Measurement of Carbon-14 and Tritium in the Effluent of a Gas Chromatography Column

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Abstract

Several methods of measuring radioactivity in the effluent of a gas-liquid chromatography (GLC) column are reviewed. If there is sufficient radioactivity in individual compounds to be measurable with acceptable precision in less than 15–20 sec, the effluent of a GLC column can be assayed for radioactivity during the course of an analysis. The effluent can be passed directly through a heated ionization chamber or proportional counter but both of these are somewhat sensitive to changes in the composition of the gas. The effluent can also be combusted to carbon dioxide and water, and the water then converted to HT for tritium assay. These gases can then be assayed in an ionization chamber, proportional counter or flow-through scintillation counter at ambient temperature. The detector volume and gas flow rate can then be large so that changes in gas composition that occur during the course of an analysis are minimized. The gas flow is adjusted for optimal speed of response. Combustion trains have been developed that minimize memory effects in tritium assays that otherwise can cause difficulties. Convenient calibration methods are available for setting up the methods and for determining their efficiencies. When there is insufficient radioactivity in the sample to be measurable during a short time interval, the effluent can be fractionated, high boiling material in each fraction can be condensed out and assayed for radioactivity for longer periods. Automatic and quantitative fraction collecting devices have been developed for use with liquid scintillation counters that make this method of radioassay accurate as well as highly sensitive.

MANY METABOLIC STUDIES using carbon-14 tritium labeled compounds can be facilitated by the use of GLC to separate the compounds that are formed from labeled precursors or that are transported from one biological compartment to another. The radioactivity can be measured in the effluent of the GLC column by a number of techniques. In this paper, several of these will be reviewed and a basis for selecting among them in a given experimental situation will be described.

In choosing a method for measuring radioactivity in a GLC effluent, many of the same considerations are involved as in choosing any other kind of detection system. Perhaps the most important consideration is sensitivity. It is desirable that the sensitivity be high since the amount of radioactivity available for analysis in most biological experiments is limited. In many experiments, the total radioactivity present in the sample may be quite high, but the specific activity may be low. In these instances, any increase in the sample handling capacity of the column increases the practical sensitivity of the radioassay. One factor in determining the sensitivity of any radiation detection system is the efficiency of the detector which may be described as the fraction of the total number of disintegrations that occur in the detector that are detected. The other factor is the time the radioactive material spends in the sensitive volume of the detector. Because of the random nature of radioactive decay, the precision of any method of radioassay is a function of the number of radioactive disintegrations that contribute to the measurement. If a compound remains in the sensitive volume of the detector longer, more events will be recorded, and the measurements will be more precise. Measuring radioactivity in samples containing low levels therefore requires longer assay times for comparable precision. This dependence of sensitivity on the time available for the measurement distinguishes detectors for radioactivity from all other kinds of GLC detectors.

In any measurement of radioactivity, more radioactive events will be recorded if the sample is present in the sensitive volume of the detector throughout the period of measurement. When any radiation detector is used with GLC, fewer events will be recorded and, consequently, some of its sensitivity will be sacrificed as the detector performs its function as a GLC detector: that of determining the distribution of radioactivity among the many compounds present in the sample. To be useful with chromatographic systems, the GLC detector must preserve as much as possible of the resolution of the chromatographic analysis. It must depict the occurrence of all maxima or peaks of concentration in the effluent of the chromatographic column since these indicate the emergence of different compounds. To detect these peaks, particularly when they occur close to one another, the detector response must be rapid. If radioactivity in the effluent is to be monitored as the gas leaves the column, the time of response of the detector is inversely related to the time individual samples of gas remain in the detector. This transit time must be short if the detector is to follow the rapid changes of concentration that may occur. However, since a short transit time limits the time available for measuring radioactivity, it limits the sensitivity. In any experiment, the choice of operating parameters is therefore determined by the transit time that can be allowed before the resolution becomes unacceptable. When the sample contains relatively high levels of radioactivity, the transit time can be reduced and better resolution can be achieved. If there is too little radioactivity, either a lower order of resolution can be accepted or the effluent can be fractionated as it leaves the column and each fraction assayed for radioactivity later. The resolution is then determined by the number of fractions collected, and the sensitivity is increased since each fraction can be assayed for a longer time period.

Since measuring the radioactivity during the course of an analysis is so much more convenient, fractionating the effluent is generally reserved for those experiments in which the level of radioactivity is too

low to permit the measurement to be done any other way. The choice between the two methods, ideally, is based on the amount of radioactivity that can be injected into the column.

Choice of Mean Transit Time

In any flow-through system, the time an average molecule spends in the effective volume of the detector, or the "mean transit time," is given by the ratio of the volume of the detector to the flow rate of gas through it. This relationship holds if the gas is completely mixed in the detector cell, so that a given concentration placed directly into the cell decreases exponentially with time; it also holds if mixing is incomplete or if virtually no mixing occurs.

Detectors in which radioactive compounds condense would tend to have large "volumes" by this definition. If a detector behaved as a GLC column, its "volume" for a given compound would be the retention volume of the compound on the column.

