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Radioassay by gas-liquid chromatography of lipids labeled with carbon-14

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SUMMARY

A method for radioassay of carbon-14 labeled substances analyzed by gas-liquid chromatography is described. The column effluent is automatically fractionated by condensing the vapor in a series of cartridges filled with anthracene crystals coated with silicone oil. The radioactivity in each cartridge is then assayed directly by scintillation counting. The considerations leading to the design and operation of an automatic fraction collector based on this principle are explored. Application of the method to several types of analyses demonstrates its high sensitivity and defines its limitations.

Several methods have been reported for assaying vapors eluted from a gas-liquid chromatographic (GLC) column during the course of an analysis. Radioactivity in the gas phase has been measured directly by the use of ionization chambers (1) or proportional counters (2, 3). The techniques of liquid scintillation counting have been applied by condensing the effluent vapors cumulatively in the presence of a scintillator system (4, 5). Under these conditions the elution of a radioactive compound from the column is indicated by a sustained increase in the counting rate of the scintillator system.

The resolving power of each of these methods of radioassay is inversely related to the time taken for the measurement of the radioactivity. Because it is desirable to take full advantage of the resolution of the analytical process, it is the usual practice to limit this time. Since the amount of material that can be analyzed on a usual GLC column without overloading it is limited, these methods are most applicable to analyses of materials having high specific radioactivity that can be assayed accurately in a matter of seconds. Materials with lower specific radioactivity require more time for accurate measurement. The additional time may be made available by fractionating the effluent and subsequently measuring the radioactivity in each fraction. When this approach is used, the measurement of radioactivity can be made for whatever time is necessary to obtain statistically reliable results, while the resolution is limited only by the frequency with which fractions are taken.

The column effluent can be fractionated by condensing the vapors from the carrier gas and collecting the condensate into a number of discrete fractions. An effective method for doing this was suggested by the mechanism of action of a gas chromatographic column in which, it is postulated, multiple condensations and evaporations occur in a relatively small volume. Accordingly, short sections of tubing containing a solid support coated with a liquid phase were tried as trapping devices. A 1-inch length of 5 mm i.d. tubing containing Celite coated with silicone oil was found sufficient, when kept at room temperature, to condense materials such as methyl laurate from a hot column effluent and to retain them quantitatively for at least 1 hour.

Including a scintillator in the trapping cartridges permits assay of radioactivity in these cartridges without further transfer of the labeled material. The use of anthracene crystals for this purpose was suggested by the work of Steinberg (6), who described the use of anthracene for assay of carbon-14 in aqueous solution. Substitution of anthracene for the solid support in the trapping cartridges did not decrease their efficiency as trapping devices. The efficiency of anthracene as a scintillator was found to be comparable to that of the liquid mixtures commonly used for liquid scintillation counting, and coating the anthracene with a number of liquids commonly used as liquid phases in gas chromatography did not appreciably lower its efficiency. Anthracene coated with silicone oil was therefore chosen as a filling material for the cartridges (5).

An automatic fraction collector was designed, through the use of which the effluent could be divided

into a large number of fractions for scintillation counting. This approach has been applied to several kinds of analytical biochemical problems to develop procedures for its effective use.

MATERIALS

Anthracene Cartridges. Anthracene (blue violet fluorescent grade)[1] was coated with liquid phase by dissolving 10 g of Dow Corning 550 silicone oil in acetone, slurrying the acetone solution with 90 g of anthracene, and evaporating the acetone under vacuum. The cartridges used consisted of 1 3/4-inch lengths of 7 mm i.d. glass tubing, each containing a 3/8-inch long cellulose filter pushed into one end.[2] The cartridges were filled by applying a vacuum to the end of the cartridge bearing the filter and dipping the other end in the coated anthracene. The top 1/4 inch of the anthracene was pressed down, clearing the top 1/4 inch of the cartridge of anthracene.

Automatic Fraction Collector. The fraction collector was designed and constructed in collaboration with the Packard Instrument Company, Inc.[3] It consists of a turntable and a gas injector. The gas injector consists of an 0.062-inch outside diameter (o.d.) thin-walled stainless steel tube enclosed in a heated brass block. A 1/8-inch o.d. brass sleeve is silver-soldered over the terminal 1/2 inch of the tube to provide a nozzle that is heated by conduction from the brass block. The nozzle is pressed through a silicone rubber gasket that, when held against the rim of the cartridge by spring tension, seals the nozzle into the cartridge. In position for delivering a sample, the terminal 1/8 inch of the nozzle protrudes below the rim of the collecting cartridge (Fig. 1a).

