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Separation of fatty acids, phospholipids and chloroplast pigments on Sephadex LH-20

The development of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) has further extended the application of gel filtration to the separation of lipid-soluble substances[1-8].

In this investigation, the potential of Sephadex LH-20 for the separation of both artificial and naturally occurring mixtures of fatty acids, phospholipids and chloroplast pigments was further explored. Some of the results have been briefly reported[9].

Materials and methods

Solvents. The solvents used were A.R. grade (British Drug Houses Ltd., Great Brittain). The chloroform as supplied contained 2% by vol. of ethanol and 0.05% by vol. of water.

Materials chromatographed. Suitable quantities of the various substances listed in Table I were dissolved in 2 ml of appropriate solvent prior to fractionation.

Column preparation. Sephadex LH-20 was suspended overnight in the eluent of choice and transferred to a separating funnel into which a glass stirrer was inserted. The gel was allowed to percolate rapidly, with constant stirring, into a vertical glass chromatography column which had been filled with solvent and across the end of which was fused a sintered glass disk (porosity 1). When the level of gel in the glass column had reached the required height, sand (1 cm approx.) was placed on top to prevent the gel floating in eluent of specific gravity greater than that of the Sephadex LH-20. Samples were applied by layering under the solvent on top of the column. The sand facilitated application of the samples without disturbing the gel. Column dimensions are given in Figs. 1 and 2, and the quantities of the material applied, in Table I. The chromatographic behavior of the polar lipids was compared with that

TABLE I

MATERIALS FRACTIONATED ON SEPHADEX LH-20

Materials	*Quantities applied to columns (mg)*	*Suppliers and sources*
Acetic acid	30	British Drug Houses Ltd., England
n-Butyric acid	40	British Drug Houses Ltd., England
Caproic acid (pure)	50	Koch-Light Labs. Ltd., England
Stearic acid (99% +)	80	Nutritional Biochemical Corp., U.S.A.
Linolenic acid	80	Nutritional Biochemical Corp., U.S.A.
Phospholipids (from bovine milk)	80	Extracted as described by DODGE AND PHILIPS[10]
Tributyrin	40	British Drug Houses Ltd., England
Tristearin	60	British Drug Houses Ltd., England
Chloroplast pigments (*Poa trivialis*)	—	Extracted as described by STRAIN *et al.*[11] from 1 g of plant tissue

of triglycerides which are apparently separated on Sephadex LH-20 by molecular sieving[5]. Fractionations were carried out at room temperature (approx. 18°) at a flow rate of 1 ml/min and the effluent was collected in 5-ml fractions.

Examination of column effluent. Fatty acids were estimated under CO_2-free N_2 by electrometric titration, to pH 10 with 0.01 *M* NaOH, of suitable quantities (containing 1–5 μequiv. of acid) of each effluent fraction, in the presence of 3 ml of propan-2-ol[12], using a Radiometer titrator, type TTT1*c*, coupled to a Radiometer titrigraph, type SBR2*c* (Radiometer, Copenhagen, Denmark). Triglycerides were estimated as described by MOORE[13]. The phospholipid content of the effluent was monitored by estimation of the phosphorus[14] in suitable portions (containing 0.2–2 mg of phospholipid) of each effluent fraction, following evaporation of the solvent and incineration on a microdigestion stand (Gallenkamp & Co. Ltd., London) for 1 h in 1 ml 60% (w/v) perchloric acid. The elution patterns of the chloroplast pigments (Fig. 2) were monitored spectrophotometrically.

Consecutive fractions corresponding to selected regions of the elution diagrams were evaporated to dryness under nitrogen and dissolved in 0.1 to 0.5 ml of solvent. The individual phospholipids[15–19] and chloroplast pigments[20] of these pooled fractions were identified by TLC on Silica Gel G (Merck).

Results

When chloroform only was used as column eluent, tristearin, tributyrin, stearic, capric, butyric and acetic acid were separated (Fig. 1) into well-defined peaks (elution volumes, V_e: 65, 85, 225, 320, 450 and 575 ml, respectively). When linolenic acid was included in the above mixture it was not separated from stearic acid. The capric acid