The volume used in this calculation is the mixing volume of the detector. This volume may not be the same as the actual volume since some parts of the detector may be out of the direct stream of flowing gas and may not turn over as rapidly as others. It may also change with changes in flow rate. The mixing volume can be determined experimentally by introducing a radioactive gas into the detector and observing its rate of disappearance with the gas flowing through at a fixed rate. The mixing volume of the detector and the mean transit time of the gas can be used to estimate how many disintegrations occur in the detector, when a radioactive compound passes through, and its efficiency.

The response time of the detector is largely determined by the transit time of the gas passing through it. The response time of the entire system, on the other hand, may also reflect contributions from transit times of the gas through other mixing volumes the gas passes through between the column and the radioactivity detector. The contribution of the detector to the response time of the system and the response time of the detector that is optimal in relation to that of the rest of the system can both be determined experimentally by adding extra or diluting gas to the detector at increasing rates and observing the effect on the shape of the peaks. The peaks become narrower with increasing flow until a flow is reached at which further increases no longer cause any decrease in peak width but only in peak height. This gas flow produces the minimal useful mean transit time. The gas flow can be decreased from this rate for the sake of increased sensitivity, but only at the expense of some loss of resolution. In many analyses performed using packed columns, the response time of the detector can be as high as $\frac{1}{4}$ to $\frac{1}{3}$ min without significant loss of resolution.

Sample Size

If radioactivity in any single compound is to be measured with a standard deviation of 10%, 100 disintegrations must contribute to the measurement. A given compound must thus have an activity of between 300 and 500 dpm, assuming 100% efficiency of detection, if 100 events are to occur in a detector in $\frac{1}{5}$ to $\frac{1}{3}$ min. When compounds in the mixture to be analyzed contain at least 300–500 dpm, which generally requires that the entire sample contain at least 15,000 or 20,000 dpm, it is reasonable to consider assaying it during the course of the analysis. If less is present, the effluent is best fractionated. In many experiments, particularly in biology, the amount of radioactivity available for analysis is not limited by the total radioactivity present in the sample, but rather by the specific radioactivity of that sample. In an analysis done with a $\frac{1}{4}$ in. packed column, injection of a sample containing more than one or two milligrams of material often results in poor resolution. Sufficient radioactivity to be measurable must be present in samples of this size or less.

Radioassay after Fractionating the Effluent

High boiling compounds can conveniently be condensed out of the effluent of a column for radioassay by scintillation counting by a number of procedures. Methyl esters of long chain fatty acids were condensed out of the effluent by Meinertz and Dole (1) by passing the effluent through an unheated glass tube containing cotton wool moistened with methanol. Hajra and Radin (2) condensed fatty acid esters by passing the effluent through millipore filters. Dutton (3) and Iddings and Wade (4) bubbled the effluent gas through cold PPO-toluene liquid scintillation counting solution. Fales and others passed the effluent gas, usually argon, through a length of tubing immersed in liquid nitrogen; the argon condensed to a liquid and the vapors condensed with it. Tritch and I described passing the hot effluent into short sections of glass tubing containing a solid support coated with a liquid phase at room temperature (5). High boiling materials condensed in these tubes while the carrier gas passed through. To facilitate counting the radioactivity, crystalline anthracene or terphenyl, both highly efficient scintillators, were substituted for the more usual solid supports in these devices (5–7).

This last approach was based on several considerations. The tubing delivering gas to the site of condensation must be kept hot if condensation of high boilers in the tubing is to be prevented. When the heated gas emerging from this tubing is delivered to a region of appreciably lower temperature in which condensation is to occur, an aerosol tends to form. A method must therefore be provided either to collect the aerosol quantitatively or to condense the vapors before the aerosol forms. It is then also necessary to insure that compounds once condensed will be retained at the site of condensation. Following its condensation, the radioactive material must be transported to the liquid scintillation counter for assay.

It is difficult to keep the tubing that conducts the vapors to the site of condensation hot to its very end if that end is immersed in a low boiling solvent. In the system designed by Popjak (8), in which the effluent of the column was condensed by bubbling it through toluene-PPO liquid scintillation counting solution, a heater at the end of the tubing was operated hot enough to boil the toluene. It was postulated that when toluene vapor condensed further along in the tubing, higher boilers condensed quantitatively along with it. The temperature of the junction was critical. Because of the high melting point and thermal stability of anthracene and terphenyl, the tubing can be maintained at close to 200C without any such difficulty.

When the effluent of a high temperature column containing a high boiling material such as cholesterol is bubbled through cold solvent, puffs of smoke may often be seen as the bubbles break the surface

of the liquid. Recovery of condensed material from the liquid is far from quantitative. Probably the easiest and most straightforward way of preventing losses from this cause is to prevent aerosol formation by dissolving the vapors before condensation can occur. This can be accomplished by keeping the bubbles passing through the column of liquid very small, but to do so makes the gas outlet very difficult to heat adequately. It can be accomplished much more easily and effectively by exposing the vapors to the large surface of nonvolatile liquid phase in a miniature GLC column.

It is doubtful that the moistened cotton wool or the millipore filter can present sufficient surface area to condense a vapor, although either may be effective in trapping an aerosol once it has formed. It thus would seem important to ensure formation of an aerosol when these devices are used. To do so would require that the tubing leading up to the cotton wool or the filter be kept cool. Vapors then condense partly in the cooled tubing, and partly in the cotton wool or millipore filter. The distribution of materials between the two will vary with the volatility of the compound being condensed.