Upon receiving an electrical signal, an electric motor raises the injector, rotates the turntable and releases the injector to seal itself over a new cartridge. This cycle is completed in less than 1/2 second. The collector can be actuated by depressing a switch manually or by a signal from an automatic timer.

The criteria to be satisfied in the design of the fraction collector included the necessity of keeping the effluent gas hot (approximately 200°) up to the point at which condensation was to occur, the desirability of an abrupt transition between hot and cold zones, a tight gas connection that could be broken and remade rapidly and easily, and the requirement that condensation of the vapors occurs entirely within the scintillator. The nozzle design described was chosen to discourage condensation on the glass of the top of the cartridge as well as in the tubing.

Cartridge Holder for Scintillation Counting. The anthracene cartridges were assayed for radioactivity by scintillation counting in a Tri-Carb automatic liquid scintillation spectrometer.[4] Adapters were made to permit the cartridges to be handled by the automatic sample changer of this instrument. These adapters ensured uniform positioning of each cartridge in the photomultiplier compartment.

Covers for the cartridges were made by partly filling 5/8-inch long sections of thin-walled polyethylene tubing with paraffin. Following collection of the sample, the top of the cartridge was pushed into this cover, inverted, and the cover pushed into a hole in an aluminum disk, 1 1/8 inch diameter and 1/2 inch high, which held it snugly. Figure 1b shows the assembled adapter and cartridge in the photomultiplier compartment of the instrument.

Gas-Liquid Chromatography. The gas-liquid chromatographs used were designed and constructed in our laboratory. The column oven was 5 feet long and consisted of an electrically heated aluminum block 2 by 2 inches in cross section surrounded by insulation. A 3-inch section at the inlet of this column oven was insulated from the remainder by 1/2 inch of insulation and was heated separately. The detector oven was also separately heated and, like the inlet section, was "in line" with the column. The column itself was a straight glass tube 65 inches long, 5 mm i.d., fitted with a glass sidearm 1 inch from the inlet end. Gas-tight connections were made between the gas tank and the sidearm of the column, and between the end of the column protruding into the detector oven and the detector inlet through the use of 3/8-inch long, 7/32-inch diameter silicone rubber seals[5] pierced by 1/16-inch diameter stainless steel tubing. Alternatively, connections between 1/16-inch diameter steel tubes in the detector oven were made using "teflon" spaghetti tubing[6], 1/16 inch i.d., that fits snugly over the 1/16-inch diameter steel tubing.

The entire oven assembly was disposed horizontally on a table, making the sample injection port, the detector oven, and all connections available for manipulation at table top height. This arrangement makes operation of efficient columns convenient; permits many different kinds of problems to be approachable in

[1] Eastman Organic Chemicals, Rochester 3, New York.
[2] Packard Instrument Company, Inc., P.O. Box 428, LaGrange, Illinois.
[3] See footnote 2.
[4] See footnote 2.
[5] Burrell Corp., 2223 Fifth Avenue, Pittsburgh 19, Pennsylvania.
[6] Pennsylvania Fluorocarbon Co., Inc., 1115 N. 38th Street, Philadelphia, Pennsylvania.

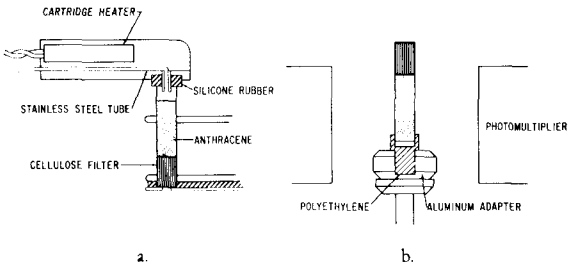

FIG. 1. The anthracene-filled cartridge (a) for collection of a sample, and (b) inverted, in its adapter, for scintillation counting in the Tri-Carb automatic scintillation spectrometer.

moderately rapid succession; makes experimentation with different kinds of columns easy; makes modification of detectors, development of new detectors, and experimentation with different methods of collecting the effluent physically convenient. The column effluent was carried from the detector oven to the injector assembly of the fraction collector in $^1/_{16}$-inch o.d. thin-walled hypodermic tubing enclosed in $^1/_8$-inch o.d., $^1/_{16}$-inch i.d. copper tubing. This tubing was heated electrically and insulated from its origin in the detector oven to the injector.

