-constants range from 0.3 to 300 sec.

The output from the ratemeter is usually displayed on a recorder with a chart speed chosen to be the same as the scanning speed. This allows the recorder chart and TLC plate to be aligned directly. Alternatively, the dot printer may be used for two-dimensional chromatograms. In this case the plate is scanned along narrows tracks (2 mm apart) and each individual pulse detected by the ratemeter triggers the printer to give a dot on pressure-sensitive paper. The result is similar in appearance to an autoradiograph. Unfortunately, background pulses are also recorded discretely and the result is not as clear as an autoradiograph, which has a more evenly distributed background. The dot-print offers the advantage that it can be obtained in an overnight run, which is considerably faster than the corresponding autoradiograph would take.

Radio-thin-layer chromatography with special reference to scanning

In order to obtain good, reproducible results from radio-TLC it is important to use plates

with uniform layers of adsorbent with the same degree of activation. Care must be taken, therefore, when plates are prepared and those with uneven or non-uniform layers should be discarded. Furthermore, the same activation procedure, or absence of activation, must be adopted throughout any experiment. As the distance between the detector of the scanner and the TLC plate is only 2–3 mm (to allow maximum detection of the weak β-emitters on the plates), there is the risk of contamination of the detector with adsorbent. This can be minimised by using ready-made plates which are now widely used. Some indication of the range of these plates now available is given in Table 4.3, which lists the major suppliers and the range of adsorbents, plate sizes, binders, etc., which can be obtained. Ready-made plates offer the advantages of uniform layer thickness and (in most cases) greater mechanical stability. Consequently they are easy to handle, the layer does not flake easily and the adsorbent layer is not easily damaged when touched. Perhaps their major disadvantage is that batch variations do occur and the degree of activation can also vary from pack to pack. For a time, adsorbent layers spread on flexible supports (polythene or aluminium foil) were available and these could be cut into strips for use in paper chromatogram scanners. In general these plates were not as popular as the rigid ones since the adsorbent tended to flake off.

When a solution of radiolabelled compounds is applied to a TLC plate it is usually put on as a spot with as small a volume as possible. In some cases, for example, where the level of radioactivity is low or where co-extractives or impurities are present in the sample, it can be advantageous to apply the solution as a band (see Fig. 4.18). This can be done by hand using a pipette or with one of several commercially available devices (*e.g.*, the Desaga unit).

After elution of the plate, care must be taken to ensure that all traces of the eluting solvent have evaporated before it is scanned. With some organic solvents, "quenching" effects could occur and these would vary as the solvent volatilised from the plate during scanning. If acidic eluting solvents are used, traces of acid left on the plate can cause corrosion of the magnetic plate on the detector.

TABLE 4.3

MANUFACTURERS OF READY-MADE THIN-LAYER PLATES

Manufacturer	Comments
E. Merck, Darmstadt, G.F.R.; Macherey, Nagel and Co., Düren, G.F.R.	Full range of analytical and preparative plates, including silica gel, alumina and cellulose, and special plates for HPTLC
Schleicher and Schüll, Dassel, G.F.R.	Full range of silica gel, alumina and cellulose plates. Preparative plates of good quality
Anacham Ltd., Luton, Bedfordshire, Great Britain	Soft-layer plates without binders, having properties similar to hand-made plates
New England Nuclear, North Haven, Conn., U.S.A.	Both soft- and hard-layer plates

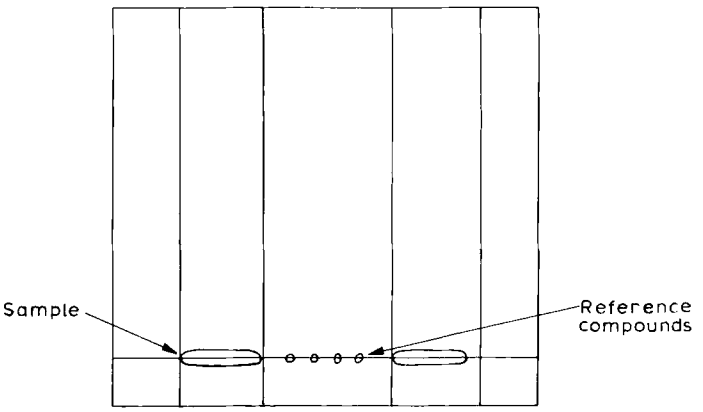

Fig. 4.18. The application of an extract of a biological sample to a TLC plate as bands. The bands are well separated to avoid overlap during elution which would affect the subsequent radioassay.

Quantitative measurements

Quantitative aspects of radio-TLC scanning have been discussed by Wood [43] who examined the variables which need consideration when reproducible, quantitative results are required.

Regarding counting statistics, it has been calculated that 40,000 cpm (*ca.* 20 nCi) must be recorded by the detector for the error in counting to be as low as 1% with a 95% confidence limit. In many biochemistry or metabolism experiments where considerably less than 20 nCi might be available for an analysis on a single chromatogram, errors up to 5% in the measurement are more common.

For low-level work low scanning speeds are preferable since this enables a larger number of counts to enter the detector heads. It has been reported [43] that when a radioactive zone is scanned by the detector then the number of counts recorded, N, will be given by the equation:

$$N = \frac{WDE(W + L)}{100\,LV}$$

where W = effective slit width (cm),
D = total radioactivity in zone (dpm),
E = detector efficiency (%),
L = length of radioactive zone in direction of scan (cm), and
V = scanning speed (cm/min).

Consequently, if the level of radioactivity and the detector efficiency are constant then a higher count-rate will be recorded if either a low scanning speed is used or if a wide slit is fitted. Whereas very low speeds (*e.g.* 30 or 60 mm/h) can be used with good effect, there are strict limitations to the width of the slit that can be used while maintaining good resolution.

Other factors that can affect quantitative scanning such as the detector voltage, detector

efficiency and time-constant are discussed by Wood [43] and the reader is referred to the original paper. However, a further important factor worthy of consideration is the detector height above the chromatogram. The position of the detector is adjustable and with isotopes such as ^{3}H and ^{14}C it is preferable to have as narrow a gap as is practical between the detector and plate. In practice, a spacing of 1.0 mm allows the TLC plate to be in close proximity to the detector with minimal risk of contact and contamination of the detector with adsorbent. This emphasises again the value of uniformly prepared TLC plates.

There are several ways in which the data from a radioscanner can be quantified. Some ratemeters have an integral mode and the radioactivity in a zone can be integrated as the plate is scanned. Alternatively an external integrator may be used in addition to a chart recorder which can be set to give a differential trace. Using the differential trace, the relative concentrations of labelled compounds can also be obtained by conventional peak area measurements or by weighing the peaks which have been cut out of the chart paper.

Autoradiography

From the discussion of autoradiography in earlier sections, it is clearly a sensitive, inexpensive method which can give very good resolution. Although there are several texts which describe the technique, greater emphasis has been given to the handling of biological samples for autoradiography and, more recently, to micro-autoradiography and electron microscopy methods. Nevertheless, the reader is referred to the comprehensive books or reviews by Fischer and Werner [23], Rogers [21] and Baserga and Malamud [44]. In addition there are several useful booklets available which outline the practical aspects of autoradiography (see Fischer [22]).

Dark-room requirements

For autoradiographic work it is necessary to have access to a photography dark-room or to a room containing a sink and running water which can be darkened completely. Fischer [22] and Kopriwa [45] have discussed the design of suitable laboratory dark-rooms equipped with double-door entrances so that people can enter or leave while the room is in use without letting in light. Although a dark-room such as this is desirable it is by no means essential to have such an elaborate facility. The need for a dark-room can be kept to a minimum by using cassette holders to maintain the film and TLC plate in contact in the dark, and the loaded cassette can be stored in a drawer or cupboard in the laboratory until it is developed. It is most important not to store the cassettes in the proximity of X-ray machines or γ-emitting sources, which can cause extensive darkening of the film. Over-warm or damp storage conditions should also be avoided. The dark-room is not needed again until the film is developed, which takes only 5–10 minutes for each film. In this way several individual workers in the same laboratory can make autoradiographs of TLC plates when only a simple dark-room is available.

Several dark-room illuminators can be used and recommendations are usually made by manufacturers of X-ray films as to which "safelights" are suitable for their particular emulsions. However, even when a safelight is used ideally it should be kept as far away from the film as possible. If the film is handled too close to an illuminator this can result in a general

background darkening which reduces the contrast on the developed film, particularly of compounds present in low concentrations.

In order to avoid the use of a dark-room altogether, Tio and Sisenwine [46] used a polaroid camera built into a specially designed cassette. Using 10 x 12.5 mm film, chromatograms with dimensions up to 9 x 12 cm could be autoradiographed. The films used were Polaroid Type 57 and 58 and it was found that, using Type 57 film, similar results were obtained to those with X-ray film and the conventional development process. Such a method suffers in that it imposes strict limitations on the number of autoradiographs that can be obtained simultaneously.

Practical considerations

The standard procedure for TLC autoradiography is to place the TLC plate in direct contact with a sheet of X-ray film such as Kodak Kodirex. Suitable film holders may be made up from two pieces of wood placed face to face and bolted together. More sophisticated cassettes designed to hold 20 x 20 cm TLC plates are available from photographic suppliers. In practice, any means of maintaining the film and TLC plate in contact (without excessive pressure) in the dark is suitable and a convenient and inexpensive way of doing this is to use the envelope and black paper in which individual sheets of X-ray film are usually supplied. As long as the plate and film are held together with tape to ensure good contact, then good results can be obtained.

One difficulty with TLC plates which does not arise with paper chromatograms is that great care must be taken not to disturb the layer of adsorbent on the TLC plate when it is lined up with the film. In this respect, most ready-made plates again have the advantage of mechanical stability. Despite this, the film most not be allowed to slide against the TLC plate surface. This can cause contamination of the film with radiolabelled adsorbent and can result in scratching of the film and spoiling the chromatogram.

This problem can be overcome by covering the TLC plate with a thin protective film of polythene or "cling-film" in order to protect it. However, this will have the effect of reducing the interaction with the film of weak β-emitters such as ^{14}C and it cannot be done if ^{3}H is being monitored. It is particularly advantageous with isotopes of higher energy such as ^{36}Cl and ^{32}P.

For development, the X-ray films are placed in a solution of a suitable developer such as Kodak DX-80. The concentrated developer is diluted according to the manufacturer's instructions and developing times range from 3 to 5 minutes depending on the temperature of the solution. The developed film is then rinsed in water and placed in a fixer solution (such as Kodak FX-40) for about 5 minutes. The film must then be thoroughly washed in running water and hung up to dry, after which it can be aligned with the chromatogram so that zones of radioactivity can be marked up.

Exposure times

Fischer [22] has tabulated the times of contact required for various amounts of ^{14}C and these data are given in Table 4.4 as an aid to the estimation of exposure times. In any mixture of labelled compounds to be separated it is likely that compounds will be present in a

range of concentrations (in terms of radioactivity). Consequently, an exposure time should be chosen which is long enough to permit compounds present in low concentrations to be detected. Fig. 4.19 shows two autoradiographs of the same chromatogram with exposure of 2 and 7 days. The compound present in lowest concentration is barely detectable on the film exposed for the short time but all of the spots can be seen clearly when a longer

TABLE 4.4

SENSITIVITY OF DETECTION OF ^{14}C ON THIN-LAYER CHROMATOGRAPHY (AFTER FISCHER [22])

Contact time (hours)	Amount of ^{14}C detectable	
	nCi	nCi/cm^2
0.5	500	2100
1	10	50
2	5	40
6	2.5	16
24	0.5	2.6
268	0.05	0.3

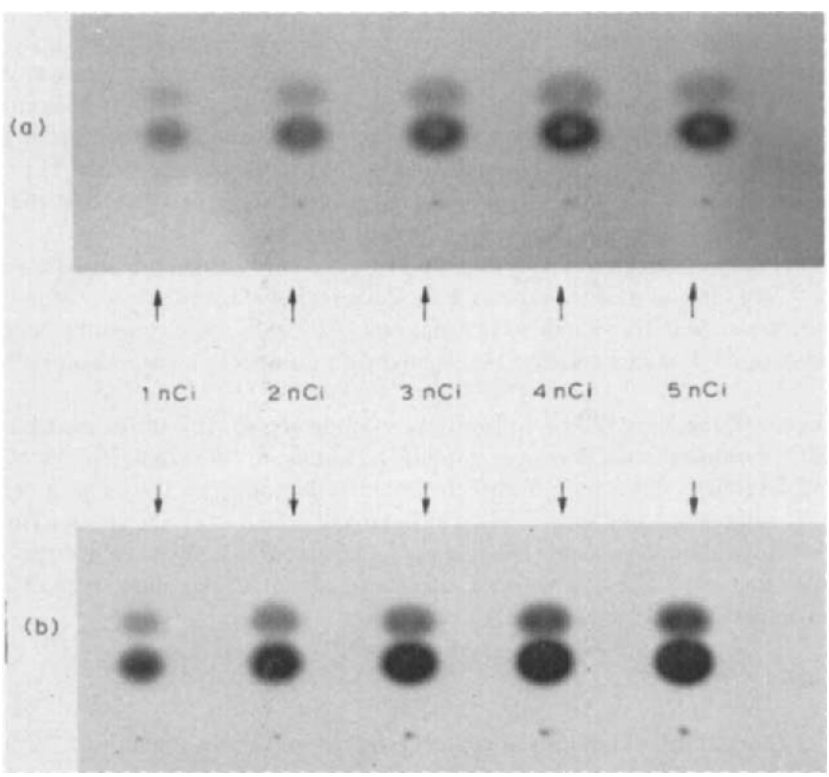

Fig. 4.19. Autoradiographs of the same TLC plate with exposure times of (a) 2 days and (b) 7 days.

exposure time is used. Unfortunately if the exposure time is too long, compounds present in higher concentration appear blurred and the resolution is not as clear. The correct exposure time can be estimated (depending on the amount of radioactivity present) as the user gains experience with the technique.

Tritium

The enhancement of weak radiation from ^{3}H by fluorographic methods has already been referred to. However, there is no reason why conventional autoradiography should not be used with ^{3}H provided that sufficiently high concentrations of tritiated compounds are present on the TLC plate and direct contact between the plate and film is ensured. If the amounts available are so small that very long exposure times become necessary then zonal analysis and liquid scintillation counting might be preferable.

Spark chamber techniques

All of the commercial spark chamber instruments are simple to operate. The dried TLC

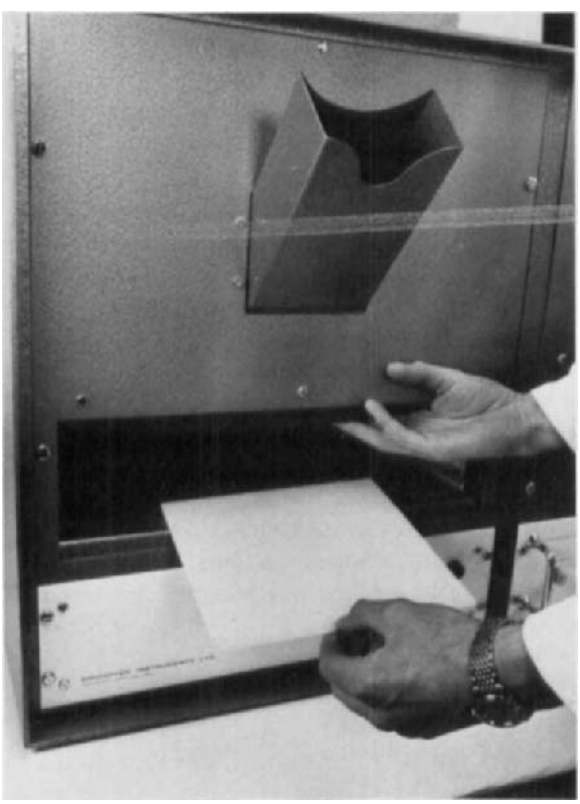

Fig. 4.20. Placing the TLC plate in position in the Birchover Radiochromatogram Spark Chamber. (Reproduced with permission from Birchover Instruments Ltd.)

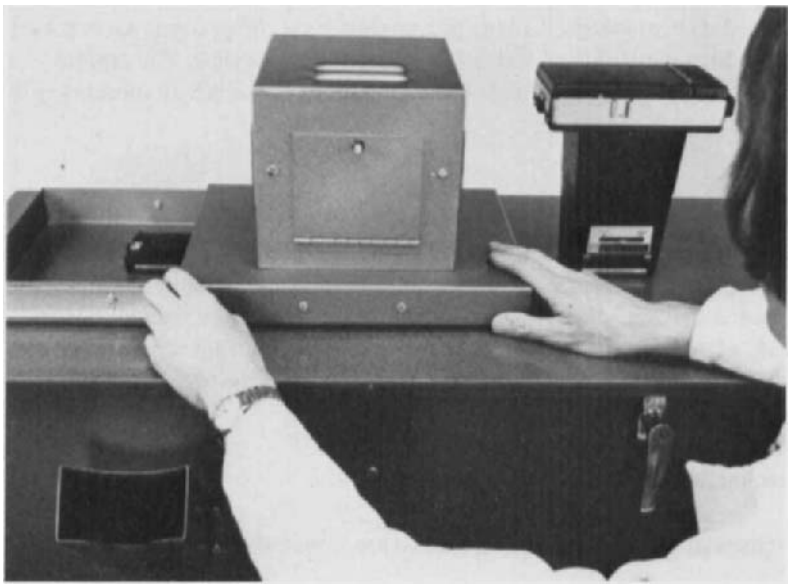

Fig. 4.21. The projector unit is removed to one side and the Polaroid camera is mounted above the projection enclosure. (Reproduced with permission from Birchover Instruments Ltd.)

plate is marked with radioactive reference spots and is placed in position (see Fig. 4.20) on the base plate. If a window is used this is then placed in position. The detection chamber is then flushed with 10% methane in argon or some other suitable mixture and after 5 or 10 minutes the high voltage supply is switched on. Suitable settings of time and exposure are selected on the polaroid camera and the shutter is opened. When the exposure is finished a polaroid print is obtained which is smaller than the chromatogram and some means of projecting the film image back onto the TLC plate is necessary. The Berthold system uses a special epidiascope for this, and with the Birchover model there is also the option of taking photographs under UV or visible light. In order to do this, the prime projector (epidiascope) unit is removed to one side and the polaroid camera is mounted above the projection enclosure as shown in Fig. 4.21. This can be a very useful attachment since a record of the presence of UV-absorbing reference compounds on a plate is obtained in addition to the radiolabelled compounds the positions of which can be marked by hand.

It is not possible to obtain quantitative data from a spark chamber and an independent method such as liquid scintillation counting or combustion of the spots should be used.

A detailed discussion of the factors affecting the sensitivity such as exposure time and operating voltage is given by Smith and Mitchell [33].

Zonal analysis

Direct counting of adsorbent by liquid scintillation counting

In a recent review of radio-TLC procedures, Snyder [3] claimed that zonal analysis and liquid scintillation counting was the most quantitative and sensitive method for detection of radioactive compounds on TLC plates. This may well be true if an automatic scraping

device is used, but a survey of several laboratories in Great Britain revealed that zonal analysis is more often carried out by hand. Consequently, skill and care are needed on the part of the operator to ensure that losses of the TLC adsorbent do not occur. Furthermore, it is not easy to scrape off very narrow bands (less than 5 mm) reproducibly and accurately although good results can be obtained if larger areas are taken.

For example, if from an autoradiograph or radioscan it is known that four radioactive zones are present on a chromatogram, then each individual zone can be scraped off for liquid scintillation assay. It is advisable to take a similar area of adsorbent from an area of the TLC plate which has not been eluted and to use this to obtain a background count-rate. This is illustrated in Fig. 4.22.

Although this procedure saves time there is always the risk that zones containing only small amounts of radioactivity might be missed, and if these are likely to be important then zonal analysis of the whole plate should be carried out.

The adsorbent taken from a zone on the TLC plate should be powdered and mixed before it is transferred to an empty counting vial. This applies particularly to silica gel and cellulose from ready-made plates, which can flake unless it is carefully removed from the plate with a sharp blade or TLC-scraper. A suitable liquid scintillator cocktail is then added to the vial (*after* the adsorbent) and the vial is capped and shaken thoroughly. The following scintillator solution is suitable:

Butyl-PDB (2-(4′-*tert.*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole)	8 g
Triton X-100	250 ml
Analar toluene	to 1 litre

One of the major problems with this technique is that the extent to which different compounds are desorbed from the TLC powder varies. As a result, radioactivity in different zones taken from the same chromatogram will be counted with different efficiencies. Moreover, since the extent to which any particular compound is desorbed cannot be known with any certainty it is difficult to correct for these differences. There are, however, several ways in which this problem can be minimised which have already been briefly mentioned. One way is to add water to the scintillator solution to try to ensure that water-soluble com-

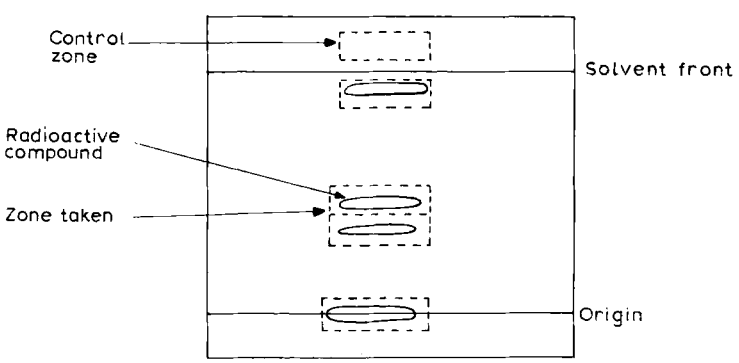

Fig. 4.22. The removal from a TLC plate of zones which have been detected by autoradiography. The radioactivity can then be quantified by liquid scintillation counting.

pounds will be desorbed in addition to less polar to toluene-soluble materials. When silica gel is the adsorbent, the water also deactivates the adsorption sites. An even better procedure is to add water (1 ml) to the silica gel powder in the vial before the Triton X-100 based scintillator (15 ml) is added. This ensures more complete desorption of the radiochemical. Alternatively, a gelling agent such as Cab-O-Sil (a finely divided silica) can be added to the scintillator cocktail using a 1 : 1 (v/v) ratio and the resulting thixotropic gel actually suspends the particles of adsorbent uniformly throughout the vial. When using agents such as Cab-O-Sil it is preferable to stir in the required quantity to a beaker of scintillator solution and then to dispense the gel to individual vials containing the TLC powder. Vigorous shaking of the vial then produces even distribution of the adsorbent. Any air bubbles present should be removed since these can have a marked effect on the subsequent counting.

Fig. 4.23 shows the results of a comparative study, using 1-cm^2 zones of a silica gel plate, of the water-addition and Cab-O-Sil methods. There was clearly a marked improvement in efficiency when the compounds were desorbed from the silica prior to addition of scintillator. It is also a more convenient method to use and avoids the handling problems of the finely divided silica in Cab-O-Sil.

The potential risk of silicosis from handling silica gel powders in the open laboratory has been referred to (p. 63) and where possible such operations should be carried out in a fume-cupboard or fume-hood although this can cause problems with handling the powder in a forced draught. The health risk is far worse with Cab-O-Sil and related products and these materials should also be handled in fume-cupboards, preferably using gloved-hands.

Yet another way of avoiding variable elution in the vial is to elute the sample from the adsorbent prior to radioassay. This may be essential if coloured materials are present on the TLC plate, since these can cause some quenching even if counted directly on the adsorbent.

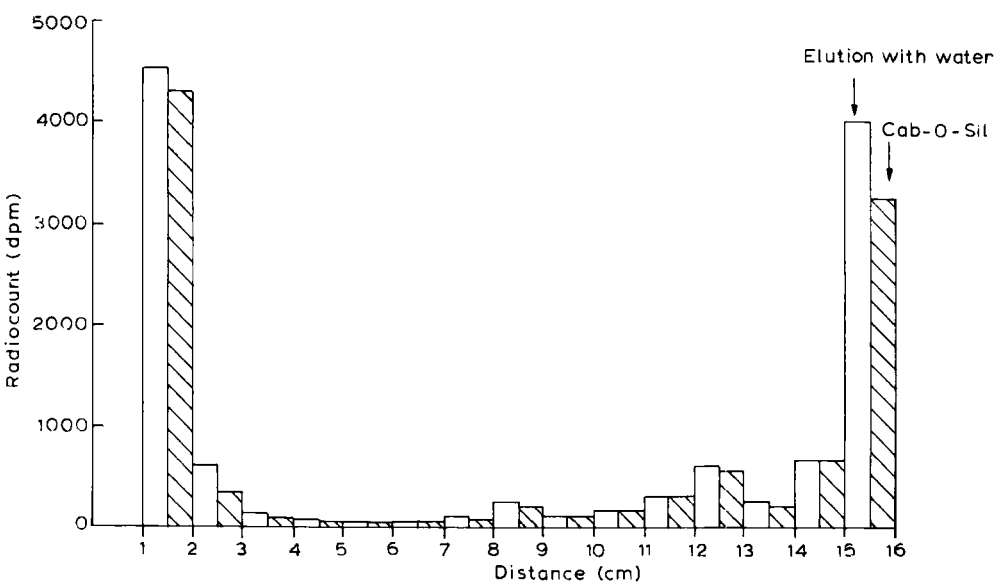

Fig. 4.23. A comparison of liquid scintillation counting using Cab-O-Sil, to suspend the adsorbent, with elution of radiolabelled material from the gel with water.

Elution from the adsorbent followed by liquid scintillation counting

In order to elute the radiolabelled material, it is first necessary to transfer the adsorbent to a tube with a sintered glass disc or frit or a tube plugged with cotton wool. This can either be done manually or with a special tube such as that shown in Fig. 4.24 [47], which is connected to a vacuum line. These tubes are available commercially from several suppliers (including Desaga and Analabs) or they can be made in the laboratory. The sample in the tube is then eluted with a solvent that will quantitatively desorb the labelled compound under investigation. In choosing a suitable solvent (or solvent mixture) one that moves the compound to a high R_F value (> 0.8) on TLC should effectively elute it from the same adsorbent [4].

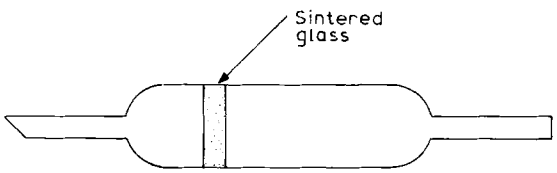

Fig. 4.24. A suction tube suitable for collection of adsorbent from TLC plates.

Elution analysis is not difficult for those compounds which are readily eluted to high R_F values on TLC, but for more polar compounds, particularly those which remain on the origin of the plate, it can be difficult to find an effective elution solvent.

After elution there are several ways of proceeding with the analysis. If the level of radioactivity in the eluate is high enough an aliquot can be taken for liquid scintillation counting in the usual way. The remainder of the solution is then available for further examination. If only low concentrations of radioactivity are present, it may be necessary to transfer the whole sample to a vial and to remove the solvent with an air-line (or at least reduce its volume to less than 0.5 ml) before addition of the scintillator solution.

Consequently, when large amounts of radioactivity are available, elution analysis is to be preferred to direct radioassay of the adsorbent since a major part of the sample is retained for further work.

Combustion analysis

The combustion of individual zones on TLC plates is used in some laboratories although it is less convenient than in PC. It is again a destructive (or semi-destructive) method which can only be used for ^{3}H- and ^{14}C-labelled samples and in most cases does not appear to offer any major advantage over direct liquid scintillation counting after elution in the vial. The exception to this is when highly coloured materials are present on the chromatogram since these would be counted with very low efficiency by the direct method and combustion analysis is a very useful way of overcoming the problem.

In practice zones of silica gel or other adsorbent are scraped off and transferred to the paper combustion boats provided with an automatic instrument. If combustion of the paper is too rapid it is possible that ^{14}C- and ^{3}H-labelled compounds present on non-combustible

adsorbents may not be completely oxidised. This problem can be avoided if the TLC adsorbent is mixed with cellulose powder in the boat as an aid to combustion.

In the author's laboratory results have shown that whereas coloured materials on silica gel plates may be counted with less than 25% efficiency (which is quite unacceptable), counting efficiencies of greater than 70% can be obtained after combustion.

APPLICATIONS OF RADIO-THIN-LAYER CHROMATOGRAPHY

Radio-TLC is now so widely used in industrial, academic and government laboratories that it is impractical to make a comprehensive review of its use. Nevertheless there are several specific areas in which it is an invaluable tool and some of these are highlighted in this section.

Radiochemical purity determinations

Before any experiment with a radiochemical is begun, it is usually necessary to check the radiochemical purity of the compound. TLC is routinely used for this, especially in the case of organic compounds. Some of the pitfalls that can occur in this apparently very straightforward procedure have been summarised by Sheppard in a review booklet [48] issued by The Radiochemical Centre, Amersham, Great Britain. A recommended method for determining radiochemical purities is given by Sheppard together with examples of artefacts that can arise by decomposition of the chemical on the plate, overloading, poor application technique and the use of insufficient carrier.

In most biochemical studies the radiochemical is usually diluted with unlabelled carrier to the specific activity which will be used in the experiment, *before* the radiochemical purity is checked, so adsorption problems arising from the lack of carrier are not often encountered. However, decomposition can occur during application or elution of the chromatogram, particularly on silica gel and alumina plates. If the structure of the compound in question is such that decomposition might be a problem then it is advisable either to carry out the analysis in a cold room or to choose a different technique for the purity determination (*e.g.* radio-GLC or radio-HPLC).

Application technique is also very important. The radiochemical should be applied to the TLC plate in as small a spot as possible to avoid the risk of double-peaking. Fig. 4.25 illustrates some of the artefacts discussed here.

In purity determinations quantitative measurements must be made of the relative concentrations of the radiochemical and any impurities present. This is usually done by zonal analysis and suspension counting or by radioscanning followed by either integration of the peaks or peak area measurements. When zonal analysis is used it is important to measure the radiochemical background count due to an area of the plate which has not been eluted (as shown earlier in Fig. 4.22). In this way any general background impurity distributed over the plate can be determined in addition to discrete impurities which are registered as peaks. The accuracy of radiochemical purity values obtained in this way is discussed by Sheppard [48] and Mellish [49].