If all compounds are to be assayed with equal efficiency, the retentivity of the trapping device for the most volatile compound in the mixture should be as good as that for the least volatile. The volatility of such compounds as methyl esters of lauric, myristic, and even palmitic acids in the presence of a flowing gas stream is appreciable even at room temperature. Varying amounts of loss will occur unless specific care is taken to prevent it. In attempting to determine what length of gas chromatography column, at room temperature, was necessary to stop and retain methyl laurate, we passed the heated effluent of a column through a foot long length of column. When the column was filled with uncoated celite, the methyl laurate was distributed over the first four inches of the column. When coated celite was used it was distributed in the first inch only. One might expect that the methods that employ millipore filters would be particularly subject to losses of relatively more volatile compounds from this cause.

Probably the most important advantage of the use of anthracene filled sections of gas chromatography column as trapping devices is the ease of automatic operation. In many analyses we do not know the identity of compounds bearing the radioactive label, and expect gas chromatography to provide us with identification of the labeled compounds as well as with the distribution of radioactivity among them. Because of the high sensitivity with which radioactivity can be detected and the variation in the specific activity of samples from biological sources, collecting fractions of a column effluent for radioassay requires a different approach than collecting them for mass spectroscopy, infrared spectroscopy or any method of chemical identification. A highly radioactive compound may not be detected at all even by a sensitive mass detector, or its identity may be obscured by the presence of large amounts of unlabeled compounds that have similar retention times on the column. Therefore, since there is no rationale for assuming that the amount of radioactivity in a given compound is necessarily related to the mass of that compound, there is no justification for assuming that the record of the radioactivity in the effluent necessarily has any relationship to the record of the mass of compounds being eluted. It follows that assaying the effluent of a column for radioactivity by condensing individual peaks, at times indicated by the record of the mass detector, is not conceptually sound if there is any doubt about which compounds the radioactivity is distributed among. To achieve reasonably accurate identification of compounds and to avoid errors due to operator bias it is preferable to fractionate the effluent into a relatively large number of *equal* fractions and assay each fraction for radioactivity. The record of radioactivity is then independent of the record of the mass detector. The radioactive compounds can then be identified, within the limits imposed by the number of fractions taken, by their retention times and by the retention times of known compounds; i.e., the same way peaks on the conventional mass record are identified.

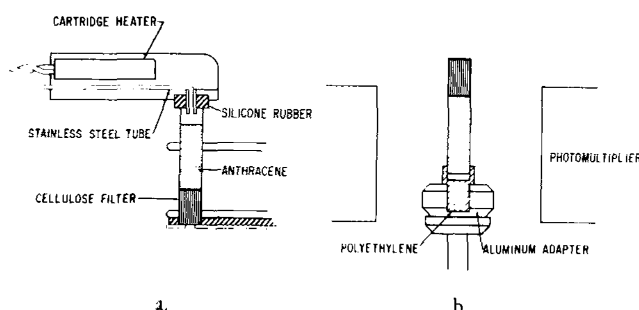

FIG. 1. A cartridge filled with crystalline scintillator; a) beneath the heated inlet tube of the fraction collector; b) inverted for counting in the liquid scintillation counter.

Since a large number of samples should be taken during each analysis, fractionating the effluent into a large number of equal fractions is best done with an automatic fraction collector actuated by an automatic timer. The procedures for handling the samples prior to counting should also be simplified or minimized. It is advantageous to avoid eluting the sample from the site of condensation. Uncertainty about completeness of elution as well as the inconvenience involved is thus avoided.

Our use of finely divided solid crystalline scintillators followed the work of Steinberg (9), who demonstrated the high efficiency with which low energy betas could be counted in aqueous solution by placing finely divided anthracene crystals in the solution. We therefore substituted coated anthracene crystals for the more usual column packing in our collection devices.

In collaboration with the Packard Instrument Company, we developed an automatic fraction collector based on the use of trapping cartridges and explored procedures for using it effectively. A leaktight seal between the hot tubing leading the effluent gas from the column and the trapping cartridge was provided by a silicone rubber gasket (Fig. 1a). Cartridges could be replaced in less than one second, which permitted cartridges to be changed even while a compound was being eluted with only minimal loss of radioisotope. Carbon-14 could then be measured in each cartridge by scintillation counting by simply placing the cartridge in a scintillation counter. No addition of solvent or transfer of material was necessary (Fig. 1b). We generally operated our columns so that all the compounds of interest emerged in one hour or less, collected fractions at 1-min intervals for the first 30 min and at either 2- or 5-min intervals thereafter. In mixtures containing 1000 dpm or less, each fraction was then counted for 10 min in an automatic Packard Tri-Carb liquid scintillation spectrometer.