Analyses were performed using 5-ft long straight glass columns containing Chromosorb W,[7] which had been treated with concentrated hydrochloric acid and alcoholic sodium hydroxide (7) and coated with an adipic acid-ethylene glycol polyester from which the esterification catalyst had been removed (8). The effluent of the column was divided by leading it into the $^1/_{16}$-inch diameter vertical member of a T-connection. The two limbs of the T were constructed of 1-inch and 7-inch lengths of 0.010-inch i.d. stainless steel capillary tubing, respectively. The capillaries were crimped using a diagonal cutter to adjust the division of the gas to a ratio of 1:10. In the series of experiments reported here, the smaller fraction was directed to the hydrogen input line of a hydrogen flame ionization detector (9), while the larger fraction was directed to the tubing leading to the fraction collector. We found the hydrogen flame ionization detector satisfactory, although its choice had been determined primarily by entirely unrelated experiments in progress at the time of this study.

Scintillation Counting. In the Tri-Carb scintillation spectrometer, the material to be assayed is placed between the opposing faces of two photomultiplier tubes with outputs arranged in circuitry for coincidence counting. The photomultiplier faces (approx. $1^7/_8$-inch diameter) are located close to the bottom of a polished aluminum chamber.

The distribution of radioactivity in a cartridge following the collection of a sample depends on the volatility of the sample, the temperature of the injector, the gas flow, and the time. Under the experimental conditions we used most frequently, most of the radioactivity was retained in the first few millimeters of the cartridge. As a result of this limited distribution volume, the location of the cartridge in the photomultiplier compartment significantly affects the counting rate, and there is a location at which the counting rate of a small volume sample is maximal. This applies to conventional solutions of DPO-toluene as well as to anthracene. In our instrument, this location is close to the floor of the photomultiplier compartment, which is close to the axis of the centers of the photomultiplier faces. The counting rate is moderately lower at greater heights above the floor of the compartment and is sharply decreased if the small amount of radioactive scintillator is placed above the level of the tops of the opposing photomultiplier faces. The heights of the electrical pulses under other than optimal conditions indicated that the decreases in counting rate were due to failure of the photomultipliers to "see" all the photons resulting from a given disintegration when it occurred in an unfavorable location, rather than the loss of the entire burst of light pulses. Nonreproducible results are obtained if successive samples are assayed at different locations; conversely, reproducible results may be obtained by keeping the position of the cartridges in the photomultiplier compartment uniform. This requirement determined the design of the cartridge holder described. By the use of this device, the anthracene crystals containing the highest concentration of radioactivity in each cartridge were placed close to the optimal position for counting (Fig. 1b).

The efficiency of anthracene as a scintillator may be compared with that of a commonly used liquid scintillation system, diphenyloxazole (DPO) in toluene. Equal quantities of C^{14}-labeled methyl laurate were pipetted into vials containing the DPO-toluene solution and into similar vials containing anthracene crystals. The vials were glass, 9 mm in diameter, and $^1/_2$ inch long. Both sets of vials were stirred. Scintillation counting was performed with the vials held close to the axis of centers of the photomultipliers. The counting rates expressed in terms of absolute efficiencies were plotted against the photomultiplier voltage settings shown in Figure 2. In this figure, the upper line of each pair represents the integral counting rate (all counts of more than 10 v pulse height) and the lower represents the counting rate of pulses of more than 10

[7] Johns-Manville Corp., 22 E. 40th Street, New York, New York.

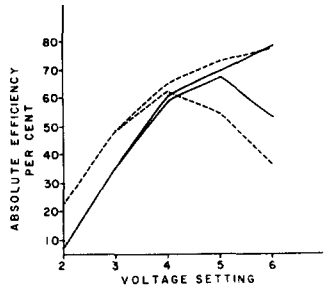

FIG. 2. Counting rates, in terms of absolute efficiencies, of equal aliquots of C^{14}-labeled ester pipetted into anthracene (dashed line) and DPO toluene (solid line) as a function of voltage applied to the photomultiplier tube. Voltage settings correspond to voltages of 730, 815, 900, 985, and 1070 v for settings of 2 through 6, respectively.

v, but less than 100 v (10–100 window).

As may be seen from these curves, the average burst of light emitted from the anthracene as a result of the impact of the carbon-14 beta particle is somewhat larger than that from the DPO-toluene.

