

# New Biochemical Separations

*Edited by*

A. T. JAMES and L. J. MORRIS

*Unilever Research Laboratory  
Colworth House, Sharnbrook, Bedford, England*

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## PREFACE

The almost frantic rate of scientific output which results in an ever increasing flow of papers in an ever increasing number of journals, has made the problem of communication of information even more difficult than it was ten years ago. In this period there has been a rapid advance in techniques of separation and in the application of these techniques, in modified forms, to a wide variety of compounds.

Unlike the industrial chemist, the biochemist has no choice in the compounds he has to study. No industry produces substances it cannot control and hence analyse, whereas living systems produce whole ranges of compounds whose complexity seems to increase with the detail with which they are studied. This complexity necessitates the development and utilization of the most refined separation techniques if one hopes to study the synthesis, breakdown and utilization of single chemical entities in living systems.

Our aim in this book is to provide up-to-date explanations of the most modern refinements in separation techniques and to include detailed experimental data of their application to biochemical problems. The contributors are acknowledged experts in the fields discussed and, in most cases, are themselves responsible for developing the refinements in separation techniques they describe. Since gas-liquid chromatography and thin-layer chromatography are still being improved and applied to a wider range of compounds, these two techniques have received most attention. We have, however, included chapters on gel filtration and its application to fractionation of proteins and polysaccharides, and on impregnated-paper chromatography and countercurrent distribution of lipids, since these also are new methods of considerable potential. Applications of these new techniques to a wide range of compounds are described. The lipids are covered in greater detail because it is in this field, in which improved techniques now enable the biochemist to tackle problems previously thought to be intractable, that the largest advances in knowledge are likely to be made in the next few years.

The book is intended for research biochemists and chemists but will also be useful to students whose courses of study (all too infrequently alas) cover modern separation techniques. If this book is successful we hope, by later editions, to keep abreast of future advances in separation techniques and we would be grateful for any suggestions from readers.

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## *Chapter 1*

# *Methods for the Detection and Estimation of Radioactive Compounds Separated by Gas-Liquid Chromatography*

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- II. Discontinuous Counting
- III. Continuous Counting
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  - 2. Scintillation systems
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  - 3. Ionization Chambers
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    - (a) Measurement of count rate
    - (b) Measurement of total counts
- IV. Selection of Counting Technique
- V. Additional Advantages of the Gas Radio-chromatogram.

## I. Introduction

The ever widening use of gas-liquid chromatography in biological chemistry, now that the technique has been applied to a variety of classes of compound including fatty acids, sugars, sterols and amino acids, has directed attention towards the detection of radioactively labelled compounds. All the common detectors of ionizing radiation have been used in conjunction with the gas chromatogram with varying degrees of success.

The system used depends on the type of ionizing radiation produced. Soft  $\beta$  emitters such as  $^{14}\text{C}$  and tritium are more difficult to deal with than hard  $\gamma$  emitters such as  $^{131}\text{I}$  or  $^{82}\text{Br}$ . Suitable detectors for both types of radiation are given in Table 1.1, together with their general characteristics (Table 1.2). The compounds eluted from the gas chromatogram may be counted either discontinuously or continuously and since the experimental arrangement is different in each case they will be considered separately.

TABLE 1.1

*Types of radiation detector and their efficiency*

Counter	Sensitive to	Efficiency for $^{14}\text{C}$	Efficiency for tritium
Geiger-Müller end window	$\gamma$ , $^{14}\text{C}$ , not Tritium	5–15%	0
Proportional counters	$\gamma$ , $^{14}\text{C}$ , Tritium	100%	60–80%
Ionization chambers	$\gamma$ , $^{14}\text{C}$ , Tritium	not reported	37%
Scintillation systems			
(a) Solid crystal	$\gamma$ only	0	0
(b) Powdered scintillator (anthracene)	$\gamma$ , $^{14}\text{C}$ , Tritium	86%	11%
(c) Plastic scintillation tubing	$\gamma$ , $^{14}\text{C}$ , Tritium	90% (as $\text{CO}_2$ )	?
(d) Liquid scintillators			
(1) Single photomultiplier	$\gamma$ , $^{14}\text{C}$ , Tritium	50%	20%
(2) Double photomultiplier	$\gamma$ , $^{14}\text{C}$ , Tritium	40–50%	15–20%

TABLE 1.2

*Effective background count rate of various detectors when used for  $^{14}\text{C}$  or tritium*

Counter	Background in counts/min.	Chromatogram moving phase limited to
Geiger-Müller end window	10–15	Any gas
Proportional counter	15–20	Argon, Helium
Ionization chambers	300	Any
Scintillation systems		
(a) Powdered scintillator (anthracene)	30–50	Any
(b) Liquid scintillator single photomultiplier	420	Any
(c) Liquid scintillator double photomultiplier	42	Any

## II. Discontinuous Counting

If the zones emerging from the gas chromatogram are condensed in collecting tubes, then the material can be counted by any of the normal methods. Suitable fraction collectors are

(i) Tubes containing loose plugs of de-fatted cotton-wool, wetted with a solvent more volatile than the substances to be condensed.

