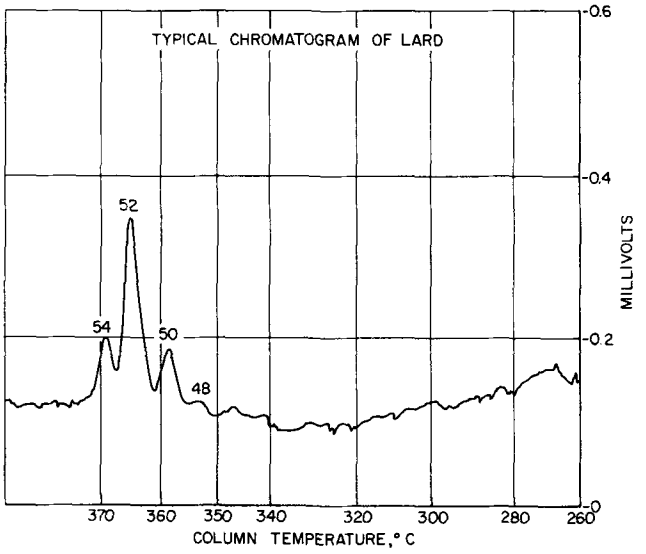


FIG. 4. Typical chromatogram of lard.

lowest molecular weight triglyceride peak found in butteroil contained 24 fatty acid carbons, even though butteroil contains a sizeable amount of butyric and caproic acids. Butteroil is apparently composed of triglycerides containing both long- and short-chain fatty acids in the same molecule.

Fig. 4 shows a chromatogram obtained from 0.6 μl. lard in a total analysis time of 12 min. The analysis of this type of very high molecular weight mixture is much more difficult than the preceding analyses. The triglycerides contained in lard have a lower thermal conductivity response and their elution temperature is close to the point where column bleeding becomes a serious problem. Even so, gas chromatography may be used to obtain useful information from this type of glyceride mixture.

REFERENCES

1. McInnes, A.G., Tattrie, N.H., and Kates, M., J. Am. Oil Chemists' Soc., 37, 7–11 (1960).
2. Huebner, V.R., J. Am. Oil Chemists' Soc., *36*, 262–3 (1959).
3. Huebner, V.R., Abstr., Am. Oil Chemists' Soc., fall meeting, Los Angeles, California, Sept., (1959).
4. Fryer, F. H., Ormand, W.L., and Crump, G.B., J. Am. Oil Chemists' Soc., *37*, 589–590 (1960).
5. Martin, A.J., Bennett, C.E., and Martinez, F.W. Presented at the 3rd Symposium on Gas Chromatography, Edinburgh, Scotland, June 8–10, 1960.
6. Vandenheuvel, W.J.A., Sweeley, C.C., and Horning, E.C., J. Am. Chem. Soc., *82*, 3481–2 (1960).
7. Horning, E.C., Moscatelli, E.A., and Sweeley, C.C., Chem. and Ind. (25) 751–752 (1959).

Monitoring Eluates from Chromatography and Countercurrent Distribution for Radioactivity[1]

H. J. DUTTON, Northern Regional Research Laboratory,[2] Peoria, Illinois

UNIQUE ANSWERS to problems as to the mechanism of organic reactions and of biological transformations are frequently provided by the use of radioactive isotopes. Their application usually involves three steps: 1) Addition of a known, radiochemically pure compound; 2) fractionation and reisolation of intermediates or reaction products; 3) isotopic assay of radiochemically pure isolated components. Chromatography affords a powerful tool for carrying out the second step. However, isotopic assay of the isolated products in step 3 has frequently posed a problem because of the difficulty of assaying radioactivity on the small amounts of individual components available.

The present review of methods for monitoring eluates for radioactivity is limited to a consideration of C^{14}- and H^3-labeled compounds and is restricted to 1) liquid-liquid chromatography (LLC), 2) countercurrent distribution (CCD), and 3) gas-liquid chromatography (GLC). Paper chromatograms have long been assayed for radioactivity, but the well-established techniques (1), which have been developed, are not discussed in this review.