There were numerous examples in the literature of the 1960s of the use of TLC to

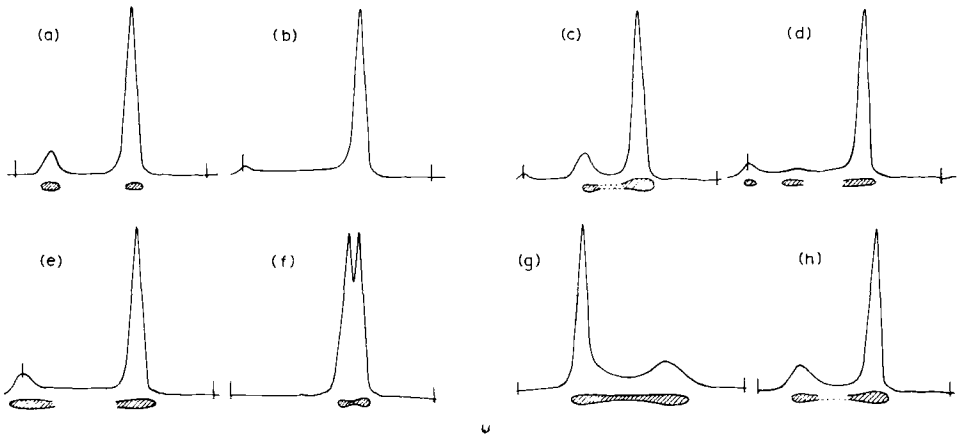

Fig. 4.25. Artefacts during the determination of radiochemical purities using TLC. (a) Decomposition during application. (b) Absorption to support due to inadequate carrier loading. (c) "Double-peaking", due to the existence of two inter-equilibrating forms. (d) Overloading and "double-peaking". (e) Overloading with carrier. (f) "Double-peaking", due to poor initial application. (g) Decomposition during elution to faster-running compound. (h) Decomposition during elution to slower-running compound.

measure radiochemical purity (*e.g.* refs. 50–58) but as the technique is now used routinely, papers devoted specifically to purity measurements are less common than they used to be. However, the wide use of radio-TLC is evident from a review of radiochemical journals such as *J. Labelled Compounds*, latterly titled *J. Labelled Compounds and Radiopharmaceuticals* (see refs. 59–61).

Biochemical studies

Several reports have appeared in the literature of the use of radio-TLC as an analytical tool in biochemical studies of lipids [62, 63]. The quantitative assay of labelled lipids formed part of a review paper by Privett *et al.* [64] in which the work up to 1965 was included. More recently, Snyder [65] recommended zonal analysis for the analysis of lipid intermediates on TLC and he was particularly concerned with detection of radiolabelled compounds for which no reference standard was available. A mixture of three lipid intermediates labelled with either ^{14}C or ^{3}H was chromatographed and a comparison was made of the zonal scans obtained when 1-cm, 5-mm and 2-mm fractions were scraped from the plate. The results shown in Fig. 4.26 clearly show the advantage of taking 2-mm samples. Similar results were obtained when a mixture of ^{14}C and ^{3}H compounds was used. In subsequent work [66] from the same laboratory dual-labelled (^{14}C and ^{3}H) samples of glycerol and glycol lipids were used and zonal scans depicting the ^{14}C and ^{3}H activity are shown in Fig. 4.27.

Webb and Mettrick [67] evaluated several techniques based on liquid scintillation counting for the quantitative radioassay of phospholipids. The ^{14}C-labelled phospholipids on

activated silica gel were assayed by liquid scintillation counting using the following scintillator cocktails:

(i) toluene-based scintillator;

(ii) toluene-based scintillator + 1.5 ml methanol (to desorb compound from silica gel);

(iii) toluene-based scintillator + 4% Cab-O-Sil;

(iv) toluene-based scintillator + 4% Cab-O-Sil + 1.5 ml methanol.

Method (iv) gave consistently high recoveries of applied ^{14}C and was recommended by the authors as the method of choice.

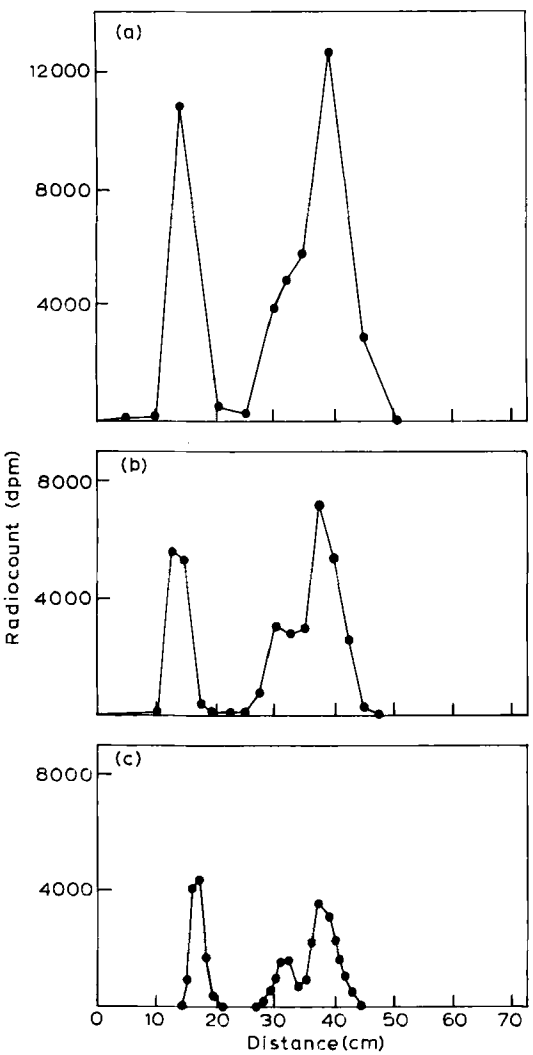

Fig. 4.26. Examples of a zonal scan in which (a) 10-, (b) 5- and (c) 2-mm sections were taken. (Reproduced with permission from Snyder [65].)

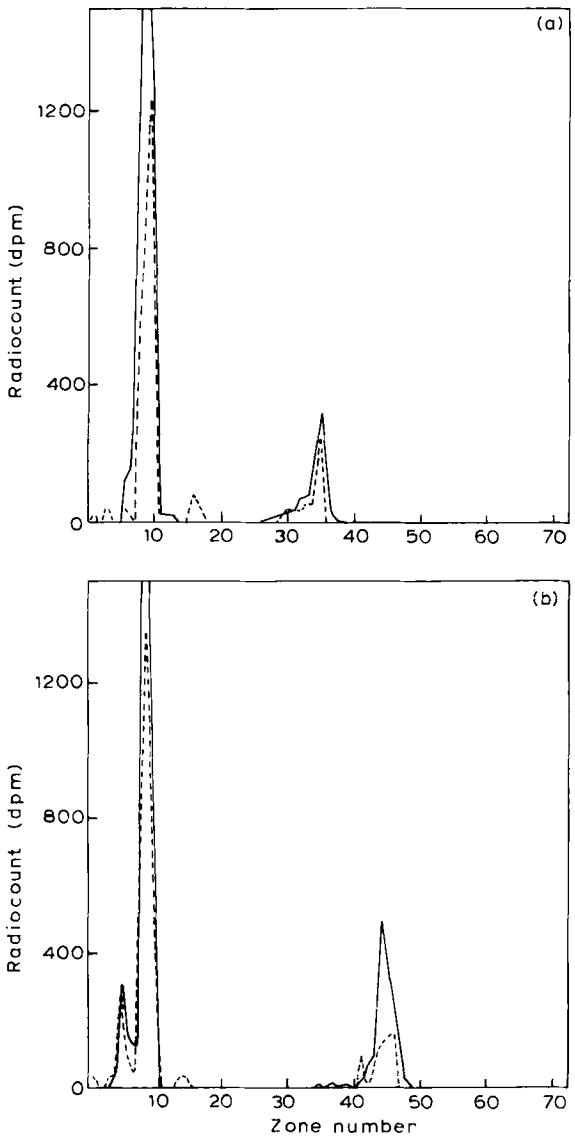

Fig. 4.27. Zonal scans of (—) ^{14}C- and (– – –) ^{3}H-labelled lipids. (Reproduced with permission from Snyder *et al.* [66].)

The use of the commercial solubiliser Aquasol (New England Nuclear) was also evaluated for ^{14}C-labelled phospholipids and it was found that better recoveries were obtained when water (10% w/v) was added to the Aquasol. For dual-labelled samples (^{14}C and ^{3}H) this Aquasol–water mixture gave consistently good results with better recoveries for ^{3}H than those obtained with the toluene–Cab-O-Sil–methanol scintillator.

Reversed-phase radio-TLC has been used for the analysis and purification, on a preparative scale, of radiolabelled fatty acids [68].

Pesticide and drug metabolism studies

Radio-TLC has been used routinely for many years in studies of the metabolism of pesticides in plants, animals, soils and micro-organisms and of the metabolism of drugs and pharmaceuticals in animals, and the techniques will be found in use in any laboratory carrying out this type of work. A brief review of the use of radiochemical techniques in drug metabolism has appeared recently [69] and Hais discussed the analysis of radiolabelled pharmaceuticals in a book edited by Macek [70]. In general, ^{14}C- and ^{3}H-labelled compounds are used but ^{32}P, ^{35}S and ^{36}Cl may be introduced into the molecule if the compound contains these elements.

It is common practice to use TLC to compare radioactive metabolites or degradation products isolated from the plant, soil or animal with unlabelled reference compounds. The references used are compounds thought to be potential metabolites of the drug or pesticide concerned. The labelled compounds are usually detected by radioscanning, autoradio-

TABLE 4.5

EXAMPLES OF THE USE OF RADIO-TLC IN PESTICIDE AND DRUG METABOLISM STUDIES

Reference	Authors	Subject	Radio-TLC methods used
72	Matsumura and Boush	Degradation of insecticides by a soil fungus	Autoradiography
73	Jordan *et al.*	Metabolism of Siduron herbicide in Kentucky bluegrass	Radioscanning and zonal elution analysis
74	Dorough *et al.*	Metabolism of methazole herbicide in cotton and beans	Autoradiography
75	Beynon *et al.*	Metabolism of benzoylprop-ethyl in wheat	Radioscanning and zonal elution analysis
76	Roberts	Metabolism of flamprop-isopropyl herbicide in barley	Radioscanning and autoradiography
77	Schuphan	Metabolism of monolinuron in Chlorella	Radioscanning
78	Savage	Adsorption and degradation of Chlorbromuron in soil	Zonal analysis
79	Tay and Sinsheimer	Metabolism of *trans*-stilbene in rabbits and rats	Radioscanning and zonal analysis
80	Bedford *et al.*	Sulphoxidation of cyanatryn by rat liver microsomes	Zonal elution
81	Hutson *et al.*	Detoxication and bioactivity of endrin in the rat	Radioscanning
82	Bedford *et al.*	The metabolic fate of endrin in the rabbit	Autoradiography
83	Melancon, Jr., and Lech	Metabolism of di-2-ethyl hexyl-phthalate in rainbow trout	Radioscanning and zonal analysis

graphy or zonal analysis but often a combination of these techniques is used. The unlabelled reference compounds may be detected by their UV absorption in which case TLC plates prepared from adsorbents in which a fluor is incorporated, must be used. Alternatively suitable spray reagents are employed.

The labelled metabolites are separated from each other and as much as possible from co-extractives which could interfere with their subsequent identification. After purification by TLC, other separatory techniques, including gel filtration, HPLC and GLC are used depending both on the nature of the metabolite and on the nature of the impurities from which it must be separated. These techniques are discussed elsewhere. The identification of metabolites purified in this way can be attempted using spectroscopic techniques, particularly mass spectrometry and nuclear magnetic resonance spectroscopy.

Radio-TLC is of particular value in the separation of "free" metabolites such as carboxylic acids or phenolic compounds. It is also very useful as a method of separating "free" compounds such as those from metabolites which are conjugated with plant or animal constituents such as glucose, glycine and other amino acids or glucuronic acid. Conjugated metabolites such as this are often hydrophilic and they are very polar in nature. Consequently they are chromatographed less readily on TLC than are the metabolites from which they are derived unless they are derivatised (*e.g.* by methylation or silylation). PC is used for the separation of conjugated metabolites and it is now recognised that other separating techniques, particularly radio-HPLC, offer great potential in the separation and purification of these materials [71].

Some recent applications of radio-TLC in drug and pesticide metabolism studies are summarised in Table 4.5. From this it is clear that most workers in this field do not rely on a single radio-TLC detection method but prefer a combination of radioscanning and either autoradiography or zonal analysis. Furthermore, quantitative results are most often obtained by liquid scintillation counting, especially when autoradiography is the primary detection method used.

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Chapter 5

Radio-electrophoresis

CONTENTS

INTRODUCTION

Electrophoretic methods are used for many biochemical separations of acidic or basic molecules either instead of or as a complement to chromatographic separations. A variety of matrices are used to effect electrophoretic separations and the major ones are listed below:

(i) paper, (ii) thin layers of silica gel or cellulose, (iii) polyacrylamide gel, (iv) cellulose acetate, (v) starch gel, (vi) agar gel.

In some cases, such as paper and thin-layer, the procedures for location of radioactivity are the same as those for chromatographic separations and the same general principles apply with minor differences. However, with other media, particularly polyacrylamide gels, special consideration is necessary for the radioassay of separated compounds. With the rapidly increasing use of polyacrylamide gels and related materials over the last decade for the separation of radiolabelled compounds, particularly nucleic acids and proteins, considerable attention has been given to methods of radioassay.

PAPER ELECTROPHORESIS

The separation of charged molecules on paper by the creation of an electric field between two electrodes was first used in the 1930s, some time before paper chromatography was discovered. Since then numerous examples of the use of paper electrophoresis have appeared in the literature for the separation of amino acids, peptides, proteins and nucleotides [1–3]. Although paper electrophoresis has essentially been superseded as a method for separation of high molecular weight compounds (particularly proteins) it is still used for some lower molecular weight separations.

In the early days of paper electrophoresis low voltages were used (less than 400–500 V) but better separations, particularly of mixtures of low molecular weight compounds, can be achieved in less time if higher voltages are used. Greater care is necessary, however, when operating voltages of 1–10 kV are used and it becomes necessary to cool the chromatogram in some way to dissipate the heat generated. In general this is done by placing the paper onto a metal plate through which cold water is passed. Details of the various types of apparatus available for paper electrophoresis and of the safety precautions recommended during its use are described by Smith [2] and by Sargent [3].

Choice of method for radioassay

The choice of methods for the radioassay of paper electrophoretograms is the same as that described in Chapter 3 for paper chromatograms and the same considerations are applicable. For example, care must be taken to ensure even drying of the paper after a run to minimise further migration of radiolabelled compounds on the paper during the drying process. When dry, the positions of labelled compounds on the paper can be located by radioscanning, liquid scintillation counting of sections, combustion of sections, autoradiography, or by means of a spark chamber. Since electrophoresis is continuously carried out on narrow strips of paper, radioscanning is a popular method. When a number of samples are run side by side on the same paper, or two-dimensional runs are made, however, an autoradiograph permits comparison between samples to be made more readily [4].

"Finger-printing"

A useful technique for the separation of complex mixtures of amino acids or peptides is to either carry out a two-dimensional electrophoresis on a square piece of paper or an electrophoretic separation along one dimension and a chromatographic separation at 90° to this (see Fig. 5.1). This technique is referred to as "finger-printing" and when applied to the analysis of protein hydrolysates the results are often referred to as peptide "maps" [5].

With such separations, if both electrophoresis and chromatography are to be used, it is advisable to carry out the electrophoresis first since this will remove from the sample salts that would interfere with chromatography. Furthermore, if the chromatography is done first then all traces of the organic and acidic or alkaline solvents must be removed since these will interfere with the electrophoresis.

When finger-printing methods are used, autoradiography is the most suitable method for location of the separated amino acids or peptides which are often present in large numbers.

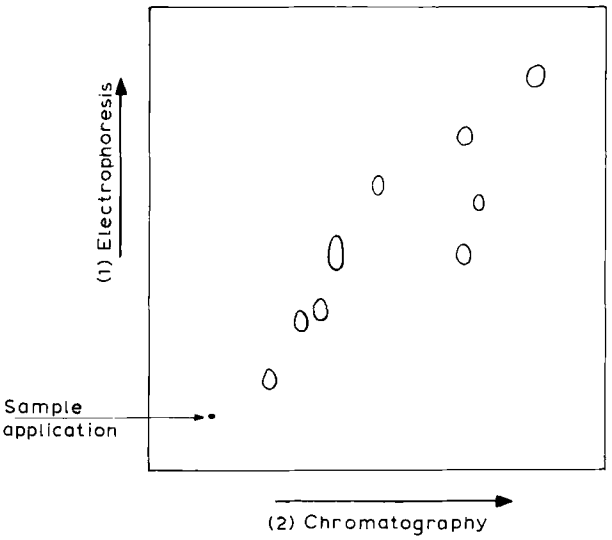

Fig. 5.1. "Finger-printing" on paper using electrophoresis in the first direction followed by chromatography at 90° to this direction.

It is impractical to cut the paper into strips for radioscanning since compounds may be located on the edge of strips. For the same reason liquid scintillation counting would be unsuitable as well as very time consuming.

Applications

There are examples in the biochemical literature of the use of paper electrophoresis for the separation of amino acids and peptides [4, 6, 7] and of oligonucleotides [8–10]. In many cases autoradiography has been used particularly for two-dimensional separations. However, radioscanning is preferred by some workers.

For the separation of complex mixtures of oligonucleotides specific finger-printing methods have been developed and used by Brownlee and Sanger [11] and by Fuke and Busch [12] and these are described on p. 100.

Brand *et al.* [8] detected ^{14}C- and ^{32}P-labelled compounds on paper by autoradiography and radioactive areas were cut out for quantitation by liquid scintillation counting. However, when working with both ^{3}H and ^{14}C in the same sample, they cut out spots from the electrophoretogram and digested these with NCS (Nuclear Chicago Solubiliser) for liquid scintillation counting.

An example of the use of paper electrophoresis in plant biochemistry is shown in Fig. 5.2 which shows the high-voltage paper electrophoresis of an amphoteric metabolite of gibberellin A in barley [13].

Paper electrophoresis is also used in pesticide and drug metabolism studies [14–18] since acidic and basic compounds are often formed as metabolites. For example [14], the drug Timolol [3-(3-*tert*.-butylamino-2-hydroxypropoxy)-4-(morpholino)-1,2,5-thiadiazole] is itself a base but some of its metabolites in animals were acidic or amphoteric. Paper

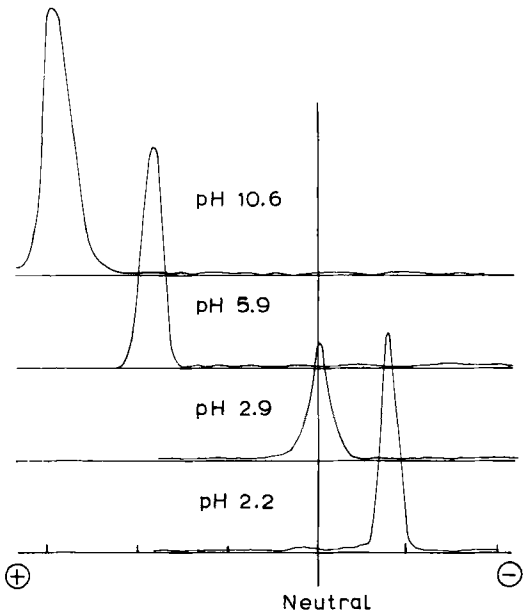

Fig. 5.2. An example of the use of high-voltage paper electrophoresis followed by radioscanning. (Reproduced with permission from Nadeau and Rappaport [13].)

electrophoresis was used to separate Timolol from its metabolites and the electrophoretogram was cut up and solubilised for liquid scintillation counting.

THIN-LAYER ELECTROPHORESIS

In general, the same advantages of TLC over paper chromatography of speed of analysis, easier handling and better resolution also apply to electrophoresis. It is somewhat surprising therefore that thin-layer electrophoresis (TLE) is not more widely used than it is at present.

A TLE apparatus is shown diagrammatically in Fig. 5.3. The TLC plate is placed on a metal block which is water-cooled. Paper wicks are used to transfer buffer from the

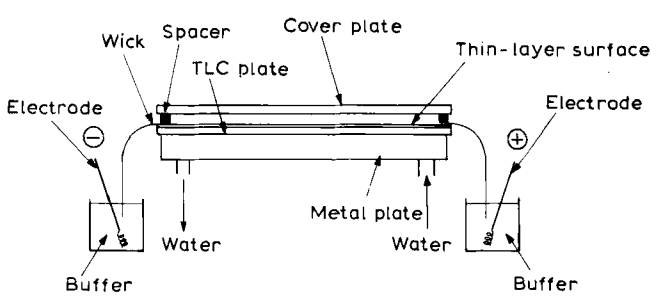

Fig. 5.3. A diagram of an apparatus for thin-layer electrophoresis.

reservoir to the plate. A glass plate is placed over the TLC plate and a spacer of PTFE or similar inert material separates the two. After application of the samples to the thin-layer plate (usually silica gel or cellulose), the plate is sprayed evenly with buffer immediately before electrophoresis is begun. Voltages of up to 1000 V are used and sufficient cooling can usually be achieved by the circulation of tap-water through the metal block.

Choice of method for radioassay

The most suitable methods for location of radioactive zones on TLE plates are autoradiography and radioscanning. Since two-dimensional separations and "finger-printing" methods are commonly used [19] autoradiography is the preferred method. Care must again be taken to avoid further migration of the separated compounds during the drying process prior to radioassay.

Applications

Examples of the use of TLE are far less common in the literature but the technique has been used to separate labelled amino acids, peptides and nucleotides. In the author's laboratory it is preferred to paper electrophoresis (because of its speed and ease of use) as a method of looking for acidic or basic compounds in mixtures of pesticide metabolites. In one instance, without the use of TLE the presence of a labile malonyl group on a plant metabolite might have been missed [20].

POLYACRYLAMIDE GEL ELECTROPHORESIS

Although polyacrylamide has been used as a medium for electrophoresis since only 1960 it now forms the basis of the most commonly used methods for separation of acidic and basic macromolecules. In fact, polyacrylamide gel electrophoresis has essentially superseded paper and starch gel electrophoresis because of its superior resolution of proteins and nucleic acids. The gels are made by the polymerisation of acrylamide monomers in the presence of methylene-bis-acrylamide or another suitable compound which serves as a cross-linking agent. Various concentrations of polyacrylamide can be used and Sargent [3] has described in detail the preparation of the gels, application of the sample and running conditions.

Polyacrylamide gels are not as easy to handle as paper and thin-layer preparations when it comes to radioassay, and considerable attention has been given to the problem of obtaining qualitative and quantitative information on the radioactive compounds present in the gel. Most methods described in the literature are based either on liquid scintillation counting or autoradiography although a semiconductographic method has also been reported.

Liquid scintillation counting methods

Since polyacrylamide gels are insoluble in solvents commonly used for liquid scintillation counting, a number of methods of digestion or solubilisation have been devised and evaluated.

Gel slicers

It is first necessary to slice the gel into sections of a suitable size which are transferred to liquid scintillation counting vials prior to treatment.

Various gel slicers have been described [21–24] which will produce slices of equal size and in most work 1- or 2-mm slices are taken in order to obtain good resolution. Gingery [25] has recently described the advantages and limitations of devices for slicing cylindrical gels and has described the construction and operation of an improved apparatus with greater flexibility in terms of the thickness of gel sections that can be obtained.

Albanese and Goodman [26] recommended a time-saving procedure in which slices were only taken of bands known to contain radioactivity (or thought to contain radioactivity from staining), rather than very small slices of regular size. In order to be certain that slices were taken from the correct region on the gel, it would be necessary to make an autoradiograph so the time-saving advantage is perhaps misleading; however, fewer samples need be prepared for scintillation counting so the method is less laborious. The slices could be cut on the basis of a suitable staining technique (for, say, proteins or nucleic acids) but there is always a risk that all the labelled compounds might not show up with the stain chosen.

The resulting histogram produced, however, is easier to interpret than a conventional histogram produced by radioassay of 1- or 2-mm sections. This is illustrated in Figs. 5.4 and 5.5 relating to the electrophoresis of ^{3}H- and ^{14}C-labelled ribosomal proteins.

In Fig. 5.4 a densitometric scan from an autoradiograph is shown together with the radiocount of each radioactive zone. When compared with the result obtained in Fig. 5.5 from radiocounting 1-mm slices, the latter results in a less clear quantitative representation of the radioactivity present.

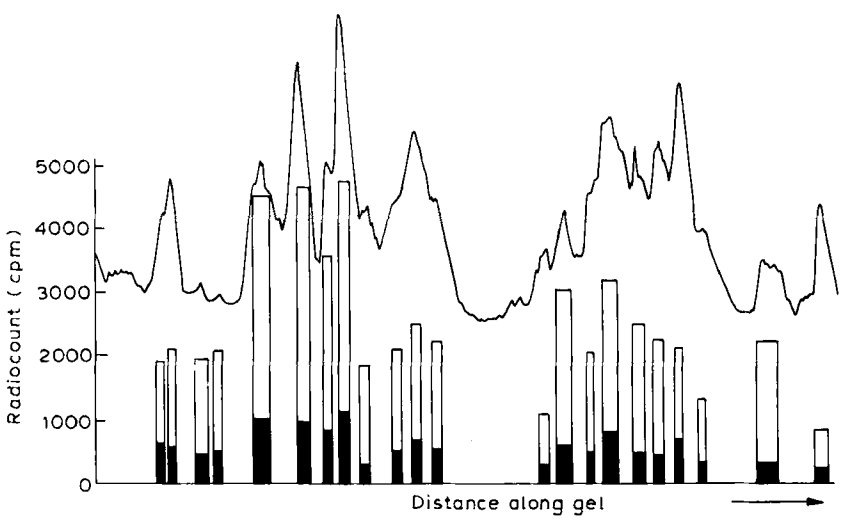

Fig. 5.4. The quantitation of ^{14}C- and ^{3}H-labelled ribosomal proteins by liquid scintillation counting of stained bands from the gel. Open bars represent ^{3}H (cpm); closed bars represent ^{14}C (cpm). The upper trace represents a densitometer scan of an autoradiograph of the intact electrophoretogram. (Reproduced with permission from Albanese and Goodman [26].)

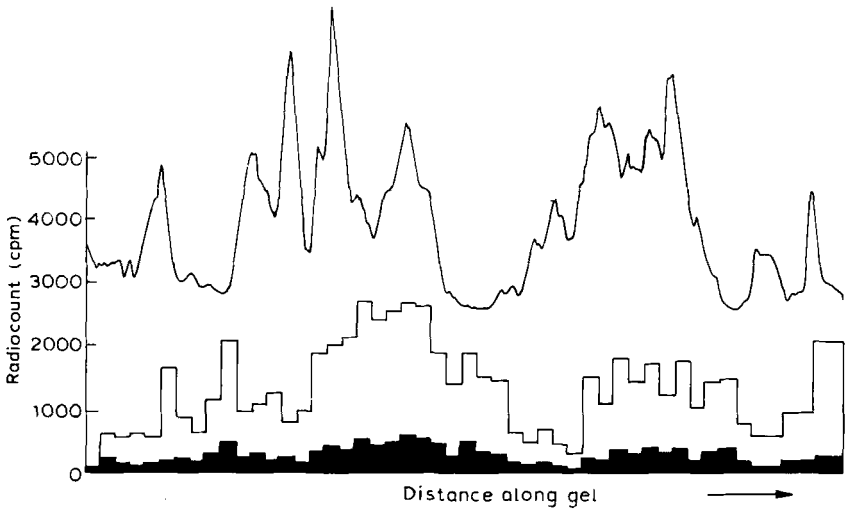

Fig. 5.5. The quantitation of ^{14}C- and ^{3}H-labelled ribosomal proteins by liquid scintillation counting of 1-mm slices of the gel. Open bars represent ^{3}H (cpm); closed bars represent ^{14}C (cpm). The upper trace represents a densitometer scan of an autoradiograph of the intact electropherogram. (Reproduced with permission from Albanese and Goodman [26].)

Solubilising gel slices

One of the original methods for solubilising gels was to treat slices with 0.1–0.5 ml of 30% hydrogen peroxide, cap the vial and heat to 50–60°C until the gel had dissolved. The vials were then cooled and a scintillator solution such as Aquasol or NCS (New England Nuclear) was added. With solubilisers of this type the digestion time required to dissolve the gel completely depends on the nature of the gel and the concentration of polyacrylamide present. A number of methods based on this general technique have been reported [27–32] although it has been criticised since volatile materials such as $^{14}CO_2$ or $^{3}H_2O$ may be lost if they are formed under the oxidising conditions used.

In order to avoid this, Spear and Roizman [33] and Goodman and Matzura [34] used alkaline hydrolysis procedures. Spear and Roizman used gels prepared with ethylene diacrylate (rather than N,N′-methylene-bis-acrylamide) as the cross-linking agent and gel slices were hydrolysed with concentrated ammonium hydroxide directly in the vial. The samples were then absorbed on glass fibre paper, dried and counted in a toluene-based scintillator with efficiencies of 15–20% for ^{3}H and 65–70% for ^{14}C. Goodman and Matzura used a mixture of 30% hydrogen peroxide and concentrated ammonium hydroxide (99 : 1, v/v).

It has been pointed out that complete solubilisation of the gel may not be essential in order to get efficient radiocounting. Gel slices actually swell in the presence of some solubilisers and the radiolabelled macromolecules are released [35–37]. At low temperatures (30–40°C) however, digestion times of up to 24 hours may be necessary before complete transfer of the sample into the solution has been achieved. Moreover, sample to sample variations may lead to inconsistencies if compounds of different structures are released from the gel slices at different rates.

This swelling procedure is also limited to the use of gels containing low concentrations of polyacrylamide. For example, Zaitlin and Hariharasubramanian [38] found that 15% polyacrylamide gels did not swell visibly in the NCS solubiliser, although swelling could be achieved by the addition of a small amount of water (10%) to the NCS reagent before it came into contact with the gel.

In 1971 Paus [39] carried out an evaluation of a range of commercial solubilisers, namely Hyamine 10-X and Soluene 100 (Packard), NCS (NEN), Bio-Solv 2 and Bio-Solv 3 (Beckman). Using 2.5% N,N'-methylene-bis-acrylamide gels and 2.5% ethylene diacrylate gels, Paus observed that the gels dissolved completely in some reagents but in some cases shrinking or swelling occurred. Shaking the gel–solubiliser mixture speeded up the solution process.

Some of Paus' results are shown in Table 5.1. Where shrinking occurred, variable counting efficiencies were subsequently obtained. Although fairly consistent results were obtained when swelling occurred, the counting efficiencies were always highest when the gel had dissolved completely. From these results (Table 5.1) it is apparent that the best overall results were obtained using Soluene 100.

One of the drawbacks of commercial solubilisers is their relatively high cost. In a recent paper, an inexpensive method of analysing ^{3}H-labelled RNA after separation by gel electrophoresis was described [40]. Gels containing 2% polyacrylamide and 0.5% agarose were first soaked overnight with 10% acetic acid and then sliced and the slices were transferred to counting vials. Absolute alcohol (5 ml) was added and after 2 h the alcohol was replaced by toluene (2 ml) for 1 h. A butyl-PBD scintillator cocktail was then added and the samples were ready for scintillation counting. The counting efficiencies achieved were comparable to those obtained from a parallel method using Soluene 350.

TABLE 5.1

AN EVALUATION OF COMMERCIAL SOLUBILISERS (AFTER PAUS [39])

Solubiliser and conditions	Result	Scintillation liquid	Counting efficiencies (%)					
			RNA				Internal standard	
			Initially		After 1 week			
			^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
1 ml Hyamine, 50°C, shaking	Swollen but not dissolved	Toluene*	14	79	15	79	18	80
1 ml Soluene, 60–65°C, vigorous shaking	Dissolved after 24 h or more	Toluene*	31	86	31	86	32	87
1 ml NCS, 60–65°C, vigorous shaking	Shrunk but not dissolved	Toluene*	40–18	76–80	17–20	81–85	32	87
1 ml Bio-Solv 2, 35°C, shaking	Shrunk but not dissolved	Toluene* with 10% 2-methoxyethanol	7–12	76–77	7–12	76–77	19	80
1 ml Bio-Solv 3, 35°C, shaking	Shrunk but not dissolved	Toluene*	7–15	76–79	9–15	76–79	24	83
0.5 ml 30% H_2O_2, 50°C, shaking	Dissolved after 3–5 h	Dioxan**	12	77	8	72	13	79
With pure scintillation liquids		Toluene*					33	87
		Dioxan**					21	85

*4 g PPO, 0.05 g dimethyl-POPOP, 1000 ml toluene.