(ii) Long tubes cooled at the far end to give a gradual decrease of temperature along the tube. The lowest temperature should be such that the partial pressure of the compound condensed should be less than 1 mm. of mercury.

(iii) Tubes packed with crystals of a scintillator such as anthracene whose surface is coated with a suitable non-volatile liquid [1].

(iv) Tubes packed with activated charcoal [2].

(v) Tubes containing a solution of a scintillator [3].

In all cases the fraction collectors should be attached to the vapour detector of the gas chromatogram in such a way that there is no opportunity for material to condense until it enters the collector tube. In some cases this will necessitate attaching a separate heater to the gas outlet of the detector. Radioactive isotopes emitting hard radiation can be counted by insertion of the whole collector tube into a suitable doped scintillator crystal [2].  $\beta$  emitters will need to be isolated from the traps, except in the case of the trap containing a powdered scintillator. In the latter case the whole tube can be inserted in a scintillation counter. This device can be obtained commercially (Packard Instruments Co., P.O. Box 428, La Grange, Illinois, U.S.A.). These discontinuous methods have the advantage that they require no other apparatus beyond conventional counting equipment

and a gas chromatogram. They all, however, suffer from the disadvantage that discrete fractions can be collected only when a peak is shown by the vapour detector. Substances of very high specific activity present as minor components of the mixture being separated on the gas chromatogram can be completely missed since the vapour detector may not be sensitive enough to detect them. The presence of such minor components can be determined by collecting fractions at equal time intervals between recorded peaks [4]. The only real advantage of these systems lies in their simplicity and cheapness. All are, of course, dependent upon the ability to condense the whole of an eluted peak and this should be checked, first of all by means of a series of collectors connected together, and secondly by using a standard sample of known weight and specific activity. Where many separations have to be done it would be most unwise to assume, in any biochemical experiment using labelled precursors, that only major components of the isolated material are labelled.

### III. Continuous Counting

All the normal types of radiation counter, with the exception of the newer semi-conductor counters, have been used to meter continuously the radioactivity of the column eluate, and many experimental arrangements have been published. The greater part of biochemical experiments involving the use of radioactive tracers are carried out with either  $^{14}\text{C}$  or with tritium, since these have the greatest applicability. Thus in this section more emphasis will be placed on apparatus for the detection of these two soft  $\beta$  emitters than on  $\gamma$  emitters. Two points should be borne in mind in determining which of the published experimental arrangements should be adopted. Firstly, the simplicity of the system, and secondly, its efficiency. The greater the complication of a system the lower is its reliability. All of the types of counter have their limitations, particularly with regard to sensitivity and efficiency.

We will now consider the various types of radiation counter. These can be operated in three ways; at the temperature of operation of the gas chromatogram to prevent any vapour condensation within the system, or by continuous condensation of the eluted materials in the counting system to give a measurement of cumulative counts, or by conversion of the eluted zones to permanent gases so that the counting system can be operated at room temperature.

#### 1. GEIGER-MÜLLER END WINDOW COUNTERS

Kokes *et al.* [5] were the first workers to pass the gas stream from the chromatogram over a Geiger window. In the design published by Roginsky

[6], the vapours were passed between two end window counters. The method suffers from the disadvantage of poor geometry and insensitivity to tritium (see Table 1.1). It is necessary to keep the counters close together so that the dead space of the system is kept as low as possible. It is always of the first importance that no material should condense in the detector, otherwise considerable memory effects will be produced. By deliberately ensuring that all the vapours condense within the detector an integral response is obtained. This was done by Blyholder [7] who used a cell