Liquid-Liquid Chromatography. Liquid-liquid chromatography may be monitored by discontinuous or continuous methods of assay. One of the simplest procedures of discontinuous assay is illustrated by Fig. 1 for the chromatography of methyl oleate exposed to tritium gas (2). This reaction yields the 9,10-tritiostearate (3). Chromatography of the fatty acids was carried out by the liquid-liquid partition chromatographic procedure of Nijkamp (4), which employs a methyl alcohol-isooctane solvent system on a silicic acid column. Alternate 1-ml. eluate fractions were (a) titrated in a nitrogen atmosphere with 2/10 *N* potassium hydroxide to a thymol blue endpoint using a Gilmont microburet and (b) diluted with 15 ml. of scintillation solution for assay of radioactivity with an Automatic Tricarb Scintillation Spectrometer (Packard Instrument Company). The time of counting may be continued over a sufficient period of time to attain the desired statistical accuracy of results. If absolute measurements of radioactivity are required rather than relative values, the presence,

[1] Lecture presented at American Oil Chemists' Society Short Course on Newer Lipid Analyses, University of Rochester, Rochester, New York, July 24–26, 1961.
[2] This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

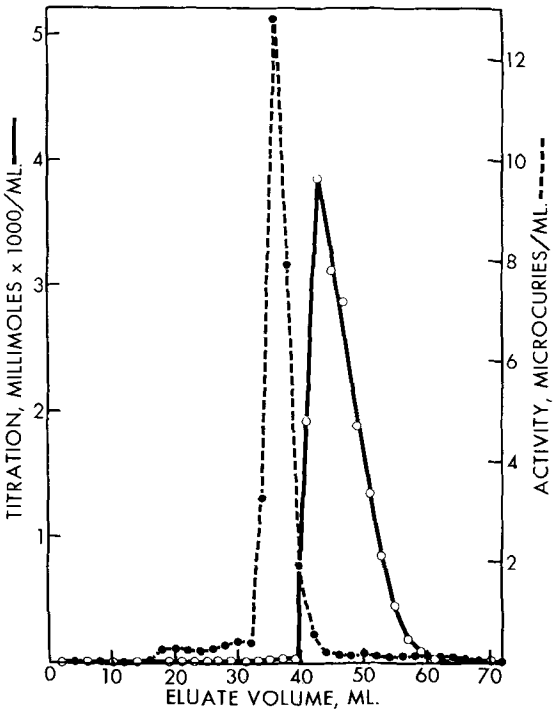


FIG. 1. Liquid-liquid partition chromatogram of a mixture of chemically purified, tritiated, oleic acid.

absence, or influence of a quencher should be ascertained in the eluate solvent phase by the usual procedure of adding known radioactivity to the sample and then determining efficiency of recovery.

In the chromatographic solvent system described, quenching by fatty acids and by the chromatographic solvent was negligible. Aldehydes, highly halogenated hydrocarbons, and nitro groups are particularly active quenchers of fluorescence; whereas water, alcohol, and hydrocarbons show a decreasing amount of interference (5). This system of chromatography is capable of isolating 9,10-tritiostearate in relatively large amounts and in high specific activity since the emergence of radioactivity is well separated from the parent oleate peak.

Several procedures for continuously monitoring liquid-liquid columns have been described, particularly for aqueous solutions. Plastic scintillators in the form of sheets have been used as the walls for flow-through cells (6). The use of plastic scintillation tubing in spiral-coiled form has also been reported (7) and of a glass tube packed with anthracene crystals. Fluorescence excited in the scintillator by radioactivity of the eluate is measured by a photomultiplier cell. The current is amplified into a ratemeter and is fed to a recorder, which plots the disintegration rate versus time, to give the differential curve for the elution of radioactive substances. In other arrangements the auxiliary equipment consists of two photomultipliers and two amplifiers, in coincidence circuit, feeding into an integrator recorder.

Use of a specially designed Geiger-Muller counting chamber has also been described (8). The eluate from the column flows over a thin mica window which covers an elongated chamber. C^{14} radiation penetrates this window, and the ion current after amplification can also be plotted versus time or volume flow through the column.

The continuous flow procedures described may, of course, be used in a batchwise fashion and aliquots collected from a chromatographic column may be passed through the cells in a batchwise system. A cell (Fig. 2) designed exclusively for batchwise operation has a vial constructed of scintillation plastic and mounted directly on the base of a photomultiplier tube (9). The solution to be measured is poured into the vial through a funnel, and the vial empties automatically in a syphoning action. An alternative batch procedure for aqueous solutions in an automatic scintillation spectrometer is that of filling the scintillation vial with plastic beads and pouring the aqueous solution over the beads (10). Counting efficiencies of 5.7 to 29.3% for C^{14} have been reported. In a gaseous phase the detection efficiency of 58.3% was obtained.