**5 g PPO, 0.1 g dimethyl-POPOP, 80 g naphthalene, 400 ml 1,4-dioxan, 400 ml xylene, 200 ml anhydrous ethanol.

Ankar [41] has described the preparation of a polyacrylamide gel which contains N,N′-diallyl-tartardiamide (DATD) as a cross-linking agent (in place of methylene-bis-acrylamide) and this product can readily be solubilised by 2% periodic acid in 20–30 min at ambient temperature. The resulting solution can be counted in water-miscible scintillation cocktails, and a scintillator containing 25% Triton X-114 in xylene [42] has been shown to give very good counting efficiencies with this gel material.

A continuous flow method

In an attempt to overcome the tedious process of handling many samples for scintillation counting, Bakay [43] used a mechanical gel fractionator [44] which extruded the gel and mixed it with a scintillator solution which was subsequently passed through a flow cell for monitoring. There was, however, a need for careful sample preparation prior to the "automatic" procedure with which a new operator would have to gain experience. The actual analysis time was less than 1 h and dual-label samples could be analysed by this method.

Combustion analysis

A combustion method for determining radioactivity in polyacrylamide gels was described by McEwen [45] in which the Schoniger flask method was used directly in counting vials. Although this is a laborious approach, combustion of gel sections in an automatic sample oxidiser is used in some laboratories. Certainly it overcomes problems of solubilisation and sample preparation encountered in direct liquid scintillation counting. However, it is claimed [26] that no improvement in counting efficiency is obtained using combustion analysis.

Autoradiography

One of the earliest reports of autoradiography in connection with gel electrophoresis was by Fairbanks *et al.* [46], who reported the detection of ^{14}C-labelled proteins on disc electrophoretograms. Disc gels were sliced longitudinally using a system of stainless-steel wires. The slices were mounted on wet filter-paper, covered with a plastic film and dried using an apparatus for filtration under vacuum. Care was taken to avoid shrinking of the gel during the drying process since this would clearly give misleading results. When dry each slice was clamped together with an X-ray film for the required exposure time.

An alternative method is to freeze the gel (after removal of staining fluids or reagents which could depress the freezing point) on a block of dry ice prior to slicing with a simple device using razor blades [47]. The frozen slices are then transferred to water to warm up without distortion after which they are dried on filter-paper for autoradiography.

With thin gels, autoradiography is more straightforward and slicing may not be necessary.

Autoradiography has since been widely used for the detection of ^{14}C, ^{35}S, ^{32}P and other relatively high-energy isotopes. Although care is needed when drying the gels and results may not be obtained as quickly as with the liquid scintillation counting methods, the tech-

nique is not as laborious and there is less risk of sample to sample variations as there is with solubilisation methods. There is also no need to vary the technique depending on the type of gel or concentration of polyacrylamide present. Moreover, the gel is retained for further work and the resulting autoradiograph can be directly aligned with a staining pattern, and this can make the interpretation of results more straightforward.

Tritium

As with TLC and PC, detection of ^{3}H by autoradiography is very inefficient and in general liquid scintillation counting methods are used. As this is destructive and can be time consuming, Bonner and Laskey [48] applied the fluorographic methods previously used in chromatography (see Chapter 4) to the detection of ^{3}H in polyacrylamide gels.

Their procedure involved first dehydrating the gel (after electrophoresis) with dimethyl sulphoxide before impregnation with the organic scintillator PPO (which is insoluble in water). The impregnated gel was then dried under vacuum and placed against X-ray film. Several types of film and temperatures of exposure were evaluated and results in agreement with those of Randerath [49] for TLC fluorography were obtained (see Table 5.2). The RP Royal "X-Omat" film used at –70°C gave the most sensitive detection conditions. In fact, a 62-fold increase in sensitivity was observed for ^{3}H when gels were exposed to –70°C rather than +22°C. A 10-fold increase in sensitivity was also obtained with ^{14}C and ^{35}S under the same conditions.

Using RP Royal "X-Omat" film at –70°C with a 24-h exposure as little as 3000 dpm of ^{3}H could be detected in a band of size 1 x 0.1 cm and as little as 500 dpm of ^{3}H could be detected after an exposure of 7 days.

A summary of the method of Bonner and Laskey is given in Table 5.3, and the procedure is also suitable for gels which have been fixed or stained.

A modification of this basic procedure was reported in 1975 [50]. It was observed that pre-exposure of the X-ray film to a flash of light considerably increased the sensitivity of ^{3}H and ^{14}C detection by fluorography and, using film exposed in this way, detection limits of

TABLE 5.2

THE AMOUNT OF ^{3}H (nCi) IN A POLYACRYLAMIDE GEL BAND NECESSARY TO FORM A VISIBLE IMAGE BY AUTORADIOGRAPHY OR FLUOROGRAPHY AFTER 24 h EXPOSURE (AFTER BONNER AND LASKEY [48])

Detection method	Type of film	Radioactivity required for visible image after 24 h exposure		
		–70°C	–20°C	+22°C
Autoradiography	RP Royal "X-Omat"			
	Kodirex Auto Process	>800	>800	>800
Fluorography with 16% (w/w) PPO	RP Royal "X-Omat"	1.4	18	87
	Kodirex Auto Process	115		227
	Blue Brand	18		
Spraying dried gel twice with Omnispray (NEN)	RP Royal "X-Omat"	>227		

300 dpm and 30 dpm for ^{3}H and ^{14}C respectively were achieved during a 24-h exposure.

Furthermore, the pre-exposure to light also resulted in a linear relationship between the radioactivity of the sample and absorbance of the image on the film. Consequently, microdensitometry measurements would be quantitative. The pre-exposure was carried out with an electronic photographic flash gun which was filtered to reduce and diffuse the light output. This pre-exposure to light caused an increase in background darkening of the film and the extent of darkening was used as a measure of the degree of pre-exposure. Preliminary experiments showed that a pre-exposure to light which caused an increase in background absorbance of 0.15 a.u. resulted in a completely linear relationship between the image intensity and the amount of radioactivity present (see Fig. 5.6). Results obtained using this method are illustrated in Fig. 5.7, which clearly shows the marked improvement in sensitivity obtained (*cf.* Figs. 5.7a and 5.7c) and the agreement between image intensity and radioactivity present as determined by liquid scintillation counting of 1-mm gel slices (Figs. 5.7c and 5.7b).

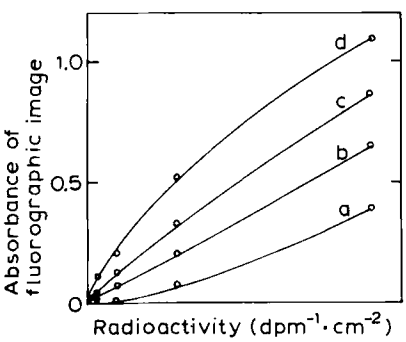

Fig. 5.6. The effect of pre-exposure of film to light on the relationship between the image absorbance and the amount of radioactivity present. Background fog absorbance increments (a.u.) achieved by pre-exposure: a, 0; b, 0.15; c, 0.3; d, 0.5. (Reproduced with permission from Laskey and Mills [50].)

TABLE 5.3

PROCEDURE FOR FLUOROGRAPHY OF POLYACRYLAMIDE GELS CONTAINING ^{3}H (ACCORDING TO BONNER AND LASKEY [48])

Step	Procedure
1	After electrophoresis, soak gel in 20 times its volume of dimethyl sulphoxide for 30 min; repeat with fresh dimethyl sulphoxide*
2	Immerse gel in 4 volumes of 20% w/w PPO in dimethyl sulphoxide for 3 h
3	Transfer gel to 20 volumes of water for 1 h
4	Dry gel under vacuum for 1 h after replacing dimethyl sulphoxide with water
5	Expose dry gel in RP Royal "X-Omat" or equivalent film and expose at −70°C

*Safety note: Protect hands from dimethyl sulphoxide, which can penetrate skin.

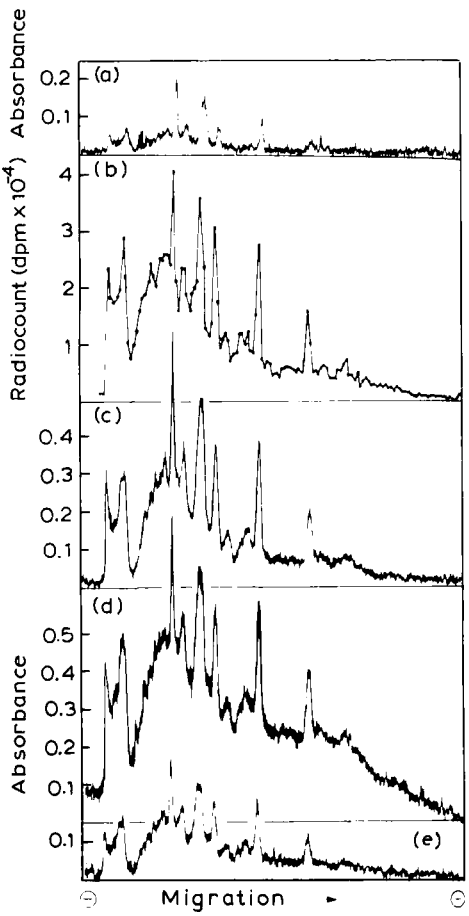

Fig. 5.7. The effect of pre-exposure on fluorographic profiles. (a) Untreated film exposed at −70°C. (b) Gel sliced into 1-mm sections for liquid scintillation counting. (c) Film pre-exposed to light to a background absorbance of 0.15 and exposed to gel at −70°C. (d) Film pre-exposed to light to a background absorbance of 0.4 and exposed to gel at −70°C. (e) Film pre-exposed to light to a background absorbance of 0.15 and exposed to gel at +22°C. (Reproduced with permission from Laskey and Mills [50].)

Double-label autoradiography

Gruenstein and Pollard [51] extended the fluorography method of Bonner and Laskey [48] to permit both ^{3}H and ^{14}C to be detected in the same sample. After electrophoresis, gel slices were obtained longitudinally and one slice was treated with PPO in dimethyl sulphoxide according to the method outlined in Table 5.3. Another slice was simply dried alongside the impregnated one and both were exposed to X-ray film. When developed after a 4-h exposure at −80°C ^{3}H bands were detected on the fluorographic film, whereas no bands were visible on the film exposed to the untreated slice. The amounts of ^{14}C present were such that they were not detectable with such a short exposure. The gels were then

painted black (to cut out photon emission) and autoradiography at room temperature was carried out for 27 days to visualise the zones containing ^{14}C. Although it is claimed that little or no "spillover" occurs between ^{3}H and ^{14}C detection methods, there is clearly a risk that this will happen if the amounts of each isotope present in individual bands is not known in advance. However, it is possible to determine which bands contain ^{3}H and ^{14}C on the same gel without resorting to liquid scintillation counting.

An image intensifier

Brisgunov *et al.* [52] were able to reduce exposure times for autoradiography by a factor of 1000 using an electronic-optical five-stage image intensifier. The gels were pressed against the input window of the image intensifier and β-particles produced luminescence on the zinc sulphide screen. The amplified image was then recorded with a 35-mm camera. As short exposure periods were used it was possible to work with wet gels and thus avoid the drying stage.

Fig. 5.8 compares densitometer tracings of autoradiographs produced by the method of Fairbanks *et al.* [46] and with the image intensifier.

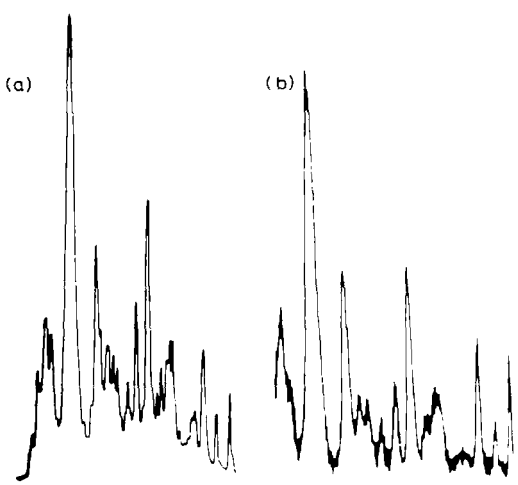

Fig. 5.8. The use of an image intensifier for autoradiography of polyacrylamide gels. (a) Without intensifier. (b) With intensifier. (Reproduced with permission from Brisgunov [52].)

A semiconductor detector

Tykva and Votruba have applied their semiconductographic method [53] to the detection of radioactivity on electrophoresis gels. A diagram of the apparatus used is shown in Fig. 5.9 and it is similar to that for TLC. Gels were cut lengthwise into four sections about 1.3 mm thick. After drying under vacuum on a glass plate for support, the gel was placed in the measuring apparatus. Radioactivity can be measured either continuously at a preset scan-

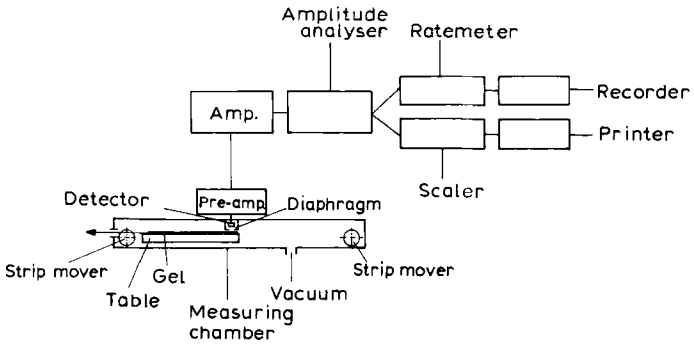

Fig. 5.9. A diagram of a semiconductographic detector for the determination of radioactivity distribution in a polyacrylamide gel. (Reproduced with permission from Tykva and Votruba [53].)

ning rate or stepwise at between 1- and 7-min intervals. Resolution comparable to that obtained by liquid scintillation counting was claimed and it was also possible to discriminate between two isotopes present in the same sample.

The semiconductor detector lacks sensitivity and this may contribute to the fact that this method has not been widely used.

Choice of method for radioassay

Of the two major methods for radioassay of polyacrylamide gels, conventional autoradiography is very suitable for detection of single isotopes other than ^{3}H. It is also useful for comparative purposes since a number of gels sliced longitudinally can be aligned on paper, dried and exposed to a single sheet of X-ray film. The fluorographic methods of Bonner and Laskey [48] and Laskey and Mills [50] are likely to increase in popularity for ^{3}H-labelled compounds, but it is unlikely that double-label autoradiography will do so because of the increased sample handling necessary.

For double-label studies, liquid scintillation counting methods are generally more suitable since quantitative data can be obtained. Since there are many ways of digesting or otherwise preparing gel slices for scintillation counting, and the composition of gels used (both qualitative and quantitative) can vary from laboratory to laboratory, it is up to the individual user to develop or modify techniques to his own work situation. This has given rise to numerous research communications, as exemplified in the following section on the evaluation of reagents, solubilisers and scintillators. The composition of the gel has also been modified in some cases to facilitate the subsequent sample preparation for radioassay.

With scintillation counting methods, consideration should be given to the size of slice taken. If small slices (say, 0.5 mm) are taken, excellent resolution will be achieved, but a large number of samples will be generated. As suggested by Albanese and Goodman [26], time can be saved by making an autoradiograph and cutting out only radioactive zones for quantitation.

It is impossible to generalise as to which method is preferable, but clearly it is advantageous to have facilities available to make the best use of both autoradiography and liquid scintillation counting.

Applications

A review of the recent biochemical literature reveals that polyacrylamide gel electrophoresis methods are now very widely used. The major areas of use are the separation of protein mixtures [54–59] and of RNA preparations [60–63]. Binding studies with nucleic acids and polypeptides [64] have also made use of the technique.

The solubilisation/liquid scintillation procedures and autoradiographic methods are both used extensively and Carter and Hakowori [65] have used the fluorographic method described earlier [48] for the determination of ^{3}H. Several authors, including Brand *et al.* [8] have checked the radiochemical purity of radiolabelled RNA preparations by gel electrophoresis since the same technique will subsequently be used to look for changes.

A comparison of results from liquid scintillation counting of both 0.5-mm and 2.0-mm slices of a gel is given in Fig. 5.10 from a recent paper by Covey and Grierson [60].

Clearly the use of polyacrylamide gels is increasing and improvements or refinements in slicing techniques and preparation of gels for liquid scintillation counting can be anticipated.

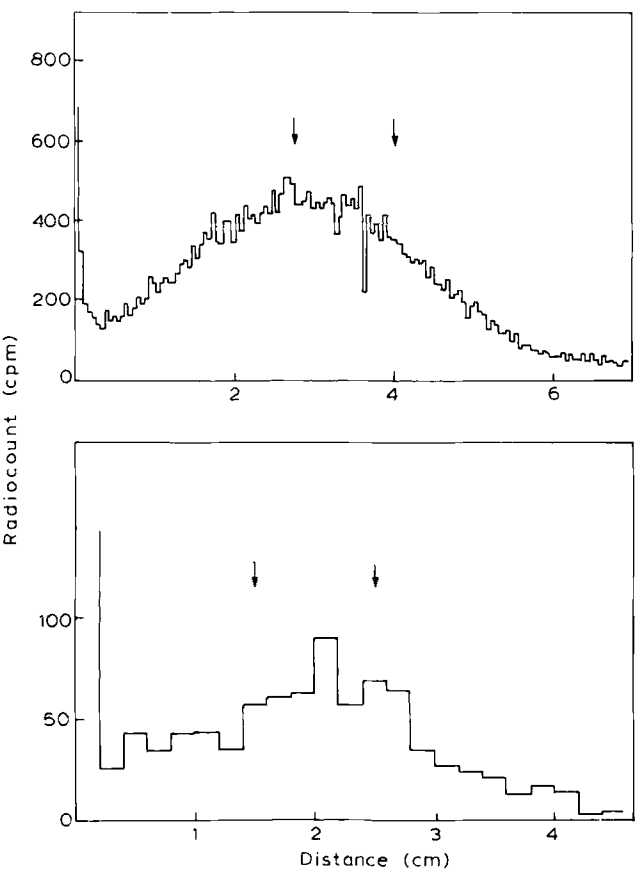

Fig. 5.10. A comparison of the liquid scintillation counting method for polyacrylamide gels in which (a) 0.5-mm and (b) 2-mm slices were taken. (Reproduced with permission from Covey and Grierson [60].)

It is also likely that the fluorographic methods for detecting ^{3}H will find wider application since only minor additional steps are necessary in the preparation of the gels for placing in contact with X-ray film.

OTHER ELECTROPHORETIC METHODS

Electrophoretic methods other than those described above have not found wide application for the separation of radiolabelled compounds. Cellulose acetate is preferred to paper for separation of proteins, peptides and nucleotides which are not absorbed by the medium. This minimises tailing effects but the low capacity of cellulose acetate makes it suitable for only small-scale separations.

Cellulose acetate has been used, however, for two-dimensional separations of radiolabelled nucleotides [11, 66]. Sanger *et al.* [66] separated the constituents of ribosomal RNA digests from *Escherichia coli*. High-voltage electrophoresis was first carried out on 3-cm strips of cellulose acetate, after which the separated components were transferred to DEAE-paper by a blotting procedure. Electrophoresis was then carried out on the paper, which was dried and autoradiographed. The result was a "finger-print" separation of the complex mixture of nucleotides. This method has been adapted and used by others [12, 67].

Agar gel is also a suitable medium for studies with radioisotopes and when agar films have been dried they can be handled in the same way as paper chromatograms and electropherograms. Examples of the use of agar gels for radiochemical studies are limited although some are described in Wieme's book [68].

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Chapter 6

Radio-column chromatography

CONTENTS

INTRODUCTION

Column chromatography in one form or another has been in use for well over 30 years and during this period there has been an increasing interest in the radioassay of column eluates. In spite of this, however, less attention has been given to the continuous monitoring of column eluates for radioactivity than to other branches of radiochromatography. It is apparently more common to collect fractions of the eluate and to measure the radioactivity in these by liquid scintillation counting. However, this situation is likely to change dramatically with the rapidly increasing interest in high-performance liquid chromatography (HPLC) of radiolabelled compounds.

Progress in the continuous measurement of β-emitters (mainly ^{3}H, ^{14}C and ^{32}P) has been reviewed by several authors, including Schram [1] who concentrated on the monitoring of aqueous solutions and McGuinness and Cullen [2] who surveyed the use of suspended scintillators.

There are, then, essentially two approaches to radio-column chromatography. Either

fractions of the eluate can be taken for radioassay or a flow-cell detector can be used. The use of liquid scintillation counting has been described in Chapter 2, and in the following section the development of flow-cell techniques is reviewed.

DEVELOPMENT OF CONTINUOUS FLOW MONITORING IN COLUMN CHROMATOGRAPHY

Flow cells based on Geiger–Müller tubes

The earliest methods used for radio-column chromatography described in the literature [3,4] were simple flow systems based on Geiger–Müller tubes. For example, Dobbs [4] built the flow cell shown in Fig. 6.1. The inlet and outlet tubes were made of brass and polythene tubing was used to connect the column outlet to the detector, care being taken to keep the dead volume to a minimum. This simple device was stable over long periods with a constant background of 13–14 cpm when housed in a 5 cm thick lead castle. The volume of the cell was 1.8 ml.

An important consideration with a flow cell is the "memory" effect or the efficiency with which the detector responds to changes in the count-rate in the flow cell. In Dobbs' apparatus the count-rate fell to 0.3% of its original value after 5 ml (*i.e.* 2.8 cell volumes) of plain solvent has passed through the cell.

In 1959 Corfield *et al.* [5] described a far more elaborate system in which fractions of

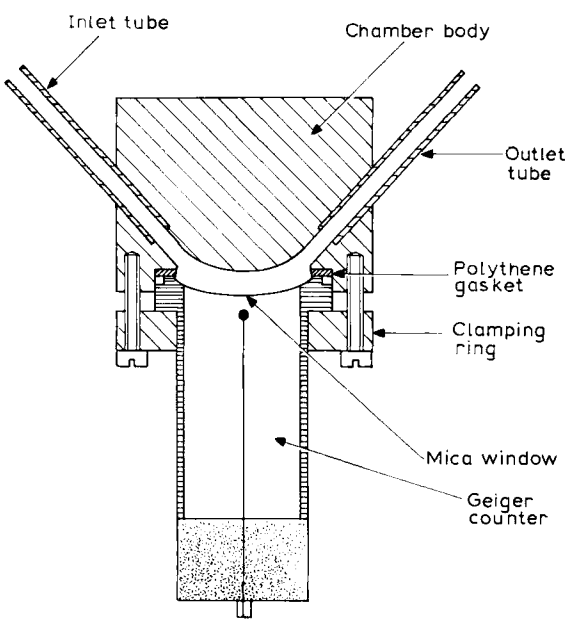


Fig. 6.1. A diagram of an assembly for monitoring radioactive eluates. (Reproduced with permission from Dobbs [4].)

the column eluate were collected and evaporated to dryness on individual planchettes. Each planchette was then held beneath a Geiger–Müller counter for radioassay. Evaporation was achieved with a small built-in heater unit comprising two spirals of nichrome wire held between sheets of asbestos paper. This heater was located beneath the planchettes and it was essential to use the correct heating rate to avoid boiling of the solvent, which could have resulted in contamination of other parts of the apparatus.

A chromatogram taken from the original paper is shown in Fig. 6.2 in which a mixture of [^{131}I] pipsylamino acids was separated. The main advantage of this automatic procedure compared with other techniques available at that time was its improved reproducibility in that the transfer of equal volumes of eluate to the planchettes and subsequent evaporation was standardised and more consistent.

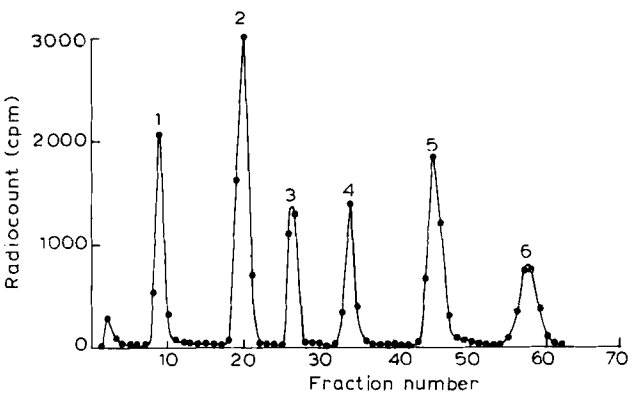


Fig. 6.2. A chromatogram of [^{131}I] pipsylamino acids. Peaks: 1 = alanine, 2 = glycine, 3 = threonine, 4 = serine, 5 = glutamic acid, 6 = aspartic acid. (Reproduced with permission from Corfield *et al.* [5].)

The use of solid organic scintillators

A flow cell actually constructed of phosphorescent material was made by Greenfield [6] for the radiocounting of ^{35}S in solution. The cell, which was made of "Pamelon" solid scintillator, formed the basis of the apparatus and the flow cell could be filled and emptied without removal from the counter assembly. The operating background was high (*ca.* 165 cpm) and the detector would probably not have been suitable for β-emitting isotopes weaker in energy than ^{35}S.

In the early 1960s several flow-cell arrangements came into use in which the eluent was passed over anthracene crystals and the resulting light emission was detected and quantified by one or by two photomultiplier tubes.

The basis of this approach was credited to Steinberg [7] who found that anthracene crystals suspended in aqueous solution gave reasonable counting efficiencies for ^{14}C. In fact, in a static system, efficiencies as high as 54% were obtained for ^{14}C when an aqueous solution containing the radiolabel was placed in a vial of anthracene crystals. In

routine use in flowing systems, however, counting efficiencies were closer to 20% for ^{14}C and the efficiency for ^{3}H was only 0.5%.

Schram and Lombaert [8] used a cell, illustrated in Fig. 6.3, made from a length of transparent polyethylene tubing (2.2 mm I.D.) packed with anthracene and coiled into a lucite vial containing silicone oil. The optimum size of the anthracene crystals was 300 μm for ^{14}C-counting and 150 μm for ^{3}H samples. The effective volume of the cell was 1 ml and counting efficiencies for ^{14}C and ^{3}H were 44% and 2%, respectively, with a background of 60 cpm. Schram and Lombaert used this detection system for the analysis of radiolabelled amino acids in biological samples by ion-exchange chromatography. An advantage of the system for this use was its high degree of stability and apparently constant efficiency with minimal quenching effects.

Piez [9] was also interested in the analysis of radiolabelled amino acids and he used an anthracene flow cell linked to an automatic amino acid analyser. The flow cell was made of quartz and its design is shown in Fig. 6.4. This design was preferred to a packed coil of plastic tubing which required too great a pressure to maintain a suitable flow-rate through the ion-exchange column. The volume of the cell was approximately 1 ml and the procedure for filling the cell is described in detail in the original paper. A Nuclear-Chicago Model 701 manual scintillation counter was used to detect radioactivity in the flow cell although minor modifications were made to the shutter assembly. The amino acid analyser used was that described by Piez and Morris [10] and the flow cell was incorporated at a stage before the ninhydrin reagent was added in order to avoid colour quenching problems.

The flow characteristics of the cell (see Fig. 6.5) were good in that about 80% of the

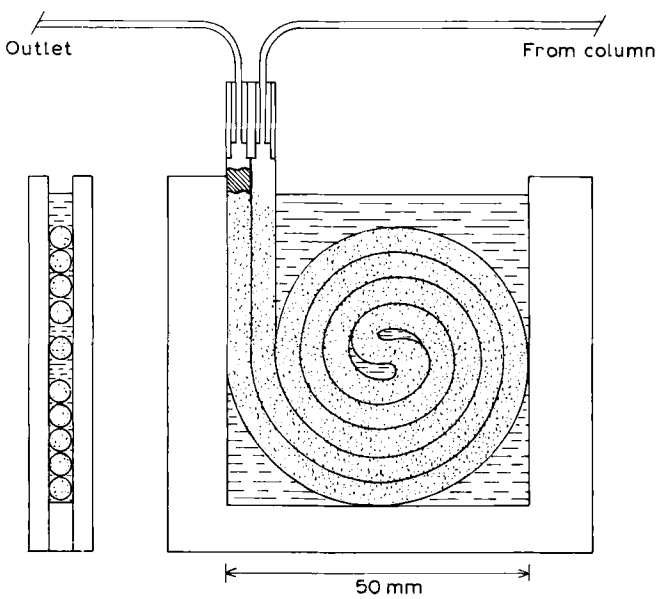


Fig. 6.3. An anthracene powder cell for scintillation counting of aqueous solutions. (Reproduced with permission from Schram and Lombaert [8].

radioactivity in solution in the cell was removed by 1 ml of fresh solution (*i.e.* in one cell volume) and in use no loss of resolution by mixing of adjacent peaks occurred. Using standard solutions of [^{14}C] glycine and [^{3}H]proline no quenching effects were observed when the buffers necessary for ion-exchange chromatography were used. Counting efficiencies for ^{14}C and ^{3}H were 38% and 0.9%, respectively, when window settings on the scintillation counter were used, which gave a background count of 18 cpm. If a narrower window was selected the background could be reduced to 7 cpm but the corresponding efficiencies were 27% and 0.7% respectively for ^{14}C and ^{3}H. An example of the output

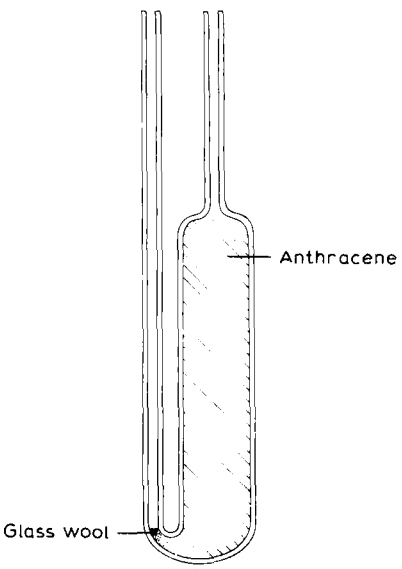


Fig. 6.4. A quartz flow cell for continuous determination of ^{14}C and ^{3}H. (Reproduced with permission from Piez [9].)

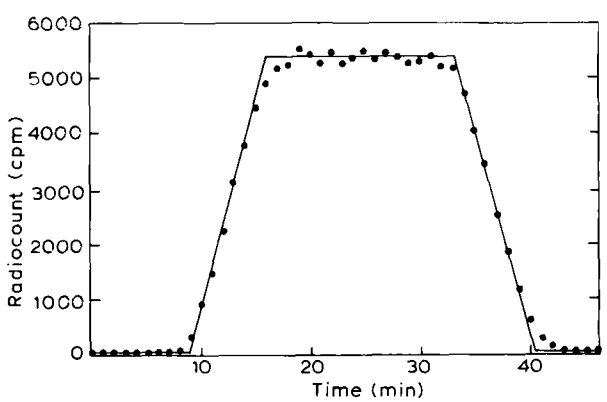


Fig. 6.5. The flow characteristics of the anthracene-packed flow cell used by Piez [9]. (Reproduced with permission.)

of this instrument is shown in Fig. 6.6. Although the minimum amount of radioactivity detectable in a single ^{14}C-labelled amino acid was less than 100 cpm, the reproducibility at such low levels was poor. However, if a peak contained more than 3000 dpm of ^{14}C-radioactivity the error in determination of radioactivity fell within the overall error of the amino acid analyser, namely 1–3%. In the case of ^{3}H at least forty times the radioactivity (than with ^{14}C) was necessary to achieve this degree of reproducibility.

