Reversed-phase partition thin-layer chromatography of rat liver lecithins to yield eight simple phosphatidyl cholines

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SUMMARY The four fractions obtained by argentation thin-layer chromatography of intact rat liver lecithins can be further subdivided by reversed-phase partition thin-layer chromatography on hydrophobic kieselguhr. The resultant eight fractions contain virtually only one saturated and one unsaturated acid each.

KEY WORDS   reversed-phase   ·   partition   ·   thin-layer chromatography   ·   rat   ·   liver   ·   lecithins   ·   unimolecular subfractions

A PREVIOUS COMMUNICATION described the separation of rat liver lecithin into four main fractions by TLC on silica gel plates impregnated with silver nitrate (1). GLC analysis revealed that approximately 50% of the fatty acids in each of the four fractions, here designated as A, B, C, and D, was made up of *two* saturated fatty acids, viz., palmitic and stearic. In fraction A the remaining 50% consisted mainly of oleic acid, in fractions B, C, and D of linoleic, arachidonic, and docosahexaenoic acid, respectively.

According to the principles underlying separation on AgNO₃-impregnated adsorbents, the total number of double bonds per molecule can be assumed to be the same for all the lecithin molecules belonging to the same fraction. It follows from this assumption and the GLC data quoted above that fraction A should largely be composed of only two kinds of lecithin molecules: palmitoyl oleoyl glycerophosphoryl choline plus stearoyl oleoyl glycerophosphoryl choline, designated PC(16:0, 18:1) and PC(18:0, 18:1), respectively. For logical reasons the possibility of appreciable quantities of molecules with other fatty acid combinations occurring in fraction A must be excluded. In the same way it can be deduced that fractions B, C, and D should also consist mainly of simple binary mixtures. Fraction B is PC(16:0, 18:2) plus PC(18:0, 18:2), fraction C is PC(16:0, 20:4) plus PC(18:0, 20:4), and fraction D is PC(16:0, 22:6) plus PC(18:0, 22:6).

The present report offers direct experimental support for this conclusion. It describes the resolution of A, B, C, and D into one palmitate- and one stearate-containing subfraction by reversed-phase partition TLC.

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography. Fatty acids are designated by number of carbon atoms: number of double bonds.

Materials and Methods. Lecithin from rat liver was obtained by preparative TLC of a chloroform–methanol extract, by the use of 0.8 mm-thick layers of Silica Gel H (E. Merck, A. G. Darmstadt, Germany) and the solvent system of Skipski, Peterson, and Barclay (2). The lecithin was then fractionated on AgNO₃-impregnated thin-layer plates according to previously described procedures (1), modified for preparative purposes as follows. The plates were coated with a 0.8 mm thick layer of Silica Gel H containing 15 g of AgNO₃ per 50 g of silica gel. Mono- and dienoic fractions were obtained on plates activated for 8 hr at 190°C and tetra- and hexaenoic fractions on plates activated at the same temperature for 2.5 hr. The solvent system for both types of plates was chloroform–methanol–water 55:35:7. Samples were applied as bands in chloroform solution by means of a micropipette. The plates were developed for 90 min, briefly dried in a stream of nitrogen and then sprayed with a 0.2% solution of 4,5-dichlorofluorescein (Fluka AG, Basel, Switzerland) in ethanol. The fractions were located under ultraviolet light, scraped off into small sintered glass funnels, and eluted with 10 ml of chloroform–methanol-acetic acid–water 50:39:1:10 into 15-ml tubes.

To each eluate was added 4 ml of 4 N NH₄OH. The tubes were shaken and then centrifuged. Two clear phases were obtained. The upper, methanol–water phase contained most of the dye and the silver ions but no lipid, which was concentrated in the lower, chloroform phase. After removal of the upper phase, the lower phase was washed once with 5 ml of the upper phase from an equilibrated mixture of 10 volumes of eluting solvent and 4 volumes of 4 N NH₄OH. The final, washed chloroform phase was taken to dryness under a stream of nitrogen. The lecithin was redissolved in methanol for subsequent fractionation by reversed-phase partition TLC. The equilibration and washing procedure caused no loss of material, as judged by determination of phosphorus or by tests with radioactively labeled rat liver phospholipids. Recovery of P from the AgNO₃-impregnated plates was 90–95%.