Countercurrent Distribution. By its very nature countercurrent distribution is a discontinuous or batch-fractionating process. Since the upper layer of solvent (the solvent that is withdrawn into the fraction collector) usually is the nonpolar phase and frequently hydrocarbon in nature, it is generally miscible with the scintillation solvent and can be counted with the same efficiency generally observed in toluene scintillation solvent systems.

The data of Fig. 3 were obtained by withdrawing 0.1 cc. of every fourth 10 ml. of upper layer fractions and introducing each fraction into 15 ml. of the scintillation solution in the solvent vial (11). Any quenching of either the solvent or the methyl ester was insignificant. Since the fatty acid methyl esters illustrated by this figure were randomly labeled by growing soybeans in the presence of radioactive CO_2, the counts plotted on the ordinate are directly related to the weight per tube. Other applications of this technique of direct elution of fractions from an automatic countercurrent distribution apparatus into scintillation solvent has been described for the glyceride structure of cocoa butter (12) and for the resolution of 9,10-tritio oleic from 9,10-tritio elaidic (13). These two compounds are formed as the result of tritium addition to sterolic acid by Wilzbach's gas exposure procedure and are separated by the silver π-complexing solvent (14). Because countercurrent distribution is a relatively slow process there has been little

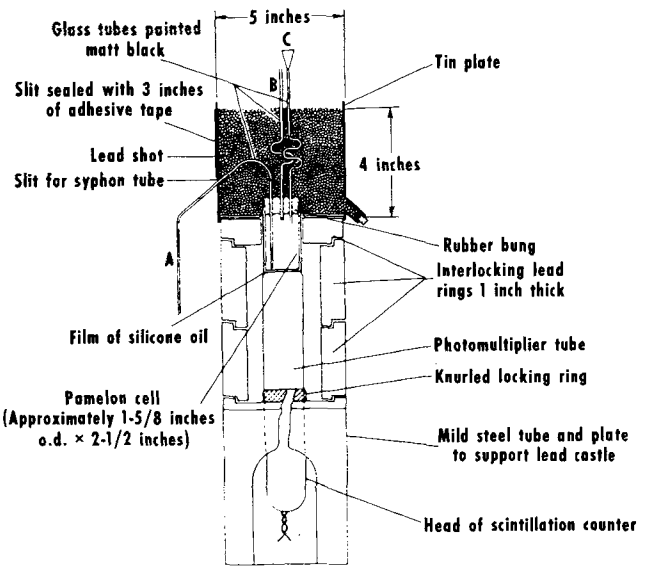


FIG. 2. A flow-through cell for use with scintillation counters.

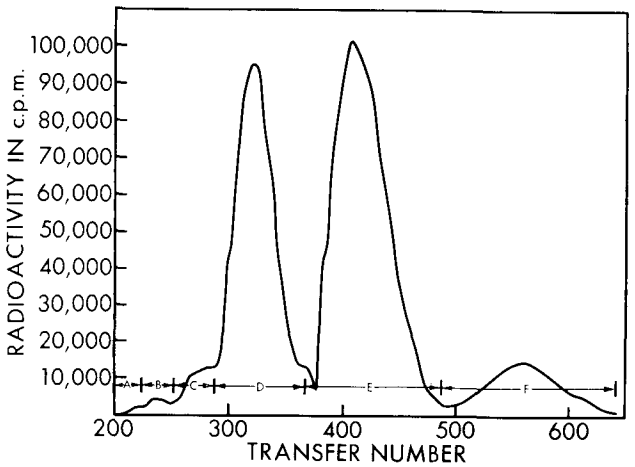


FIG. 3. Countercurrent distribution of soybean esters biosynthetically labeled with C^{14}: (C) stearate, (D) palmitate-oleate, (E) linoleate, and (F) linolenate.

demand as yet for complete automation for measuring radioactivity. The problem of monitoring is analogous to that discussed under the continuous monitoring of liquid-liquid chromatography, and undoubtedly the procedures described above would apply with minor modification.

Gas-Liquid Chromatography. Because of its ease of operation and versatility, gas-liquid chromatography is becoming increasingly popular as an analytical tool in the lipid field. The very fact that it is a micro method, however, poses distinct problems for the collection, monitoring, and assay of radioactivity of isolated components. Much biological and organic research is conveniently or necessarily carried out at low levels of specific activity. For example, if 4 microcuries of C^{14}-labeled linoleic acid are administered in human experimentation, many orders of dilution will be experienced if complete equilibration of the C^{14}-labeled linoleic acid occurs in the body's linoleic acid. The amount of radioactivity subsequently isolated in a mg. sample for chromatography becomes extremely small.