Another pressure-resistant flow cell for use with an amino acid analyser was described more recently by White and Mencken [11]. The flow cell, which was constructed of Plexiglas was housed in a commercial Nuclear-Chicago Model 6353 Chroma-cell detector with its associated ratemeter, integrator system and digital output. Using this system an analysis of tritiated amino acids from a protein hydrolysate is illustrated in Fig. 6.7.

A flow cell is generally, although not always, used without a fraction collector for column chromatography but Lloyd-Jones and Skerrett [12] used a plastic spiral flow cell, photomultiplier tube and scaler to measure the amount of radioactivity in the eluate as it was collected in the glass siphon of a fraction collector. A print-out was then obtained of the amount of radioactivity in each fraction. Such an approach is likely to be of only limited interest however.

Porcellati and Di Jeso [13] used an automatic radiochromatography system based on an anthracene flow cell for the purification of ^{14}C-labelled phospholipid phosphoric esters after radiosynthesis. The system comprised a 1-ml lucite flow cell which had an effective volume of 0.4 ml when packed with anthracene crystals of 25–50 mesh size. A thin capillary tube connected the flow cell to the chromatography column and the flow-cell outlet was passed to a fraction collector. The cell itself was housed in a Nuclear-Chicago liquid scintillation counter.

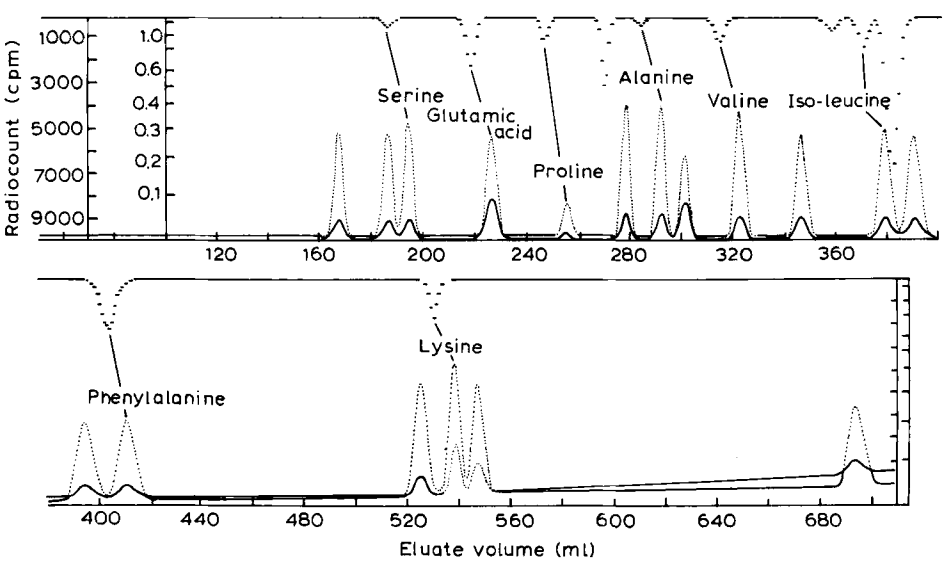


Fig. 6.6. An example of the output from an automatic amino acid analyser with radioactivity detection. (Reproduced with permission from Piez [9].)

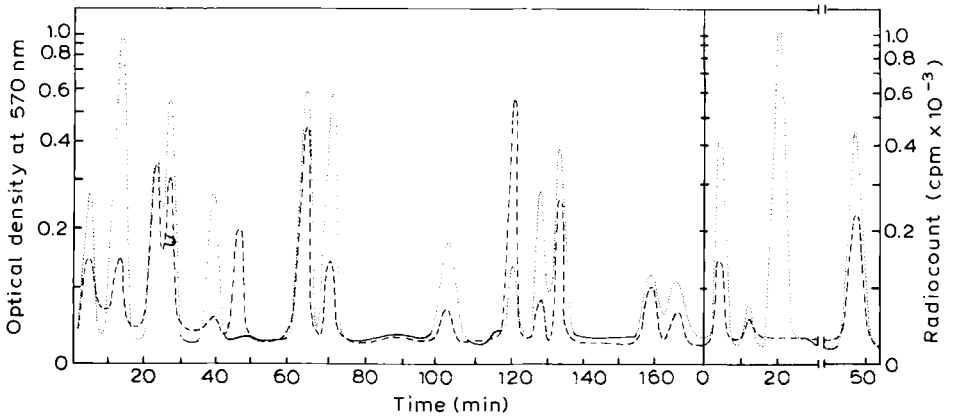


Fig. 6.7. The analysis of tritiated amino acids by White and Mencken [11]. (Reproduced with permission.)

This method was preferred to the alternative purification procedure of repeated crystallisation of the radiolabelled product because of its speed and specificity. Furthermore it yielded a very pure product which could be used directly for metabolic studies.

The use of scintillating glass beads and solid inorganic scintillators

As interest in the column chromatography of radiochemicals increased it became apparent that there were a number of limitations to the versatility of anthracene as a scintillator for use in flow cells. Experience had shown that some compounds were adsorbed or retained by anthracene crystals and this gave rise to memory effects which could not always be predicted. Another limitation was that only aqueous samples could be used with anthracene because of its solubility in organic solvents. Furthermore, its efficiency for monitoring ^{3}H was very low.

For these reasons, in the late 1960s there appeared in the literature reports of several flow monitoring techniques using either solid glass or inorganic scintillators or alternatively liquid scintillators. (The latter are discussed in the next section on p. 111.)

Clifford *et al.* [14] developed a versatile flow counter using a lithium–cerium glass scintillator (Nuclear Enterprises NE 901). The flow cell, which is shown in Fig. 6.8, was made from a 1 mm deep optical cell of borosilicate glass, which was packed with 250–300-μm beads and the cell was mounted between two photomultiplier tubes coupled to a ratemeter equipped with coincidence circuiting.

A shortcoming which arose in practice was that lithium–cerium glass was phosphorescent for long periods after exposure to light and it could take 1–2 days of storage in the dark before an acceptable background count-rate could be achieved. The problem can be avoided, of course, by minimising the handling of the cell and avoiding direct exposure of the scintillator to light. As an alternative the inorganic scintillator europium-activated calcium fluoride did not exhibit phosphorescence and furthermore it gave higher efficiencies for ^{14}C (approximately 50% compared with 21–30% for the NE 901 scintillating glass).

In 1970 a rather complex instrument called a "liquid spectro-radiochromatograph" was described by Davies and Mercer [15]. As shown in Fig. 6.9 it comprised a column chromatograph equipped with gradient elution, a UV spectrophotometer, a scintillation counter and a fraction collector, all with chart recorder outputs. The radioactivity detector was a commercially available Nuclear Enterprises NE 808 flow cell housed adjacent

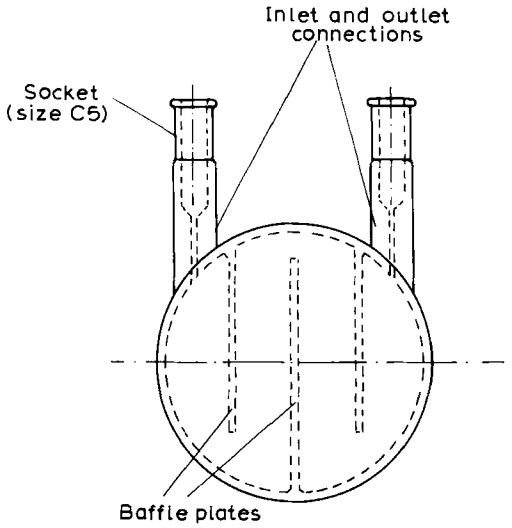


Fig. 6.8. A diagram of the flow cell used by Clifford *et al.* [14]. (Reproduced with permission.)

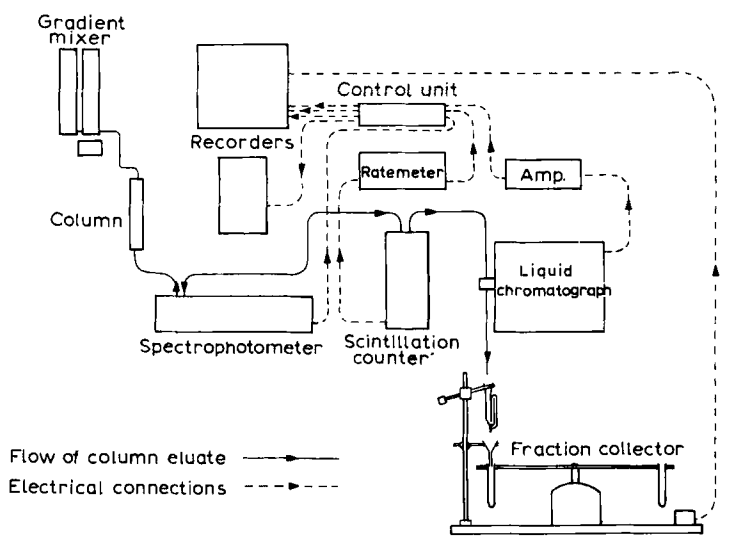


Fig. 6.9. A schematic diagram of the arrangement of a "liquid spectro-radiochromatograph" designed by Davies and Mercer [15]. (Reproduced with permission.)

to the face of an IDL (Isotopes Developments Ltd.) scintillation counter, good contact being established using a film of white oil. The flow cell, which had a capacity of 0.5 ml, was a flat glass spiral packed with europium-activated calcium fluoride crystals. Counting efficiencies were not high, namely 16.6% for ^{14}C and 1.6% for ^{3}H with background count-rates of 180 cpm and 80 cpm respectively. This instrument was used for the routine qualitative analysis of radiolabelled lipids.

The use of liquid scintillators

An early system used by Sarpenseel and Mencke [16] suffered from the drawback that the whole column eluate was mixed with liquid scintillator solution and the compounds present could not easily be recovered for further work. Hunt [17] developed an apparatus in which a small part of the eluate was split off and combined with the liquid scintillator while the major part of the eluate passed through a UV detector into a fraction collector (see Fig. 6.10). A flow cell was specially made from polyethylene tubing, which was coiled and mounted inside an outer case made from a polyethylene scintillation counter vial. Stainless-steel fittings and a Packard Kel-F adaptor were used to complete the flow cell and to make liquid-tight connections. The column eluate was combined with scintillator solution in a mixing chamber in which any bubbles formed could escape. The flow cell itself was housed in a cooled cabinet containing a Packard Model 3022 dual-channel liquid scintillation counter and Model 3041 flow monitor.

The apparatus was evaluated with a range of liquid scintillator cocktails, and efficiencies of up to 14% were obtained for ^{3}H and 70% for ^{14}C. The lower limit of detection was of the order of 100 dpm and this was achieved with eluates containing at least

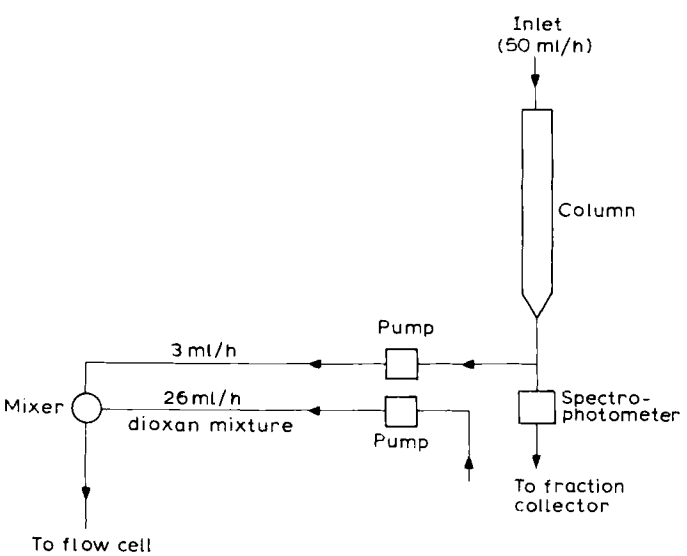


Fig. 6.10. A flow diagram of the column chromatograph used by Hunt [17]. (Reproduced with permission.)

0.9 nCi/ml of ^{3}H or 0.2 nCi/ml of ^{14}C. The flow characteristics of the cell were excellent and there was virtually no memory effect.

A valuable feature of systems like that of Hunt is that both ^{3}H and ^{14}C could be monitored simultaneously by setting up suitable channels on the liquid scintillation spectrometer, and this is in addition to the measurement of UV absorption. Using a liquid scintillation counter increases the cost considerably but it clearly also gives more sensitivity and versatility.

Radio-high-performance liquid chromatography

In recent years the use of HPLC has increased dramatically and its application to the analysis of mixtures of radiolabelled compounds warrants particular consideration [18, 19]. Although a considerable number of books on HPLC technique have appeared in the last 5 years (see refs. 20–22), reference to the use of radioactivity detectors is rare [20].

If a continuous flow monitor is used it is particularly important with HPLC to use a cell of low effective volume so that the good resolution obtained in the column is not lost by remixing of the separed compounds in the flow cell. Schutte [19] carried out a useful comparison between liquid chromatography using a glass scintillator flow cell and a homogeneous system based on liquid scintillation counting of part of the eluate. Block diagrams of these systems are shown in Figs. 6.11 and 6.12, respectively. The radiocounter used was the ICN Tracerlab Coruflow Model SCE 542 with twin photomultiplier tubes and a coincidence circuit. A UV detector was used in series with the radiodetector in both systems.

For the glass-filled cell a number of designs were evaluated and a flat U-shaped cell was found to give the best overall performance. It was also easier to fill and to handle than a coiled or helical glass cell. Schutte also commented on the phosphorescence problems with the cerium–lithium glass beads although with careful handling away from direct light, a background of about 140 cpm was obtained. Typical efficiencies were 17% for ^{14}C and 0.5% for ^{3}H. (A system similar to that shown in Fig. 6.11 has been used in the author's laboratory for several years and this is discussed in detail later in this chapter.)

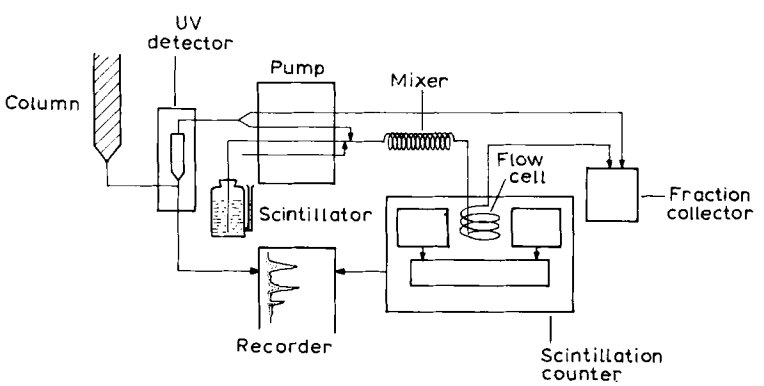


Fig. 6.11. A schematic diagram of the homogeneous counting system described by Schutte [19]. (Reproduced with permission.)

In Schutte's homogenous system (Fig. 6.12) one-fifth of the eluate from the column was mixed with a liquid scintillation solution containing toluene, Triton X-100, PPO and POPOP. After mixing, the solution was passed through a helical flow cell with a volume of 1.4 ml. It was possible to collect fractions of the solution emerging from the flow cell for more accurate radioassay in a liquid scintillation counter if required. With this flow cell very good counting efficiencies were possible, namely 80% for ^{14}C and 30% for ^{3}H.

Using a standard solution of three ^{14}C-labelled compounds the two detection systems were compared (see Fig. 6.13). Despite the differences in basic counting efficiency the

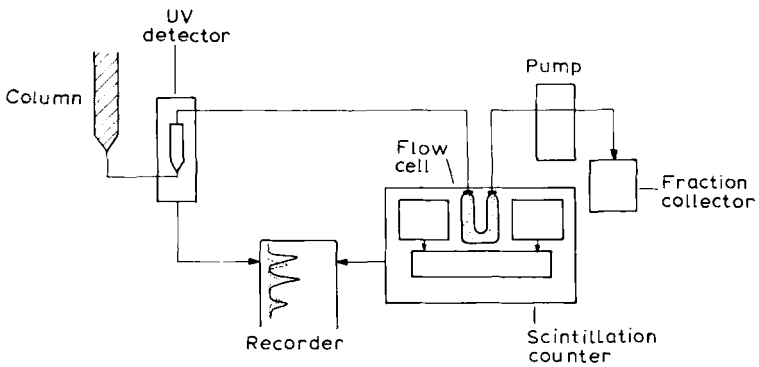


Fig. 6.12. A schematic diagram of the heterogeneous counting system described by Schutte [19]. (Reproduced with permission.)

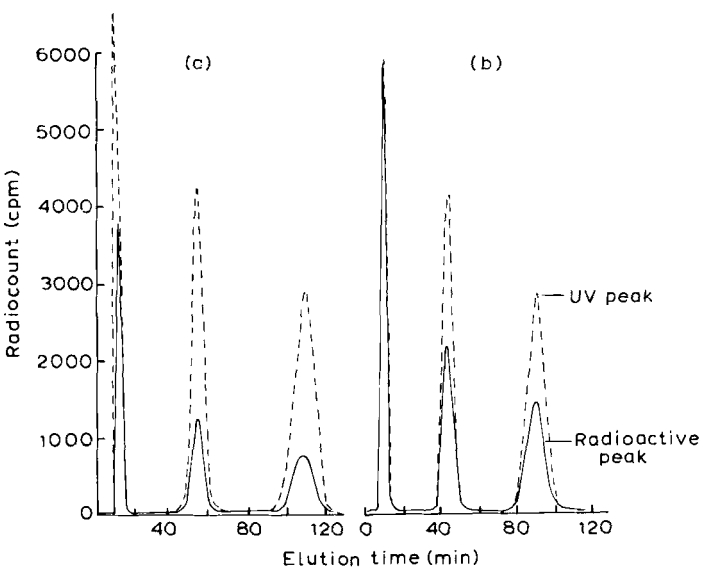


Fig. 6.13. A comparison of the homogeneous and heterogeneous counting systems described by Schutte [19]. (Reproduced with permission.)

lower limit of detection was similar for both detectors since in the homogeneous system only one-fifth of the radioactivity in the sample was measured. The lower limits were claimed to be as high as 10 nCi for ^{14}C and 1 nCi for ^{3}H. These are surprisingly high considering the good counting efficiencies quoted by Schutte.

Sieswerda and Polak [23, 24] have also used HPLC with radioactivity detection and constructed a simple low-volume flow cell suitable for use with HPLC systems. A diagram of the cell is shown in Fig. 6.14 and the apparatus into which the cell was incorporated is shown schematically in Fig. 6.15. The radiodetector was an early model of the Tracerlab Coruflow with two photomultiplier tubes and coincidence circuitry. Both a strip-chart recorder and a multichannel analyser were used to display the output. The characteristics of the flow cell were measured using standard ^{3}H and ^{14}C compounds and several organic scintillators and a lithium–cerium glass scintillator were evaluated. For the determination of efficiencies, 150 nCi of a standard was injected directly into the detector and the number of counts observed was recorded in the multi-channel analyser. The results, which are summarised in Table 6.1, clearly demonstrate the higher efficiency of the organic scintillators, especially PPO (2,5-diphenyloxazole) and PTP (*p*-terphenyl). However, both these materials gave rise to packing problems in that PPO could not be packed uniformly and unacceptably high back-pressures were encountered with PTP. Consequently, the crystalline scintillator POPOP was used. Although its effi-

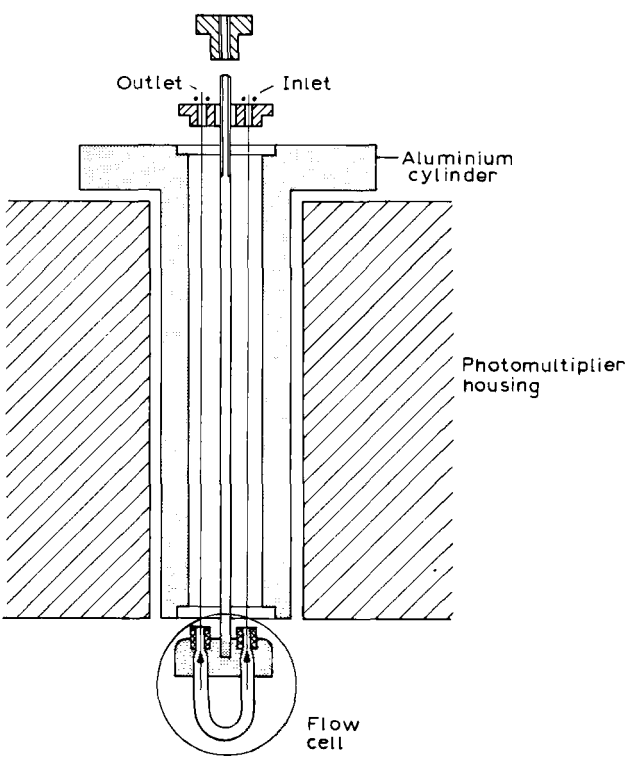


Fig. 6.14. A diagram of the flow cell used by Sieswerda and Polak [23]. (Reproduced with permission.)

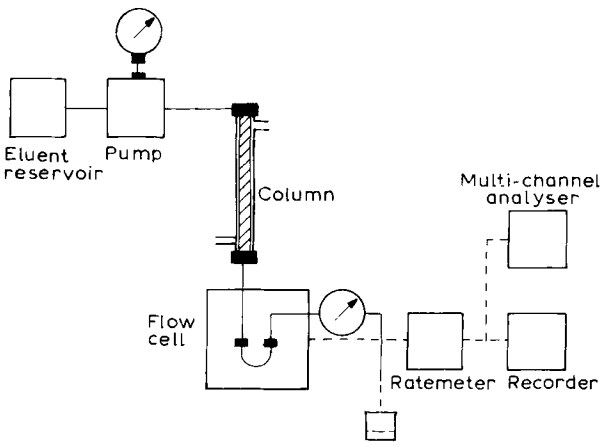


Fig. 6.15. A schematic diagram of the apparatus used by Sieswerda and Polak [23]. (Reproduced with permission.)

ciency was somewhat lower (54%) than PPO or PTP it was possible to obtain a constant flow-rate and radioactivity in aqueous solution was monitored using this anthracene-packed flow cell. The flow cell had operating efficiencies of 51% for ^{14}C and 5% for ^{3}H respectively.

A versatile column radiochromatography instrument designed by Figgi *et al.* has recently been introduced onto the market by Berthold as the BF5025 system (Fig. 6.16). The versatility stems from the provision of two detector systems of which one is a flow cell containing scintillating glass beads and the other is a flow-through detector in which a portion of the column eluate is mixed with a liquid scintillator. The instrument is intended for use primarily with the liquid scintillator system since this inherently has the

TABLE 6.1

DETERMINATION OF THE RELATIVE STATIC EFFICIENCY OF SEVERAL SCINTILLATORS FOR ^{14}C-LABELLED ETHANOL (AFTER SIESWERDA AND POLAK [23])

Scintillator	Activity		Counts (cpm)	Flow-rate (ml/min)	Volume (ml)	Relative efficiency (%)
	(nCi)	(10^5 dpm)				
Anthracene	150	333	26890	0.40	0.52	34.9
PPO	150	333	57260	0.60	0.58	100
DPS	150	333	62980	0.18	0.41	46.7
Naphthalene	150	333	4800	1.03	0.49	17.0
ANPO	150	333	118550	0.10	0.58	34.5
POPOP	150	333	46620	0.40	0.58	54.3
trans-Stilbene	150	333	11810	1.22	0.56	43.4
PTP	150	333	246600	0.10	0.53	78.6
LiCe	150	333	2920	1.75	0.35	24.6

highest sensitivity of detection (95% for ^{14}C and 50% for ^{3}H are claimed compared with figures of 12% for ^{14}C and 0.1% for ^{3}H with the glass scintillator).

In practical terms, however, whereas there is a real gain in sensitivity for ^{3}H detection, there is little real advantage for ^{14}C since again only a portion of the eluate is monitored for radioactivity in the liquid scintillation system.

In Fig. 6.17 schematic diagrams of the two systems of the BF5025 are shown. The same photomultiplier tubes, ratemeter, scaler and electronics are used for both detectors. With the liquid scintillator system (Fig. 6.17a) the eluate from the column passes through a splitter. The major part of the eluate can then pass through a mass detector to a fraction collector. The smaller part of the eluate together with the scintillator solution is pumped through a mixing chamber to the radioactivity detector. The split ratio can be varied according to the requirements of the isotope and sample being analysed.

Two different designs of flow cell are supplied with the instrument (see Fig. 6.18), one packed with cerium-activated lithium glass (effective volume 0.7 ml) and the other for use with liquid scintillator (effective volume 0.16 ml). The design of the latter is not suitable for use with a solid scintillator because of the back-pressure that would build up.

A single-sample liquid scintillation counter, which could be fitted with a flow cell for column radiochromatography, was marketed by Intertechnique Ltd. This was the SL20 instrument which is now no longer available commercially. Flow cells of various sizes could be packed with any solid scintillator and data presentation was either in the form of a strip-chart recorder trace of the ratemeter output or a digital output using an automatic lister.

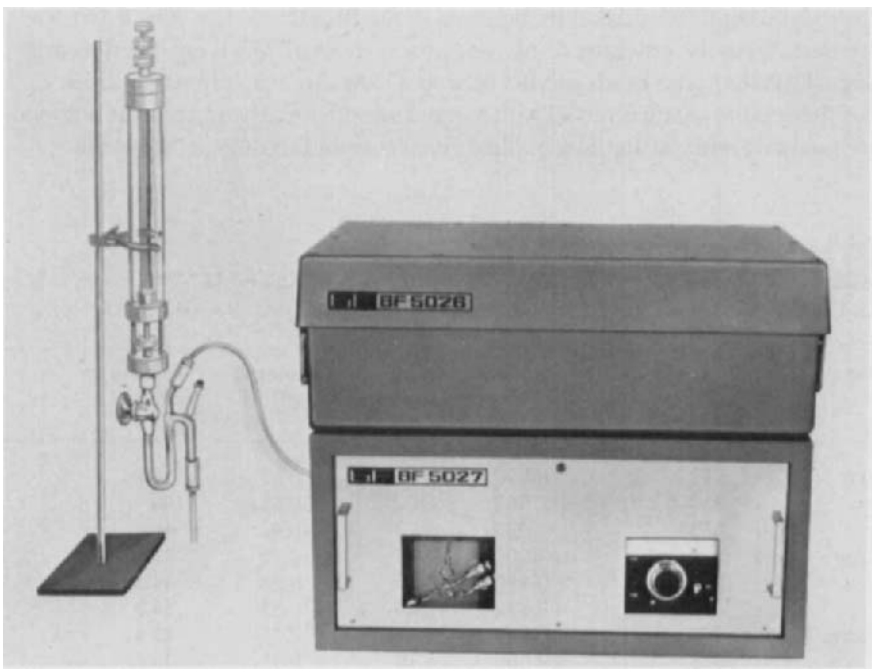


Fig. 6.16. The Berthold column radiochromatography measuring system. (Reproduced with permission from Intertechnique Ltd.)

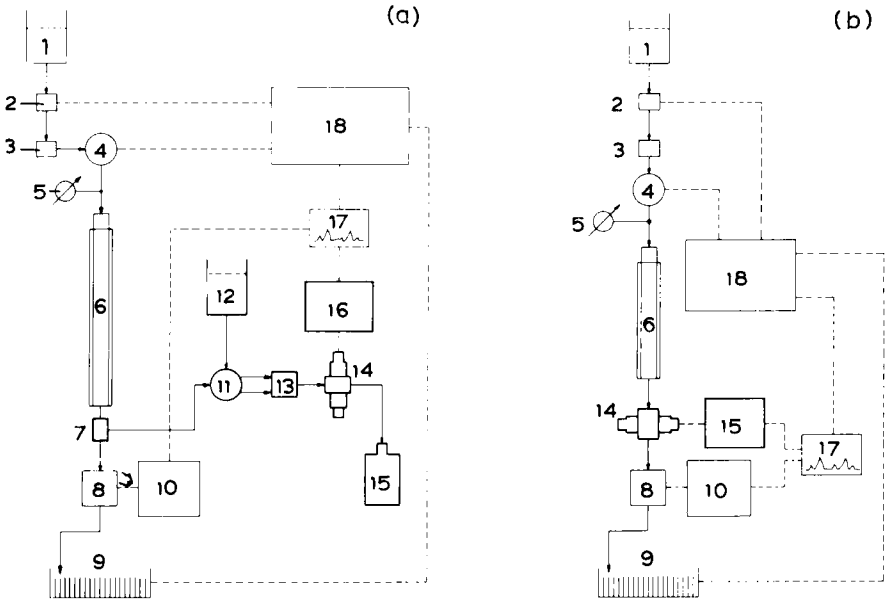


Fig. 6.17. A schematic diagram of Berthold systems for continuous monitoring of column eluates. 1 = Eluent reservoir, 2 = regulating valve, 3 = bubble trap, 4 = pump, 5 = pressure gauge, 6 = column, 7 = eluate splitter, 8 = mass detector, 9 = fraction collector, 10 = amplifier for mass detector, 11 = pump, 12 = scintillator storage, 13 = mixing chamber, 14 = flow-cell detector, 15 = collecting flask, 16 = amplifier for radiodetector, 17 = dual-pen recorder, 18 = automatic control equipment.

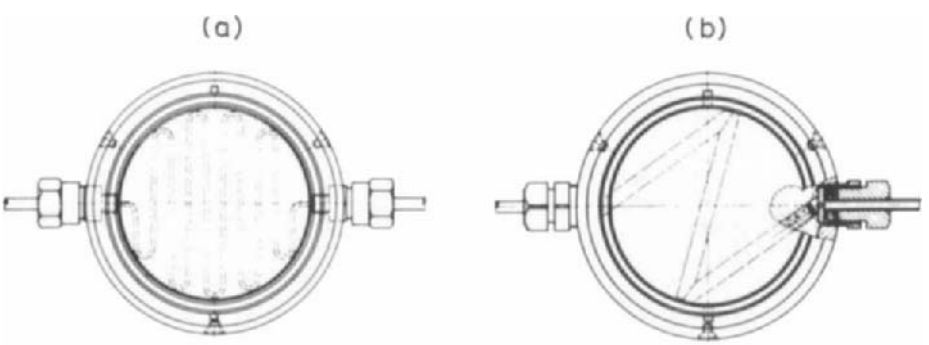


Fig. 6.18. A diagram of some of the flow cells for use with the Berthold BF5025 column radiochromatography system. (a) For use with liquid scintillator; (b) for use with solid scintillator.