Voltage settings for scintillation counting can be determined from a graph such as this. The setting at which the number of counts per minute "in the window" (lower line in the graphs) is at a maximum is usually selected. However, because of differing degrees of volatility of substances in any given analysis, there may be some variability in the position of the condensed radioactivity within the cartridge. To compensate for this effect, the voltage on the photomultipliers was increased, and integral counting was used. This has the effect of amplifying smaller light pulses to values above the minimum selected as baseline, thus including the smaller as well as the larger pulses in the measurement. The voltage chosen was higher by two "taps" (approximately 170 v) than the optimal voltage for assaying carbon-14 in a 15-ml sample of liquid scintillator. The background counting rate of the anthracene was comparable to that of DPO-toluene at the same photomultiplier setting and was primarily a function of the photomultiplier tubes used. During the time in which the results reported here were obtained, the background counting rate at the setting described (Tap 6) varied between 20 and 50 cpm.

APPLICATIONS

This method of radioassay has been applied to several experimental situations.

Analysis of Mixtures Containing Small Quantities of Highly Radioactive Material. An artificial mixture of methyl esters was prepared from arbitrarily unequal amounts of five commercially obtained fatty acids with high specific radioactivity. To aid in identifying the components, a mixture of nonradioactive fatty acid esters was added prior to the analysis. The effluent gas was divided so that 10% was delivered to the mass detector (hydrogen flame ionization detector) and 90% was delivered to the fraction collector. Cartridges were changed at 1-minute intervals through the use of the automatic timer. The results of triplicate analyses are shown in Table 1.

A similar approach, except for the omission of unlabeled carrier, was used in an analysis of a mixture of esters obtained from an *in vitro* enzymatic synthesis of fatty acids from a labeled short-chain precursor. Table 2 shows the result of this analysis. Cartridges were changed at 1-minute intervals for the first 15 minutes, and at 2-minute intervals thereafter.

Reproducibility of Analysis of Low Specific Activity Mixtures. Approximately equal quantities of C^{14}-palmitic and C^{14}-linoleic acids were administered to rats, and the method described was used to determine the ratio of these acids in several lipid fractions of the chyle. Analyses were done on two aliquots of each sample, but because of the limited amount of material available, the aliquots were not always of equal size. The assumption was made that no significant amount of radioactivity was present in fatty acids other than palmitic and linoleic. The *total* effluent of the column

TABLE 1. TRIPLICATE ANALYSES OF A MIXTURE CONTAINING METHYL DECANOATE, DODECANOATE, TETRADECANOATE, HEXADECANOATE, AND OCTADECANOATE PREPARED FROM C^{14}-LABELED FREE FATTY ACIDS*

Time†	Methyl Ester‡	Retention Time (center of peak)	Width of Peak at Half Height	Analysis I	Analysis II	Analysis III
min		*min*	*min*	*cpm*	*cpm*	*cpm*
1				0	0	0
2	10:0	1.9	0.12	4334	4390	4360
3	12:0	2.7	0.16	5374	5100	5169
4				188	297	199
5	14:0	4.4	0.24	4543	3958	4379
6				193	181	230
7				1052	856	851
8	16:0	7.4	0.4	3343	3400	3548
9				157	120	158
10				108	95	115
11				39	40	60
12				206	60	77
13	18:0	12.9	0.7	3440	3318	3260
14				410	490	530
15				54	80	66
Total				23441	22385	23002

* Total injected included 100 μg of unlabeled methyl esters and trace quantities of labeled esters assaying approximately 26,000 cpm. Approximately 90% was collected. The remainder was bypassed to the mass detector.

† Solvent appeared 0.9 minute after collection began.

‡ Refers to the number of carbon atoms in the fatty acid from which the ester is derived. and the number of double bonds.

TABLE 2. ANALYSIS OF A MIXTURE OF FATTY ACIDS PRODUCED BY *in Vitro* ENZYMATIC SYNTHESIS FROM A C^{14}-LABELED PRECURSOR

Time	Methyl Ester*	Retention Time (center of peak)	Width of Peak at Half Height	Analysis
min		*min*	*min*	*cpm*
1				70
2	12:0	2.0	0.16	1175
3				1249
4				346
5	14:0	4.3	0.24	1180
6				100
7				286
8	16:0	7.5	0.4	1638
9				312
10				68
11				32
12				16
13	18:0	13.5	0.7	860
14				776
15	18:1	15	0.7	738
16				98
18	18:2	18	0.8	182
20				60
22				20
24	20:0	24	1.2	265
26				140
28				125
33				28
38				24
43	22:0	45	2.4	36
48				44
53				100
58				18

* Refers to the number of carbon atoms in the fatty acid from which the ester is derived, and the number of double bonds.

was collected into two equal fractions, one of which contained methyl palmitate and the other methyl linoleate. As shown in Table 3, reproducible results were obtained for the radioactivity attributed to palmitate and linoleate despite the low total radioactivity.