Reversed-Phase Partition TLC. An inert and nonadsorptive support for reversed-phase partition TLC was obtained by repeatedly washing Kieselguhr G (E. Merck, A. G., Darmstadt, Germany) with 2 N HCl until it was free from sulfate, tested by the addition of a few drops of a saturated solution of Ba(NO₃)₂ to a centrifuged aliquot of the kieselguhr suspension. The sulfate-free material was then repeatedly suspended in distilled water until free from chloride (no precipitate with a saturated silver nitrate solution). The sulfate- and chloride-free kieselguhr was then washed with methanol and dried at 120°C. The dry powder was exposed to the vapors of dichlorodimethylsilane (Fluka AG, Buchs SG, Switzerland) in a desiccator. When completely hydrophobic

(after 1–2 days and judged from shaking a sample with water in a test tube) the powder was again washed with methanol on a sintered glass filter under suction. The filter cake was then suspended in diethyl ether. All lumps and coarse particles that rapidly sedimented from the ether suspension were rejected. The remaining material was air-dried and stored until needed for preparing plates. To 50 g of this kieselguhr was added 20 ml of undecane (Kistner AB, Gothenburg, Sweden) and enough hexane to give a rather thin slurry, which was spread to a thickness of 0.5 mm on 10 $\times$ 15 cm glass plates. The plates were left at room temperature for 2 hr and then stored in a desiccator (the closed vessel prevents evaporation of undecane).

Aliquots of the four lecithin fractions previously obtained were applied to the plates as spots or bands. The load of phosphorus was 0.5–1 μg per spot or 20–25 μg over a band, 7 cm long. 7 parts of methanol–water 9:1 that had been equilibrated with undecane in a separatory funnel was diluted with 3 parts of pure methanol–water 9:1 and the mixture used as developing solvent. Development took place at 18°C in tanks lined with filter paper that was moistened with the moving phase. Immediately after development, while the plate was still wet, the fractions could be seen as grayish areas against a white background both in reflected and transmitted light. The bands were scraped off into conical centrifuge tubes containing 10 ml of chloroform–methanol 2:1. The tubes were shaken and centrifuged. The clear supernatant solution was decanted into a second tube and the solvent was evaporated under nitrogen.

The fatty acid composition of each fraction was determined by GLC after methanolysis in 2.5% H_2SO_4 in methanol at 65°C for 5 hr in stoppered tubes. The extracted methyl esters were purified on thin-layer plates and analyzed by GLC with an ethylene glycol succinate polyester as the stationary phase. Phosphorus content of the lecithin fractions isolated from the $AgNO_3$-impregnated plates was determined by the method of Chen, Toribara, and Warner (3).

Results and Discussion. As illustrated in Fig. 1, each lecithin fraction applied to the undecane-impregnated plate was resolved into two subfractions. From the GLC analysis in Fig. 2 it is evident that the four pairs of spots on the plate in Fig. 2 represent the eight major molecular species found in rat liver lecithin.