Higher-energy isotopes

The foregoing discussion has centred on the detection of weak β-emitters in column eluates. A much more simple detection system can be used when isotopes of higher energy such as ^{32}P are to be monitored. For example, Jordan [25] used a flow cell for ^{32}P work which consisted simply of a block of PTFE containing a spiral groove covered with

a thin Mylar window. This was placed in contact with a Geiger–Müller tube connected to a ratemeter and recorder. The cell had a volume of 1 ml and this relatively high volume might render it unsuitable for high-performance chromatography. The flow characteristics of the cell are shown in Fig. 6.19.

Conventional liquid scintillation counting can, of course, be used with ^{32}P-labelled compounds but it is also possible to use Cerenkov counting with higher-energy isotopes. Matthews [26] collected fractions of an aqueous eluate from chromatography columns in plastic counting vials and was able to determine the ^{32}P radioactivity directly, with an efficiency of 32%, by Cerenkov counting in the absence of a scintillator. Excellent reproducibility of measurements was obtained with this method. Cerenkov counting has the advantage that as no liquid scintillator is added to the sample, all of the sample is retained for further examination. However, Matthews somewhat over-emphasised the fact that problems of quench correction are overcome with Cerenkov counting since this is not a problem with the liquid scintillation counting of higher-energy isotopes.

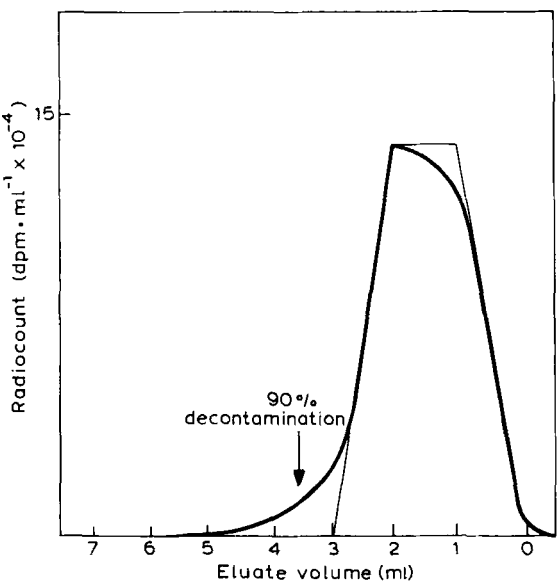


Fig. 6.19. The flow characteristics of a cell used by Jordan [25]. (Reproduced with permission.)

CHOICE OF METHOD FOR RADIO-COLUMN CHROMATOGRAPHY

The method chosen for radioassay of the eluate from a chromatography column will depend on the isotope(s) to be detected, amounts available, resolution, the time factor and funds available for equipment. In addition, special consideration may need to be given to possible interaction of the sample with the scintillator.

Range of isotopes

With "high-energy" β-emitting isotopes such as ^{32}P very simple flow cells based on Geiger–Müller tubes can be used to monitor column effluents. However, with weaker isotopes, including ^{14}C and ^{35}S, any of the recent flow-through methods described above, or fraction collecting and liquid scintillation counting of fractions can be used. When working with ^{3}H, very low counting efficiencies are obtained with solid organic, inorganic and glass scintillators and this may cause problems if low levels of ^{3}H are being used. In this case liquid scintillation counting should be used, either in a flow-through system or separately on individual fractions.

A liquid scintillation counting method may also be preferred if more than one isotope is present in the eluate. Provided that the β-energies are sufficiently different to permit different channels to be set up (as is the case for ^{3}H and ^{14}C) then discrimination between the isotopes can be readily obtained.

Sensitivity and efficiency

The static counting efficiency can be determined by filling the flow cell with a solution containing a known amount of radioactivity and measuring the number of counts per minute registered. The effective cell volume must be determined by one of the techniques referred to on p. 106.

The counting efficiency of the detector when in use as a flow cell will vary with the flow-rate and the effective cell volume according to the following equation:

$$E = \frac{\text{cpm}}{\text{dpm}} \cdot \frac{v}{V}$$

where E = efficiency in %, v = flow-rate in ml/min, and V = cell volume (liquid volume) in ml.

In practice a compromise must be made between cell volume and flow-rate. The higher the cell volume and the lower the flow-rate, then the higher will be the sensitivity of detection. However, under such conditions, loss of resolution may occur.

Resolution

With column radiochromatography it is particularly important to choose a detection technique in which the resolution obtained on the column is not lost during radioassay. In this respect fraction collecting followed by radioactivity determination is more suitable for "low-efficiency" chromatography such as ion exchange or gel filtration on large-particle-sized materials in which high resolution is seldom achieved.

With flow-through systems, however, it is important to ensure that the volume of the cell and its associated tubing is compatible with the column separatory technique in use. The importance of this in radio-HPLC cannot be over-emphasised. An illustration is shown in Fig. 6.20 in which the isomers of a ^{14}C-labelled sample of the pyrethroid insecticide NRDC 149 were separated on Partisil-5 (5-μm silica).

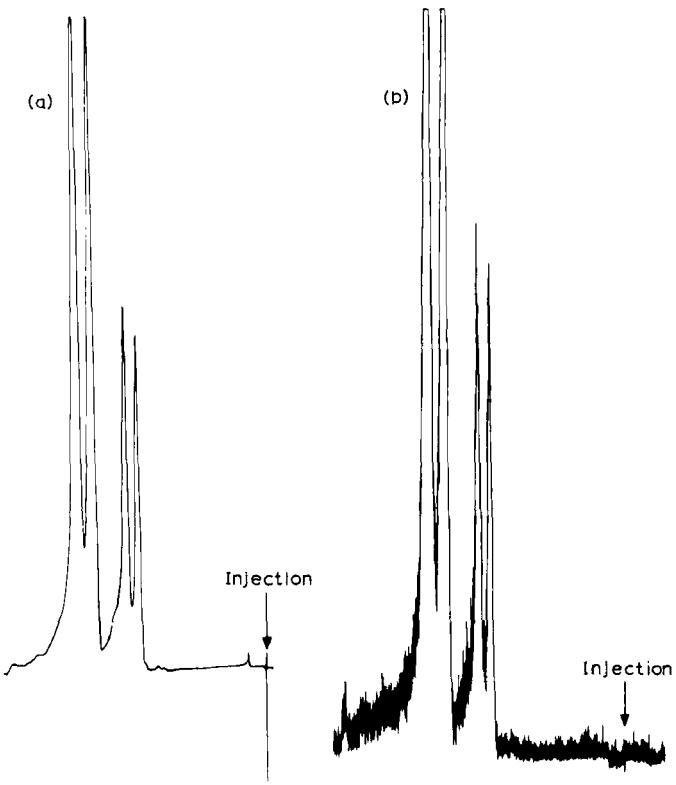


Fig. 6.20. The separation of isomer pairs of NRDC 149 by HPLC with (a) UV detection and (b) radio flow-cell detection.

The effective cell volume was approximately 700 μl, which was sufficiently small to retain the resolution of the geometrical isomers and partial resolution of the optical isomers obtained on the HPLC column.

Speed of analysis

Although the analysis of fractions of column eluates is the most routinely used method of radioassay it is also the most laborious and time consuming. Furthermore, if the number of fractions taken is large then a very high proportion of the time available on a liquid scintillation counter can be taken up by the analysis of column fractions. For these reasons flow-through techniques offer a positive advantage in terms of speed of analysis. If a fraction collector is incorporated after a flow cell containing a solid scintillator (through which the whole eluate passes), it should be clear from the output of the flow cell in which fractions the radiolabelled compounds are to be found. Alternatively, individual labelled compounds may be collected manually as they emerge from the flow cell.

Liquid scintillator flow-cell systems such as the Berthold BF5025 are somewhat more

complex to set up than the solid scintillator systems and on average probably need more attention to ensure that, for example, the liquid scintillator does not run out and that there are no problems with the stream splitter.

Cost and availability of equipment

Assuming liquid scintillation counting time is available then clearly the cheapest method (in capital terms) of carrying out column radiochromatography is to use a fraction collector. Even if a liquid scintillation counter is not available it is possible to carry out TLC on fractions and check for radioactivity content by autoradiography [27]. This would be essentially qualitative and is not likely to have a wide appeal. Alternatively, if cost is a problem, an inexpensive flow-through system can be built from low-cost components or from existing radiochemical equipment such as photomultiplier tubes and ratemeters or scalers. Nuclear Enterprises supplies a range of flow cells including the NE 806(A) containing anthracene crystals and the NE 808 containing europium-activated calcium fluoride. These can be used with suitable existing equipment or with a manual liquid scintillation counter head (fitted with a flow-cell adaptor) and a scaler–timer or scaler–ratemeter, all supplied by Nuclear Enterprises. Further details are given in the Appendix.

Alternativley, a flow cell could be fitted into an automatic liquid scintillation counter for a time but this would prevent the use of the counter for conventional samples.

When considering commercial instruments, the cost of an ICN Tracerlab Coruflow and flow cells is about the same as that of a thin-layer radioscanner, in 1977 Great Britain prices. The Berthold BF5025 dual measuring system is more expensive (about 1.5 times the cost of a TLC scanner). It may also be necessary to add the cost of a fraction collector to these basic costs if one is not already available.

Needless to say, running costs are lower if solid scintillators are used since (particularly the glasses) these have a long life and there is a much lower expenditure on scintillation cocktails.

Recovery of labelled material

The only totally destructive on-line method is one in which the total eluate is combined with a liquid scintillator solution from which it would be very difficult to recover the labelled materials. In the Berthold system and others like it in which only a portion of the column eluate is mixed with scintillator, only about 20% of the sample is lost. This may not be serious unless supplies of the radiochemical in question are extremely limited. In our experience with the chromatography of radiolabelled pesticide metabolites which have been isolated from plant or animal tissues, the amounts available are often very limited and any loss of material is to be avoided. Consequently, we prefer to use a completely non-destructive system, namely a flow cell containing a solid scintillator.

If fraction collecting and liquid scintillation counting are used one can again expect to lose 10–20% of the sample during radioassay, depending on the amount of radioactivity present.

Sample and solvent compatibility

Before deciding on a column chromatography detection system consideration must be given to the nature and variety of solvents that will be used. Not only must the solvent resistance of the tubing and mechanical parts be considered but also the compatibility of the solvent(s) with the scintillator to be used. Obviously if organic solvents are to be used then the use of organic scintillators is ruled out. Inorganic scintillators (for example, europium-activated calcium fluoride) can be used with most solvents except dilute mineral acids or aqueous alkali. Only the glass scintillators possess a more universal resistance to a full range of common solvents.

Under some circumstances the physical and chemical nature of a radiolabelled compound to be chromatographed may rule out the use of a particular technique. If highly coloured materials are involved, colour quenching effects may prevent the use of a liquid scintillation counting method. Similarly, if the chemical reacts with or is absorbed by an organic scintillator, this will give rise to either a short-lived memory effect or a more permanent contamination of the cell. In this context biopolymers are known to complex with some organic scintillators.

Flexibility

In most laboratories in which radiochemicals are analysed there are likely to be a variety of column chromatography techniques in use. These could include gel filtration and ion exchange, which use aqueous eluents, common adsorption and partition chromatography using organic eluents and high-performance chromatography, which could involve the use of both organic and aqueous solvents. Therefore a versatile technique should be adopted which can be operated under a wide variety of conditions. The use of pressurised systems in HPLC should also be considered.

Ignoring costs, perhaps the most versatile apparatus available commercially is the Berthold BF5025 since this can be used with a liquid scintillator flow cell or with a flow cell packed with glass beads. However, versatility can also be obtained if a range of flow cells containing different solid scintillators and with different effective volumes is obtained. Having flow cells of different physical designs is also of value since, for example, the coiled designs tend to build up more of a back-pressure than say a U-shaped design. Consequently the latter is more suitable for systems used under pressure.

RADIO-COLUMN CHROMATOGRAPHY IN PRACTICE

The radioassay of column eluates by liquid scintillation counting does not warrant detailed discussion and this section will be devoted to the use of flow cells. Particular emphasis will be given to the considerations which need to be made when setting up a radio-HPLC system and to some of the problems encountered in its operation.

Radio-high-performance liquid chromatography

A radio-HPLC apparatus which has been used in the author's laboratory for several years is illustrated in Fig. 6.21 and a block diagram of the system is shown in Fig. 6.22. The solvent is pumped from the reservoir using a DCL micropump (Series III) which has been fitted with a stainless-steel high-pressure pump head with PTFE seals and is rated at 3000 p.s.i. (20.7 MPa). A degree of pulse damping is achieved with 2 large-pressure gauges and 25 m of coiled stainless-steel capillary tubing. Although the radiodetector is not affected by pulsing some damping is desirable to obtain an acceptable baseline with the UV detector. It is essential to use an in-line filter between the pump and the damping coil in order to prevent transfer of PTFE from the pump to the capillary and a subsequent blockage.

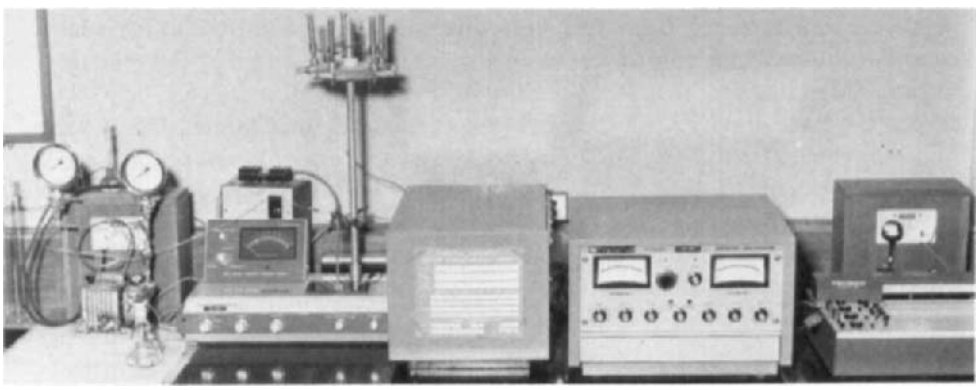


Fig. 6.21. A radio-HPLC apparatus currently in use in the author's laboratory.

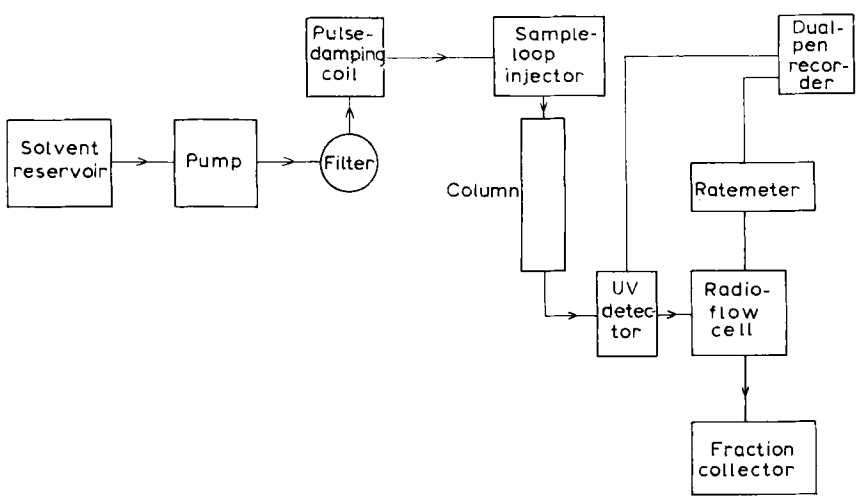


Fig. 6.22. A schematic diagram of the radio-HPLC apparatus used in the author's laboratory.

In general, an external loop injection system is used with loops of between 50-μl and 1-ml capacity. In this way the sample can be introduced onto the column while the system is under pressure and septum problems from syringe injections are avoided. A range of stainless-steel columns is used for analytical and semi-preparative-scale separations. These range in size from 0.1 m × 4.5 mm to 0.5 m × 9.5 mm I.D. with minimal column dead volumes. Consequently, it is vital to use piping and connections between the column and radiodetector with very small dead volumes. The columns themselves are packed at Sittingbourne Research Centre [28] and they are made with zero-dead-volume fittings which may either be obtained commercially or drilled out specially, and PTFE capillary tubing is used in place of the wider-diameter tubing which is more generally used.

The column eluate is first passed through a Cecil CE212 variable-wavelength UV monitor equipped with a cell of 10 mm pathlength with a volume of 8 μl. In order to minimise the length of PTFE tubing connecting the column and the UV cell, the cell cover has been slightly modified (see Fig. 6.21). In a more recent modification the foot of the column actually fits directly into the UV cell compartment which reduced the dead volume further. The radiodector is an ICN Coruflow SC-542 and the flow cell (Fig. 6.23) is U-shaped and made of quartz and is packed with a cerium-activated lithium glass powder. This cell is housed in a sliding drawer between a matched pair of low-noise photomultiplier tubes. Part of the signal from the photomultiplier tubes is fed to a fast coincidence circuit while the remaining signal is fed to summation amplifiers. The signal is mixed again in a slow coincidence circuit and the output is displayed, together with that from the UV monitor on a dual-pen recorder.

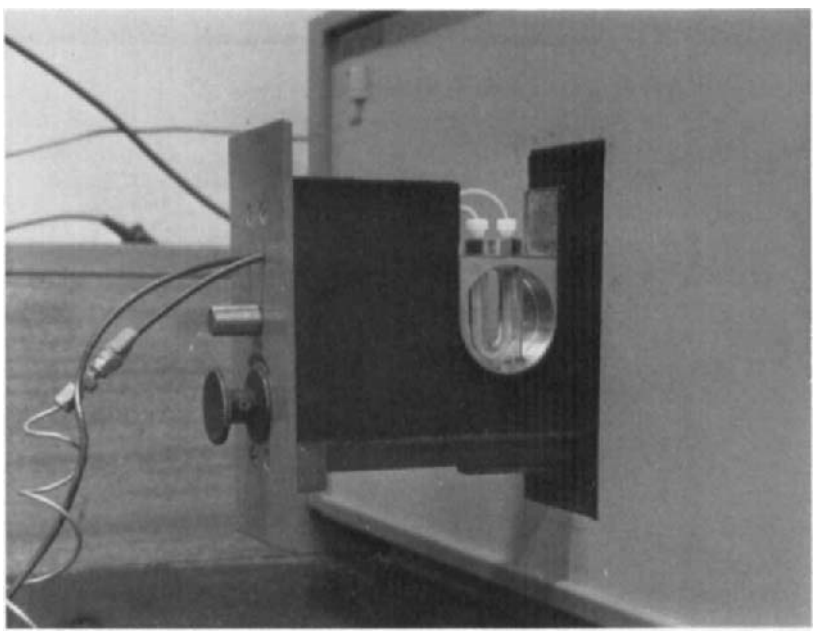


Fig. 6.23. The flow-cell arrangement in the ICN Coruflow detector used in the apparatus shown in Fig. 6.21.

The outlet from the Coruflow is discarded into a waste receiver if analytical columns are used but for semi-preparative work either a fraction collector is used or, more commonly, separated radiocomponents are collected manually in individual tubes.

Fig. 6.24 illustrates the importance of minimising the dead volume of the system. In Fig. 6.24a a chromatogram of the triazine herbicide, cyanatryne, and one of its metabolites is shown and this was obtained before the dead volume of the system was reduced. After the modifications had been made the result in Fig. 6.24b was obtained and there is a clear improvement in the peak shape from the radioactivity detector, which indicates that no loss of resolution had occurred between the UV cell and the glass scintillator flow

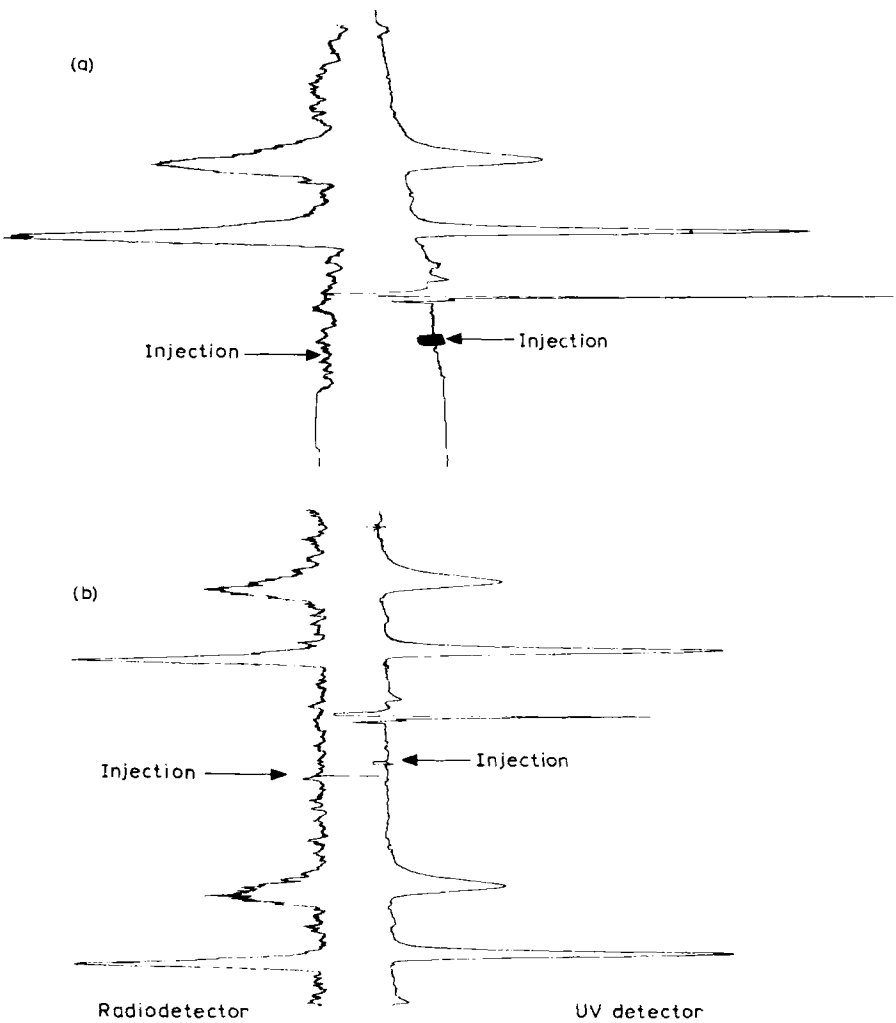


Fig. 6.24. Radio-HPLC analysis of cyanatryn and one of its metabolites. (a) Conventional chromatogram; (b) chromatograms obtained after the dead volume of the system had been reduced.

cell. In fact the effective volume of the system between the outlet of the UV monitor and the *outlet* of the radioactivity flow cell has been determined and found to be approximately 800 μl.

In use a background count-rate for the radiodetector of 20–30 cpm is usually obtained when ^{14}C is monitored. Soon after the apparatus was built, a problem of high background count-rates (100–200 cpm) was encountered. This was found not to be due to contamination or "memory" effects within the flow cell but to a "fibre optic" effect with the PTFE tubing at the outlet of the radioactivity flow cell. As a result, sufficient light was able to penetrate the dark shields of the photomultiplier tubes to cause an increase in background. The problem was resolved by sheating the PTFE tubing with black polyvinyl chloride.

With the versatile HPLC system described here, flow-rates of 7–8 ml/min can be used. However, there is a strict limitation to the flow-rate when the radioactivity flow cell is in use. This is due in part to the back-pressure of the cell packed with solid scintillator and to the fact that if the residence time of the radioactive material within the cell is too short, then the counting efficiency will be low. In practice, flow-rates of 0.8–1.0 ml/min are used and under these conditions efficiencies as high as 40% for ^{14}C can be obtained. With a system similar to this which is in use at the Radiochemical Centre at Amersham, efficiencies for ^{14}C and ^{3}H have been found to be 45% and 1.3% respectively. The lower limit of detection of our system for ^{14}C is approximately 500 dpm.

A further example of the retention of resolution throughout the system is shown in Fig. 6.20 which shows the separation of four diastereoisomeric pairs of the insecticide NRDC 149. This separation was achieved in a 0.2 m × 4.5 cm column of Partisil-5 using petroleum spirit (b.r. 62–68°C)–dichloromethane (85:15, v/v) as eluent and there is clearly little or no loss of resolution between the UV and radiodetectors.

APPLICATIONS OF RADIO-COLUMN CHROMATOGRAPHY

Amino acid analysis

There has been a continuing interest in the analysis of radiolabelled amino acids using automatic analysers since Piez [9] published his comprehensive paper in 1962. As amino acids are usually chromatographed on ion-exchange columns and aqueous buffers are used as eluents, in most cases flow cells containing anthracene were incorporated into the flow stream of the analyser [1,9,11,29,30]. In high-efficiency systems it is important to use a pressure-resistant flow cell as illustrated by White and Mencken [11] who built a plastic cell capable of operation at the pressures involved when using a modified Phoenix automatic amino acid analyser. An example of the output from White and Mencken's system is shown in Fig. 6.7.

In a more recent report, De Belleroche *et al.* [31] described an automatic method for monitoring amino acids and related compounds in striatial synaptosomes. Tissue extracts were analysed by cation-exchange chromatography and the column eluate was initially passed through a UV detector after which it was split using a micro T-piece from which one-third was pumped through a fluorimeter and two-thirds to a radioactivity detector.

The latter comprised a perspex flow cell packed with plastic phosphor spheres (100 μm in diameter) and a Packard Scintillation Spectrometer. This apparatus was used to determine the specific activity of dopamine in corpus striatum synaptosomes after incubation with radiolabelled DOPA (3,4-dihydroxyphenylalanine).

Biochemical studies

All methods of column chromatography are used in general biochemical studies, the most common being gel filtration and ion-exchange chromatography. A review of the recent literature has shown that the most common method of radioassay of column eluates is to collect fractions for liquid scintillation counting. Perhaps the major reason for this is that with these low-efficiency chromatography techniques, particularly gel filtration, relatively large fractions (often as much as 10–15 ml) of the eluate are collected and there is little loss of material if 0.5–1.0 ml of each fraction is sub-sampled for radioassay.

For example, Willox *et al.* [32] in their studies of the metabolism of aniline in arachnida chromatographed water-soluble metabolites on Sephadex G-15 eluted with water. In this case, even though 15-ml fractions were collected, liquid scintillation counting of aliquots clearly showed the presence of two major metabolites. Gel filtration, of course, is frequently used for the analysis of macromolecular complexes and Ihl [33] examined extracts containing indole-acetic acid bound to protein from plants on Sephadex G-25 or G-75 columns. Smaller fractions (approximately 2 ml) were collected from these columns from which only 0.2 ml was taken for radioassay. Nelson *et al.* [34] used Sephadex G-10 to separate the products formed when [^{14}C]acetylhydrazine was incubated with liver microsomes. Fractions of 2 ml were taken and sub-sampled for radioassay. Klein *et al.* [35] chromatographed [^{35}S]heparan sulphate on Sephadex G-100 and G-200 and again the fraction collecting method was used. Gel filtration is also used for the chromatography of polysaccharides and an example of its use in the separation of radiolabelled β-glucans is given by Howard *et al.* [36].

These recent examples from the literature emphasise the very limited use of flow-through methods in biochemical studies. This is somewhat surprising considering the availability of commercial flow-cell systems, especially since in most of the cases cited above, the amounts of radioactivity present in the column eluates would have been readily detectable using a solid scintillator.

In the author's laboratory gel filtration on Sephadex G-10 and G-15 using water as eluent and Sephadex LH-20 using organic solvents has been carried out on extracts of plants treated with radiolabelled pesticides. Although a fraction collector is used from time to time, it has been found preferable to use a U-shaped flow cell packed with cerium-activated lithium glass and housed in a Nuclear Chicago Chromacell. This set-up has been used mainly for the separation of labelled compounds of relatively low molecular weight from non-labelled impurities of higher molecular weight.

For some situations HPLC is now being used in addition to the well established column chromatography methods. For example, Morris *et al.* [37] used HPLC when working with plant cytokinins and Nagels and Parmentier [38] also used HPLC during kinetic studies of the biosynthesis of chlorogenic acid in *Cestrum poeppigii.* Desrosiers

et al. [39,40] used high-performance ion-exchange chromatography for the separation of ^{14}C- and ^{3}H-labelled nucleosides and nucleotides. In all of these examples, liquid scintillation counting of 0.1–1.0-ml fractions of the column eluates was the method of radioassay.

Pesticide and drug metabolism studies

The use of column chromatography techniques in studies of pesticide and drug metabolism is less common than other radiochromatography techniques, particularly radio-TLC, and the methods used are similar to those described in the previous section. However, in the past 2–3 years there has been an increasing interest in molecular size separations and in newer approaches to ion-exchange chromatography together with the completely new possibilities created by the introduction of HPLC. With the exception of radio-HPLC, in most laboratories radioactivity in eluates from columns is usually measured without the use of a flow-through system. This is probably because these techniques are used at irregular intervals and the cost and time necessary to set up a continuous monitoring system is apparently not justified.

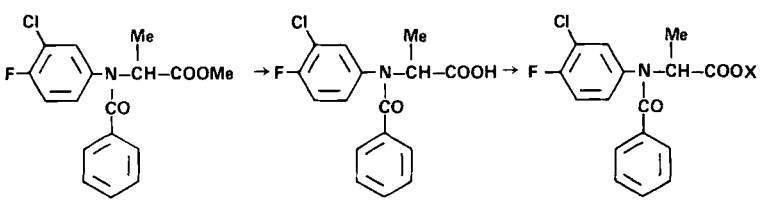
With the introduction of radio-HPLC into the metabolism laboratory, however, it is preferable to couple a commercial flow-through system onto a liquid chromatograph. One reason for this is that, in order to get established in HPLC it is necessary either to buy a complete liquid chromatograph or to build a system using a suitable pump, column and mass detector (usually a UV monitor). Consequently, a flow-cell system could be incorporated into the instrument at the same time. Furthermore, the potential of HPLC as a valuable tool in metabolism studies is so apparent that in many laboratories it is already established as a complement to radio-TLC and radio-GLC, and if the technique is to be used routinely then liquid scintillation counting of fractions can become very time consuming and costly.

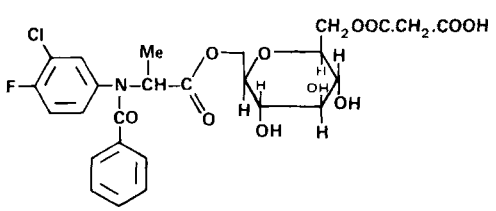
There are several reasons why radio-HPLC is of such importance in metabolism studies. There now exists a whole range of column packings which cover liquid–solid adsorption, liquid–liquid partition, reversed-phase partition, anion and cation exchange and gel permeation. This means that a column packing and eluent suitable for the separation of most mixtures of compounds can be found. The reversed-phase mode (in which the stationary phase is non-polar and the mobile phase is polar) is particularly valuable in the present context since the most polar components of a mixture will elute from the column before the less polar ones. Pesticides (and drugs) are usually metabolised to more polar products so a reversed-phase system capable of separating a mixture consisting of a relatively non-polar pesticide and all of its metabolites formed in plants or animals is of considerable value. Moreover, the serious problem of retention of polar compounds on the column is overcome by the use of reversed-phase chromatography.

With the introduction of microparticulate column packings with particle sizes of 5 μm or 10 μm, the capacity of HPLC columns has increased enormously with the result that relatively large-scale separations (milligram amounts) can be carried out. High capacity is also necessary for the analysis of extracts of biological samples containing radiolabelled compounds in the presence of large amounts of unlabelled co-extractives.