Several rat epididymal fat pads were incubated in different solutions containing C^{14}-acetate to determine which fatty acids were synthesized under various conditions. Following incubation, the fats were extracted and methyl esters prepared from the fatty acids. Aliquots containing less than 2000 cpm in 2 mg were analyzed.

These analyses were performed at a somewhat lower temperature than those shown in Table 1, so that methyl linoleate was eluted in 50 minutes rather

TABLE 3. DUPLICATE ANALYSES OF A MIXTURE CONTAINING METHYL PALMITATE AND METHYL LINOLEATE BOTH LABELED WITH C^{14}

Sample*	Aliquot I (cpm)	Ratio	Aliquot II (cpm)	Ratio
1	A. 816 B. 975	0.84	A′ 805 B′ 978	0.82
2	A. 1519 B. 1536	0.99	A′ 1268 B′ 1345	0.94
3	A. 1246 B. 1327	0.94	A′ 1285 B′ 1345	0.95

* Each sample analyzed contained approximately 3 mg of methyl esters.

than 15 minutes. The effluent was fractionated every minute as before. The comparative increase in resolution that resulted is shown in Figure 3, where the mass curve represents the output of the hydrogen flame ionization detector and the bar graph represents the counts per minute minus background in successive 1-minute fractions.

Analysis to Determine the Purity of a Labeled Compound. Cholesteryl palmitate-1-C^{14} was first purified by silicic acid chromatography, and the methyl esters of the fatty acids were prepared and analyzed in the presence of an added mixture of unlabeled methyl esters. Cartridges were changed at 1-minute intervals. The results of the analysis are shown in Table 4.

DISCUSSION

Recovery and Quantification. The relationship between the quantity of radioactivity injected into the column and the quantity measured in the anthracene depends on several factors: the portion of the amount injected that is delivered to the anthracene, the effectiveness with which the radioactivity is retained in the anthracene, and the efficiency with which the radioactivity is measured. Although these factors combine to yield the apparent recovery figure, they are distinct and can be individually approached experimentally and individually discussed.

The efficiency of anthracene as a scintillator has been described above. As a result of the voltage settings used for scintillation counting, relatively more counts per minute are recorded using anthracene than are recorded using diphenyloxazole in toluene at its own optimal setting. Recoveries should be judged, therefore, on the basis of comparison with a similar aliquot pipetted directly onto anthracene.

The efficiency with which the radioactive vapors are condensed and retained in anthracene coated with silicone oil has been documented previously (5). The

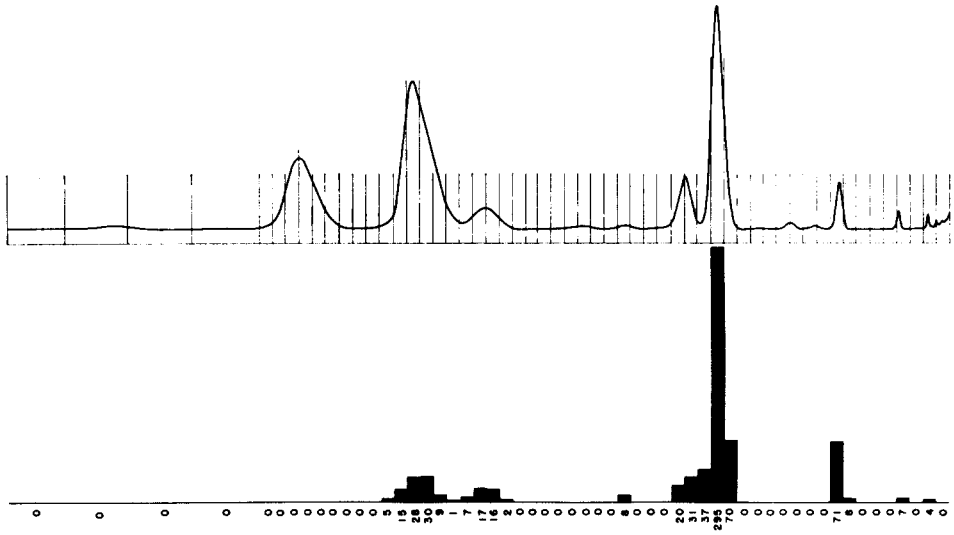

FIG. 3. Analysis of approximately 2 mg of the methyl esters of the fatty acids of a rat epididymal fat pad that had been incubated with acetate labeled with carbon-14. The curves shown represent the output of the hydrogen flame ionization detector. The vertical lines on those curves represent the times at which the fraction collector sample changing device was actuated. The bar graph and figures beneath them represent the radioactivity in each fraction in counts per minute, determined by a 10-minute count. Background of 50 cpm has been subtracted.