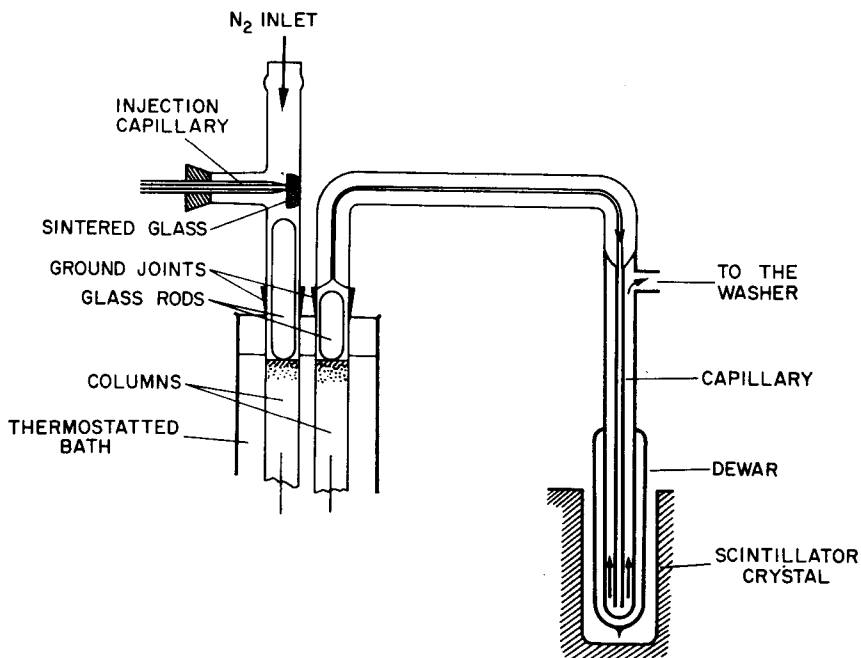


FIG. 1.1. Experimental arrangement for counting  $\gamma$ -emitters with a solid crystal (after Moussebois and Duyckaerts [8]).

cooled in liquid nitrogen. One of the cell walls was sufficiently thin to permit the radiation from  $^{14}\text{C}$  to pass on to a thin end window counter strapped against the window. This device, however, has the inherent fault of all end window systems of poor geometry and poor efficiency. The Geiger system with its insensitivity to tritium could, however, be used in conjunction with a more sensitive counter to allow a simultaneous recording of both  $^{14}\text{C}$  and tritium.

## 2. SCINTILLATION SYSTEMS

(a) *Solid crystal scintillator.* As already explained solid crystal scintillators are sensitive only to  $\gamma$  emitters. The experimental arrangement used by

Moussebois and Duyckaerts [8] is shown in Fig. 1.1, and a typical result obtained with  $^{131}\text{I}$  labelled *n*- and *iso*-alkyl iodides is shown in Fig. 1.2. The counting efficiency of this system is low but quite adequate for the separation described. Herr *et al.* [9] passed the gas stream from the column through a glass spiral placed on a thallium doped sodium iodide crystal and were able to detect organic bromides labelled with  $^{82}\text{Br}$ . When

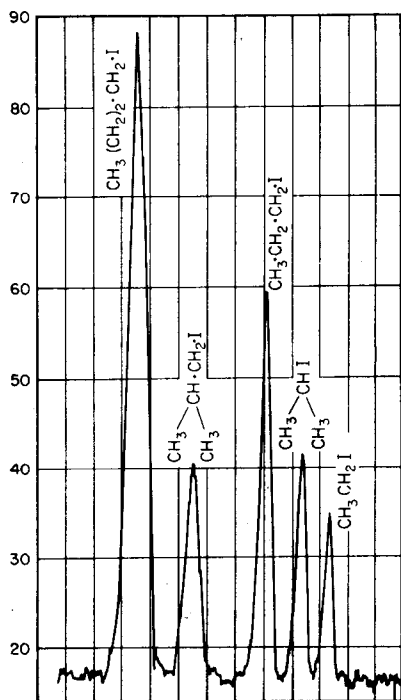


FIG. 1.2 Result obtained in the separation of *n*- and *iso*-alkyl iodides labelled with  $^{131}\text{I}$  using the apparatus shown in Fig. 1.1.

columns can be operated at room temperature or below, emitters can be detected by placing a scintillator crystal around the column itself [10].

(b) *Powdered crystal systems.* The anthracene packed condensers described in Part II have also been used by Karmen *et al.* [1] for continuous counting, by placing a U-shaped packed tube between the faces of two photomultiplier tubes. Each zone as it emerges from the counter is trapped on the silicone oil coated anthracene crystals and a record of the cumulative count rate can be obtained. The counting efficiency of the system is shown in Table 1.2. It suffers from the disadvantage of accumulation of material so that the device cannot be used at its maximum sensitivity if many counts are built up by condensation of fast moving materials of high activity. It is, however, simple to construct and is now commercially available (Packard Instrument Co.).