In our laboratory radio-HPLC has been in use for several years, mainly using the system

described in detail earlier (see Fig. 6.21), and broadly speaking there are two major applications of the technique. Firstly, it is used for comparative chromatography of pesticide metabolites and authentic reference compounds as part of the preliminary identification of the metabolites. In order to do this it is necessary to use another detector, in addition to the radiodetector, which can monitor the presence of the unlabelled reference compounds in the eluate, and a UV detector is usually used prior to the solid scintillator flow cell. Some examples of this are given in a recent study of the degradation of the pyrethroid insecticide NRDC 149 in soil [41]. In this work the *cis*- and *trans*-isomers of the insecticide were separated on a Partisil-5 column using 20% dichloromethane (50% water saturated) in petroleum spirit (b.r. 62–68°C) and the *cis*- and *trans*-isomers of one of the degradation products (2,2-dimethyl-3-(2′,2′-dichlorovinyl)cyclopropanecarboxylic acid) were separated on the same column using 1% v/v dioxan and 0.1% v/v acetic acid in petroleum spirit. In each case the retention volume of the radiolabelled degradation product was compared with that of an authentic standard of the relevant isomer. Another example of this is shown in Fig. 6.24 in which a metabolite of the triazine herbicide cyanatryn has been separated from cyanatryn itself. Secondly, it is used for the purification of relatively large amounts of metabolites which are conjugated to plant sugars or amino acids so that characterisation of the conjugate in the pure form can be attempted by, for example, derivatisation and mass spectrometry or possibly field-desorption mass spectrometry directly on the conjugate without prior derivatisation. The latter approach is exemplified in some recent work on the wild-oat herbicide flam-





Conjugate characterised after purification by reversed-phase HPLC

Fig. 6.25. The metabolism of the herbicide flamprop-methyl in wheat [42].

prop-methyl [42]. When the metabolism of this herbicide in wheat was studied the major metabolites were conjugates of the carboxylic acid, "flamprop", derived from flamprop-methyl (Fig. 6.25). Larger amounts of these conjugated metabolites were biosynthesised in wheat seedlings and one component of the mixture was purified by reversed-phase HPLC on a 20 cm x 9.5 cm column packed with Spherisorb silica-5-ODS (5 μm particle size) using 30% v/v ethanol in water as eluent. This was shown by field-desorption mass spectrometry to contain malonyl derivatives of the glucosyl and galactosyl esters of "flamprop".

Comparisons between radiolabelled substances and unlabelled standards on HPLC should be made with caution especially when tritiated compounds are involved. As a result of the highly efficient separations achieved by HPLC, isotopic separation of ^{3}H-labelled compounds from their unlabelled (^{1}H) analogues has actually been observed [43]. This effect was discovered when ^{3}H-labelled materials were found to have slightly shorter retention times than those of the unlabelled standards to which they were believed to correspond [44].

The use of HPLC in the identification and quantitation of drugs and their metabolites in general has been reviewed [45] and the comments made on pesticides are, of course, applicable to drugs. The value of reversed-phase chromatography is shown by Twitchett *et al.* [45] who separated metabolites of tetrahydro-cannabinal (thought to be conjugates) in rabbit urine on Partisil-5-ODS before and after hydrolysis with an enzyme or with alkali. The formation of less-polar hydrolysis products is apparent from the elution order.

HPLC has been used to separate metabolites of the carcinogen 1,2-dimethylhydrazine in the rat [46,47] using ion exchange. Highly reproducible separations of 1,2-dimethylhydrazine, azomethane, azoxymethane, methylazoxymethanol, methylazoxymethanol acetate, formaldehyde and methanol were achieved and used for kinetic studies. Once again, a flow cell was not used for radioassay, which was carried out by liquid scintillation counting.

There are a number of examples in the literature where HPLC has been used for pesticide or drug metabolism studies using radiolabelled compounds but in which only a UV detector has been used [48–51]. In this situation full use of the radiolabelled substrates is clearly not being used and the incorporation of a radioactivity monitoring technique would be a major improvement.

Purification of radiochemicals

As discussed in Chapter 4, TLC is commonly used to check the radiochemical purity of compounds prior to their use but it is only used to actually purify radiochemicals when small amounts are being handled. Large-scale purifications are carried out by suitable column chromatography and silica gel or alumina columns are commonly used to mimic TLC separations. HPLC has also been used for radiochemical purifications and Norris and Still [52] used a Waters Associates Carbowax 400 on Porasil C (36–57 μm) eluted with chloroform to purify [^{14}C]-4-nitrocatechol, which had been prepared by photolysis of [^{14}C]-4-nitrophenol. No radiochemical detection was used, however, and the reaction product was detected in this case with a UV monitor.

Analytical HPLC has also been used to determine the radiochemical purity of separate *cis*- and *trans*-isomers of a ^{14}C-labelled insecticide, which were not separable on TLC [41].

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Chapter 7

Radio-gas–liquid chromatography

CONTENTS

INTRODUCTION

Gas–liquid chromatography (GLC) emerged as a powerful analytical technique in the 1950s and it became very widely used in the 1960s. Since 1955 various approaches to the application of GLC to the separation of mixtures of radiolabelled compounds have been used. In general, the methods used fall into two categories, namely "continuous" methods in which the concentration of radioactive compounds in a gas stream is monitored continuously and "discontinuous" methods in which the resolved compounds in the effluent are

trapped as they emerge from the GLC column for separate radioassay. In the latter case, once the compounds have been separated their radiochemical content can be determined by the most suitable method available.

Radio-GLC has been featured in a number of reviews, including those by Adloff [1] (1962), James [2] (1964), Karmen [3,4] (1967, 1969), Scott [5] (1967), Snyder [6] (1969), Cram [7] (1970), Lubkowitz [8] (1972) and Matucha and Smolkova [9] (1976). Most reviews have covered continuous methods and Snyder [6] also discussed the commercial instruments available in 1969. The most recent review [9] discusses not only detection methods and output of data, but also lists many applications of radio-GLC in synthesis, "hot atom" chemistry, organic chemistry and biochemistry.

In this chapter the chronological development of continuous and discontinuous methods of radio-GLC is described before consideration is given to the choice of a method for any particular problem. The emphasis has been concentrated on the analysis of weak β-emitters in organic-chemical and biochemical applications, and reaction gas chromatography is not within the scope of the material reviewed.

DEVELOPMENT OF RADIO-GAS–LIQUID CHROMATOGRAPHY

Continuous methods

One of the earliest reports of a continuous method was made by Wolfgang and Rowland [10] in 1958. Their method first involved the separation of a mixture of labelled compounds by GLC using helium as carrier gas after which the gas stream was passed through a thermal conductivity detector. At this point methane was injected into the gas stream to produce a quenching gas before the effluent was passed through a gas-flow proportional counter for radiochemical detection. Compounds emerging from the proportional counter could be condensed in a cold trap for further examination. A diagram of the apparatus is shown in Fig. 7.1.

Wolfgang and Rowland also discussed the important relationship between such factors as flow-rate, sensitivity and counter volume for proportional counters. Although increased sensitivity could be obtained using a low flow-rate and large counter volume, such conditions also led to peak broadening and the possibility of loss of the resolution obtained with GLC due to remixing of adjacent compounds in the counter. The optimum volume for the proportional counter was calculated to be between 10 and 20 ml.

Early liquid scintillator systems

In connection with their work on the GLC of ^{14}C-labelled fatty acids, Popják and coworkers [11] designed a radio-GLC system based on liquid scintillation counting since this has an inherently high sensitivity. The gas stream from the GLC column was passed into a circulating, cooled solution of a scintillator and, in this instrument, a single photomultiplier tube was used to determine the radioactivity present. For details of the design and construction of the instrument the reader is referred to the original paper. The performance of the instrument was assessed by using a standard mixture of the methyl esters

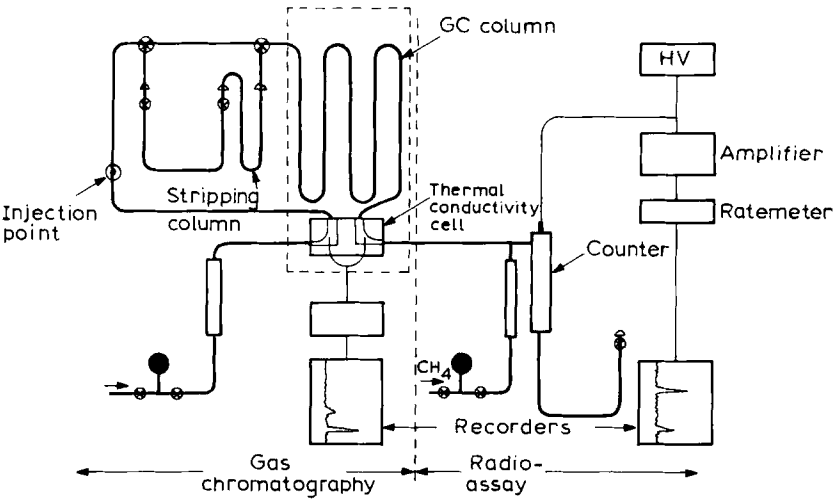

Fig. 7.1. A diagram of the radio-gas chromatograph used by Wolfgang and Rowland [10]. (Reproduced with permission.)

of four ^{14}C-labelled fatty acids (C_{10}, C_{12}, C_{14} and C_{16}). A potential disadvantage of this type of instrument is that radiocounts from separated compounds are accumulated in the scintillator solution so that if a minor component followed a major one it could be difficult to quantify accurately, as shown in Fig. 7.2. In practice, however, this did not appear to be a serious problem, particularly when mixtures of compounds containing similar amounts of radioactivity were analysed.

It was found that approximately 150–200 cpm above the background count-rate could be detected. However, as only a single photomultiplier tube was used the instrument had a high background of about 420 cpm.

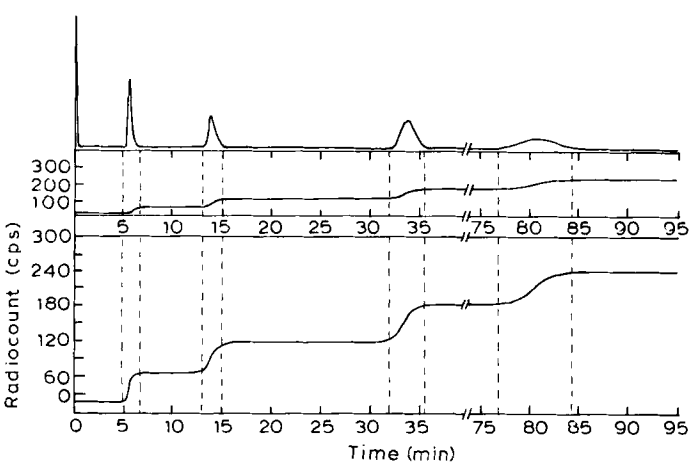

Fig. 7.2. The output from the radio-gas chromatograph designed by Popják *et al.* [11]. (Reproduced with permission.)

This early instrument was developed further [12] so that the output from the scintillator was monitored by a pair of photomultiplier tubes connected through a coincidence circuit. Moreover, with pulse height analysis it was possible to monitor two isotopes simultaneously in the same sample. The instrument behaved consistently and reproducibly with a variation of ±3% when used for the analysis of ^{14}C-labelled fatty acids. The background count-rate of this modified radio-GLC apparatus was about one-tenth of that observed when a single photomultiplier tube was used and a ^{14}C-labelled component containing as little as 15–30 cpm above background could be detected. Both ^{3}H and ^{14}C were monitored in the detector although there was an overlap of 16% of the ^{14}C counts into the ^{3}H channel. The true ^{3}H count could then be obtained either by correcting numerically or an electrical subtracting circuit could be used.

A weakness of the systems described above was that variations in counting efficiency might occur during a run due to quenching of the liquid scintillator. This could happen if, for example, coloured material emerged from the GLC column or if the stationary phase bled off the column directly into the liquid scintillator.

Although this did not appear to be as serious a problem as expected with the type of sample (natural products) analysed by Popják *et al.* [12], the problem could be minimised by using a combustion procedure.

Combustion methods

In 1962 Karmen and co-workers [13] developed a flow-through radio-GLC method in which labelled compounds in the column effluent were oxidised over hot copper oxide to carbon dioxide and water. If ^{3}H was present, the tritiated water produced was then reduced to $^{3}H_2$ over hot iron. The effluent containing $^{14}CO_2$ and/or $^{3}H_2$ was then passed through a cartridge of anthracene crystals and a scintillation counter continuously monitored the radioactivity in the anthracene detector. The apparatus is shown schematically in Fig. 7.3. Karmen *et al.* studied the effect of varying several detector parameters, including the size of the detector. When the cell diameter was increased, a decrease in counting efficiency was observed although this was less marked for ^{14}C than for ^{3}H. The detector efficiency was measured at different photomultiplier voltages and ranged between 65 and 86% for ^{14}C and between 10 and 12% for ^{3}H. The background count-rate was rather high, namely 30–50 cpm for ^{14}C- and 100 cpm for ^{3}H-counting conditions. It was found that although the photomultiplier tubes contributed largely to the high background, other factors such as the condensation of traces of radioactivity onto the anthracene during cooling periods (when the instrument was not in use) also contributed. The lower limit of sensitivity of the instrument was 1000 dpm in a single injection.

Drawert and Bachman [14] reviewed detection methods available for radio-GLC in 1963 and they also evaluated several detectors. For continuous measurement of radioactivity a proportional counter tube was preferred and hydrogen–methane was used as counting gas. The proportional counter was built to the design of Curran [15] and its plateau characteristics were determined for a range of hydrogen–methane compositions. The optimum range was found to be 80–60% hydrogen and 20–40% methane. The internal volume of the proportional tube was 63 ml which is very high relative to those currently in use. Such a volume clearly permitted a long residence time to be obtained for compo-

nents in the gas stream but the risk of mixing of adjacent peaks in the counter was higher.

With this apparatus, which is illustrated in Fig. 7.4, both the combustion method (to $^{14}CO_2$ and $^{3}H_2O$) and a cracking procedure (of hydrogen over a Raney nickel catalyst at 420°C) prior to the proportional counter were used.

James and Piper [16,17] also favoured the approach of converting radiolabelled compounds into $^{14}CO_2$ or $^{3}H_2O$ so that the proportional counter could be operated at ambient temperature. Various experimental designs were evaluated, including the continuous

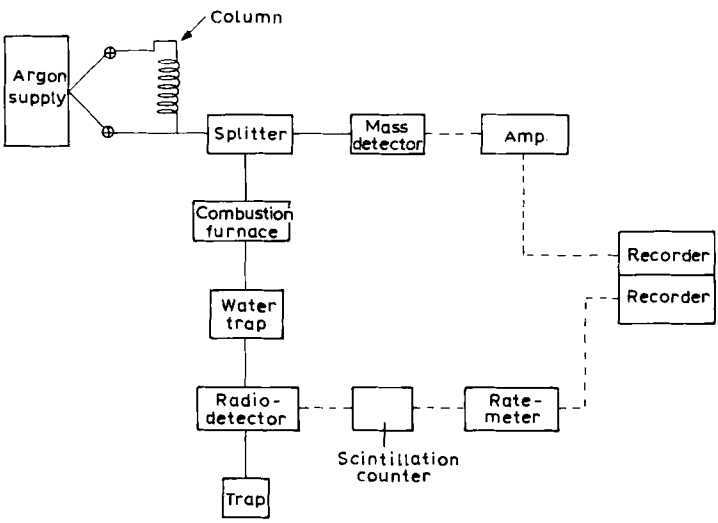

Fig. 7.3. A schematic diagram of the integrated gas chromatograph–scintillation counter designed by Karmen *et al.* [13]. (Reproduced with permission.)

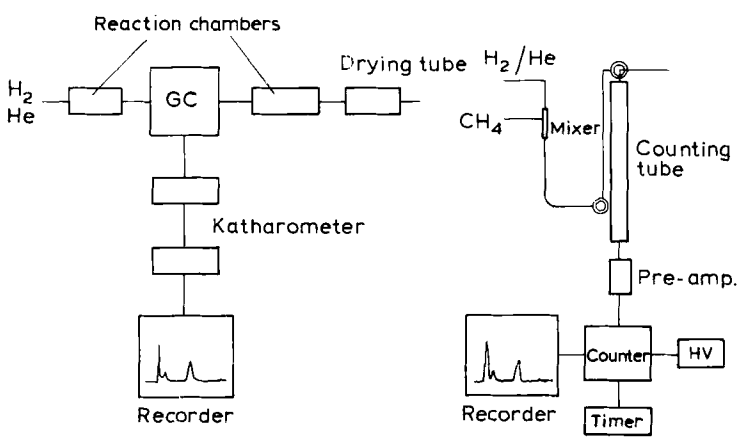

Fig. 7.4. A diagram of the radio-GLC system used by Drawert and Bachman [14]. (Reproduced with permission.)

system shown in Fig. 7.5. An earlier system [16], which consisted of a combustion train connected to a GLC outlet and coupled to a proportional counter, was available commercially for several years (Pye Scientific Instruments). The improved arrangement shown in Fig. 7.5 comprised a metal or glass column coiled around a heated aluminium former. The column was again attached to the combustion furnace, which contained copper oxide and iron powder. Argon was used as carrier gas for GLC and 5% CO_2 was added to the gas stream before it entered the proportional counter. As the diagram shows, a katharometer detector was used for hydrogen arising from the reduction of water vapour.

The apparatus as described was suitable for monitoring either ^{14}C or ^{3}H and an alternative arrangement (Fig. 7.6) was set up when simultaneous counting of both isotopes was required. The first counter measured both $^{14}CO_2$ and $^{3}H_2$ after which the $^{14}CO_2$ was absorbed so that the second counter detected only tritium. A typical output from the instrument is shown in Fig. 7.7. In routine use the background count-rate was 15–20 cpm for ^{14}C. Further improvements were described by James and Hitchcock [18] and the application of the instrument to fatty acid analysis is illustrated later in this chapter.

At a symposium on radioisotope measurement techniques in 1965, Simon and coworkers [19] described an instrument based on a Perkin-Elmer GLC apparatus and Berthold counting tubes, again based on combustion and proportional counting. However, it was recommended for use mainly with combustion under reducing conditions to give more efficient determination of ^{3}H. For operation under reducing conditions the combustion tube was packed with a mixture of zinc, iron and oxides of cobalt, nickel and vanadium. The tube was operated at 620–700°C depending on the nature of the sample. The instrument was available commercially for several years and counting tubes of various

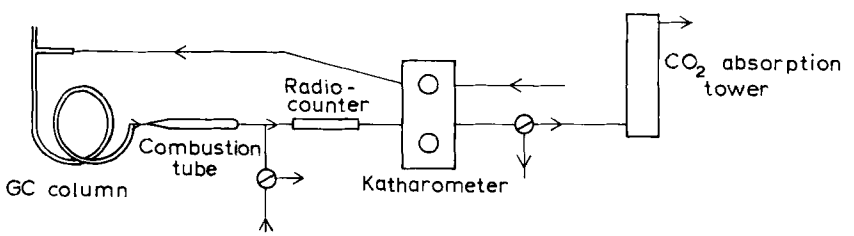

Fig. 7.5. A schematic flow diagram for either ^{14}C or ^{3}H counting using the radio-GLC system of James and Piper [17]. (Reproduced with permission.)

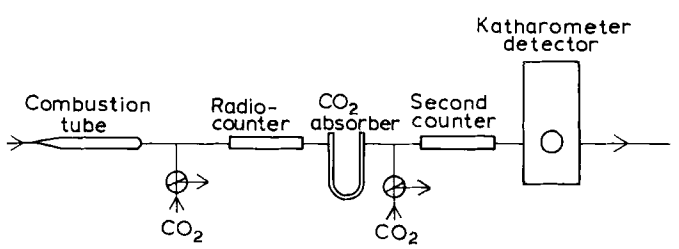

Fig. 7.6. A schematic flow diagram for simultaneous ^{14}C and ^{3}H counting using the system of James and Piper [17]. (Reproduced with permission.)

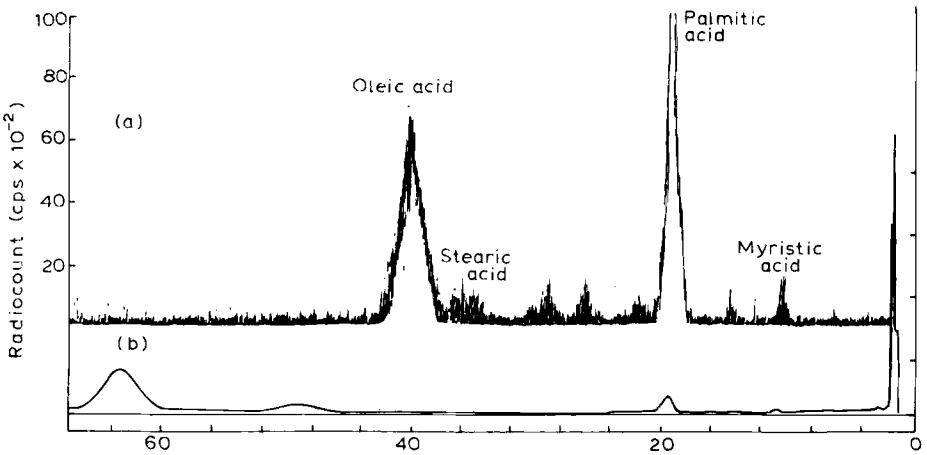

Fig. 7.7. A typical output from James and Piper's radio-gas chromatograph [17]. (a) Radioactive trace, (b) FID trace. (Reproduced with permission.)

volumes ranging from 10 to 100 ml could be used. Although the high-volume tubes were unsuitable for mixtures of compounds with similar retention times, they did serve to extend the detection limit of the instrument.

A criticism of some of the radio-GLC systems already described was made by Swell [20] in 1966 who felt that existing methods lacked sensitivity, had operating temperature limitations or were not very efficient for the analysis of ^{3}H. Swell designed a system with two columns and detectors for simultaneous, accurate measurement of mass as well as radioactivity. From the diagram of the apparatus (Fig. 7.8) it is apparent that it was similar to a system described by Karmen [3] but it had greater versatility. By changing the composition of the combustion tube, either ^{14}C or ^{3}H or both isotopes could be determined as shown in Table 7.1. The counting efficiency of the proportional counter was found to be, on average, 92.5% for ^{14}C and 61.5% for ^{3}H and the background count-rate was 40–50 cpm. The instrument was very sensitive with at best lower limits of detection of 75 dpm and 100 dpm respectively for ^{14}C and ^{3}H although 3–4 times these amounts were preferred as a minimum.

In continuous methods based on combustion which have been described so far, the labelled compound in the gas stream is oxidised either after it has passed through the mass detector or, more commonly, after the gas stream has been split between the mass detector and the radiodetector. In an instrument described by Martin [21] the total effluent from the GLC apparatus was combusted *prior* to mass and radioactivity measurement at ambient temperature. This was done by using a microthermistor detector to determine the total carbon dioxide produced. Propane was then added to the helium gas flow to produce a counting gas suitable for monitoring the radioactive content of the carbon dioxide.

The major advantages of this design were that it was very sensitive and no correction for splitting (or care to avoid problems associated with a stream splitter) was needed. Its

use was limited, though, to ^{14}C-labelled compounds only and furthermore the background for the unshielded detector was approximately 60 cpm, which was high for an instrument used for the analysis of small quantities. Presumably the background could be reduced for low-level work by suitable shielding of the proportional counter.

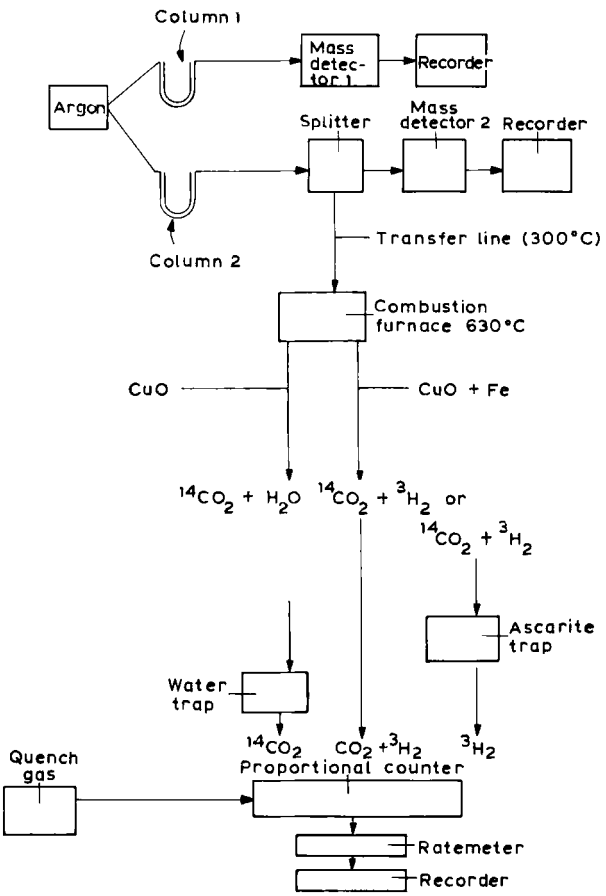

Fig. 7.8. A block diagram of the radio-GLC system used by Swell [20]. (Reproduced with permission.)

TABLE 7.1

THE DETECTION OF ^{14}C OR ^3H ALONE OR IN THE PRESENCE OF BOTH ISOTOPES (AFTER SWELL [20])

Isotope to be determined	Isotopes in sample	Combustion tube contents
^{14}C	^{14}C	Copper oxide + magnesium perchlorate
^{14}C	$^{14}C + {}^3H$	Copper oxide + magnesium perchlorate
^3H	^3H	Copper oxide + iron filings
^3H	$^{14}C + {}^3H$	Copper oxide + iron filings followed by an ascarite trap to remove $^{14}CO_2$

A low-background detector

The introduction of a very low background detector for radio-GLC in 1968 may be regarded as something of a breakthrough for low-level work. Simpson [22] used a radio-gas chromatograph design based on proportional counting after combustion and he incorporated, amongst other features, an anti-coincidence circuit with the detector. This resulted in a background count-rate of only 1–2 cpm compared with values of at least 25 cpm with detectors reported previously. A diagram of the apparatus described by Simpson is shown in Fig. 7.9.

In detail, the detector comprised a proportional counter, a cosmic guard counter, a cylinder of plastic phosphor, a graded shield, an anti-coincidence circuit and an output display. The signal from the proportional counter was fed to the input of the anti-coincidence gate which rejected those signals which were concident with signals from the cosmic guard counter (which must have originated from outside the proportional counter). The anti-coincidence signal was then passed to a ratemeter the output of which was usually displayed on a chart recorder.

The volume of the proportional counter was 12 ml and its counting efficiency was calculated from the following formula to be 94.5% for ^{14}C and 64% for ^{3}H.

$$E\,(\%) = \frac{\text{observed cpm} \times \text{flow-rate (ml/min)} \times 100}{\text{applied radioactivity (dpm)} \times \text{counter volume (ml)}}$$

The background count-rate was measured under different operating modes to find out how effective different parts of the counter were in reducing the background. When the proportional counter was unshielded the background was 39 cpm. The lead/steel/copper shield reduced this to 15 cpm and the anti-coincidence circuit reduced it further to the 1-cpm level. It is interesting to note that the contribution arising from cosmic events was 8–9 cpm.

Instruments based on this design are commercially available from Panax Instruments (with Pye Unicam) and, more recently, from ESI Nuclear. Two early examples of the

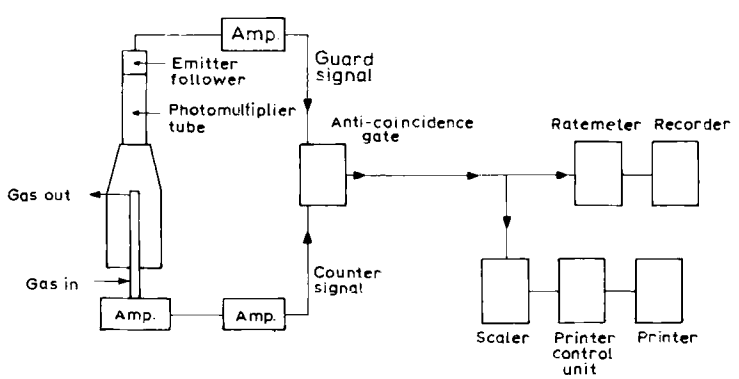

Fig. 7.9. A block diagram of the radio-gas chromatograph with a low-background detector designed by Simpson [22]. (Reproduced with permission.)

Panax Instruments were described by Strong and co-workers [23]. The background count-rate of one system was found to be 3–4 cpm when in general use and it remained constant at this level over a period of 10 months. When the column temperature of the gas chromatograph was increased, however, the background often increased to 8–9 cpm. When a low flow-rate was used (10 ml/min) the counting efficiency for ^{14}C was 95% but with the higher flow-rates which were more commonly used (*ca.* 50 ml/min) the overall efficiency dropped to around 20%. This was a direct result of reducing the residence time of $^{14}CO_2$ in the counter and emphasises the need for lower flow-rates with radio-GLC than with conventional GLC. Typical chromatograms from the Panax instrument [23] are shown in Fig. 7.10.

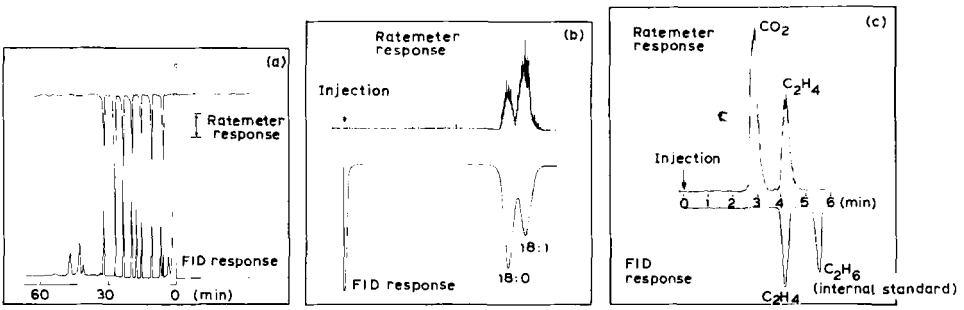

Fig. 7.10. (a) Radio-gas chromatogram of methyl esters of ^{14}C-fatty acids on a 1.5 m long column packed with diethylene glycol adipate on 100–120 mesh Diatomite C. (b) Radio-gas chromatogram of methyl esters of ^{14}C-stearic acid (18:0) and ^{14}C-oleic acid (18:1) on a 1.5 m long column packed with 5% polyethylene glycol adipate on 80–100 mesh Chromosorb G. (c) Radio-gas chromatogram of ^{14}C-labelled CO_2 and C_2H_4 produced in tracer experiments. (Reproduced with permission from Strong *et al.* [23].)

Strong *et al.* also considered the operating conditions of radio-GLC which gave the optimum performance in terms of sensitivity. Regarding the split ratio between the radio-detector and the mass detector, calculations showed that the counts due to the sample were proportional to both the counting efficiency and the counter volume. If argon or helium is used as carrier gas for GLC a small concentration of a quenching gas such as CO_2 must be added to the GLC effluent. Alternatively argon containing 5% CO_2 is available in cylinder form and can be used throughout.