During the course of this study several types of plates were tested. It soon became evident that a nonadsorptive support for the stationary phase was an absolute prerequisite. As expected, silica gel was entirely unsatisfactory. Also, on plates made by conventional procedures from untreated kieselguhr, severe trailing obscured possible separations. This necessitated the somewhat tedious washing and silanizing procedure. Although the coating
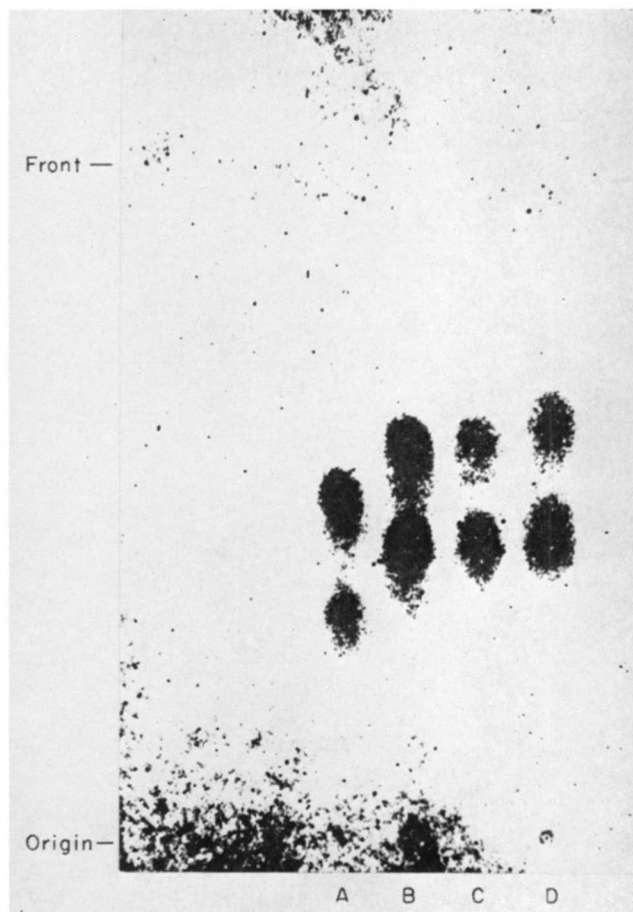

FIG. 1. Reversed-phase partition TLC of the four subfractions from rat liver lecithin, previously obtained by preparative argentation TLC. Stationary phase, 0.5 mm thick layer of dichlorodimethylsilane-treated kieselguhr which contained 20 ml of undecane per 50 g of kieselguhr. Mobile phase, methanol–water 9:1, partially saturated with undecane. Total sample load per spot, 0.5–1 μg P. Detection for this photograph, phosphomolybdate spray after evaporation of undecane.

A, monoenes; B, dienes; C, tetraenes; D, hexaenes.

layer of the plates presently described is much softer than that of the usual thin layer of silica gel, it adheres well to a clean glass surface and has not posed any special problems in the handling of the plates.