(c) *Plastic scintillators.* A different experimental arrangement which has

the merit of simplicity is to use a tube of plastic scintillator coiled in a spiral to give a long path length and fitted to the face of a photomultiplier tube. This system was first used by Funt and Hetherington [11]. A slightly different experimental arrangement was used by Cameron, Boyce and Taylor [12] who had a hollow cylinder with a dead volume of 0.5 cc., also attached to a photomultiplier. The spiral was used for detection of  $^{14}\text{C}$  and the small cylinder for tritium. This system is, of course, not applicable to high temperature operation because of the low melting point of the plastic tubing. Nevertheless, for compounds of low molecular weight,

this device could easily be made from an existing scintillation counter. It is important to have the output of the rate meter fed to a recorder, as a decatron presentation necessitates continuous readings by an observer.

(d) *Counting in solution.* Relatively few workers have done a great deal of work with gas radio-chromatography. The most striking work has undoubtedly been carried out by Popjak and his group. They designed and constructed a continuous liquid scintillator system capable of being attached directly to the outlet of the vapour detector of the chromatogram (in their case a gas density balance). The success of their system merits a detailed description. In their first paper Popjak and his group [13] described a counter associated with a single photomultiplier tube. In a later publication [14] they described a modification whereby two photomultiplier tubes were used with a greater light collecting efficiency.

The gas stream from the vapour detector passes into a continuously circulated and cooled solution of diphenyloxazole in toluene or xylene.

The solution of the scintillator is contained in a cell of the form shown in Fig. 1.3. The gas stream from the vapour detector enters at the capillary inlet I, the gas bubbles produced at the gas liquid interface pass up tube A into the escape channel E. Here the bubbles break and the gas emerges, with the liquid draining back through tubes B and C. The current of gas ensures a constant circulation of liquid by its pumping action. The photomultiplier scanning tube is placed at the bend between B and C. Complete mixing of absorbed vapour takes place in the body of solution within 5 seconds. In the experimental arrangement, the section between B and C is replaced by a shallow circular chamber of almost the same diameter as the photomultiplier tube. The volume of this chamber is 18 ml and the total volume of the whole circulation system is 25 ml, so that the photomultiplier scans 72 per cent of the circulating liquid. The faces of the photomultiplier tubes are held against the open ends of the chamber by a clamp and plastic ring. In this way the scintillator liquid is in direct contact with the face of the photomultiplier tube and no extra light-absorbing surfaces are introduced. The photomultiplier assembly consists of a light-tight and waterproof case,

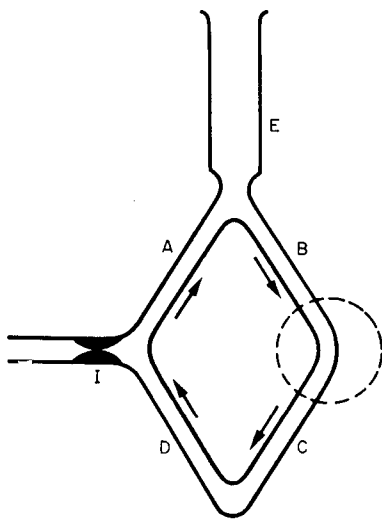


FIG. 1.3 Schematic diagram of the absorption cell used for continuous liquid scintillation counting (after Popjak *et al.* [13]).

inside lead shielding, and connection to the gas density balance is by means of heated sleeving. Full details and drawings of this apparatus are provided in the original communications [13, 14].

In the single tube apparatus the output from the photomultiplier tube is fed to a preamplifier whose output is measured with a rate meter (type 1172, EKCO Electronics Ltd., Southend-on-Sea, Great Britain). The ranges of the rate meter are modified to give two outputs of relative sensitivity 3.3:1, the absolute value of each range being selected with a switch and lying between 10 and  $10 \times 10^4$  at stages of 10. An automatic range

