notes on methodology

Purification of neutral lipid fractions by thin-layer chromatography on aluminum oxide

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SUMMARY Two-stage thin-layer chromatography on Aluminum Oxide G has been used to separate triglycerides, cholesterol, cholesterol esters, and diglycerides from contaminating fatty acids present in the lipid mixtures. Negligible hydrolysis of glycerol esters occurs. The method is particularly useful in metabolic experiments conducted in vitro, in which radioactive fatty acids are employed as precursors.

KEY WORDS two-stage thin-layer chromatography · Aluminum Oxide G · separation · neutral lipids · triglycerides · cholesterol · cholesterol esters · glycerides · free fatty acids

In studying the incorporation of labeled fatty acids in vitro into individual phospholipids or triglycerides, the greatest difficulty arises in freeing these lipids from small amounts of labeled fatty acids with high specific activity that are present in the medium. Rates of incorporation into the glycerides are often low, and the problem of contamination by the precursor becomes an extremely serious one.

Recent publications concerned with the separation of neutral lipids by thin-layer chromatography (TLC) describe the use of silica gel and solvent systems in which the triglycerides migrate with R_F values very near to those of the free acids (1) or the cholesterol esters (2). In our hands, these have not proved satisfactory in removing traces of radioactive fatty acids used as precursor materials, and other means to accomplish this were sought.

Alumina, well known for its capacity for adsorption of fatty acids (3), has found some application in column chromatography of neutral lipids, but its hydrolytic effects on glycerol esters restricted its use (4). Aluminum oxide has been used also in spread layer chromatography (7), in which loose layers of the adsorbent, without binder, were spread onto the plates with a glass rod. This technique is obviously not suitable for routine quantitative determinations.

The present report describes the use of aluminum oxide containing 15% of calcium sulfate as a binder (Aluminum Oxide G) to separate neutral lipids from contaminating fatty acids. It has proved especially useful for studying the incorporation of fatty acids into triglycerides, for the latter are not significantly hydrolyzed in the short time periods involved. Two solvent systems of weak polarity are employed. With the first, the fatty acids, phospholipids, and other relatively polar substances remain at the origin, whereas cholesterol, diglycerides, triglycerides, cholesterol esters, fatty acid methyl esters, and hydrocarbons migrate with R_F values increasing in that order. The last four classes are not well separated at this stage.

A final separation of triglycerides from the less polar cholesterol esters, fatty acid methyl esters, and hydrocarbons is achieved by the use of a second solvent system of weaker polarity, in which only the latter three lipid classes migrate. The entire procedure requires 2–2.5 hr.

Experimental Method. Glass plates (20 × 20 cm) were coated with a layer, 250 μ thick, of a suspension of 30 g of Aluminum Oxide G (Merck, Darmstadt, Germany, distributed by Brinkmann Instruments, Inc., Westbury, N.Y.) in 70 ml of distilled water, by means of a Desaga applicator (Brinkmann Instruments, Inc.). The layer was allowed to dry at room temperature for 1 hr and the plates were used without further activation.

Samples of lipids dissolved in benzene or chloroform were applied, with the aid of micropipettes, in spots on a line 2 cm from the bottom of the plate. The spots were confined to an area of less than 5 mm by evaporating the solvent with a stream of nitrogen as the sample was applied. The following standards were obtained from the Hormel Institute: 1-monopalmitin, a mixture of 1,2- and 1,3-dipalmitin, tripalmitin, triolein, cholesterol, and cholesteryl palmitate.

Chromatographically pure tripalmitin, labeled in the glycerol moiety, was prepared from glycerol-1,3-^{14}C (0.19 μc/mg, Volk Radiochemical Co., Chicago, Ill.) and palmitic acid (chromatographically pure) by a modification of the method of Hartman (5). Drierite was used to remove the water evolved during the reaction. The lipids were extracted from the reaction mixture with petroleum ether, and the fatty acid was removed from this extract by repeated washing with 0.05 N NaOH in 50% ethanol. The petroleum ether extracts containing the glycerides were chromatographed on a column of silicic acid according to Horning, Williams, and Horning (8). The eluted fractions containing the triglyceride were evaporated and rechromatographed on silicic acid. The operation was monitored by TLC and the tripalmitin was finally recrystallized from petroleum ether.