distribution of radioactivity in a cartridge 1 foot long was determined following collection of C^{14}-labeled methyl laurate in it and passage of heated (200°)

TABLE 4. ANALYSIS OF METHYL ESTERS DERIVED FROM CHOLESTERYL PALMITATE-1-C^{14}

Sample*	Methyl Ester†	Retention Time (center of peak)	Width of Peak at Half Height	Analysis
		min	*min*	*cpm*
1				0
2	10:0	2	0.12	0
3	12:0	2.8	0.16	0
4				4
5	14:0	4.3	0.24	55
6				380
7	16:0	7	0.4	90,660
8				5,830
9				1,240
10				640
11				405
12	18:0	12.3	0.7	317
13				194
14				188
15				120

* Total injected included 100 μg of unlabeled methyl esters and trace quantities containing 110,000 cpm. Approximately 90% was collected; the remainder was bypassed to the mass detector.

† Refers to the number of carbon atoms in the fatty acid from which the ester is derived, and the number of double bonds.

column effluent through the cartridge for 1 hour. The methyl laurate radioactivity was distributed in the first inch, at the end of 1 hour, when the anthracene used was coated with 10% (by weight) of silicone oil. The distribution was more widespread when uncoated anthracene was used.

It is difficult to predict exactly how long a shorter chain ester will be retained on the anthracene, since this will be influenced by the temperature in the cartridge. This temperature is influenced by multiple factors, including the temperature of the gas injector block, the time in contact, the materials used, the flow rate of gas, and the circulation rate of the air around the cartridge. If more volatile compounds are studied, retentivity should be determined experimentally.

Quantification of the radioassay, in our experience, is primarily influenced by the portion of the injected radioactivity delivered to the fraction collector. The connections between the column and the fraction collector must have no gas leaks, since the pressure-drops across the cartridges, although small, are definite. When a mass detector is used in such a way that the entire column effluent passes through the mass detector before reaching the fraction collector, it is possible to insure the leak-tightness of the system by including a flowmeter in the column inlet; obstruction of the outlet at the fraction collector should cause the inlet flow to drop to zero. This method, of course, is not applicable when part of the column effluent is

delivered to the fraction collector and part to the mass detector, unless the connection to the mass detector is also obstructed.

When a stream-splitting arrangement is used, it is desirable that the pressure-drop across the bypass be appreciably greater than the pressure-drop across the cartridges. The record given by the mass detector is then not distorted when the collecting cartridges are changed, and variation in the quantitative nature of the bypass caused by variation in the pressure-drop from cartridge to cartridge is minimized.

Another avoidable potential source of difficulty is the presence of large "dead" volumes in the connecting tubing between the column and the fraction collector. This can result from the inclusion of a mass detector with appreciable volume in the line or by the presence of a cold spot in the line. Either of these conditions causes a disparity between the time the component reaches the fraction collector and the time it reaches the mass detector. Connecting tubing is therefore kept short, well heated, and insulated, and detector volumes are kept small. Detectors are operated at somewhat higher temperatures than usually required and higher rather than lower gas flow rates are chosen whenever possible.

Another potential cause of failure to obtain quantitative recovery of injected radioactivity is retention of the radioactivity in the column. Incomplete esterification of the fatty acids results in retention of the free fatty acid for longer than the retention time of the corresponding methyl ester and may result in the emergence of the free fatty acid during a subsequent analysis. The presence of labeled, less volatile components other than fatty acids, such as cholesterol, in a mixture of methyl esters can cause similar effects.

Failure of methyl esters to be eluted from the column can be attributed to "adsorption" on the solid support or to transesterification with polyesters of the liquid phase. Among the methods used to minimize adsorption on the solid support are treatment of the solid support with acid and with alkali washes, and the use of phosphoric acid mixed with the polyester as recommended by Metcalfe (10) for the analyses of free fatty acids. The possibility of transesterification was reduced by ion exchange treatment of the polyester liquid phases to remove esterification catalysts (8).