Recent liquid scintillator systems

Most of the "continuous" methods described so far have favoured the use of a proportional counter but Schutte and Koenders [24] reported in 1973 another system based on liquid scintillation counting. These authors once again emphasised the limitation of combustion methods in that they are applicable only to ^{14}C- and ^{3}H-labelled compounds and a method involving passing the GLC effluent into a scintillator solution is more versatile.

Schutte and Koenders developed another system in which the GLC effluent was dissolved continuously in a stream of scintillator solution. Their system is shown schematical-

ly in Fig. 7.11. It is based on a Hewlett-Packard 5750 gas chromatograph with a splitter leading to a flame ionisation detector and a scintillation spectrometer (Tracerlab Cornflow Model SCE-542) with a dual-pen recorder to display the output. Fractions were subsequently collected as they left the flow cell so that the radioactivity in a peak could be determined more accurately using a liquid scintillation counter. The counting efficiency of the flow cell was calculated using the formula on p. 141.

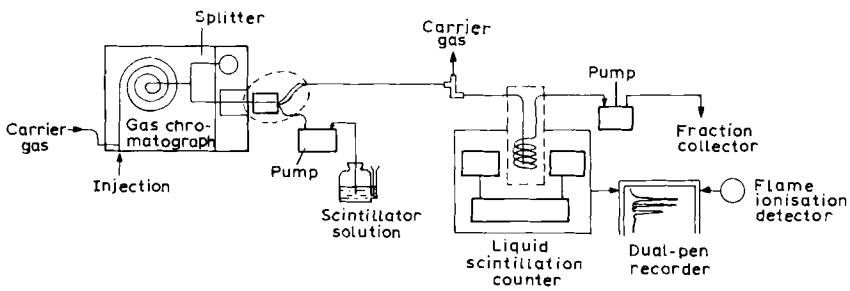

Fig. 7.11. A schematic diagram of the apparatus for continuous radio-GLC described by Schutte and Koenders [24]. (Reproduced with permission.)

Typical results using ^{14}C and ^{3}H standards are shown in Fig. 7.12a; a separation of a mixture of organic standards is shown and it can be seen that the limit of detection of the scintillation flow cell was approximately 0.2 nCi. In Fig. 7.12b the ability of the instrument to deal with both ^{3}H and ^{14}C in the same sample is shown.

It is clear from these results that the instrument is efficient, sensitive and versatile in the variety of isotopes that can be detected without the necessity of a combustion step. Perhaps its major disadvantage lies in the high cost of the radiodetector and the back-up use of a liquid scintillation counter, although this is not essential.

Semiconductor detection

Another economical and versatile radio-GLC method based on a rather different detection principle was recently described by Tykva and Šeda [25]. The radioactivity in the gas stream was monitored directly with a semiconductor detector which can be used for all β-emitters (*e.g.*, ^{3}H, ^{14}C, ^{35}S and ^{32}P). In addition to this isotope versatility, semiconductor detectors have such advantages as low background and stability in operation but suffer from lack of sensitivity, with a detection limit of approximately 150 nCi. However, the value of this simple device was that it could be fitted to most conventional gas chromatographs and could be considered for the analysis of samples containing relatively high concentrations of radioactive compounds boiling in the approximate range 30–200°C.

Buffer storage

In an attempt to retain the convenience of continuous-flow radio-GLC but to enhance

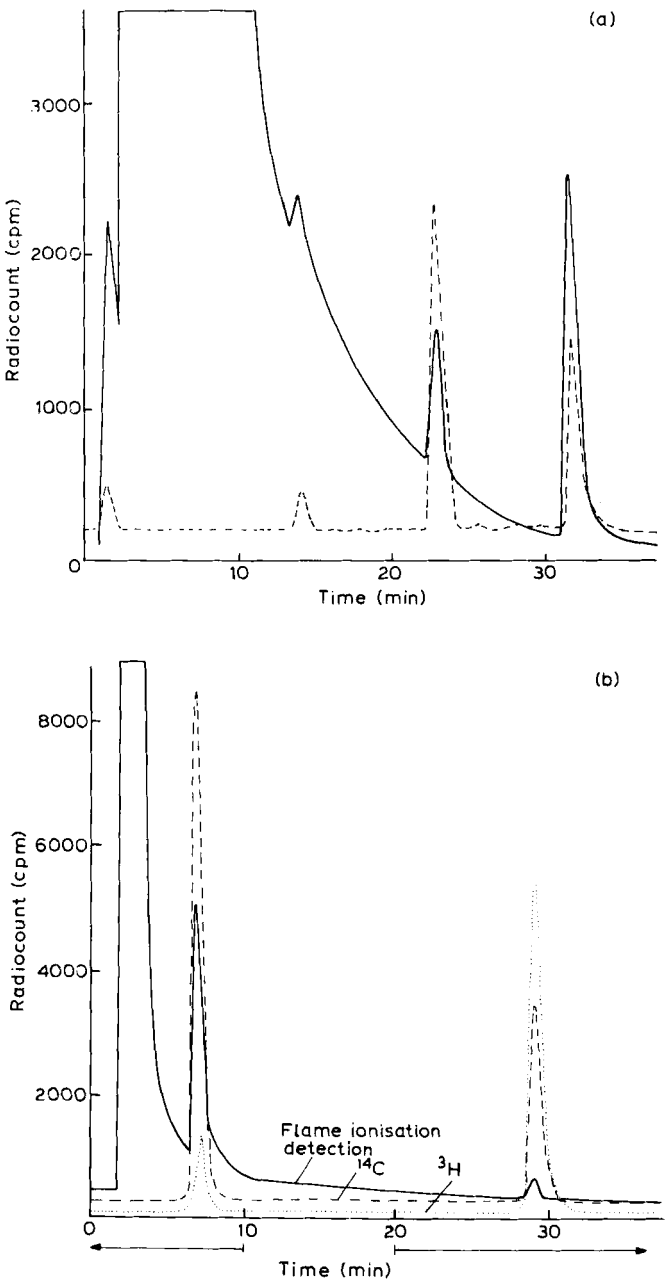

Fig. 7.12. (a) The analysis of ^{14}C-labelled standards using the radio-GLC system of Schutte and Koenders [24]. ——, Flame ionisation detection; – – –, radiodetection. (b) Dual-label detection with the radio-GLC system of Schutte and Koenders [24]. (Reproduced with permission.)

its sensitivity, Karmen and Longo [26] reported a "buffer storage" procedure. The idea was to use a conventional combustion method to form $^{14}CO_2$ or $^{3}H_2O$ and to trap the labelled vapours in sodium hydroxide, which was channelled through a length of narrow tubing for storage. In this way the high resolution of the GLC column would be retained. For ^{14}C-compounds, two methods of trapping were evaluated. The more convenient method was to use a 7 cm × 3 mm I.D. glass tube containing either Chromosorb W (80–100 mesh) or Chromosorb T polytetrafluorethylene (30–60 mesh) held in place with glass wool plugs. This was connected through a simple debubbler to 3 mm I.D. polymeric tubing for storage, and a tube of anthracene placed in a Packard Tri-Carb flow counter was used to detect the radioactivity. The use of the storage tube did not result in loss of resolution or peak broadening and had the advantage that eluates containing low concentrations of radiolabelled compounds could be pumped slowly through the flow cell to obtain better counting statistics.

An alternative method of radioassay suggested by Karmen and Longo was to continuously precipitate the $^{14}CO_2$ as barium carbonate onto a moving paper strip which could subsequently be assayed for radioactivity using a paper chromatogram scanner. Such a method is likely to suffer from mechanical and handling problems, however.

Discontinuous methods

Perhaps the major criticism of continuous flow-through methods of radio-GLC is that they are inherently less sensitive than discontinuous or trapping methods because of the relatively short counting time. When radiolabelled compounds are trapped out from the gas stream a radio-counting technique and counting time can be chosen to give more precise quantitative data than would be obtained from a continuous system. Moreover, trapping techniques can be simple and inexpensive and existing radiochemical equipment (such as a liquid scintillation counter) can be used for the radioactivity determination.

Numerous approaches have been used including trapping labelled compounds in liquid scintillator [27–29] on anthracene coated with a silicone oil [30,31] or some other hydrocarbon followed by scintillation counting [32], or in cooled traps [33]. Alternatively, when ^{14}C-labelled compounds are being separated, combustion of the compounds in the gas stream could precede the trapping stages [34,35]. For example, the method of Trenner *et al.* [35] involved a GLC/combustion step followed by trapping of the $^{14}CO_2$ formed in Hyamine contained in vials, followed by liquid scintillation counting. The approach was devised in an attempt to combine the advantages of the combustion technique with the high sensitivity of liquid scintillation counting.

In practice, the effluent from the gas chromatograph was split with the minor part (25%) being passed to flame ionisation detector while the major part (75%) was passed to the combustion oven, which contained a quartz tube packed with copper oxide and steel wool. The gas stream containing $^{14}CO_2$ was dried in a magnesium perchlorate trap after which it was passed to a series of vials containing trapping solution (a mixture of Omnifluor (NEN, 4 g/l) and hydroxide of Hyamine 10-X (NEN) in the ratio of 3:1.8). Each vial had its own manually operated solenoid valve and the gas stream was passed through a given vial for the required time, after which it was diverted to a new vial con-

taining fresh solution. Gas flow-rates of up to 75 ml/min could be used with essentially quantitative trapping.

When using this type of manual switching procedure, allowances must be made for the time lag between the mass detector and the combustion oven outlet (because of the closer proximity of the flame ionisation detector to the GLC column), especially when closely resolved radiocomponents are emerging from the column.

This method of Trenner *et al.* proved to be a very sensitive technique, and samples containing less than 0.5 nCi could be determined satisfactorily.

Douglas and Black [36] recently described another highly efficient trapping method using Pyrex glass tubes filled with 3% Dow Corning DC 200 to 80–100 mesh Florisil. This economical method gave 90% recoveries and was used for trapping radiolabelled steroids, biogenic amines and alkaloids.

In 1969 Thomas and Dutton [37] described a radio-GLC method in which fraction collection and liquid scintillation counting were automated and therefore combined the advantages of both continuous and discontinuous methods. A stainless-steel collection tube was fitted to the GLC outlet and this was housed in an aluminium block heated to 250–260°C (Fig. 7.13). This was connected to a T-piece, and compounds in the gas

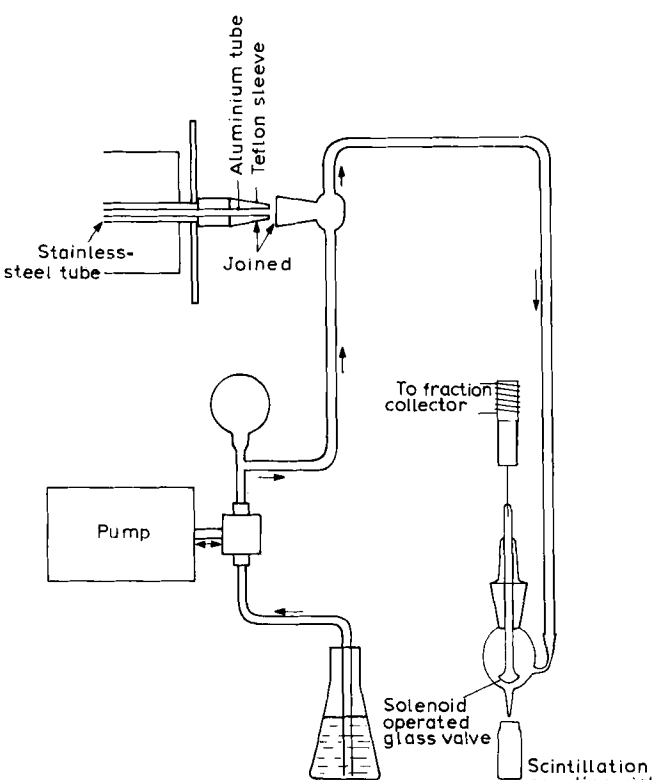

Fig. 7.13. An automatic system for the condensation of GLC effluent in liquid scintillator solution designed by Thomas and Dutton [37]. (Reproduced with permission.)

stream were collected in a stream of liquid scintillator which was passed to a fraction collection system operated on a time basis. The results from several repeat runs were processed by a computer and a profile of the average was plotted out. Samples with specific activities of only 1–10 nCi/mg could readily be analysed using this apparatus.

CHOICE OF METHOD FOR RADIO-GAS–LIQUID CHROMATOGRAPHY

From the foregoing survey it is apparent that the radio-GLC techniques which have been developed for the analysis of organic compounds are mostly variations of three basic approaches, namely:

(i) The effluent from the GLC column is continuously monitored for radioactivity using a gas-flow proportional counter either before or after a combustion step.

(ii) The effluent from the GLC column is continuously monitored for radioactivity by passing it into a liquid scintillator solution.

(iii) The compounds after separation on the GLC column are trapped individually for separate radioassay and analysis.

Each of these approaches has its advantages and its limitations and no single method is superior to the other for all applications. It is necessary for the individual user to decide which technique will best solve his problem and which apparatus would best complement his existing radiochemical equipment. Such a decision will be based on the considerations discussed below.

The only commercial radio-GLC instruments currently available in Great Britain (supplied by Panax Instruments and ESI Nuclear) are based on combustion and proportional counting.

Range of isotopes

All of the continuous and discontinuous methods described above are suitable for the analysis of ^{14}C-labelled compounds and most methods are also suitable for detecting ^{3}H. With both trapping and continuous liquid scintillation counting methods both ^{3}H and ^{14}C can be determined simultaneously but when a proportional counter is used separation of the isotopes is necessary to radioassay. The major limitation of modern instruments based on combustion and proportional counting are that only ^{3}H and ^{14}H can be detected with them and these instruments are not suitable for use with ^{36}Cl, ^{35}S and ^{32}P.

Sensitivity

Discontinuous methods followed by radioassay of fractions are inherently more sensitive than flow-through techniques and this has been discussed earlier in this chapter. There is, therefore, more control over the precision of radiochemical detection when fractions are radiocounted. With continuous systems, however, the difference in ultimate sensitivities of liquid scintillation flow systems such as those of Popják *et al.* [11,12] and Schutte and Koenders [24] and proportional counter instruments [22,23] are not very different.

As the sensitivity of detection in a flow-through system is dependent on the flow-rate,

conditions can be chosen to optimise sensitivity provided there is no loss of resolution when the flow-rate is reduced.

Experience with the commercial radio-GLC instruments supplied by Panax Instruments and ESI Nuclear (which have a particularly low background count-rate) has shown that less than 1 nCi of ^{14}C can be readily detected and often as little as 0.4–0.5 nCi is detectable in a single peak.

Resolution

The most effective way of retaining the resolution of the GLC column during subsequent radioassay is to trap very small fractions although this can be laborious and time consuming. As discussed previously, a proportional counter with a volume of 10–12 ml will not result in serious remixing of compounds passing through it as long as the flow-rate is not too low.

There are limitations (in terms of resolution) with those liquid scintillator systems in which the effluent is passed continuously into the same scintillator. An integral output is produced in this situation and the amount of radioactivity in the scintillator increases with time. Consequently, it can be difficult to detect and quantify a minor component if it follows a major component (see Fig. 7.2).

The resolution achieved by the automated liquid scintillation method of Thomas and Dutton [37] illustrates the good resolution and precision that can be achieved with a fraction collecting method.

Speed of analysis

The commercially available radio-GLC instruments referred to earlier are very straightforward to use and useful results can be obtained within an hour of switching on and setting up the apparatus. Results are obtained directly from these instruments and from other continuous methods. One of the disadvantages of trapping techniques is that considerable time may need to be devoted to the subsequent handling and sub-sampling of fractions and the necessary radioassay, which is usually by liquid scintillation counting. This can be minimised if a collection method is chosen in which only individual compounds are collected as they leave the gas chromatograph, rather than a large number of fractions which are collected on a time basis. This is not easy to do unless a mass detector such as flame ionisation detector is used to indicate the emergence of a peak although it will not necessarily be known at that stage whether it is radioactive or not.

Some of the radio-GLC methods described in this chapter clearly involved a considerable amount of development time and this may not be feasible in many non-academic laboratory situations.

Cost and availability of equipment

In terms of capital costs the most inexpensive detection methods are those based on trapping methods, especially if ancillary radiocounting equipment is already available, since no dedicated detector need be linked directly to a chromatograph. However, the

cost in terms of time and chemicals of the radioassay of fractions can be relatively high and if radio-GLC is to be used in a laboratory on a regular basis then the purchase of a commercial instrument should be considered.

Recovery of radiolabelled materials

Continuous radio-GLC methods are destructive whether combustion analysis or liquid scintillation is used, but the amounts of material needed for an analysis are usually very small. In those situations where the amounts available for analysis are very limited or the cost of producing the sample is high, a trapping method would ensure that all of the material is recovered. However, some loss of material is inevitable when sub-sampling for radioassay.

RADIO-GAS–LIQUID CHROMATOGRAPHY IN PRACTICE

There are numerous approaches to discontinuous methods of radio-GLC and the GLC technique is not often affected by the collection method used. With flow-through methods, however, some special consideration to chromatographic technique is worth while in order to ensure the best results.

Commercial instruments

Most of the author's experience has been obtained using the radio-gas chromatograph shown in Fig. 7.14 which is based on a Perkin-Elmer 452 GLC instrument and a Panax radiodetector and, latterly, with the ESI Nuclear instrument (Fig. 7.15) with a Pye 104 gas chromatograph. A more recent version of the Panax detector is shown in Fig. 7.16.

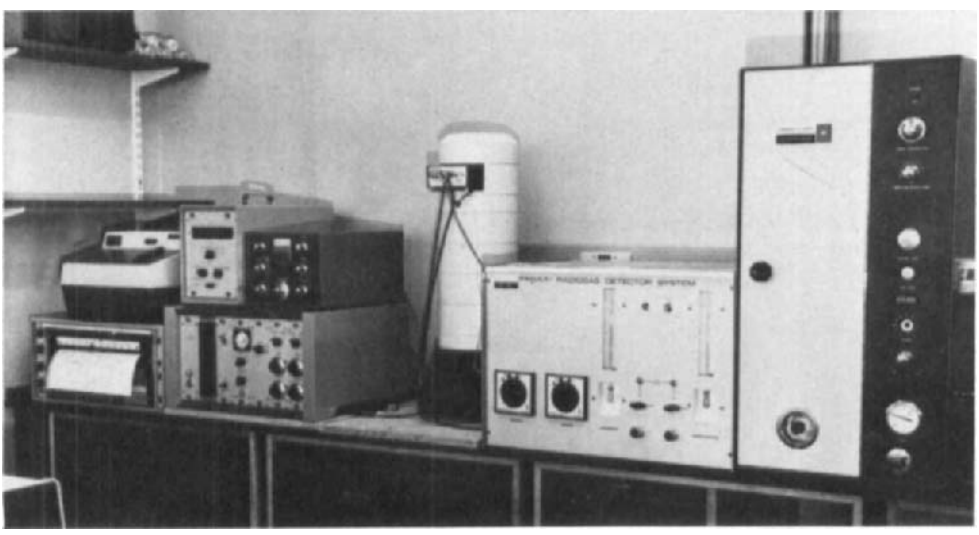

Fig. 7.14. A Panax radio-GLC system in use in the author's laboratory.

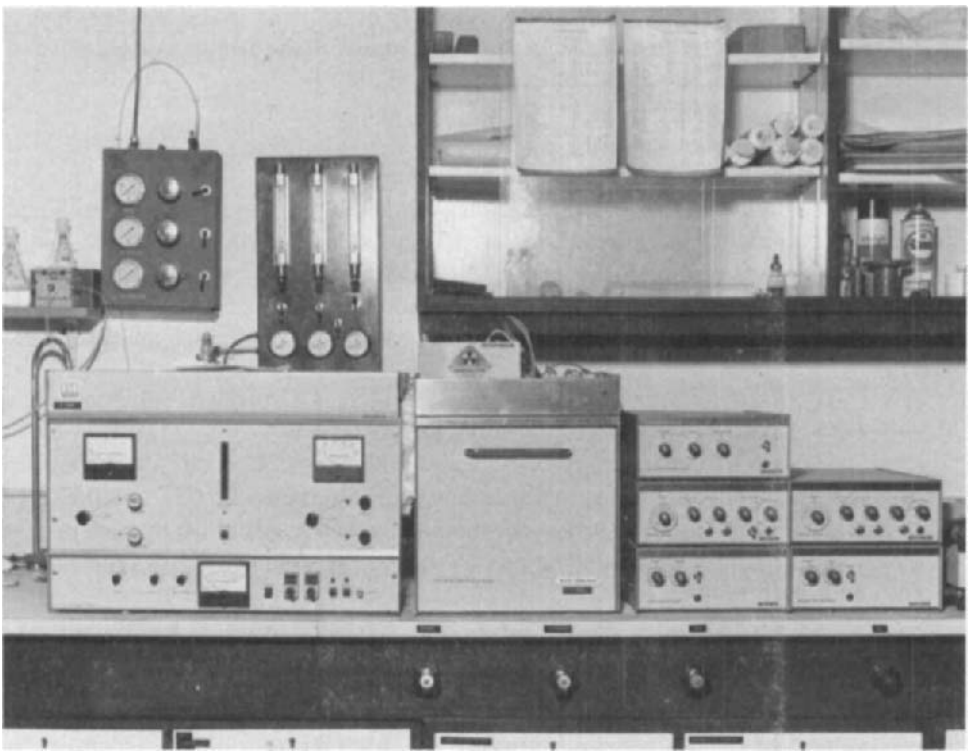

Fig. 7.15. The ESI Nuclear radio-GLC system. (Reproduced with permission from ESI Nuclear Ltd.)

Fig. 7.16. A recent version of the Panax radio-GLC instrument. (Reproduced with permission from Panax Equipment Ltd.)

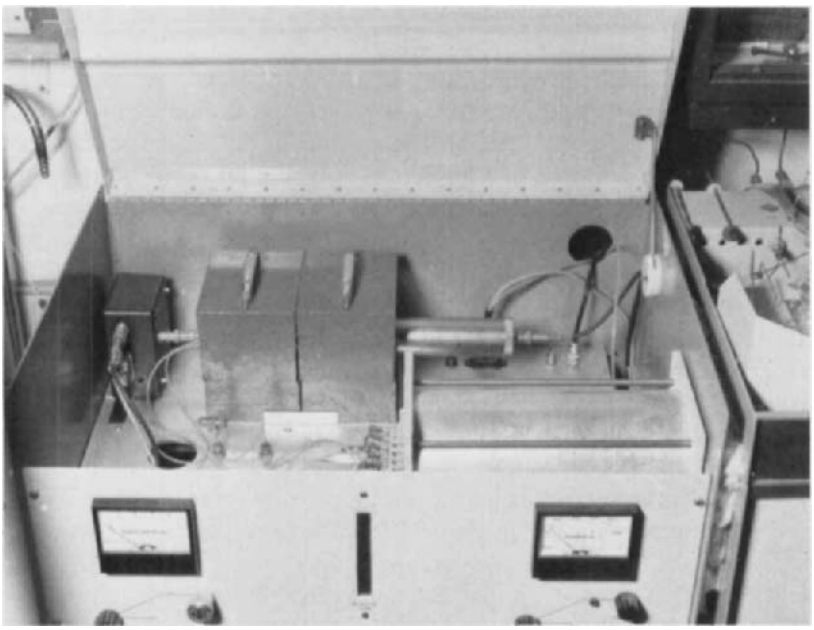

Fig. 7.17. The arrangement of the proportional counter and combustion oven in an ESI Nuclear radio-GLC instrument.

Both the Panax and ESI Nuclear instruments are very similar in design although the lay-out of the ESI instrument has been simplified with the proportional counter and lead casing housed within the furnace unit, as shown in Fig. 7.17. A flowmeter is also supplied with this instrument.

With either instrument it is common practice to split the effluent from the GLC instrument so that part is passed to a mass detector (usually flame ionisation or electron-capture detector) and the other part is passed to the radiodetector. A split ratio should be chosen to channel suitable amounts of the separated compounds to each detector. In the apparatus shown in Fig. 7.14 an electron-capture detector with a ^{63}Ni source is used and this has a much greater sensitivity than has the proportional counter detector. Consequently only 5–10% of the GLC effluent was passed to the electron-capture detector and 90–95% to the radioactivity detector. When the less sensitive flame ionisation detector is used on the Pye 104 a 1:1 split ratio is more suitable.

One problem with the electron-capture detector is its high sensitivity in that compounds with a good electron-capture response may overload the detector when amounts are present in a concentration suitable for the proportional counter. As a result, the electron-capture detector does not always operate in the linear range and its response cannot always be used to obtain accurate quantitative data. This is not a serious drawback since the output of the radioactivity detector can be used for quantitation. It does mean, however, that the electron-capture data could not be used for specific activity measurements. In this case, separate analyses of a diluted solution on a conventional gas chromatograph is necessary.

Gas–liquid chromatographic operating conditions

In continuous radio-GLC methods incorporating a combustion furnace a flow-rate lower than in normal GLC is common. The main reason for this is to increase the residence time of compounds in the proportional counter. The presence of the packed combustion tube tends to create some back-pressure and this results in typical flow-rates of between 60 and 100 ml/min. It is important to check the flow-rate regularly during a run since variations can occur and the efficiency of the proportional counter varies with flow-rate. In this respect, the flowmeter incorporated into the ESI Nuclear instrument is very useful.

GLC operating conditions should be selected to give as efficient a separation as possible and this is particularly important in low-level work. Each component of a mixture will then pass through the proportional counter in as small a volume as possible and the best signal-to-background noise response will be obtained.

Some consideration must be given to the carrier gas and the use of a suitable quenching gas for operation of the proportional counter. If argon is the carrier gas there is provision with the Panax and ESI Nuclear detectors for the introduction of a measured proportion (usually 5%) of carbon dioxide to make up a suitable counting gas. This is done after the GLC column and before the combustion oven. A simpler alternative is to use an argon–carbon dioxide (95:5, v/v) mixture which is available in cylinder form.

The stationary phase in the GLC column and the oven temperature should be chosen so that bleeding of the phase into the combustion furnace tube is kept to a minimum since this can lead to contamination and eventually to blocking of the packed tube. As contamination of the catalyst builds up, the flow-rate will fall and complete combustion of the labelled products may not be achieved.

The furnace tube

For the combustion of ^{14}C-labelled compounds to $^{14}CO_2$, copper oxide is used as catalyst and iron filings are used for the reduction of $^{3}H_2O$ to tritium gas. The tube is packed first with copper oxide and then with iron (separated by quartz wool plugs) and finally magnesium perchlorate is introduced to absorb any water vapour in the combusted effluent. The tube should be packed quite tightly to ensure that there are no air spaces in which mixing of consecutively eluting compounds could occur. However, a tube packed too tightly will cause a reduction in the overall flow-rate through the system.

Once the combustion tube is connected to the GLC outlet (via a short, heated connection) a freshly packed furnace tube should not be connected to the proportional counter tube until it has been maintained at temperature for 1–2 hours by which time any volatile material which might contaminate the proportional tube will have escaped.

From our own experience it is not necessary to repack the furnace tube very often. The magnesium perchlorate tends to solidify as it absorbs water vapour but it can be removed and replaced easily and quickly when the radio-gas chromatograph is used intermittently. As long as the end of the furnace tube is closed when the instrument is not in use there are rarely "start-up" problems and results can be obtained within 2 hours of first switching the furnace on. Since it is recommended to increase the temperature of the furnace in a stepwise manner, some time is required for this.

Nature and purity of samples

The nature of the sample to be injected into the radio-gas chromatograph will vary from ideal samples such as pure radiochemicals to radiolabelled compounds isolated from reaction mixtures or biological material. The latter samples, which will almost certainly be contaminated with non-radioactive impurities, may require extensive clean-up before they can be introduced into the radio-gas chromatograph. Experience has shown, however, that although unlabelled impurities present in solution can introduce additional peaks on the mass detector side, their presence in low concentrations rarely interferes with the detection of the labelled compounds by the proportional counter. In general, though, it is undesirable to introduce impurities onto the GLC column if they can be removed by a prior clean-up such as solvent–solvent partition or column chromatography.

Background count-rates

The background count-rate claimed by Simpson [22] for his radiodetector was 1.01 cpm in the original paper and sales literature from Panax and ESI Nuclear claims backgrounds of 1–2 cpm and less than 3 cpm, respectively. Such low background count-rates can be achieved but in general the operating background is 5–6 cpm, when the detector is set up according to the recommended procedure. Although this is higher than the figures claimed it is perfectly acceptable even for low-level work where peaks containing less than 1 nCi are being measured.

The background count can increase for a number of reasons, the most common being incorrect adjustments of the high tension on the guard tube or minor electronic faults. When in use for long periods or when labelled compounds are held up on the GLC column (due to the use of incorrect conditions) then bleeding of radioactive material from the column can occur and this leads to a gradual increase in background. If this occurs the GLC tube should be disconnected from the radiodetector and either purged to remove the contamination or changed for a freshly packed column. This problem can arise when mixtures of unknown labelled compounds are chromatographed and involatile compounds are present in the mixture.

The proportional counter wire itself can become contaminated to give a rise to a higher background. Since it is not an easy job to take the proportional counter apart to clean and replace the wire, all other possible sources of high background should be looked into first. Finally, the presence of γ-emitting source in the vicinity of the radiodetector can be a cause of high background which is often overlooked. For example, the ^{60}Co source used for determination of the plateau region of the proportional counter should be well shielded and stored away from the radio-gas chromatograph.

Efficiency

The efficiency of the proportional counter used in these commercial instruments should be at least 90% for ^{14}C and at least 60% for ^{3}H. Simpson determined the values using $^{14}CO_2$ generated from $Na_2{}^{14}CO_3$ and [1,2-^{3}H]*n*-hexadecane and observed efficiences of 94.5% (±1% S.D.) for ^{14}C and 64% (±1.5% S.D.) for ^{3}H.

The efficiency is calculated from the following equation:

$$\text{Efficiency (\%)} = \frac{\text{observed cpm} \times \text{flow-rate (ml/min)} \times 100}{\text{applied radioactivity (dpm)} \times \text{counter volume (ml)}}$$

APPLICATIONS OF RADIO-GAS–LIQUID CHROMATOGRAPHY

Radio-GLC has been used to determine radiochemical purities although it is less commonly used than radio-TLC for this purpose. Specific activities can be readily measured using radio-GLC with a mass detector as well. Other areas in which the technique has found application include drug and pesticide metabolism, general biochemical studies, lipid and fatty acid analysis and trace analysis. Reaction radio-GLC does not fall within the scope of this chapter and its applications are not discussed.

It has been pointed out by Neumann and Schenk [38] that, in common with other radiochromatography techniques, a comprehensive review of the applications of radio-GLC is very difficult to make because the method itself is rarely indexed as a keyword. However, representative examples of radio-GLC applications are given below to its use and its limitations as an analytical tool.

In the majority of cases, continuous flow-through methods for radio-GLC are used and trapping techniques are less frequently referred to. Furthermore, the use of continuous radio-GLC is on the increase as judged from the number of reports in the literature in which the technique has been applied.

Radiochemical purity determinations

Radio-GLC can be a useful complementary method to radio-TLC for checking the purity of radiochemicals after synthesis and before they are used for experimental purposes since, for example, the presence of volatile impurities would not be apparent from radio-TLC data alone. For this reason, a range of separatory techniques should be used for the measurement of radiochemical purity.