Although carbon-14 can be counted with high efficiency on coated anthracene, the efficiency of tritium counting is very low because of absorption of the energy of the betas in the coating and tends to vary with the specific activity of the compounds because of self-absorption. Exploring the possibility of transferring the anthracene crystals to liquid scintillation solution revealed that anthracene *in solution* was a very inefficient scintillator and that it interfered strongly with scintillation counting in PPO-toluene. We therefore substituted coated terphenyl crystals for the coated anthracene. At the conclusion of an analysis each cartridge is placed directly in a standard liquid scintillation counting vial, 10 cc of PPO-toluene solution is added, and the vial is shaken slightly. The terphenyl, coating and all, flows out of the cartridge, and almost completely dissolves in the toluene, leaving only a small portion of the crystals at the bottom of the vial. Tritium, as well as carbon-14, can then be assayed by conventional liquid scintillation counting. There was no interference from the glass tube and no detectable difference between the efficiency of tritium counting this way and the efficiency when the tritium-labeled solution was pipetted directly into PPO-toluene solution (7). It was greater than that obtained using white Chromosorb, a widely used gas chromatographic solid support, in the trapping cartridges. When Chromosorb was added to a liquid scintillation counting solution, the efficiency of tritium counting decreased, possibly because some of the tritium-labeled material was adsorbed on the Chromosorb, as well as because of light absorption by the Chromosorb. It seemed reasonable to expect that variable degrees of adsorption would cause the efficiency to vary from one compound to another. It therefore seemed preferable to use a solid support that would dissolve in the scintillation solution without interfering with the measurement. Because of its high boiling point, *p*-terphenyl is ideally suited for this purpose. Although it is a good primay scintillator, when dissolved in toluene, its solubility is limited at low temperatures. For this reason, we used PPO-toluene solution with terphenyl, rather than terphenyl-toluene alone.

Radioassay by Monitoring the Gas During the Analysis

Radioactivity in the effluent of a GLC column has been monitored using flow-through ionization chambers (10–13), proportional counters (14–16) and scintillation counters (17–21). Each detector has been used at ambient temperature for assaying radioactivity in low boiling compounds and at elevated temperatures for high boiling compounds. Each has also been used in conjunction with combustion trains which convert organic materials in the effluent into carbon dioxide and water and then reduce the water to hydrogen gas for tritium assay. The carbon dioxide and hydrogen are then assayed for radioactivity in a detector at ambient temperature. Equipment for performing the radioassay by each of these methods is now available commercially from a number of suppliers.

In choosing a method of monitoring, one of the first decisions to be made is whether or not to subject the effluent to combustion. One of the approaches described earliest was to use an ionization chamber or a proportional counter designed for high temperature operation just as one would use a conventional gas chromatographic detector. When this approach is used, the possibility always exists that polar compounds will be adsorbed on the wall of the detector. Radioactive compounds that condense or are adsorbed on the wall of a detector may still be counted with two pi geometry. It is thus even more important to prevent absorption or condensation in the radiation detector than in the usual mass detector in which material condensed on the wall would not be detected. This difficulty is avoided when the effluent is combusted.

A second difficulty is encountered when markedly electronegative compounds or efficient quenching agents are present in the effluent, whether these come from the column bleed or the sample. Marked changes in efficiency of radiation detectors are often observed when electronegative compounds pass through the detector.

In their study of the behavior of the proportional counter used for monitoring the effluent of the chromatography column for tritium, Lee et al. (15) described the effect of nonradioactive vapors on the characteristics of the counter. The passage of a peak of either air or ethylene through the counter reduced the efficiency markedly. The ionization chamber is also sensitive to the changes in the composition of the gas, responding to small quantities of unlabeled compound as though they were labeled (21). Both of these effects can be minimized by: 1) subjecting the effluent to combustion prior to delivering it to the detector; 2) using a relatively large volume detector in conjunction with a large diluting flow of gas.

A convenient and effective combustion train for carbon-14 analysis consists of a combustion tube, a 7-in. length of quartz tubing, 5 mm I.D., filled with copper oxide wire, which is maintained at 700C, followed by a water trap, a somewhat shorter tube filled with magnesium perchlorate crystals.

For tritium analysis, the effluent of the copper oxide tube is passed through a similar tube filled with steel wool, which is also kept at 700C. In this tube the water produced in the combustion reaction is reduced to hydrogen. The effluent of the tube, containing carbon dioxide and hydrogen labeled with tritium, is then delivered to the detector. Our use of this combustion train was based on the work of James and Piper (16). One difficulty was encountered. Oxygen gas released from the copper oxide apparently formed a small quantity of ferric oxide on the steel wool which strongly absorbed water. After several tritium labeled compounds were analyzed, a significant quantity of tritium was absorbed. When unlabeled compounds were combusted, the water formed released the tritium from the iron oxide by exchanging with it and these compounds were then detected as though they were labeled. In order to eliminate this highly undesirable effect, we added a stream of hydrogen gas at the rate of approximately 2 to 3 ml per minute between the copper oxide and the steel wool (Fig. 2). The hydrogen kept the steel

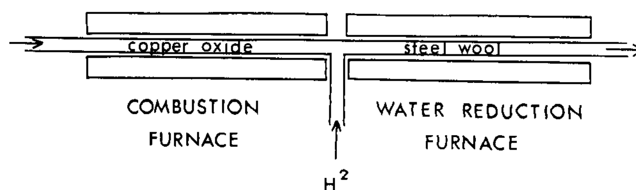

FIG. 2. Diagram of the combustion train. The gas passed first through heated copper oxide wire, then through heated steel wool. Hydrogen was added to it as shown to keep the steel wool reduced.

wool reduced and completely eliminated the memory effect. The steel wool was then considered to function as a catalyst for the exchange reaction between the added hydrogen gas and the tritium labeled water whereby the hydrogen gas emerged as tritium labeled hydrogen, HT. No memory effects were observed in subsequent tritium assays (Fig. 3).