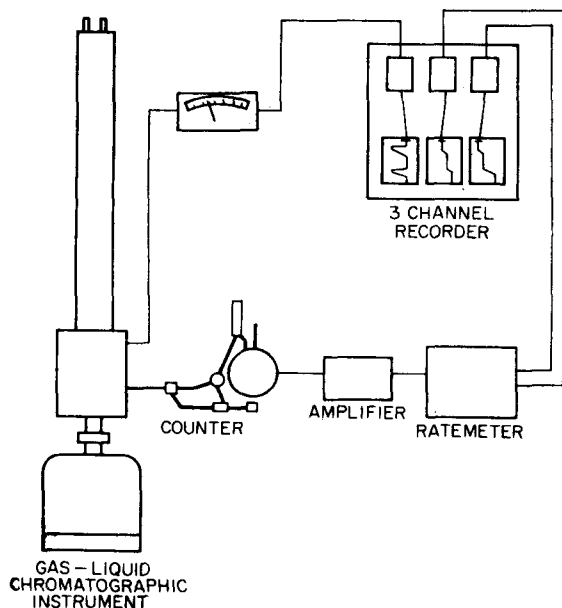


FIG. 1.4 Schematic layout of single photomultiplier counter (after Popjak *et al.* [13]).

changing switch is included so that when the rate meter output reaches 40 per cent of the maximum level the range is automatically switched to one-tenth of the sensitivity. A three channel recorder is used, one channel for recording the gas density balance output and the other two for recording the two levels of radioactivity. The experimental arrangement is shown schematically in Fig. 1.4. Since all the compounds accumulate in the counter the apparatus may switch automatically to a lower sensitivity and so provide insufficient sensitivity for slow moving components of low specific activity. This can be obviated by using a suitable manifold to empty and refill the counter during a run.

An integral response is obtained and a typical result is shown in Fig. 1.5,

demonstrating how by use of these dual ranges the record is always kept on scale at a suitable sensitivity level. The background count lies between 7 and 10 counts per second and the counting efficiency is 50 per cent for  $^{14}\text{C}$ . The integrating time constant of the rate meter is kept at 1 second for ranges of 30–100 counts/sec. and 0.2 sec. at higher ranges. In the later modification the scintillator chamber is modified to accept a photomultiplier tube from each side, the total volume being slightly lower than before. The counter is kept, as in the previous model, in a refrigerated chamber at  $-5^\circ\text{C}$ . By the use of these two counters and anti-coincidence counting, the background level is only 10 per cent of that of the previous model (see Table 1.2). The circuitry of the system has been altered to allow a simultaneous recording of  $^{14}\text{C}$  and tritium (see Fig. 1.6). This is carried out by the use of discriminating circuits whose performance is shown in Table 1.3. It is easy to remove the tritium counts from the  $^{14}\text{C}$  channel but very difficult to remove  $^{14}\text{C}$  counts from the tritium channel to a high degree of accuracy. The tritium counts are obtained by electronically subtracting 16 per cent of the  $^{14}\text{C}$  counts. An example of this is shown in Fig. 1.7(a) which demonstrates a simultaneous recording of tritium and  $^{14}\text{C}$ .

The recorders used for the three channels are of the moving coil galvanometer type which is so highly damped and sluggish that it filters out a good deal of rapid electronic noise and adds a finite time constant. The integrating time constants used in the rate meter of the later device were as follows:

- 5 secs. in the counting range 0–3 counts/sec.
- 1 sec. in the counting range 0–10 and 0–30 counts/sec.
- 0.2 secs. at all higher ranges.

Figure 1.7(b) shows a recording of radioactivity of a mixture of fatty acid methyl esters in which the scintillator liquid was removed during the

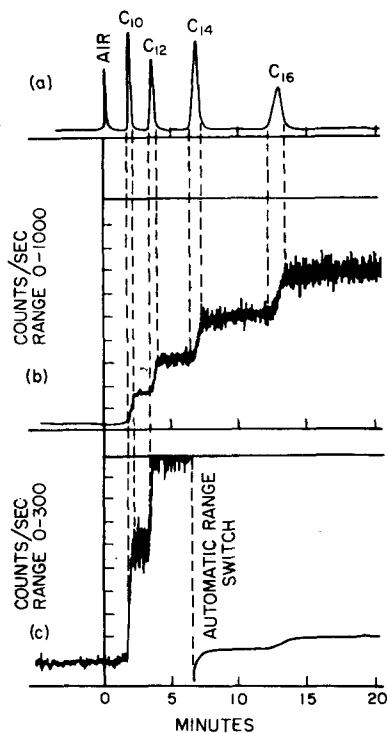


FIG. 1.5 Gas-radio-chromatograms of saturated acids using continuous scintillation counting (after Popjak *et al.* [13]).