A procedure to condense eluates in a stream from gas chromatographic columns upon anthracene crystals coated with silicone oil has been described by Karmen (15). The anthracene serves both as the condensation surface and as the scintillation crystal. Successive collections on the anthracene crystals are introduced into the counting chamber of the liquid scintillation counter and the counting continued over as long a period as required. Automatic fraction changing equipment for carrying out this operation is available commercially.

Collection techniques, which are experimentally much simpler, consist of direct condensation of solutes in the gas streams into the scintillation solvent by bubbling the effluent stream through a scintillation solvent. This procedure has been applied successfully both as a manual technique (16) and as an automatic collection technique.

The manual collection system, illustrated in Fig. 4, provides for passing a gas stream from the gas chromatographic column exit to filled vials of scintillation solvent. Glass wool is wrapped about the portion of the joint extending from the chromatographic instrument and up to the side arm to reduce heat loss and

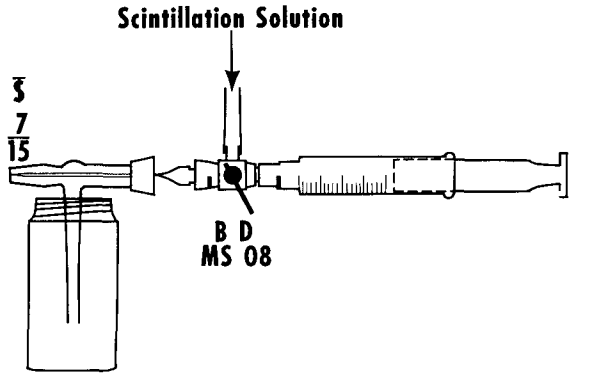


FIG. 4. Manual collection system for condensation in a scintillation solvent.

condensation of solutes in this portion of the collection tube. Since a temperature gradient along the glass tube is inevitable, the expedient used for quantitative removal of the condensed solutes is to inject 0.5 ml. of scintillation solution from the syringe just prior to removing the vial and replacing it with a new one. Normally, replacement of collecting vials are made on either at 1 min. or at 30 sec. intervals. Some care must be taken while injecting the flushing solvent so that it does not find its way into the detection unit of chromatograph. On the other hand, injection should be sufficiently rapid so as to flush down all glass surfaces. A temporary or normal back pressure at the time of injection serves to mark the thermoconductivity record at each sample change. Three-way syringe valves are available to facilitate the filling of a syringe and subsequent injection of the sample into the chromatographic equipment. An example of the application of this manual collection procedure is given in Fig. 5 for the gas chromatographic separation of products from tritiated methyl oleate.

A more sophisticated procedure involving automatic collection, Fig. 6, provides for 1) continuous condensation of solutes from the gas stream of the chromatogram in scintillation solvent; 2) continuous concurrent flow of scintillation solvent and its collection in an automatic fraction collector; 3) refinements in indexing the chromatogram corresponding to sample collections; 4) sharp cut-off of the solvent flow be-

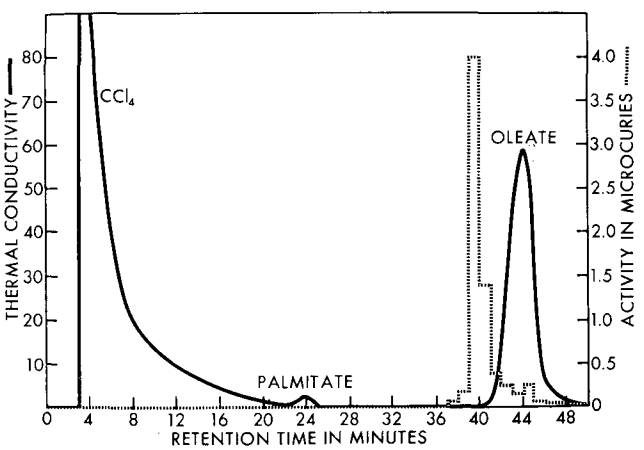


FIG. 5. Gas chromatogram of methyl oleate immediately after exposure to tritium gas.