Converting organic materials to carbon dioxide and hydrogen is so convenient and the analysis of radioactivity so accurate and reproducible that it is difficult to conceive of any applications in which the radioactivity ought to be analyzed without combustion. Even when the effluent is to be collected for subsequent characterization, a stream splitting device can be used and a reasonably large fraction of the column effluent can be collected without passing it through the combustion train.

The effluent of the combustion-water reduction train can be passed through any of the flow-through radiation detectors. A procedure we have found convenient is to deliver the effluent of the column to a stream splitting device that provides approximately a 10:1 division. The smaller fraction is delivered to an ionization detector. We prefer the hydrogen flame ionization detector for this application because it contains no radioactive source to interfere with the action of the radiation detector, is highly sensitive, and can operate with very low gas flows. Since it combusts the sample to carbon dioxide, contamination of the equipment with carbon-14 labeled nonvolatile materials is reduced. The larger fraction is delivered to the combustion train. If carbon-14 alone is to be assayed, the effluent of the copper oxide tube is passed through a short section of tubing containing magnesium perchlorate in which the water is trapped. In tritium assays, the effluent of the tube containing steel wool is passed through a similar water trap to dry the gas before delivering it to the detector.

We have evaluated each of the different flow-through radiation detectors with GLC effluents that have been subjected to combustion. The scintillation counter (20) consisted of a transparent tube, 10 ml volume, filled with anthracene crystals which was mounted between two photomultiplier tubes for coincidence counting (Packard Instrument Company Model 317 and 320 Flow Monitor Systems). Separate channels of pulse height information were recorded for the carbon-14 and tritium scintillations. The ionization chamber was a 275 ml flow-through ionization chamber which was used with the Cary Model 33 Vibrating Reed Electrometer (Applied Physics Corporation). Approximately 1200 ml of argon was added per minute to reduce the response time of the chamber (12).

The proportional counter was a 50-ml thin-window proportional counter ordinarily used for counting radioactivity deposited in planchets. The carrier gas used was carbon dioxide. Enough argon was added to the gas before it was delivered to the counter to make the composition of the gas in the counter 10% carbon dioxide in argon. A planchet containing a ^{14}C solid source was placed beneath the counter, and the voltage was adjusted for maximum counting rate.

The results of these experiments may be summarized as follows: The sensitivities of all of the detectors for carbon-14 were comparable. Tritium was detected with 3- to 5-fold higher efficiency by the proportional counter than with either the ion chamber or the scintillation counter. Both the ionization chamber and the proportional counter were found to be sensitive to changes in composition of the gas while the scintillation counter was not. This sensitivity was manifested in different ways. The ionization chamber responded to the passage of unlabeled material through it (Fig. 4). Although subjecting the effluent to combustion reduced the magnitude of this effect it was still evident when samples approaching milligram size were analyzed. The responses are 10^{-15} ampere high (21). They thus become quantitatively significant with samples containing relatively low levels of radioactivity and low specific activities.

The sensitivity of the proportional counter system to changes in the composition of the gas is manifested by decreases in counting rate when air peaks pass through the detector and when marked changes

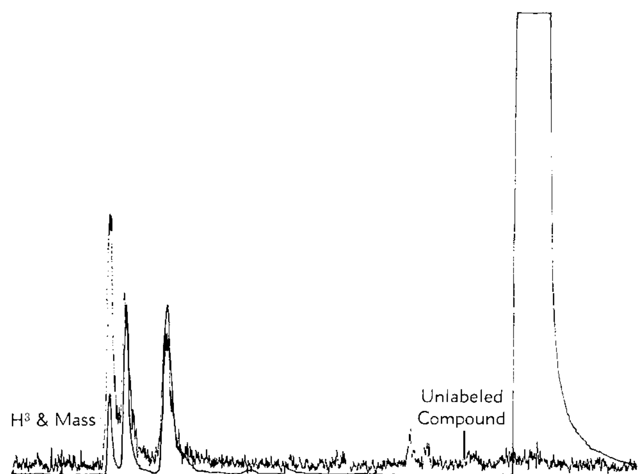

FIG. 3. Analysis of a mixture of ^{3}H labeled methyl, ethyl, and propyl acetates followed by injection of unlabeled isooctane. The record of the mass detector is superimposed on the record of the scintillation detector. No "memory" effects were noted in the tritium record when hydrogen was added to the water reduction tube.