Palmitic acid-1-^{14}C and linoleic acid-1-^{14}C (Volk Radiochemical Co.) were freed from neutral lipids before use by a procedure similar to that employed to purify the tripalmitin, except that the petroleum ether extracts

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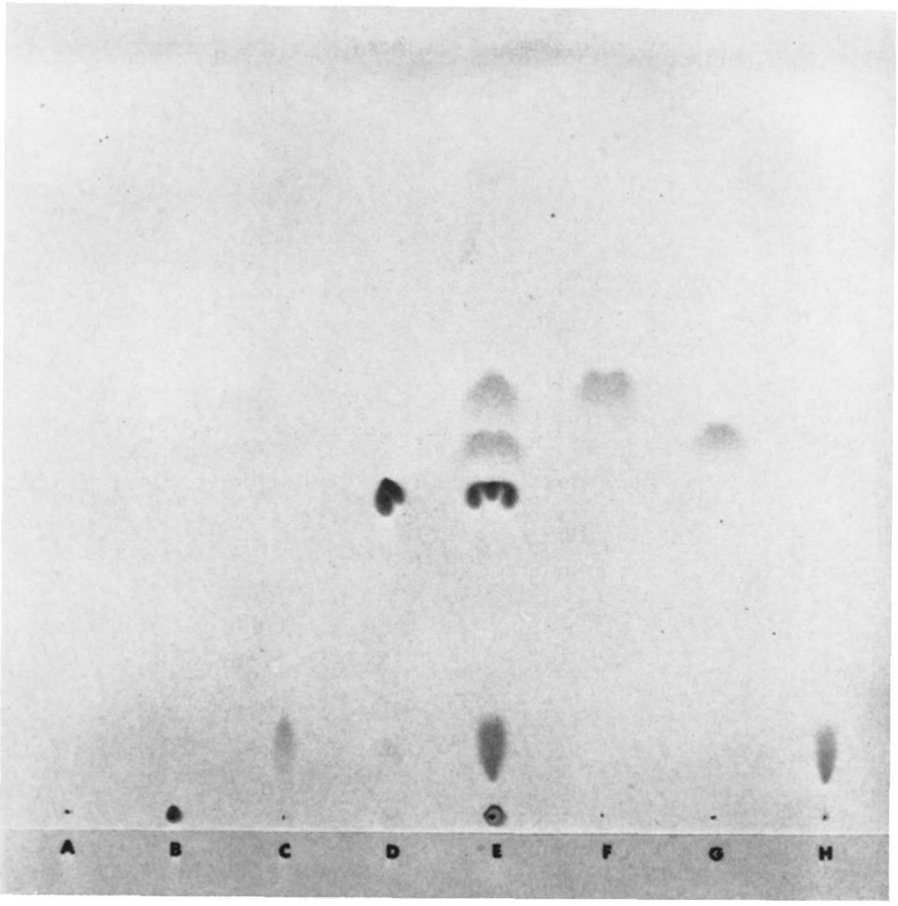

FIG. 1. Two-stage thin-layer chromatography of various lipids on Aluminum Oxide G. The chromatogram was developed and the lipids were stained as described in the text. *A*, palmitic acid; *B*, monopalmitin; *C*, dipalmitin; *D*, triolein; *E*, mixture of *A–H*; *F*, cholesteryl palmitate; *G*, cholesteryl acetate; *H*, cholesterol.

were rejected. The aqueous alcoholic layer was acidified and extracted with petroleum ether to recover the fatty acids.

The solvent mixtures used in the development of the plates were: solvent I, benzene–ethyl acetate 9:1; solvent II, hexane–benzene 9:1. The solvents were reagent grade and were distilled before use.