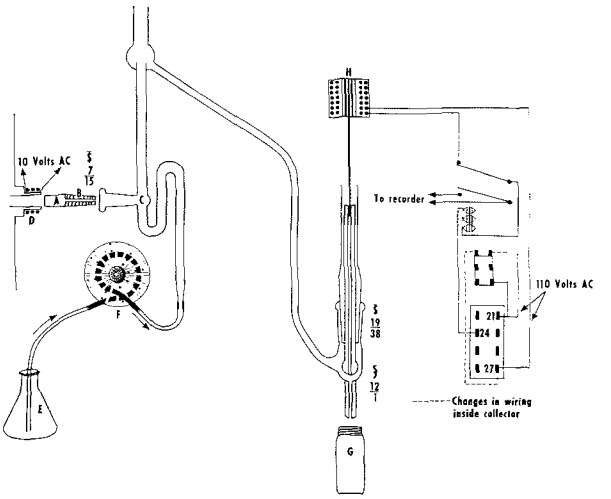


FIG. 6. Automatic system for condensation in scintillation solvent.

tween fraction collections; 5) maintenance of the high temperature of the gas stream up to the point of its contact with the scintillation solvent.

In following the sequence of events, the gas stream issuing from the thermal conductivity cell of the chromatographic instrument passes through standard taper brass plug A and exits through the narrow end encased in the Teflon sleeve B of standard taper 7/15. This sleeve, it will be noted, is formed to cover the front face of the brass tube A and thus during gas flow to exclude solvent from direct contact with brass. The standard taper of the Teflon sleeve fits the glass standard-taper joint of the glass condensation and delivery tube C. During operation glass wool surrounds this brass delivery tube, the ground glass joint, and the nichrome heating wire D. This heater wire, dissipating 50 watts, assures that during operation the temperature out to the tip of the brass plug remains at 150°C. or higher. Scintillation solvent contained in flask E is pumped by means of Sigma pump F through condensation tube C where it mixes with the stream of gas from the column. In the bulb, bubbles of gas phase and scintillation solvent separate, the gas stream exits out of the top and the scintillation solvent passes through the side arm and valve to the scintillation vials G in the collector. A solenoid activated ball-joint serves as a valve to stop solvent flow while scintillation vials are changed. Solenoid H receives its power from the normally closed contact of relay I. This relay is activated during the time of vial transfer from the fraction collector as supplied with power by pin 24 on the Jones plug of the collector.

To provide uninterrupted power leads, two modifications in electrical wiring of the collector were made as indicated by the dotted lines in Fig. 6. These leads from the switch were brought to appropriate points of the Jones plug. A second set of normally open points on relay I served to short out the terminals of the recording potentiometer during the second interval of transfer and place a pip upon the thermal conductivity curve.

Scintillation vials are held in a wheel made of ¾ in. plywood and drilled at appripriate positions to hold one row. The collector is operated under time flow conditions, and the desired collection time is set on its interval clock. This technique is illustrated in Fig. 7 with a mixture of methyl oleate, methyl

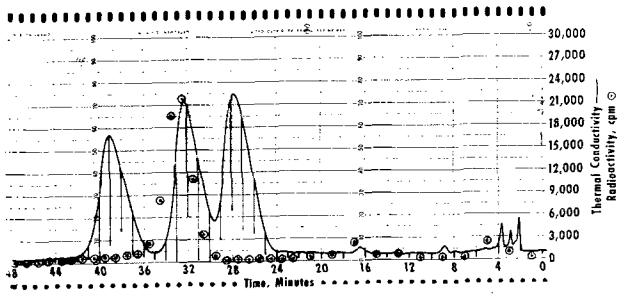


FIG. 7. Gas chromatogram obtained on a mixture of methyl oleate, C^{14}-labeled methyl linoleate, and methyl linolenate.

linoleate, and methyl linolenate with added C^{14}-labeled methyl linoleate.

The relative merits of manual versus automatic operation are primarily those of simplicity of equipment as against the convenience and uniformity of machine sampling. Somewhat greater flexibility is permitted under manual operation where the operator at his discretion may collect an entire peak in a single tube if he so desires. This feature is particularly valuable for very low-level radioactivity samples where splitting the peak into several fractions increases the relative importance of background error.