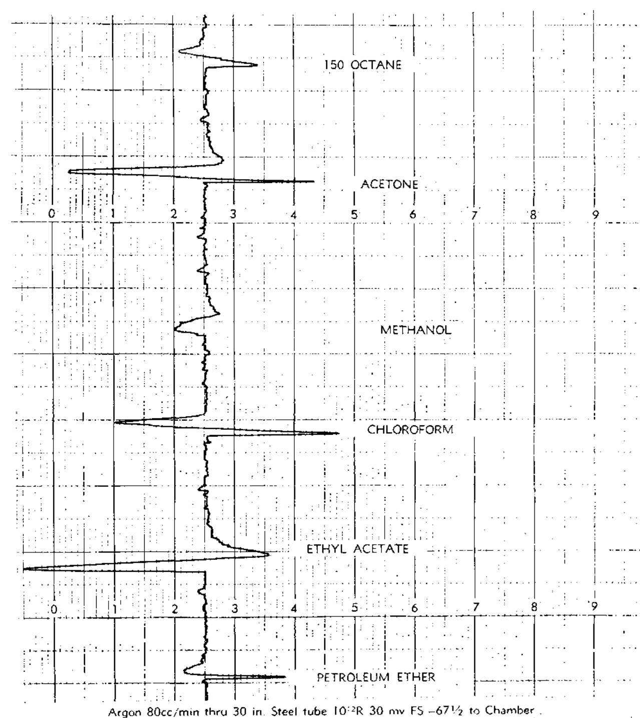

FIG. 4. Response of an ionization chamber to unlabeled organic vapors.

in the composition of the gas occur as a result of the passage of large mass peaks. These effects have been well documented by Lee and co-workers (15). They are particularly significant when low specific activity samples are being analyzed. The combination of carbon dioxide carrier gas, combustion of the effluent, and a large chamber with a brisk flow of diluting gas was chosen to keep the composition of the gas in the chamber essentially constant during the analysis. The same measures can be used with ionization chambers.

The scintillation counter did not show these effects. In addition, it offered the capability of performing simultaneous carbon-14 and tritium assays through the use of pulse height analysis. Although it appeared to be somewhat more expensive than either the ionization chamber or the proportional counter, it provided a convenient method for assaying an aliquot of the entire sample for radioactivity prior to the analysis that more than made up for its increased cost.

It should also be noted that radioactivity in high-boiling material emerging from a column has been measured by passing the effluent through cold liquid scintillator that is monitored continuously (19). The high-boiling materials, as they sequentially condensed, increased the counting rate of the detector in stepwise fashion. It seemed reasonable to conclude that the retained radioactivity could be measured during the entire period between the emergence of one compound and the next. The system thus appeared to offer appreciably greater sensitivity than any of the flow-through methods. However, since the background counting rate was increased by the emergence of each compound, it took increasingly longer times to detect the emergence of a new compound. As a result, the resolution decreased during the course of the analysis. When we compared the sensitivity of this method with that of the flow-through methods with comparable resolutions, the sensitivity of this method was less.

It should be possible to replace the liquid scintillator continuously during the analysis and thus transform this into a flow-through system. To my knowledge, this has not been done.

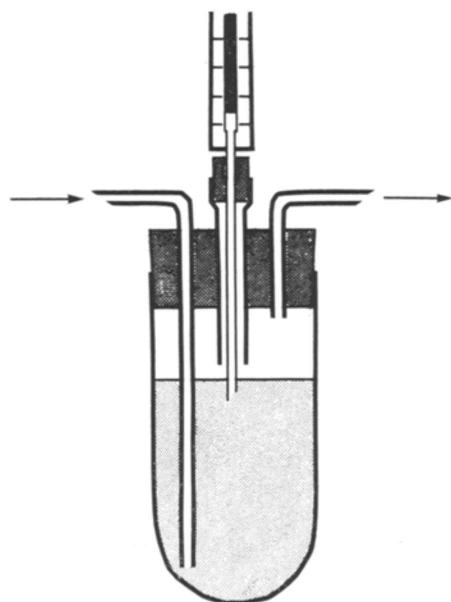

FIG. 5. Apparatus for generating $^{14}CO_2$ from $NaH^{14}CO_3$.

TABLE I
Reproducibility of $^{14}CO_2$ Generator

No. of samples	Average of counts recorded	S.D. observed	S.D. predicted
6	10,071	203.2	100
4	5,522	119	74
3	4,229	84	65
6	2,752	102	53
6	9,654	105	97
3	5,382	119	73
4	15,852	123	125

Calibrating Flow-Through Detectors

Calibrating flow-through detectors is frequently a problem because of the difficulties involved in handling radioactive carbon dioxide or tritium gas and of delivering known quantities of these gases to the detector. Carbon-14 labeled sodium bicarbonate is relatively inexpensive and is readily available. A convenient method of calibrating a flow-through detector is to inject an assayed aliquot of an aqueous solution of labeled sodium bicarbonate, which may be handled without significant losses, into a carbon dioxide generator such as shown in Figure 5. Reproducible quantities of carbon-14 labeled carbon dioxide were conveniently passed through flow-through detectors using this system. The efficiencies of two different flow-through detectors, a proportional counter, and an anthracene scintillation counter were compared under the same conditions by the use of this technique (Table I and Fig. 6).

