Journal of Chromatography, 78 (1973) 141–147

CHROM. 6474

RADIOISOTOPES — INTRODUCTION

DETECTION AND CHROMATOGRAPHIC RESOLUTION OF LABELED LIPID INTERMEDIATES FORMED IN ENZYME SYSTEMS

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SUMMARY

At present, zonal profile analysis carried out with automatic scraping equipment and liquid scintillation radioassay provides the ultimate resolution and sensitivity for detecting and quantitating labeled metabolic intermediates. The application of this approach to the analysis of metabolites involved in the biosynthesis of O-alkyl glycerolipids is illustrated in this paper.

INTRODUCTION

The detection of initial substrates and final products labeled with ^{14}C or ^{3}H that are present in metabolic systems is generally possible with most radioassay instrumentation and techniques that are currently available. In contrast, the detection of the small amounts of radioactivity found in many metabolic *intermediates* often presents a difficult and challenging problem. This is particularly true if the functional groups on the intermediary molecular species are very similar. Except for liquid scintillation assay and scraping techniques (1- and 2-mm zones), most radiometric procedures and instrumentation are incapable of detecting activity levels that are only slightly above the background or of resolving barely separable components with statistically good quantitation of their radioactivity.

This introductory paper on radioisotopes is organized so as to illustrate how very closely related labeled molecules produced in biochemical systems can be detected after chromatography on thin layers of silica gel.

GENERAL BACKGROUND INFORMATION

A number of earlier papers[1–9] have thoroughly reviewed the original literature on the various procedures that are available for the radioassay of thin-layer chromatograms. The methods include: (a) elution and subsequent radioassay; (b) strip-scanning with Geiger–Müller detectors; (c) strip-scanning with phototube detectors; (d) autoradiography; (e) fluorography; (f) sublimation and distillation autography; (g)

* Under contract with the U.S. Atomic Energy Commission.

combustion and subsequent radioassay of products; (h) beta camera photometric analysis; (i) spark-chamber photo-detection; and (j) zonal profile scanning using liquid scintillation detection.

Some of the methods are most helpful for rapidly locating radioactive areas on the chromatograms and therefore represent useful prerequisite steps before proceeding with more tedious, but more quantitative, techniques. Perhaps the best instrumentation of this type is the spark-chamber device described by PULLAN[10]. It is an extremely sensitive and rapid procedure, which produces a spark when beta-particles cause the ionization of gas (10% of methanol in argon) between a series of parallel electrodes consisting of 50 individual spiral cathodes containing a central anode wire; a photograph of the sparks produces an image similar to an autoradiograph. The radioactive areas located on the chromatogram can then be marked for liquid scintillation radioassay in order to obtain the resolution and quantitation desired. The radiochromatogram spark-chamber is commercially available from Birchover Instruments Ltd. (Great Britain).

We have found that for quantitation, the liquid scintillation radioassay of minute zones (zonal profile analysis) facilitated by automatic scraping instruments provides the best resolution and sensitivity for the detection of biochemical metabolites. Most adsorbents, visual indicators and related materials that are used in thin-layer chromatography have no influence on the quantitative aspects. Furthermore, low-level samples can be counted for long periods of time so as to gain statistically good quantitative results. Zonal profile scans are especially helpful in exploring labeling patterns of compounds in systems that are being studied for the first time, as the scans can reveal peak areas of isotopic distribution that are not associated with reference compounds. It is in this manner that metabolic intermediates have been found; an example of such data obtained in our laboratory has been the identification of newly discovered alkyl glycerolipids that contain ketone groups[11]. The application of the zonal profile procedure is illustrated for the analysis of three molecular species of lipids that are closely related metabolically and chromatographically.

ESSENTIAL FEATURES OF THE PROCEDURE USED TO PREPARE ZONAL PROFILE SCANS

After chromatography of the sample, the dried chromatoplate is exposed to iodine vapor (or dichlorofluoroscein) so that areas of sufficient mass can be noted in relation to standard compounds resolved on an adjacent lane. Next, the chromatoplate is placed in a scraping device[12] that is capable of automatically collecting small zones of the adsorbent layer along the entire chromatographic strip in counting vials used for liquid scintillation radioassay. A scintillation solution (see Table I) is then added and the activity in each vial is determined in a liquid scintillation spectrometer. Plotting of the data obtained from sequential vials along the entire chromatographic lane results in a zonal profile scan (Figs. 1 and 2). The entire procedure of scraping, collection, dispensing of the scintillation solution, plotting and analyses of the zones can be carried out automatically[13].