The continuous monitoring of gas chromatography has been accomplished by a variety of procedures. Two systems give rise to an integral rather than a differential curve for radioactivity. A system of mixing a scintillation solution with the exit gas stream from a chromatographic column and of circulating the solution over the face of a photomultiplier tube has been described by Popjak (17) and is shown in Fig. 8. In this way he obtained an integral curve for radioactivity concurrently with a differential curve for thermal conductivity data. A similar type of representation is obtained by allowing the solutes to condense upon the anthracene crystals and to meas-

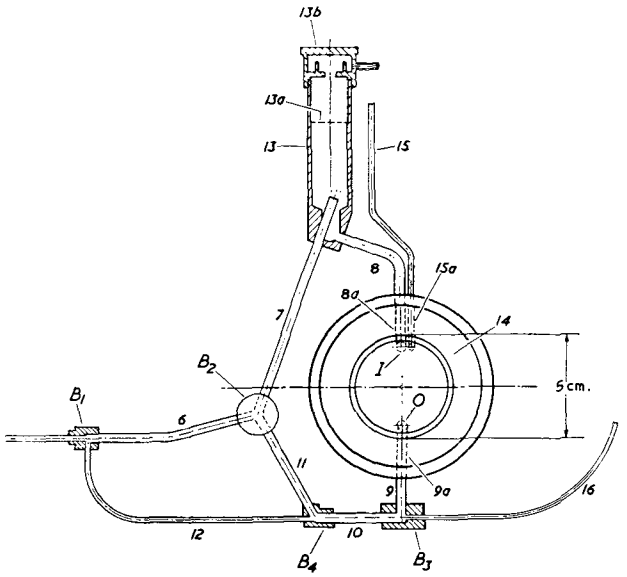


FIG. 8. Construction of a scintillation counter for measuring radioactivity of vapors.

ure the intensity of fluorescence continuously as the chromatogram develops (15). Still a third procedure of obtaining the integral radioactivity curve has been employed by Emmett (18) who condenses the radioactive materials out on the surface of a liquid nitrogen finger. The activity of the finger is continuously assayed by a thin window Geiger-Muller tube, ratemeter-recorder system. For these three systems, in common with all continuous methods, the time of counting is necessarily limited to either the time of peak elution or the time between elution of peaks.

Monitoring high-temperature gas chromatography with a proportional countering equipment has been described by Wolfgang and Rowland (19). Helium is used as carrier gas in chromatography and is converted to counting gas by the continuous injection of methane following its passage through the thermal conductivity cell. Differential-type curves are obtained with the use of a counting ratemeter and recorder.

James and Piper (20) have used proportional counting but have introduced a copper oxide combustion tube in the exit gas stream to convert organic vapors to CO_2 and water. The gas stream is dried, 5% CO_2 in injected, and the mixture is passed into the counter at room temperature. Gas density and radioactivity recordings on yeast lipids are shown in Fig. 9.

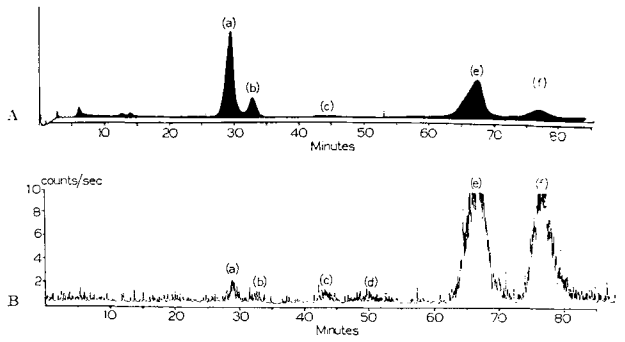


FIG. 9. Comparison of simultaneous record of vapor detector (gas density meter) and proportional count rate, from analysis of fatty acids isolated from yeast grown in the presence of 2-C^{14}-stearic acid.

The use of an ion chamber to monitor radioactivity of eluates from gas chromatograms was first described by Riesz and Wilzbach (21). Two recorders simultaneously plot the thermal conductivity current and the ion current to give the mass and radioactivity in differential-type curves. Although this system was highly successful for relatively low-boiling hydrocarbons, at 150°C. and above strain currents in the insulator limited its applicability. A high-temperature ion chamber useful for monitoring the gas chromatography of fatty acid methyl esters is shown in Fig. 10 (22). The principal feature of this system is its Teflon insulator, which does not develop strain currents at the high temperatures employed (21). An example of its application to the same tritiated oleate system shown in Figures 1 and 5 is given in Fig. 11. Recently two similar designs of ion chambers for use with high-temperature gas chromatography have been published (23, 24). One limitation of all ion-chamber electrometer recorder systems is that

