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Note

Separation of naturally occurring lecithins according to fatty-acid chain-length and degree of unsaturation on a lipophilic derivative of Sephadex

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The main molecular species of naturally occurring lecithins can be separated by counter-current distribution[1,2] or by a combination of argentation and reversed-phase partition thin-layer chromatography (TLC)[3,4]. However, these procedures are rather tedious and, for preparative work, both their capacity and resolving power need to be improved. Almé *et al.*[5] recently demonstrated (on the microgram scale) the separation of synthetic lecithins and lysolecithins on a lipophilic ion exchanger. The lecithins in the test mixture consisted of dioleyl-lecithin plus fully saturated lecithins having the same fatty acid residue at positions 1 and 2. The chromatographic behaviour of mixed-acid lecithins of the naturally occurring type containing residues of one saturated and one unsaturated fatty acid was not studied. In the present investigation, a non-ionic partially alkylated Sephadex derivative was found to be particularly suitable for the fractionation of lecithins isolated from egg yolk and rat liver.

With a simple binary mixture of methanol and water as the mobile phase, the various molecular species were separated according to the total number of carbon atoms and double bonds in the lecithin molecule.

EXPERIMENTAL

All solvents and chemicals were of reagent grade and were used without further purification. Sephadex G-25 superfine was purchased from Pharmacia (Uppsala, Sweden) and was freed from its finest particles by the back-washing technique of Hamilton[6]. Nedox 1114 (Ashland, Columbus, Ohio, U.S.A.) which is a mixture of C_{11}–C_{14} olefin oxides was kindly supplied by Professor J. Sjövall. The hydroxypropyl derivative of Sephadex G-25 was synthesized according to Ellingboe *et al.*[7] and was allowed to react as described by the same authors with various amounts of Nedox 1114 to form hydrophobic products which contained 25–55% (w/w) of C_{11}–C_{14} alkyl chains.

Chromatography was performed in water-jacketed glass columns (I.D. 10 mm). A slurry of the alkylated Sephadex derivative in the mixture of methanol and water used as mobile phase was poured into the column and beds of various heights were packed by gravity flow. The lecithins, 5–15 mg, were dissolved in 0.1–0.5 ml of the mobile phase and applied to the top of the gel bed. The mobile phase consisted of methanol containing 10–20% (v/v) of water; this solvent had been found suitable for

the fractionation of rat-liver lecithins by reversed-phase partition TLC on Kieselguhr impregnated with undecane[4]. The mobile phase was made to flow through the column packing by the hydrostatic pressure obtained by placing the solvent reservoir 0.75–1.25 m above the column exit; the solvent flow-rate was 5–10 ml/h. A refractive index detector (Varian RI detector) was used for automatic monitoring of the column effluent. Column and detector were maintained at the same constant temperature by circulating water from a thermostated water bath (Colora Ultra-Thermostat NB-DS).

Lecithins from egg yolk and rat liver were obtained by preparative thin-layer chromatography[8]. Methyl esters for fatty acid analysis by gas–liquid chromatography (GLC) were prepared by trans-esterification in methanol–H_2SO_4 as described previously[3].

A Varian Aerograph 600 D equipped with a flame ionization detector and an electronic integrator was used for GLC analysis.

RESULTS

The alkyl-group content of the Sephadex derivative was found to be of critical importance. With the fully alkylated derivative initially tested, the lecithins emerged from the column in one peak close to the solvent front. However, satisfactory results were obtained by lowering the alkyl-group content from 55 to 20–35% (w/w). Increase in the amount of water in the mobile phase resulted in larger retention volumes and increased separation factors. For the fractionation of egg-yolk and rat-liver lecithins, 80% (v/v) methanol in water was suitable. By increasing the column temperature from 25° to 42°, the retention volumes were reduced without affecting the resolving power of the column.

Fig. 1 shows the separation of 15 mg of egg-yolk lecithins into four fractions of differing fatty acid composition; entirely analogous separations were obtained with rat-liver lecithins. The lecithins applied to the column were quantitatively recovered in the eluate.

As can be inferred from the results in Table I, separation takes place according to both the length and the degree of unsaturation of the two acyl chains in the lecithin molecule. Lecithins in the same peak on the chromatogram are characterized by the

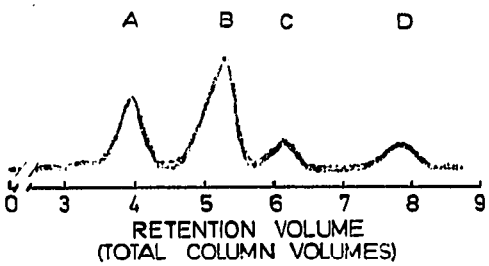

Fig. 1. Separation of 18 μmoles of egg-yolk lecithins on a column (33 cm × 1 cm) of hydroxyalkoxypropyl-Sephadex with a hydroxyalkyl (C_{11}–C_{14}) group content of 22% (w/w). The mobile phase was methanol–water (4:1, v/v); flow-rate 8.7–9.7 ml/h; temperature 41.6°. The HETP values (mm) for the peaks were 0.55 (A), 0.57 (B), 0.30 (C) and 0.33 (D).