The results obtained by reversed-phase partition chromatography are dependent upon the temperature at which the chromatography is carried out. Development at 4°C increases the degree of resolution of all four lecithin samples into their subfractions, although the monoenoic lecithins (sample A in Fig. 1) occasionally exhibit some streaking, perhaps due to a lower solubility in the mobile phase at this temperature. At 25°C the R_f values of all fractions are increased to more than 0.9. At 18°C separations are satisfactory so long as the water content of the mobile phase is between 5 and 15%. Below 5% water the R_f values become too high, above 15% too low, for resolution.
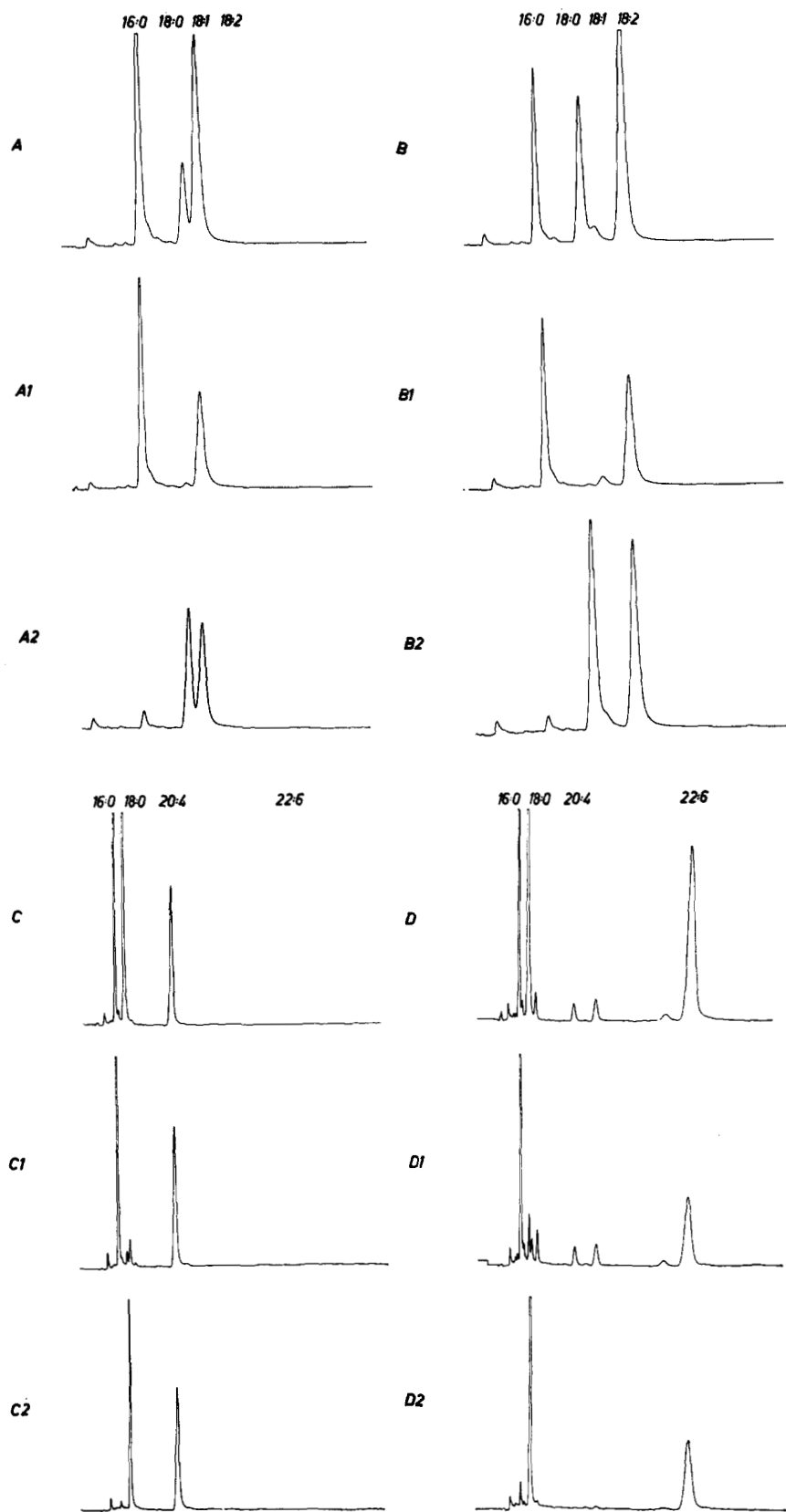
FIG. 2. GLC analysis of rat liver lecithin subfractions. A, B, C, and D, correspond to the samples applied to the reversed-phase thin-layer plate in Fig. 1 and represent the four fractions of rat liver lecithin obtained after argentation TLC. 1 and 2 refer to the upper and lower spot respectively of corresponding samples in Fig. 1.

It is noteworthy that the unsaturated fatty acid moiety of the lecithin molecule has little influence on the R_f values, especially of the polyunsaturated lecithins (samples B, C, and D in Fig. 1). Thus, the partition coefficient in an undecane–methanol–water system for the type of lecithins studied here is largely determined by the saturated fatty acid at the 1-position. A similar observation has been made by Collins (4, 5) for the countercurrent distribution of rat liver lecithins in carbon tetrachloride–methanol–water. Although all the lecithin appeared in one peak, fatty acid analysis revealed a difference in the distribution of palmitic and stearic acid. This allowed the calculation of different partition coefficients for palmitate- and stearate-containing lecithins, not taking the unsaturated acyl chain into consideration. As a corollary to this finding one could not expect to obtain pure subfractions from a complex mixture of lecithins by partition only. In this respect the described combination of argentation and reversed-phase partition TLC forms a very potent fractionation procedure, at least when applied to a lecithin preparation with a high degree of positional specificity in the distribution of saturated and unsaturated fatty acids, like rat liver lecithin.

REFERENCES

1. Arvidson, G. A. E. 1965. *J. Lipid Res.* **6:** 574.
2. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. *Biochem. J.* **90:** 374.
3. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. *Anal. Chem.* **28:** 1756.
4. Collins, F. D. 1963. *Biochem. J.* **88:** 319.
5. Collins, F. D. 1966. *Biochem. J.* **99:** 117.