Retention of radioactive methyl esters on the column was encountered by us on two occasions. Both occurred when we used polyester phases coated on acid- and alkali-treated Chromosorb W, which had also been siliconized by treatment with dimethyldichlorosilane prior to coating with polyester (11). The effect noted was more pronounced with highly radioactive, very small samples that failed to be eluted; partial recovery of injected radioactivity was obtained by including unlabeled carrier in the sample injected. We no longer use the silicone pretreatment for the solid support when using polyester coatings.

We have been unable to effect an increase in the rate of release of radioactivity from a column after completion of an analysis by the injection of unlabeled material, although we have repeatedly looked for the effect.

Resolution. Since the radioactivity of components does not necessarily vary with the mass of these components, any approach in which the radioactivity is collected at intervals determined by the appearance of mass peaks may lead to erroneous interpretations. In most of the experiments reported here, the effluent gas was fractionated into equal aliquots. This yields a record of radioassay that is independent of the record of mass. The two records are then compared and the compounds bearing the radioactive label identified. The accuracy with which the radioactive components are identified is a function of the resolving power of the radioassay.

The resolution offered is exemplified in the analysis of the multicomponent mixture shown in Table 1. Changing cartridges every minute did not permit quantification of the radioactivity in the 10 and 12 carbon methyl esters and indeed was not adequate to show that the radioactivity was in these fatty acid esters rather than in unsaturated or branched-chain fatty acid esters.

Identification of the radioactive fatty acids of Table 2 is similarly qualified. The resolution was adequate to establish the presence of radioactivity in methyl oleate but was inadequate to distinguish it quantitatively from the radioactivity in methyl stearate. Evidence for synthesis of linoleate rests on the greater activity in sample 18 than in sample 16.

In the experiment recorded in Table 3, only two aliquots were taken in each analysis. This was done on the assumptions that there was no radioactivity except in methyl palmitate and linoleate, that the radioactivity of each was well separated from the other, and that for purposes of the experiment there was no exchange of radioactivity *in vivo*. The validity of these assumptions determines the reliability of the result.

In the determination of the purity of cholesteryl palmitate (Table 4), it was not possible to state that there was radioactivity in methyl palmitoleate (which would have been present in samples 8 and 9) nor was it possible to rule out its presence. Because of the increase in counting rate after the emergence of palmitate, the

presence of any trace quantities of radioactivity in fatty acid esters emerging after palmitate also went undetected. It should be noted that the increase in counting rate is a very small percentage of the activity in the major component. More than 90% of the injected radioactivity was recovered during the time of the analysis. Since the effluent stream was divided about 1:10 in the detector oven, this represented essentially quantitative recovery.

In each of these three examples, the resolution shown was adequate for the requirements of the experiment. Collecting two or three times as many samples in the same time period would have permitted sharper identification of the components. Alternatively, collection of a sample every minute would have been more adequate had the time of the GLC analysis been extended to double or triple the time used. Either alternative is readily available. Figure 3 demonstrates the utility of this approach. In this series of analyses, minor radioactive components were identified despite the low specific radioactivity of the materials analyzed.

Although it is not obvious in Table 1 that there is any time difference between the emergence of a component as detected by the mass detector and the collection of that component by the fraction collector, the presence or extent of any disparity can be more accurately determined if the frequency of collection of samples is increased. The results of other experiments in which the radioactivity in a single cartridge was monitored continuously during an analysis indicated that bypass systems such as described here may result in a 5- to 10-second difference between the start of a peak on the mass record and the detection of the radioactivity. It also has been occasionally observed that the "trailing" of the radioactivity following the emergence of a radioactive component may be apparently greater than the trailing of the mass of the component. It may be difficult to determine whether this last effect reflects the behavior of the ester on the column or is an artifact introduced by the fraction collection system. Two experimental tests are possible. The temperature of the outlet tubing may be increased and the flow rate of the gas may be increased. If either of these causes the record of radioactivity and the mass record to become more nearly coincidental, it is assumed that their previous lack of coincidence was an artifact.

This possible difficulty also serves to emphasize the desirability of collecting components at equal intervals rather than by reference to the record of the mass detector. Since the record obtained is then independent of the mass record, the resolution offered is apparent and mistakes in interpretation are less likely.