TABLE I

FATTY ACID COMPOSITION OF EGG-YOLK AND RAT-LIVER LECITHIN FRACTIONS OBTAINED BY COLUMN CHROMATOGRAPHY AS DESCRIBED IN FIG. 1

The lecithins in peaks A and C of the sample originating from rat liver were further separated by argentation chromatography[3] into fractions A_2, A_3 and A_4 and C_1, C_2, C_3 and C_4.

Lecithin source	*Peak (see Fig. 1)*	*Fatty acid, mole%**							
		16:0	*16:1*	*18:0*	*18:1*	*18:2*	*20:4*	*22:6*	*Unidentified*
Egg yolk	A	42.3	—	—	3.6	34.6	2.9	16.7	—
Rat liver	A	44.2	1.1	—	6.6	25.9	14.6	7.7	—
Rat liver	A_2	43.9	1.5	—	5.6	49.1	—	—	—
Rat liver	A_3	45.6	—	0.4	4.7	0.6	48.6	—	—
Rat liver	A_4	39.6	—	0.7	6.1	0.9	3 9	41.7	7.1
Egg yolk	B	45.4	—	0.7	52.0	1.9	—	—	—
Rat liver	B	30.5	—	11.8	34.7	2.9	—	—	20.2
Egg yolk	C	1.5	—	43.0	6.8	30.6	8.8	9.3	—
Rat liver	C	1.4	—	49.4	2.5	22.1	18.1	6.5	—
Rat liver	C_1	16.9	5.0	19.4	33.1	6.9	—	—	18.7
Rat liver	C_2	1.1	—	49.6	1.7	47.6	—	—	—
Rat liver	C_3	—	—	49.4	—	—	50.6	—	—
Rat liver	C_4	0.8	—	49.2	—	—	—	43.1	6.9
Egg yolk	D	2.4	—	46.1	51.4	—	—	—	—
Rat liver	D	3.5	1.1	47.6	47.9	—	—	—	—

* Percentage of total GLC detector response is given in fractions containing unidentified fatty acids.

same "equivalent carbon-atom number", defined as the total number of acyl-chain carbon atoms minus the total number of double bonds. The equivalent carbon-atom numbers of peaks A, B, C and D in Fig. 1 are 32, 33, 34 and 35 respectively.

DISCUSSION

The present results do not permit strict evaluation of the parameters affecting the described separation of different kinds of lecithins. For this, further work is required in which the fatty acid structure of the lecithin molecules is varied systematically. In the procedure of Almé *et al.*, methanol–water–chloroform was used as mobile phase[5]. Such mixtures create good reversed-phase systems with alkylated Sephadex gels[7]. A comparison of the present results with those of Almé *et al.* shows that the two systems have similar properties. Accordingly, the present procedure may be regarded as a kind of reversed-phase partition chromatography involving a lipophilic stationary phase covalently linked to its support. However, from a theoretical point of view it may also be regarded as adsorption chromatography in which hydrophobic effects are operating.

According to the results presented here, there is no need for incorporating ionic groups into the stationary phase as far as the separation of lecithins is concerned. Possibly this may be explained by the formation of an intramolecular salt-bridge between the phosphate group and the quaternary ammonium group of the lecithin molecule. Ionic groups in the stationary phase may well prove to be of crucial importance for the separation of other phospholipids.

The high capacity and resolving power of the system described here make it very suitable not only for analytical purposes but also for preparative work. In a recent experiment, 35 mg of rat liver arachidonyl-lecithins (prepared by argentation TLC) were applied to the column described in the legend to Fig. 1, yielding in one run approximately 15 mg each of palmityl-arachidonyl- and stearyl-arachidonyl-lecithin. Quantities in this range are usually sufficient for many types of physico-chemical studies, *e.g.*, differential scanning calorimetry, determination of surface pressures, X-ray diffraction studies and so forth.

The absence of chloroform from the solvent mixture used as the mobile phase is also of some practical importance, since ordinary column-chromatographic equipment is usually resistant to methanol, but not to chlorinated hydrocarbons.

Phosphatidylethanolamines in the form of their triethylammonium salts are soluble in methanol, as originally shown by Brockerhoff[9]. In this form, naturally occurring phosphatidylethanolamines can be fractionated by reversed-phase partition TLC with undecane as stationary phase and methanol–water as mobile phase[10]. Preliminary results indicate that the separation of lecithins described here is also applicable to phosphatidylethanolamines when chromatographed as their triethylammonium salts.

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