An important concern in the application of this procedure is to ascertain that the total radioactivity in the labeled compounds applied to the chromatogram is accounted for by the integral of all the zones collected from the origin through the solvent front. Quantitative recovery is possible only when self-absorption (due to

TABLE I

COMPOSITION OF SCINTILLATION SOLUTION USED FOR RADIOASSAY OF THIN-LAYER CHROMATOGRAPHIC FRACTIONS

Component	*Percentage*
Scintillator [2,5-Bis-(5′-*tert.*-butylbenzoxazolyl)-2′-thiophene] (w/v)	0.36
Toluene (v/v)	54
Naphthalene (w/v)	7
Methyl Cellosolve (v/v)	36
Water (v/v)	3

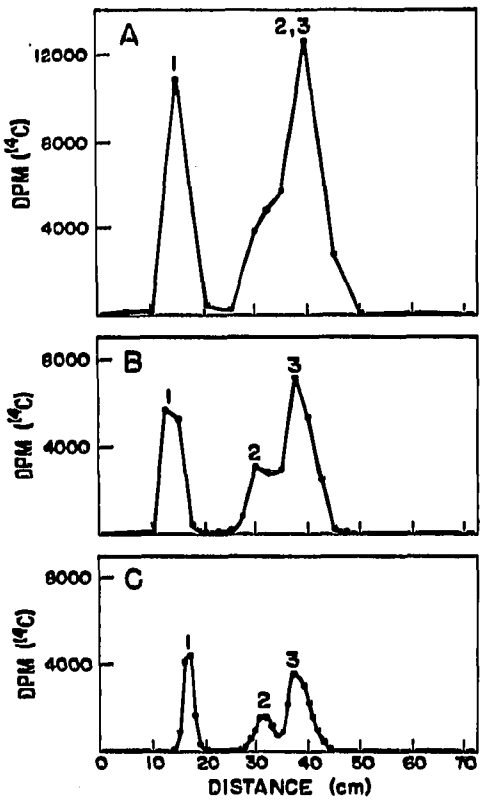

Fig. 1. A ^{14}C-zonal profile scan of a model mixture of standards consisting of: (1) [1-^{14}C]hexadecylglycerol; (2) [1-^{14}C]hexadecanol; and (3) [1-^{14}C]hexadecyldihydroxyacetone. Chromatography was carried out on thin layers of Silica Gel G developed in a system of hexane–diethyl ether–methanol–acetic acid (80:20:5:1). The letters A, B and C designate the 1-cm, 5-mm and 2-mm scans, respectively.

adsorption of the labeled component on adsorbent particles and the glass surface of the vial) and other quenching phenomena are absent. In our laboratories, we have found that relatively polar scintillation solutions containing a constant proportion of water permit the quantitative recovery of carbon-14 and tritium associated with

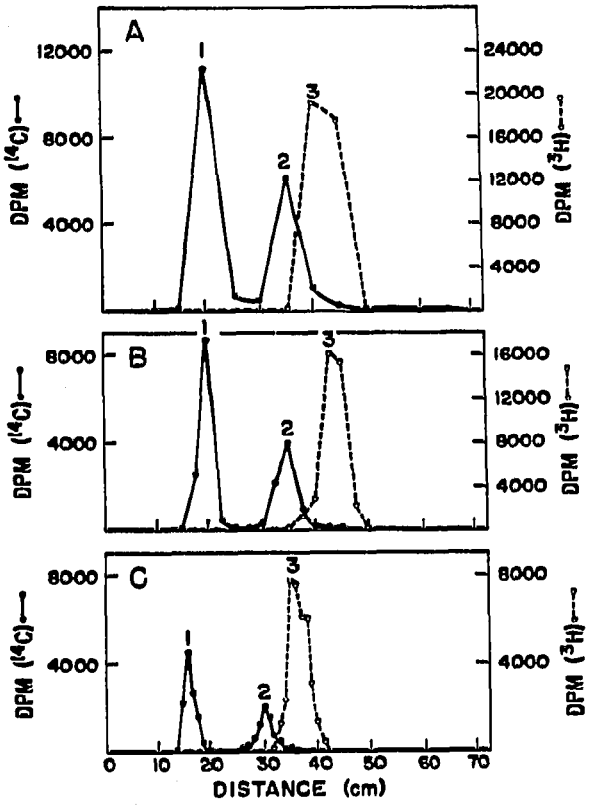

Fig. 2. A ^{14}C, ^{3}H-zonal profile scan of a model mixture of standards consisting of: (1) [1-^{14}C] hexadecylglycerol; (2) [1-^{3}H]hexadecanol; and (3) [1-^{14}C]hexadecyldihydroxyacetone. The chromatographic system used is described in Fig. 1. The letters A, B and C designate the 1-cm, 5-mm and 2-mm scans, respectively.

both non-polar and polar lipids. The constituents of these scintillation solvents and how they prevent self-absorption losses of radioactivity are described elsewhere[2,9]; the composition of one of the most economical and practical solutions is given in Table I.