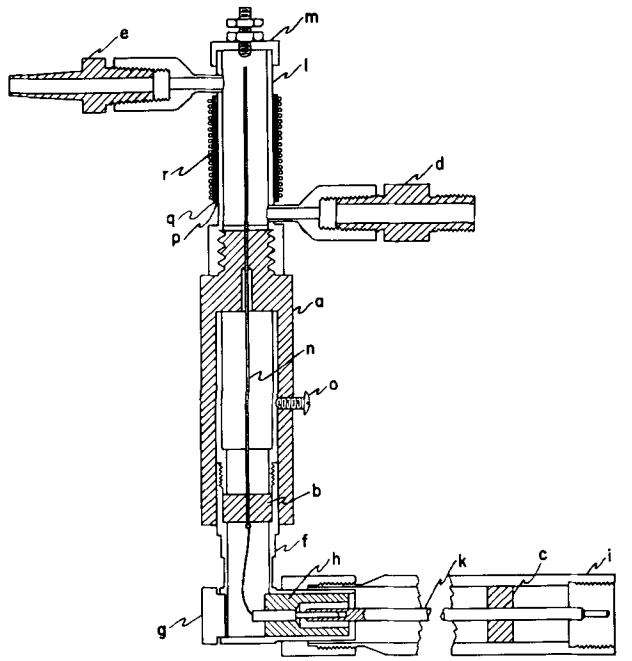


FIG. 10. Ionization chamber for high-temperature gas chromatography.

reaction products of relatively high specific activity must be used, i.e., 0.1 microcurie per mg.

The most critical single factor in all of the monitoring procedures, and one most likely to be overlooked in the use of these techniques, is that of supplying adequate heat. A temperature of 150°C. throughout the gas stream conduction and ion chamber itself must be maintained. Cool areas will condense and accumulate radioactivity and will reduce the percentage recovery. Particularly when considering the radioactive volatiles in scintillation solvent is it important that the temperature of the conducting tube be maintained up to the point of the gas solvent interface.

The advantages of the continuous monitoring system, such as with an ion chamber, are obvious in that one readily obtains concurrent chart records of mass and radioactivity in their usual differential form of presentation. Any continuous flow and/or differential registration procedure, of course, suffers because the rate of disintegration may be assessed only during the actual period of residence in the detector. The principal advantage of monitoring with liquid scintillation procedures over the ion chamber is one of

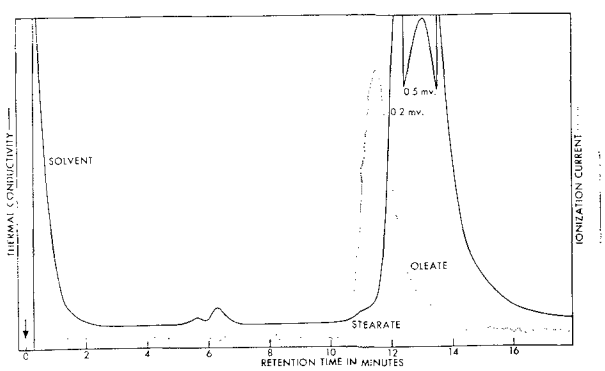


FIG. 11. Gas chromatogram of tritiated methyl oleate after saponification, alcohol exchange, acidification, and methylation.

sensitivity. By condensing in a scintillation solution, the radioactivity may be subsequently observed at the convenience of the operator and for sufficiently long periods of time to obtain the desired statistical results.