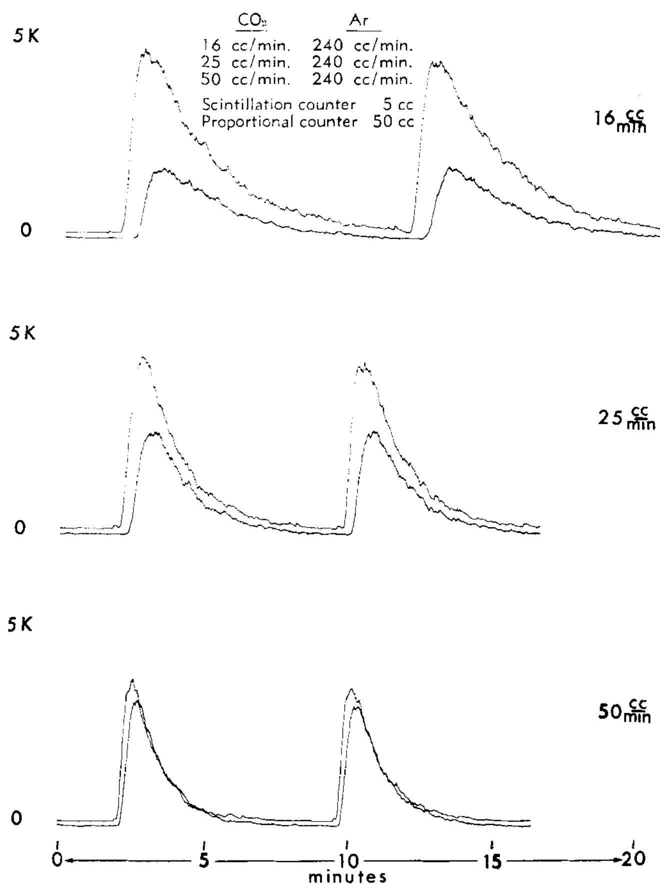

FIG. 6. The effect of gas flow rate on the responses of a crystalline anthracene flow-through scintillation counter (upper curves in each test) and a flow-through proportional counter (lower curves) connected in a series as determined by injecting samples of $NaH^{14}CO_3$ into the generator. The gas flow through the 5 cc scintillation counter was 16, 25, and 50 cc/min to obtain the upper, middle, and lower records respectively. An additional 240 cc/min was introduced between the two detectors.

Applications

The kinds of study for which analysis of radioactive materials by gas chromatography is particularly suitable fall into several general classifications. One of the most frequent uses is to study a biosynthetic pathway; to determine what compounds are intermediates in the biosynthesis of a given compound or class of compound. This kind of study is generally performed by incubating a labeled precursor with a tissue slice, or other source of enzymes, or injecting the precursor into an intact animal. After a selected time period the slice, enzyme mixture, or organ is extracted, subjected to chemical purification to isolate the class of compounds to be studied, and aliquots of the purified mixture are analyzed by GLC. Many studies of fatty acid and sterol synthesis have been performed in this way (22). Since the fatty acids in a given lipid in a given tissue may come from several sources, the gross composition does not necessarily reflect the activity of the synthetic mechanism alone. The distribution of radioactivity among these fatty acids, on the other hand, is a direct reflection of synthesis in the tissue.

These techniques are also particularly suited to studies of the relative rates of metabolism of several compounds. In studies of fatty acid absorption, for example, mixtures of several carbon-14 labeled fatty acids were administered simultaneously. Thoracic duct chyle and serum were collected, lipids extracted, and the fatty acid composition and distribution of radioactivity among the fatty acids were determined by GLC. If radioactivity were distributed in a given lipid fraction in the same proportions as in the administered fat, the mechanism responsible for synthesizing that fraction could be said to utilize exogenous fatty acid nonspecifically; i.e., each fatty acid in proportion to its concentration. If the distribution were different, this would indicate that a greater fraction of certain fatty acids were being utilized than others and that these fatty acids were preferred by the synthetic mechanism. In several experiments, it was found that fatty acids were absorbed and converted into triglycerides in direct proportion to their relative concentration in the fed fat (Fig. 7). They were also incorporated into cholesterol esters and phospholipids in direct proportion to their concentration in the fed fat, but with proportionality constants that differed from one acid to another (23,24). The relative rates of incorporation of different fatty acids into lipids of red blood cells and white blood cells in vitro (Fig. 8), and the rates of transfer of different fatty acids across the placenta have also been studied using a similar approach.

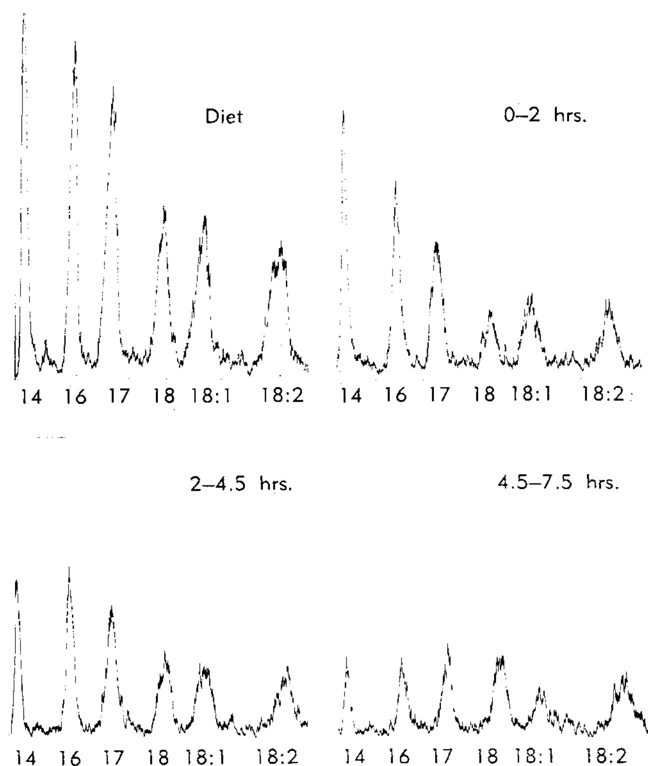

FIG. 7. Patterns of distribution of radioactivity in fatty acids of diet and of total lymph lipids at various times following the feeding of this diet, as determined by flow through scintillation counting.