THE USE OF ZONAL PROFILE SCANS TO DETECT INTERMEDIATES FORMED IN ENZYMIC REACTIONS

A specific group of labeled lipids has been chosen so as to emphasize the importance of analyzing small zones of adsorbent layers when one is attempting to detect labeled metabolic products that differ only slightly in polarity. The model mixture of compounds used to obtain the zonal profile scans (2-mm, 5-mm and 1-cm) illustrated in Figs. 1 and 2 consisted of [1-^{14}C]hexadecanol or [1-^{3}H]hexadecanol, O-hexadecyldihydroxyacetone and O-hexadecylglycerol.

Figs. 1 and 2 show that the separation of O-hexadecyldihydroxyacetone and the long-chain fatty alcohol is not clearly visible except in the scan based on the analysis of 2-mm zones. The components depicted in the scan of Fig. 2 are identical with

those in Fig. 1, except that in the former, [1-^{3}H]hexadecanol was substituted for [1-^{14}C]hexadecanol. Chromatography of a standard ("spike") labeled with a different isotope than that of the other components of the mixture can be extremely helpful in identifying closely related molecules. The data in Fig. 2A vividly demonstrate that even in the analysis of a large zone (1 cm), the heterogeneous distribution of the ^{3}H- and ^{14}C-labeled components indicates that the apparent single peak observed in the ^{14}C-zonal profile (Fig. 1A) could not possibly be misidentified as a fatty alcohol.

The dual isotope zonal profile scans shown in Fig. 2 also emphasize the problem of isotopic cross-contamination caused by trailing of radioactivity from one compound into an adjacent zone containing a different compound. Techniques for the detection of radioactivity are extremely sensitive compared with the detection mass; therefore, the trailing of components is sometimes not obvious when the separated components on a chromatogram are made visible with spray reagents or iodine vapor. On the other hand, zonal profile analysis of the isotopic distribution along the lane will clearly show gross contamination of unlabeled components in the system. Unless the trailing of a radioactive peak is recognized, one would mistakenly assume that the radioactivity associated with a particular "spot" on a chromatogram is derived from that component made visible with the mass detecting reagent. The shape of the radioactive zonal profile scan reveals maximal information about cross-contamination on the chromatogram. Different sensitivities in procedures used to detect the asymmetry of a fatty acid ([1-^{14}C]palmitic acid) after thin-layer chromatography are illustrated in Fig. 3 when charring, autoradiographic and zonal profile scanning techniques are used for detection.

The specific lipids used in these experiments are metabolically related according to the scheme shown in Fig. 4; a more complete account of the enzymic reactions

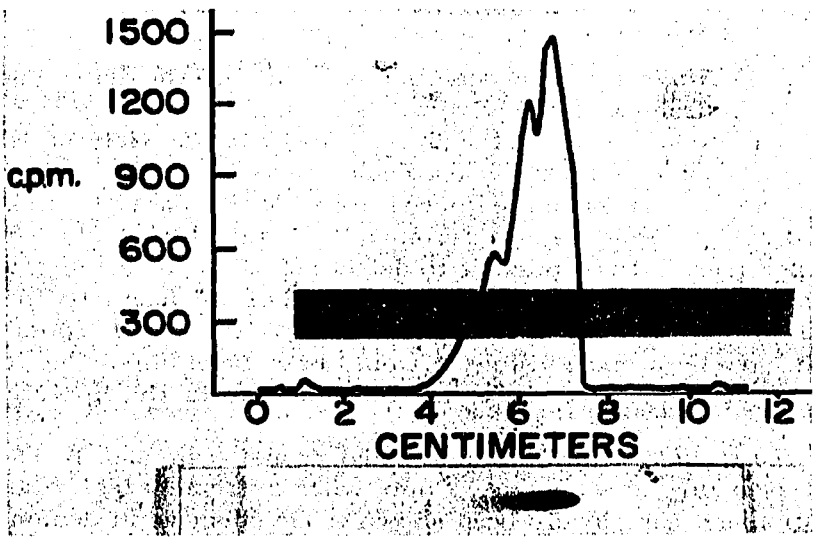

Fig. 3. Detection of the asymmetric distribution of palmitic acid (labeled with ^{14}C) by three different methods of analysis after chromatography of the fatty acid on a thin layer of Silica Gel G in hexane–diethyl ether–acetic acid (50:50:1). The zonal profile scan is shown as the line drawing, the autoradiogram is depicted as an overlay over the scan and the rendering of the palmitic acid spot visible by charring (H_2SO_4 and heat) is shown at the bottom of the figure.

involved in the metabolism of ether lipids has been published elsewhere[14]. The fatty alcohol is the precursor of the O-alkyl moiety in glycerolipids, and in actual enzymic incubations it is always present in much larger amounts than the intermediate, O-alkyldihydroxyacetone, or the product, O-alkylglycerol. Therefore, if areas are scraped according to the spot size of standards, the radioactivity representing the alkyldihydroxyacetone would be included with the area designated as the fatty alcohol substrate used as precursor.