Sensitivity. In many experiments the quantity of radioactivity that can be put on a column is limited not by the lack of radioactive material but by its low concentration in the material to be analyzed. Advances in the technology of "preparative" gas-liquid chromatography can therefore be expected to increase the "sensitivity" of our methodology. In our experience, the use of approximately $^1/_4$-inch diameter columns permitted us to inject samples with up to 1 mg per component without serious loss of resolution and up to 5 mg with only moderate loss of resolution. The amount that can be analyzed is to some extent a function of the slowness with which the analysis is carried out.

The sensitivity of the radioassay, in terms of the minimum quantity of radioactivity that can be detected or measured in a given component, is theoretically very great. A given set of samples can be counted for whatever length of time is necessary to get statistically reliable results. As may be seen from several of the tables, however, the tendency of radioactivity to continue to be eluted from a column after the emergence of a radioactive compound is not negligible. This tends to make the system of radioassay somewhat more sensitive at the beginning of an analysis than further along in the course of an analysis.

Use of a column for low-level radioassay following an analysis involving high levels of radioactivity is often difficult or entirely impractical. Because of the many processes that tend to cause radioactivity to be retained on the column (e.g., from higher boiling radioactive compounds), the rate of emergence of "background" radioactivity from a column depends on the previous history of the column. A determination of the background radioactivity prior to analysis is therefore desirable. It is also desirable to have chromatography columns that are easily packed and quickly installed so that they may be considered disposable after relatively few analyses.

Because of the design of a series of experiments in which labeled free fatty acids were to be used, the absence of traces (1 part in 10,000) of radioactivity in components other than the given free fatty acid was critical. Because of the difficulties shown in Table 4, it was difficult to guarantee their absolute purity by analytical means and impossible to detect the presence of nonvolatile impurities at this concentration range; we therefore chose to purify by gas-liquid chromatography rather than merely to analyze.

Free fatty acids were purified by solvent extraction and were then analyzed and prepared pure on a microscale using 5-mm i.d. columns packed with ethylene glycol-adipate on Chromosorb W. The maximal amount of free fatty acid that could be analyzed without

impairing the resolving power of the column was determined prior to the analysis using unlabeled acid. This amount, usually 5 mg of labeled acid, was then analyzed. The effluent was fractionated so that the material emerging before the acid of interest was collected in one cartridge, the acid itself in a second. For this purpose, uncoated Chromosorb W was used to fill the cartridge in place of the anthracene. Since the samples following the emergence of the acid showed large amounts of radioactivity for at least several hours, the column was removed from the oven and discarded as radioactive waste. (Each column was used for only one radioactive analysis.) The fatty acids trapped in the cartridges were quantitatively eluted with two 5-ml aliquots of toluene.

Some of the columns used in these preparations were packed with Chromosorb W 78%, phosphoric acid 2%, and ethylene glycol adipate 20% (10). Others were packed with Chromosorb W 90%, and ethylene glycol adipate 10%.

Determination of Specific Radioactivity. The radioassay system can be used for the determination of specific radioactivity since the response of the mass detector may be calibrated to read in terms of micrograms of material. It is also possible to determine the total quantity of material present in a sample from an aliquot through the use of an internal standard. Since simultaneous recordings of mass and radioactivity are available, information concerning relative and absolute specific radioactivities is immediately available. When a column effluent is split between the mass detector and the collection system, it may be of interest to have reliable quantitative data concerning the fractional distribution of the gas stream to the two pathways. This may be determined experimentally by comparing the response of the mass detector to a given known mixture with and without the stream-splitting device in the circuit.

Choice of a Mass Detector. One has considerable latitude in the choice of a mass detector to be used with this system of radioassay. In many experiments, the addition of unlabeled material to the sample prior to injection was desirable. This makes detection possible by passing the entire sample through a relatively insensitive detector such as a katharometer. For this kind of operation the detector cell must be of leak-tight construction. Since it offers the most quantitatively accurate results, a detector of this kind is ideal for analyses of relatively large amounts of materials with low specific radioactivity. On the other hand, the use of ionization detectors with an arrangement for splitting the effluent between detector and collector offers the advantage of generally easier operation and greater versatility. Because of their high sensitivity, ionization detectors may be more easily adapted to analyses of materials with little mass when, for one reason or another, it is undesirable to add unlabeled carrier.

Ionization detectors are not easily used without splitting the stream, since they either degrade the sample to more volatile products, contaminate it with radioactivity, or have a limited dynamic range which unnecessarily limits the quantity of sample that may be analyzed. Each of these detectors has sensitivity adequate for most purposes even when only $^1/_{10}$ of the total sample is sent through the mass detector.

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