A number of examples of purity determination by radio-GLC have appeared in the literature including ^{14}C-labelled hydrocarbons [39], detergents [46] and lipids [41].

Specific activity measurements

In order to measure specific activities of radiolabelled fatty acids, Bishop *et al.* [42] converted ^{3}H-labelled fatty acids into their methyl-^{14}C esters and ^{14}C-labelled acids into their methyl-^{3}H esters. For a known specific activity of a methyl group, the ^{14}C:^{3}H or ^{3}H:^{14}C ratio in the esters separated by radio-GLC was a direct measure of the specific activity of the fatty acids. This approach, which avoids the necessity of quantifying mass- and radio-peaks, is discussed in more detail on p. 155.

It is more common, however, to use separate mass and radioactivity measurements and the need for caution in the use of data from a mass detector which may not be operating with a linear response in a continuous-flow radio-GLC has already been referred to.

Analysis of fatty acids and lipids

Within the area of fatty acid chemistry and biochemistry, radio-GLC has found more frequent application than in any other single area of research [12,42–51] and some highlights of this work are discussed below. This work has centred on the biosynthesis of fatty acids and on their conversion into lipids.

Popják *et al.* [11,12] in fact designed their radio-GLC instrument for fatty acid analysis. Since other analytical techniques available at that time (such as paper and partition column chromatography) were either time consuming or inefficient for the analysis of fatty acids, the GLC analysis of their methyl esters was evaluated. Although the suitability of GLC for this type of separation of homologous fatty acid esters had been realised for some time [52], a convenient detection system for labelled derivatives was not available. The original Popják instrument [11] was used for separating ^{14}C-labelled fatty acid esters and the modified instrument [12] was used for simultaneous assay of ^{14}C- and ^{3}H-labelled compounds.

Blomstrand and Gurtler [44] purified fatty acids by radio-GLC without derivatisation and they developed a precise technique [53] for introducing an accurately known amount of labelled compound onto the gas chromatograph with the use of a platinum spiral.

In 1968 Kolb and Wiedeking [46] compared the radio-GLC analysis of ^{3}H- and ^{14}C-labelled compounds using combustion under reducing conditions (to give $^{14}CH_4$) with a gas chromatograph linked to a proportional counter. Using *n*-hexadecane as a standard, excellent counting efficiencies were obtained with this system (see Table 7.2).

In a separate experiment, a comparison was made between the use of oxidising or reducing conditions during the radio-GLC analysis of some ^{14}C-labelled fatty acids. When the specific activities of the methyl esters of a mixture of fatty acids from a plant extract were determined, very good agreement was found between the two operating modes. The resulting GLC traces are shown in Fig. 7.18 and the corresponding specific activities are given in Table 7.3. The results are clearly independent of the type of catalytic reaction used during combustion.

Further work was carried out on fatty acid separations by Syroezhko *et al.* [47] who established a procedure for quantitative radio-GLC analysis of mixtures of C_2–C_8 monocarboxylic acids and also for a mixture of isomeric 2-, 3- and 5-decanones.

A novel technique was described by Bishop and co-workers [42] for the accurate determination of specific activities of fatty acids based on dual labelling with ^{14}C and ^{3}H. One approach involved the analysis of the methyl esters of a mixture of ^{3}H-labelled fatty acids and then repeating the analysis *after* the addition of the ^{14}C-labelled ester of the acid whose specific activity was required. The first separation was carried out on an analytical

TABLE 7.2

COUNTING EFFICIENCES DETERMINED USING THE SYSTEM OF KOLB AND WIEDEKING [46]

Compound	Specific activity (μCi/g)	Activity determined (dpm)	Counting efficiency	Standard deviation
[1,2-^{3}H] *n*-Hexadecane	2.30	4500	98.4	±3.5
[1-^{14}C]*n*-Hexadecane	0.78	1500	102.4	±3.6

TABLE 7.3

SPECIFIC ACTIVITIES DETERMINED UNDER OXIDISING OR REDUCING CONDITIONS (AFTER KOLB AND WIEDEKING [46])

Compound	Specific activity (μCi/g)	
	Determined under oxidising conditions	Determined under reducing conditions
Methyl palmitate-^{14}C	30.0	30.5
Methyl oleate-^{14}C	21.6	20.0
Methyl linoleate-^{14}C	22.9	25.6

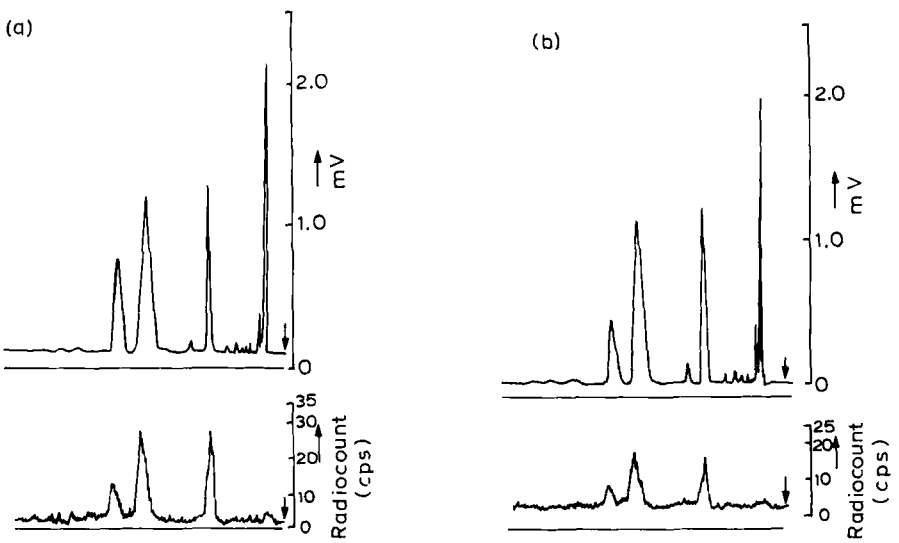

Fig. 7.18. Analysis of the methyl esters of ^{14}C-labelled fatty acids using (a) oxidising conditions and (b) reducing conditions during combustion of the GLC effluent. (Reproduced with permission from Kolb and Wiedeking [46].)

column whereas the second separation was made on a preparative GLC column. From the ^{14}C:^{3}H ratio of the eluted ester (determined by liquid scintillation counting) and from the peak areas in both separations the specific activity of the tritiated ester could be calculated. Their method suffered from the limitation that its accuracy depended on peak area measurement, which is difficult to do precisely.

For this reason a different approach was used, namely to use the ^{14}C label in the carrier as an indication of the recovery of the methyl ester through the analysis. When [^{14}C]-methanol of known specific activity was used for the methylation, the specific activity (with respect to the ^{3}H label) of the esters after GLC separation was directly proportional to the ^{3}H:^{14}C ratio.

For the calculation, the following simple formula was used, assuming the same sample of [^{14}C]methanol with constant specific activity was used throughout:

$$S\chi = \frac{R_\chi}{R_s} S_s$$

where S_s and $S\chi$ were the specific activity with respect to ^{3}H of the standard fatty acid and of the sample, respectively, and R_s and R_χ were the corresponding ^{3}H:^{14}C ratio only that is determined, quantitative trapping of the esters from the GLC column is not necessary.

The structural analysis of triglycerides has received considerable attention in recent years [49,50] and useful progress in analytical technique has been made. This area of analysis is a complex one and the procedure available for compositional analysis of substituted triglycerides involves several steps with possible losses at each stage. This creates a particular problem when radiolabelled triglycerides are being analysed because of the difficulty in relating the amounts of radioactivity found in each of the products to each other and to that in the original triglycerides. This problem was overcome again by the use of a suitable "carrier" or internal standard throughout the analysis. Details of the progress and results obtained with this basic approach were given in a recent review [50].

Biochemical studies

Radio-GLC has been applied to the analysis of radiolabelled sugars [54] and amino acids [24] after derivatisation, steroids [20,55] and other natural products [56–59]. It has also proved to be a useful technique in biosynthesis studies with ^{14}C-labelled substrates [60–63]. In this context Matucha *et al.* [60] studied the formation of higher fatty acids and amino acids formed when algae were grown in a $^{14}CO_2$ atmosphere and Hatanaka *et al.* [64] described some recent work on the biosynthesis of *trans*-2-hexenal in chloroplasts from plants.

In their work on the radio-GLC of steroids Derks *et al.* [55] used an unusual discontinuous method in which compounds were collected from the GLC effluent onto a TLC plate which was moved at a constant rate or in a stepwise mode. After the run, the TLC plate was scanned with a radioscanner in the usual way (see Chapter 4).

Drug and pesticide metabolism

Neumann and Schenk [38] have reviewed the radio-GLC analysis of drug metabolites. A major advantage of analysing complex mixtures which have been isolated from biological samples such as animal tissue is that relatively small quantities of radiolabelled material can be readily detected. The excess of unlabelled material which is co-extracted from the sample should not interfere with the radiochemical analysis.

It is clear from the recent literature that interest in the use of radio-GLC for drug metabolism studies is increasing [35,38,65–71]. Neumann and Schenk have outlined in detail the processing of extracts of different tissues prior to radio-GLC analysis. Rats were dosed with ^{3}H-labelled drugs and the various tissues were isolated and extracted. TLC and alumina column chromatography were used to clean up extracts of lymph, fat and brain

prior to radio–GLC analysis and a typical chromatogram is shown in Fig. 7.19. As tritiated compounds of high specific activity (200 nCi/mmole) were used it was possible to identify 1-ng quantities of the drug metabolites. The use of unlabelled "carriers" also gave better resolution and sharper peaks.

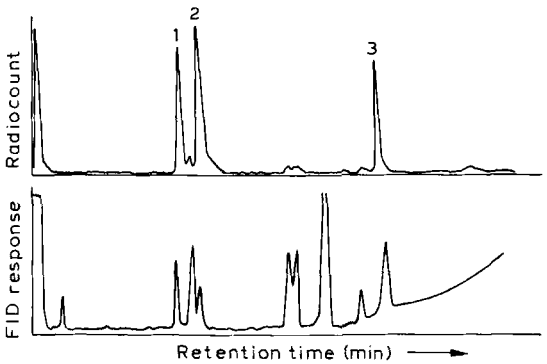

Fig. 7.19. A radiochromatogram of a lymph extract of a rat treated with ^{3}H-dimethylaminobibenzyl. Peaks: 1 = dimethylaminobibenzyl; 2, 3 = metabolites. (Reproduced with permission from Metsler and Neumann [65].)

There are fewer examples in the literature of the use of radio–GLC in pesticide metabolism studies. However, radio-GLC can be used to solve problems which are not amenable to radio-TLC or column methods. For example, when studying the fate in soil of the three major constituents of the nematicide D-D, namely *Z*- and *E*-1,3-dichloropropenes and 1,2-dichloropropane, which are volatile liquids, ^{14}C-labelled samples were prepared and initially their radiochemical purities were determined by radio-GLC [72]. The individual compounds were added to soil in glass flasks, and these were sealed and stored for the required incubation period. When a treated soil was to be analysed the flask was first cooled to below 0°C before extraction of the soil by shaking with acetone. The acetone extracts were chromatographed directly on the radio-gas chromatograph as shown in Fig. 7.14 and some typical chromatograms are illustrated in Fig. 7.20. The presence of the 3-chloroallyl alcohols in extracts of soils treated with the corresponding 1,3-dichloropropene can clearly be seen and the build-up of these compounds with time was monitored. In contrast, the 1,2-dichloropropane was apparently stable in soil as only the unchanged parent compound was present in the extracts.

Soil extracts from these experiments were also analysed by radio-TLC to obtain information on non-volatile degradation products and the complementary use of these techniques provided information on the overall fate of these rather volatile materials in soil.

Other examples can be found in the literature of pesticide metabolism experiments in which radio-GLC has provided information on the identity of radiolabelled metabolites [73–76] as well as a check on radiochemical purity before experimental work is begun [75,76].

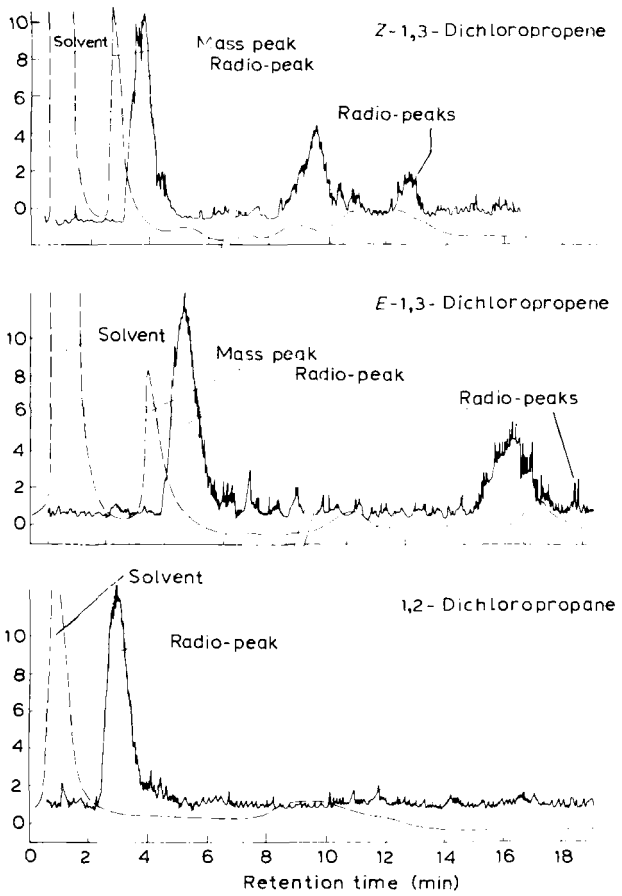

Fig. 7.20. Radio-GLC analysis of extracts of soils treated with *Z*- and *E*-1,3-dichloropropenes and 1,2-dichloropropane [75].

Pesticide residue analysis

Cannizzaro *et al.* [77] analysed soil around apple trees by radio-GLC for residues of a ^{14}C-labelled miticide which had been applied to the trees as part of a metabolism study. These workers emphasised the usefulness of radio-GLC for this work in that, as stated earlier in other examples, rigorous clean-up of extracts was unnecessary, which simplified the analysis considerably.

The radio-GLC was calibrated with a solution of the labelled miticide of known radioactivity content in the same way as a conventional GLC instrument is calibrated with solutions containing known weights of compound. Excellent agreement was found between the total radioactivity found in the soil extracts by liquid scintillation counting and that found by radio-GLC followed by peak area measurements.

It is surprising that more experiments of this type have not been reported since most pesticides are amenable to analysis by GLC. With the increasing tendency to carry out pesticide metabolism studies using radiolabelled compounds under controlled outdoor conditions [78] quantitative information on the persistence of such pesticides under "field" conditions could be obtained.

RADIO-GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

In recent years combined gas chromatography–mass spectrometry (GC–MS) has proved to be an extremely valuable technique for the separation and identification of unknown compounds from biochemical and metabolic studies and other sources. An exciting development of GC)MS which is being evaluated is to combine the technique with the specificity of radioactivity detection in a radio-GLC–MS instrument.

An instrument build in the Biochemistry Department, University of Pittsburgh was described recently [79] and other interfaces between radio-gas chromatographs and mass spectrometers have been referred to [80,81]. The Pittsburgh instrument was built from a Packard 894 proportional counter (Fig. 7.21) and an LKB 9000 GC–MS instrument. The oxidising furnace was fitted to the GLC oven housing of the LKB 9000 and the gas flow to the furnace passed through a heated inlet which penetrated into the GLC oven itself and it was linked through a splitter to the GLC–MS gas flow stream. The dual-pen recorder output displayed total ion current from the mass spectrometer and the ratemeter output from the proportional counter. Mass spectra could be obtained on any peak of interest (mainly radioactive ones) and any non-radioactive impurities could be ignored.

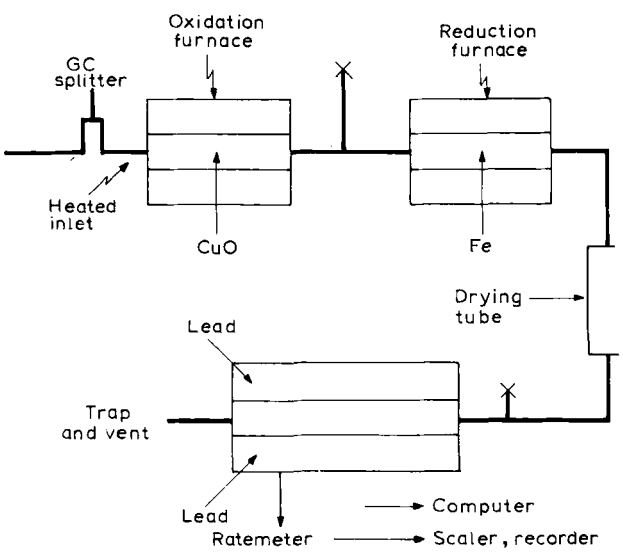

Fig. 7.21. A block diagram of the gas-flow counter used to build a radio-gas chromatograph–mass spectrometer by Nulton *et al.* [79]. (Reproduced with permission.)

Typical operating conditions were flow-rates of 100 ml/min through the GLC column and 10 ml/min of propane gas through the proportional counter with a 1:4 split ratio (MS:PC). The apparatus was used to identify biosynthetic intermediates in extracts of *Penicillium brevicompactum* and initially extracts were injected onto the radio-GLC–MS instrument (after suitable clean-up by gel filtration) to obtain a preliminary scan of the positions of mass and radioactive peaks. From this, optimal operating conditions and sample sizes for injection were obtained and a second injection was made from which mass spectral data were obtained. Identification of metabolites could then be made from comparisons of GLC retention time data and mass spectra with those of authentic samples.

More recently Yackovitch and Swann [81] listed the following criteria for an interface between a gas-flow proportional counter and a mass spectrometer:

1. It must be simple and convenient to use.
2. It must be adaptable to both chemical ionisation (CI) and electron impact (EI) systems.
3. It must not adversely affect the performance of either the mass spectrometer or the proportional counter.

The low-background radio-GLC system (Panax Instruments) was chosen and this has been successfully interfaced with a Finnigan Model 3100 D GC–MS apparatus. The proportional counter system used was found not to be sensitive to changes in flow-rate.

An important feature of this radio-GLC–MS instrument is that the effluent from the gas chromatograph flows through a glass-lined microvalve which, when closed, directs all the effluent to the mass spectrometer. As the valve is opened, part of the effluent is directed to the radiodetector. Consequently, the mass spectrometer can be used normally when non-labelled compounds are being analysed.

For operation of the gas proportional counter, argon and carbon dioxide are metered, combined and fed into the GLC oven in a stainless-steel coil in order to preheat the gases. The GLC effluent and the combined gases are then fed into the combustion furnace and the transfer line between the GLC oven and the furnace is maintained at 20°C above the GLC oven temperature. This prevents condensation of the GLC effluent in the transfer line. The effluent is then combusted in the usual way.

The instrument has been evaluated using real samples (with minimal clean-up) of ^{14}C-labelled herbicides extracted from rat faeces and it has been shown to meet all the requirements listed above.

The combined technique of radio-GLC–MS clearly has great potential as a method of obtaining structural identifications of labelled compounds in biological samples more quickly and with less clean-up than is necessary for conventional GC–MS.

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Chapter 8

Miscellaneous applications related to radiochromatography

CONTENTS

INTRODUCTION

There are a number of applications of radioisotopes which involve separations and which are, therefore, related to radiochromatography. Several of these have been collected together in this chapter since they illustrate the usefulness of radioisotopes in biological studies, and the methods of detection used are similar to those described in earlier chapters.

SOIL LEACHING STUDIES

Soil columns

Radioisotopes have been used to study the movement of fertilisers and inorganic ions or foreign compounds, particularly pesticides, through soil. Possingham [1] reviewed some early studies with ^{32}P in which ^{32}P-labelled superphosphate was actually applied to field plots. Samples were taken from different depths and assayed for ^{32}P either with a Geiger–Müller counter or by autoradiography of samples placed in small containers.

Although γ-emitting isotopes in soil columns have been monitored using an external Geiger–Müller counter, with ^{14}C- and ^{3}H-labelled compounds it is necessary to treat the column with the radiolabelled sample, elute with water and collect fractions from the foot of the column for liquid scintillation counting. A semi-automatic apparatus for studying the leaching behaviour of pesticides in soil which is in use at Shell Biosciences Laboratory is illustrated in Fig. 8.1.

Glass columns (45 cm × 4.5 cm I.D.) fitted with stopcocks were plugged with glass wool and packed with soil. They were then filled with water slowly from the bottom and allowed to drain. The radiolabelled compound was then applied to the top of the soil column which was covered lightly with glass wool. Water from an automatic dispenser was applied to the column at regular intervals (*e.g.*, 2 ml/h) and the column eluate was collected in a fraction collector. With the apparatus shown in Fig. 8.1 a number of columns could be eluted simultaneously and samples of the eluate fractions were taken for liquid scintillation counting. The experiments are either continued until all the compound has

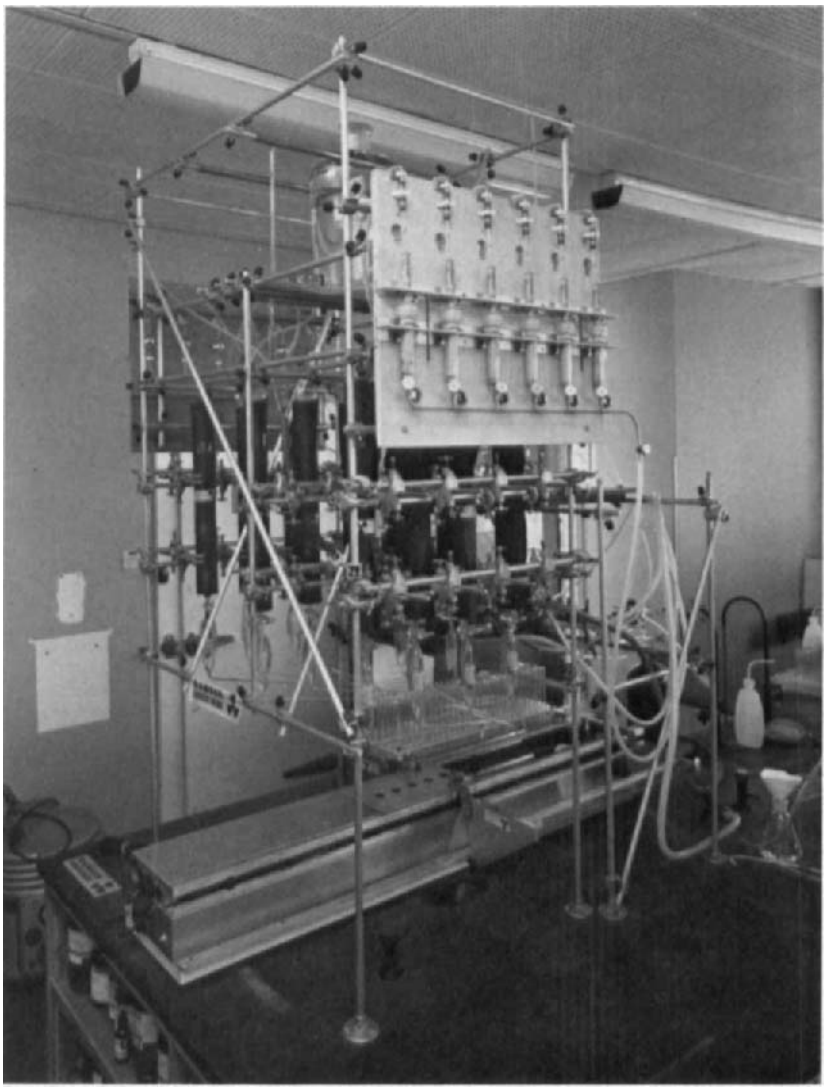

Fig. 8.1. An apparatus for studying the leaching of pesticides through soil columns.

leached or until a fixed volume of water (usually 1 litre which is equivalent to 600 mm of rainfall) has been applied. If the latter is done then the soil column is allowed to drain before it is exuded and cut into sections for combustion analysis to determine where the residual radioactivity is located.

Although laboratory soil columns do not reproduce the conditions which occur in the field of upward and downward movement of water, a standardised procedure involving the use of the automatic apparatus does permit comparative data to be obtained for a range of compounds.

Soil thin-layer chromatography

Since the preparation and elution of soil columns can be a tedious and slow procedure involving frequent attention, soil thin-layer chromatography is preferred in many laboratories as a method of determining pesticide movement. Helling has published several papers on the technique [2–5] including a discussion of some of its applications [4].

The elution technique is the same as that for conventional TLC but considerable care is necessary to prepare the plates. The soil is first sieved through a 250-μm sieve and water is added to produce a smooth slurry. Using a TLC spreader, the slurry is applied quickly to clean glass TLC plates. Alternatively the soil can be smoothed over with a glass rod. Radiolabelled compounds are then applied to the air-dried plates, which are eluted with water using either ascending chromatography or horizontal elution with a wick.

After elution the positions of radioactive zones can be located by autoradiography or radioscanning.

CONTINUOUS MONITORING OF RADIOACTIVITY IN ANIMALS

It is always difficult to relate *in vitro* experiments precisely to *in vivo* situations with animals. This has led several workers to carry out *in vivo* experiments with radioisotopes in which the location or concentration of a radioisotope is monitored continuously.

For example, Jacobs [6] studied intestinal absorption and transport of ^{14}C-labelled amino acids in rats using a flow cell packed with anthracene. A saline solution containing the amino acids was circulated through the intestinal lumen of the anesthetised animal and a portion of the "perfusate" was taken off for direct scintillation counting. The rate of absorption of amino acids can be monitored directly as it is proportional to the observed count-rate in the flow cell.

In a similar way Page and Baines [7] developed a method for monitoring the concentration of ^{14}C in blood using an ICN "Coruflow" scintillation counter in which a U-shaped cell was packed with a plastic scintillator. This experiment was carried out with a baboon and it was necessary to give heparin at regular intervals to prevent the blood from clotting in the flow cells. Two detectors were used, one for venous blood and the other for arterial blood. Counting efficiences were in the range 1.5–3% depending on the cell used. This system was found to be suitable for monitoring ^{14}C-concentrations in flowing blood and should be applicable to other related problems.

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Appendix

A brief list of the major manufacturers of instruments used in radiochromatography is given below. Since prices vary from country to country and rarely remain stable for long periods, these have not been included.

LIQUID SCINTILLATION COUNTERS

Instrument	Manufacturer	Comments
Intertechnique SL 30 Series	Intertechnique Ltd., Cottrell House, Wembley, Middlesex, Great Britain.	Series of 200–300 sample belt changers with varying degrees of computation/data handling.
Intertechnique SL 4000 Series	Intertechnique Ltd.	Multi-user; tray changer system.
Kontron MR 300 liquid scintillation counter	Kontron International, Bernerstrasse-Süd 169, CH-8048 Zürich, Switzerland.	300 samples; rack changer; multi-user instrument.
Philips PW 4540 liquid scintillation counter	Philips Analytical Dept., Pye Unicam Ltd., York Street, Cambridge CB1 2PX, Great Britain.	300 samples; tray changer, direct conversion to dpm; multi-user instrument.
LKB-Wallac 1210 UltroBeta liquid scintillation counter	LKB Instruments Ltd., 232 Addington Road, Selsdon, South Croydon, Surrey CR2 2YD, Great Britain.	200 samples; belt changer; direct conversion to dpm; multi-user instrument; pneumatic sample changer.
Packard Model 2650 Tri-Carb liquid scintillation counter	Packard Instrument Co., 2200 Warrenville Road, Ill. 60515, U.S.A.; and 13–17 Church Road, Caversham, Berkshire RG4 7AA, Great Britain.	450 samples; tray changer; direct conversion to dpm.
Packard 3000 Series Tri-Carb liquid scintillation counter	Packard Instrument Co.	A range of 300-sample instruments with varying degrees of computation.
Searle Model 6880 Mark III liquid scintillation counter	Searle Analytic, P.O. Box 75, Wiegerbruinlaan 75, Uithoorn, The Netherlands.	300 samples; belt changer; multi-user; conversion to dpm.

Searle Mark II liquid scintillation counter	Searle Analytic.	300 samples; belt changer; multi-user.
Searle Delta 300	Searle Analytic.	300 samples; simpler and more economical than other Searle instruments.

PAPER AND THIN-LAYER CHROMATOGRAM SCANNERS

Instrument	Manufacturer	Comments
Berthold thin-layer Scanner II	Laboratorium Prof. Berthold, 7547 Wildbad, Schwarzwald, G.F.R.	Two-dimensional scanner; attachment for paper strip scanning optional.
Berthold paper chromatogram scanner LB 280	Laboratorium Prof. Berthold.	
Panax thin-layer radio-chromatogram scanner, system E.0111/RRD-1	Panax Equipment Ltd., Willow Lane, Mitchum, Surrey CR4 4UX, Great Britain.	Integral recorder; attachment for paper strip scanning optional.
Packard radio-chromatogram scanner Model 7201	Packard Instrument Co.	Scans paper strips and 5-cm TLC plates.

SPARK CHAMBER AND CAMERA SYSTEMS

Instrument	Manufacturer	Comments
Berthold Beta-Camera LB 290 B-E	Laboratorium Prof. Berthold.	Recently modified grid design.
Birchover radio-chromatogram spark chamber System 450 B and 450 A/UVP	Birchover Instruments Ltd., The Spirella Building, Letchworth, Herts. SG6 4ET, Great Britain.	Optional UV illumination for viewing TLC plates.
Panax Beta-Graph	Panax Equipment Ltd.	Incorporates an oscillating spiral cathode.

AUTOMATIC SAMPLE OXIDISERS

Instrument	Manufacturer	Comments
Intertechnique IN 4101 sample oxidiser	Intertechnique Ltd.	Uses catalyst during oxidation.
Packard 306 Tri-Carb sample oxidiser	Packard Instrument Co.	
Searle Model 6550 combustion system	Searle Analytic.	

RADIO FLOW-CELL SYSTEMS FOR COLUMN CHROMATOGRAPHY

Instrument	Manufacturer	Comments
Berthold radioactivity monitor LB 5025-HP	Laboratorium Prof. Berthold.	System can be used with solid scintillators or liquid scintillators.
ICN Coruflow CMF-101 with CMF-103 flow-cell kit	ICN Instruments, 277 Antwerpse Steenweg, 2800 Mechelen, Belgium.	Used only with solid scintillators.
Nuclear Enterprises manual liquid scintillation counter LSC 1 with NE 801A, NE 802A flow cells	Nuclear Enterprises Ltd., Sighthill, Edinburgh EH11 4EY, Great Britain.	Manual counter for use with a range of flow cells.

RADIO-GAS–LIQUID CHROMATOGRAPHY

Instrument	Manufacturer	Comments
ESI Nuclear Model 504 radio-detector for gas chromatography	ESI Nuclear Ltd., Klempfern House, Holmsdale Road, Reigate, Surrey RH2 0BQ, Great Britain.	Similar design to Panax instrument with simplified lay-out.
Panax radio-gas chromatography system	Panax Equipment Ltd.	Similar to ESI Nuclear instrument but retains lead castle arrangement.

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