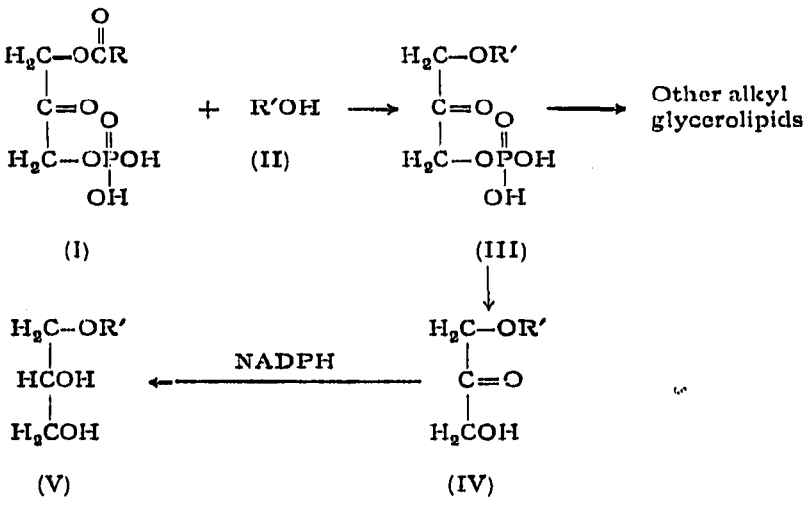

Fig. 4. The biosynthesis of alkyldihydroxyacetone and alkylglycerol from a long-chain fatty alcohol. The compounds shown are acyldihydroxyacetone phosphate (I), fatty alcohol (II), alkyldihydroxyacetone phosphate (III), alkyldihydroxyacetone (IV) and alkylglycerol (V).

The application of zonal profile scans for detecting enzymic intermediates has also been illustrated for the hydrolysis of triacylglycerols by pancreatic lipase[13,15]. In the lipase reaction, the final products (besides the unused substrate triacylglycerols) include diacylglycerols, acylglycerols and fatty acids. Such a mixture can be further complicated by the non-enzymic formation of the isomeric forms of the diacylglycerols (1,2- and 1,3-isomers) and acylglycerols (1- and 2-isomers)[15]. Under the incubation conditions used in one experiment[13], it was possible to detect less than 50 disintegrations/min in the zone of maximum activity representing the peak of the diacylglycerol fraction. With other methods or by scraping areas corresponding to the size of "spots" (1–2 cm in diameter) that have been made visible, it would have been impossible to detect the minute amounts of diacylglycerols, a key intermediate in the lipolytic reaction.

The capabilities of the technique for obtaining zonal profile scans can be extended to the analysis of even smaller zones, *e.g.*, 1 mm, when extremely high resolution is required. This approach has already been shown to be effective in demonstrating the isotopic fractionation effect of tritium and carbon-14 labeled molecules[16]. These experimental data also indicate the high resolving power of adsorption chromatography carried out on thin layers, even though under most circumstances such a high degree of resolution is undetectable.

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DISCUSSION

HAIS: Your determination of sulfuric acid-charred lipids, by quenching and especially when an external source is used, is a sort of photometric procedure. What are the advantages when compared with the photometers of the conventional design, as far as sensitivity, reproducibility and linearity of response are concerned?

SNYDER: The capabilities of the commercial liquid scintillation spectrometer for automatic transport, counting, timing, recording and calculations make this arrangement very attractive for measuring the mass of a compound by its quenching effect on a light source. In combination with the automatic scraper, a large series of samples can be analyzed very rapidly. If the radioactive source used to assess the degree of quenching has a high count-rate, it is sufficient to count the samples for only a few seconds with good statistical accuracy. I do not claim any advantages in accuracy or sensitivity over spectrophotometric methods. In a previous study, the standard deviation of a series of parallel determinations of lipid mass was 0.57% of the mean value [see *Anal. Biochem.*, 28 (1969) 503]. The color yield depends on the chemistry, *e.g.*, degree of unsaturation, of the compounds, and it is not necessarily simply proportional to the carbon content of a compound. This quenching technique has also been applied to the colorimetric measurement of sulfhydryl groups (see *Organic Scintillators and Liquid Scintillation Counting*, Academic Press, New York, 1971, pp. 419–424.). The quenching response is linear up to 200 nmole of a sulfhydryl compound and up to approximately 100 μg of lipid mass.