REFERENCES

1. Arnoff, S., "Techniques of Radiobiochemistry," The Iowa State College Press, Ames, Iowa, (1957).
2. Dutton, H. J., Jones, E. P., Mason, L. H., and Nystrom, R. F., Chem. and Ind., No. *36*, 1176–1177 (Sept. 6, 1958).
3. Jones, E. P., Mason, L. H., Dutton, H. J., and Nystrom, R. F., J. Org. Chem., *25*, 1413–1417 (1960).
4. Nijkamp, H. J., Anal. Chim. Acta, *10*, 448–458 (1954).
5. Kerr, V. N., Hayes, F. N., and Ott, D. G., Intern. J. of Appl. Radiation and Isotopes, *1*, 284–288, (1957).
6. Schram, E., and Lombaert, R., Anal. Chim. Acta, *17*, 417–422 (1957).
7. Funt, B. L., and Hetherington, A., Science, *129*, 1429–1430 (1959).
8. Bangham, D. R., Biochem. J., *62*, 552–553 (1956).
9. Greenfield, S., The Analyst, *83*, 114–116 (1958).
10. Steinberg, D., Nature, *182*, 740–741 (1958).
11. Dutton, H. J., Jones, E. P., Scholfield, C. R., Chorney, W., and Scully, N. J., J. Lipid Research, *2*, 63–67 (1961).
12. Dutton, H. J., Scholfield, C. R., and Mounts, T. L., J. Am. Oil Chemists' Soc., *38*, 96–101 (1961).
13. Dutton, H. J., Jones, E. P., Nystrom, R. F., J. Org. Chem., in press.
14. Dutton, H. J., Scholfield, C. R., Jones, E. P., Chem. and Ind., in press.
15. Karmen, A., and Tritch, H. R., Nature, *186*, 150–151 (1960).
16. Dutton, H. J., ms. in preparation.
17. Popjak, G., Lowe, A. E., Moore, D., Brown, L., and Smith, F. A., J. Lipid Research, *1*, 29–39 (1959).
18. Emmett, P. H., Abstract of Papers, 135th Meeting, Am. Chem. Soc., Boston, Mass., April 5–10, 1959, p. 3Q.
19. Wolfgang, R., and Rowland, F. S., Anal. Chem., *30*, 903–906 (1958).
20. James, A. T., and Piper, E. A., J. Chromatog., *5*, 265 (1961).
21. Riesz, P., and Wilzbach, K. E., J. Phys. Chem., *62*, 6–9 (1958).
22. Mason, L. H., Dutton, H. J., Bair, L. R., J. Chromatog., *2*, 322–323 (1959).
23. Cacace, F., and Inam-Ul-Haq., Science, *131*, 732–733 (1960).
24. Dobbs, H. E., J. Chromatog., *5*, 32–37 (1961).

Fatty Acid Structure Determination by Chemical Means

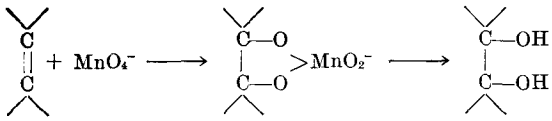
ROBERT A. STEIN, Department of Physiological Chemistry, University of California, Los Angeles, California

THE UTOPIAN GOAL for a chemist performing a structure determination is to possess an unknown compound in a high state of purity and to command reactions that proceed in an unambiguous manner. In practice, a compromise with either of these ideals still makes it possible to adequately determine a structure, but the lack of both of them will severely cloud the interpretation of the data.

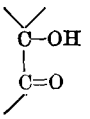
The various chromatographic techniques are effective in preparing pure samples of fatty acids that are initially contaminated with homologues or dissimilar compounds, but greater stress is placed on such techniques in separating closely allied isomers. Obvious examples of these are the isomeric unsaturated fatty acids (including geometric as well as positional isomers) and positional isomers of branched chain acids. The extent to which small quantities of these isomeric compounds can be found depends on the specificity of the chemical reagents used in the structure determination. For this reason, this discussion will consider the qualitative nature and quantitative limitations of some reactions useful to the lipid chemist in solving identification problems not easily solved by chromatographic techniques alone. Further, consideration will be limited to the fatty acids with a hydrocarbon chain and will not include those with other functional groups.

Potassium permanganate is useful in cleaving fatty acids at centers of unsaturation. An examination of the mechanism and experimental conditions used with this reagent will assist in understanding the limitations or sources of difficulty in using it.

A simplified view of a thorough permanganate oxidation of a double bond shows the products as two carboxylic acids with a concomitant reduction of the Mn(VII). The reaction however is not simple, and inevitably other products are formed; the course of the reaction is determined by pH conditions. An investigation of the oxidation of oleic acid using O^{18}-labeled permanganate (1) in alkaline solution confirmed the theory that the first step of the oxidation involved the formation of a cyclic ester between the olefin and permanganate ion which was followed by hydrolysis in the alkaline solution to the glycol. The recovery of high yields of the glycol indicates it is stable toward oxidation under these conditions.



Oxidation under less alkaline conditions results in a further oxidation of the olefin to the ketol or acyloin (2).



Conditions (pH 9) that give the ketol in good yields (75%) from oleic acid do not oxidize the glycol to the ketol (3). To reconcile this apparent anomaly, it is considered (1) that the cyclic ester is a common intermediate in both reactions and undergoes rapid hydrolysis to the half ester. At high pH, hydrolysis is complete, resulting in glycol formation, while in somewhat less basic medium the ketol formation involves a further oxidation of the Mn(V) in the ester which then undergoes a concerted elimination reaction with base.