The method consists in developing the chromatogram in solvent I until the solvent front has traveled approximately 6 cm. The plate is then removed from the chamber and dried in a stream of nitrogen, and the development is continued in the same direction, in a chamber containing solvent II, until the solvent front reaches the top of the plate. The plate is air dried and sprayed with a solution of 0.2% 2′,7′-dichlorofluorescein in 95% ethanol. The spots are viewed under ultraviolet light and circumscribed with a glass stylus. The triglycerides and the other lipids are located with the aid of standard chromatograms of the known lipids mentioned above. Figure 1 shows the degree of resolution, which is highly reproducible.

The areas containing triglycerides are scraped off with a sharp spatula and transferred to vials containing a scintillation mixture which consists of 200 ml of dioxane (reagent grade), 16 g of naphthalene (reagent grade), 1.0 g of 2,5-diphenyloxazole (scintillation grade, Packard Instrument Company, LaGrange, Ill.), 10 mg of α-naphthylphenyloxazole (scintillation grade, Packard

TABLE 1 EXTENT OF HYDROLYSIS OF TRIPALMITIN (GLYCEROL-1,3-^{14}C) ON ALUMINUM OXIDE G AND SILICA GEL G

Adsorbent	Radioactivity Applied	Areas* 1	2	3	4	5	6	7	Recovery
		Radioactivity Recovered							
	cpm	*cpm*							%
Aluminum Oxide G	44,000	43,230	0	6	72	220	0	13	98.9
Silica Gel G	44,000	44,380	106	0	0	0	0	5	101.0

* The portions of the chromatograms scraped and counted are numbered from top to bottom. They include the following lipid classes: area 1, triglycerides; areas 2 and 3, unknown; areas 4 and 5, diglycerides; area 7, monoglycerides and free fatty acids.

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Instrument Company), 9.2 g of Cab-O-Sil (Cabot Corporation, Boston, Mass.), and 30 ml of methanol. Radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer (Model 3214) with an efficiency of 55% in a 500–1000 V window and 14% gain at a temperature of −2°.

Results. The following representative experiment demonstrates the extent to which triglycerides are hydrolyzed during chromatography on Aluminum Oxide G. A 1% solution of tripalmitin (glycerol-1,3-^{14}C) in benzene was applied in several spots on each of two plates (20 × 20 cm), one coated with Aluminum Oxide G and the other with Silica Gel G. Standard chromatograms were prepared on the same plates, using mixtures of monopalmitin, 1,2- and 1,3-dipalmitin, tripalmitin, and palmitic acid, and the plates were developed at the same time, with the procedure given above. After the plates had been sprayed with 2′,7′-dichlorofluorescein reagent, the chromatograms of the labeled tripalmitin were divided into sections corresponding to different lipid classes according to the behavior of the reference compounds. The sections were scraped off and collected, and the radioactivity was assayed (6). The results are shown in Table 1. It can be seen that a maximum of 0.5% of the triglyceride chromatographed on the aluminum plate is hydrolyzed in the process. Data for silica gel, where no detectable hydrolysis occurred, show, at the same time, the radioactive purity of the labeled tripalmitin.

The degree of separation of radioactive fatty acids from other lipids on Aluminum Oxide G plates was examined as follows. Mixtures of either palmitic acid-1-^{14}C or linoleic acid-1-^{14}C with each of the following substances were chromatographed as described: tripalmitin, cholesteryl palmitate, triolein, and dipalmitin. In no instance was radioactivity found in the portions of the chromatograms containing diglycerides, triglycerides, or cholesterol esters, and 97–102% of the radioactivity was recovered in the spots containing the fatty acids.

Similar experiments were carried out in which either palmitic acid-1-^{14}C or linoleic acid-1-^{14}C was added to whole rat liver lipid preparations, and the mixtures were chromatographed. Approximately 50 μg of total lipid mixture was applied to each spot on the chromatogram. Again 98–103% of the radioactivity remained at the location of the fatty acids, and none was associated with any of the other lipid classes on the chromatograms.

In all such experiments, the system was assayed for quenching effects due to the presence of Aluminum Oxide G in the scintillation mixture. No quenching has been observed.

The technical assistance of Miss Elvira de Castro is gratefully acknowledged.

This work was supported in part by PHS Research Grant CA-04720 from the National Institutes of Health, U.S. Public Health Service.

Manuscript received February 24, 1965; accepted May 24, 1965.

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