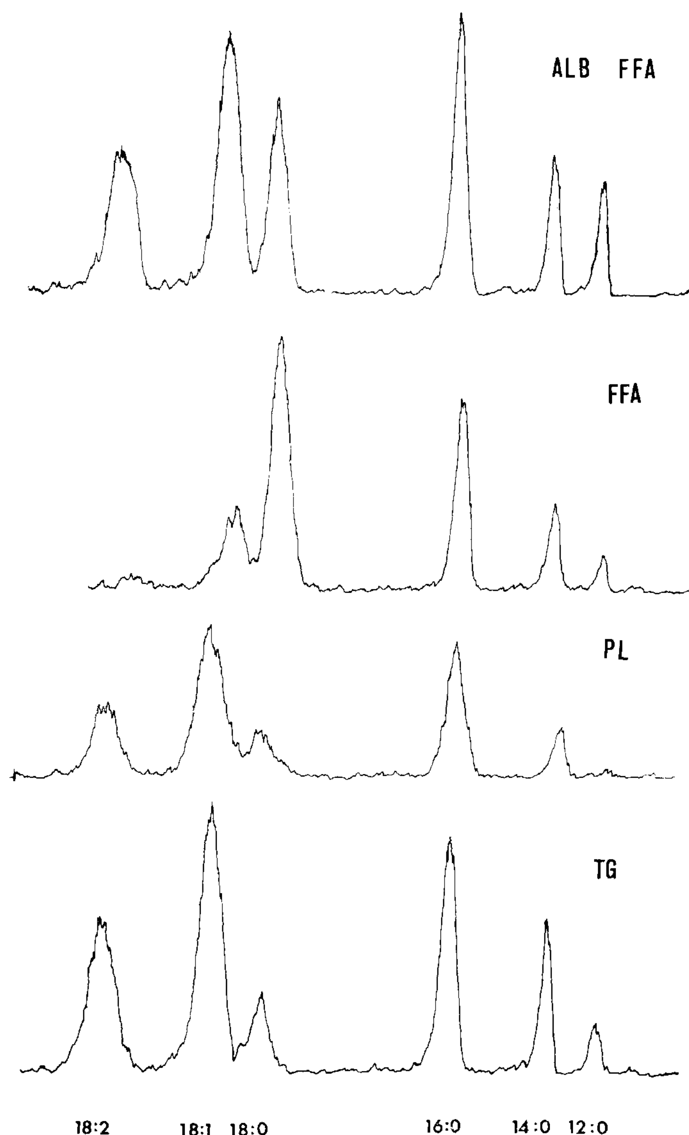

FIG. 8. The patterns of distribution of ^{14}C radioactivity in the fatty acids bound to serum albumin, in the free fatty acids associated with erythrocytes in equilibrium with the albumin, and in the erythrocyte phospholipids and triglycerides synthesized by the erythrocytes from those fatty acids.

GLC analysis can also be used for isotope derivative assays. In a characteristic analysis, radioactive derivatives of certain classes of compounds, such as alcohols, are prepared using carbon-14 labeled reagents, such as ^{14}C-acetic anhydride. The labeled derivative of the compound to be analyzed is then isolated, usually by chromatography. To correct for

losses in preliminary purification steps and in derivative formation, a small quantity of the compound to be analyzed that is labeled with tritium is added to the original mixture and carried through each step in the procedure. Purification is judged adequate when further purification results in no change in the $^{14}C/^{3}H$ ratio. The quantity of the compound of interest that was present in the original mixture is then calculated from this ratio, and the specific activities of the ^{14}C-labeled reagent and the ^{3}H-labeled compound. GLC analysis can substitute for other chromatographic methods of purification in isotope derivative assays. It offers an advantage over other microassays by GLC in that a quantity of unlabeled material can be added as a carrier. When a sample in the nanogram range is analyzed by GLC, a significant fraction may be absorbed on the column and retained. Addition of carrier can eliminate this loss.

A similar isotope derivative procedure is to add to the sample a compound that has the same reactive group as the compound to be analyzed as an internal standard. Carbon-14 or tritium labeled derivatives are then prepared using a suitable reagent, and the mixture is analyzed by GLC. Assuming that both derivatives are formed and are chromatographed equally well, the ratio of the radioactivity in the standard to that in the unknown is the same as the molar ratios of the two compounds in the original mixture (25).

These isotope derivative procedures can provide methods of analyzing compounds with specific reactive groupings in the presence of larger quantities of other compounds with similar behavior on GLC columns that interfere when nonspecific detectors are used.

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