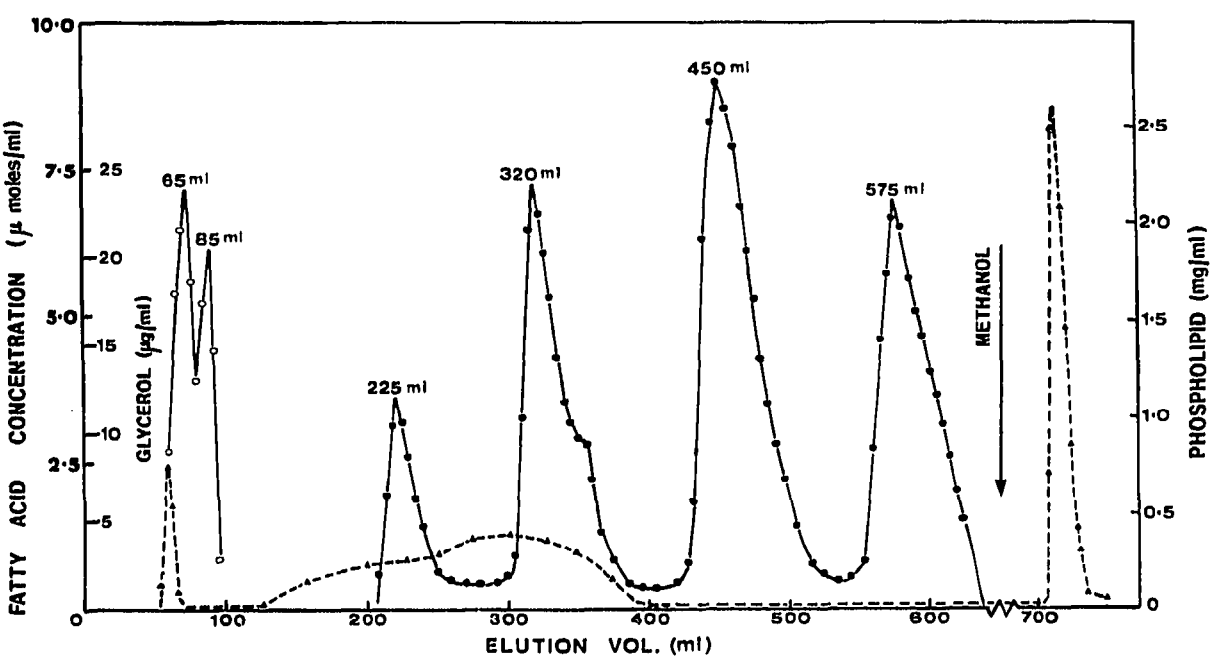

Fig. 1. Fractionation on Sephadex LH-20 columns (54 cm × 2.4 cm I.D.) equilibrated with chloroform, of an artificial mixture of bovine milk phospholipids (▲- - - -▲), triglycerides (○—○) and fatty acids (●—●). Following removal of the fatty acids the column was eluted with methanol. Elution volumes: tristearin, 65 ml; tributyrin, 85 ml; stearic acid, 225 ml; capric acid, 320 ml; butyric acid, 450 ml; acetic acid, 575 ml. Further details are given in the text.

appears to have contained a fatty acid contaminant as indicated by the inflection in its elution curve (Fig. 1), which was also observed on chromatography of capric acid on its own. The recoveries of the individual fatty acids and triglycerides were approximately 85%.

On fractionation of a preparation[10] of milk phospholipids (Table I) on its own, or in the presence of fatty acids (Fig. 1), the phosphatidylinositol was eluted as a sharp peak (<10% of phospholipid applied) near the void volume (V_e, 60 ml) followed by a wide phospholipid band (V_e, 140 to 395 ml; 50% of phospholipid applied) containing the sphingomyelin and most of the lecithin applied. On subsequent elution of the column with methanol (Fig. 1) or 20% by vol. of methanol in chloroform, a further 40% of the phospholipids applied were eluted as a large peak which contained the phosphatidylserine, phosphatidylethanolamine and some lecithin.

On columns equilibrated with a solvent of higher polarity, *viz.* chloroform containing 20% by vol. of methanol and 1.25% by vol. of water[21], very poor resolution of the polar lipids was obtained. Over 90% of the phospholipids of milk were eluted as a single peak (V_e, 55 ml) near the void volume, closely followed by the fatty acids. Stearic, capric and butyric acid were eluted as three ill-defined peaks (V_e, 70, 85 and 96 ml, respectively). On the other hand the separation of the triglycerides was largely unaffected by the polarity of the eluent.

On fractionation (Fig. 2) of an extract[11] containing chloroplast pigments (Table I) on columns equilibrated with petroleum ether (b.p. 40–60°), β-carotene and the chlorophyll artifact phaeophytin *a* emerged near the void volume (V_e, 24 ml), followed by a wide carotenoid band (V_e, 30 to 60 ml) containing lutein. On subsequent elution of the column with petroleum ether–diethyl ether (80:20) a large peak (V_e, 82 ml), containing chlorophylls *a* and *b* as well as violaxanthin and neoxanthin, was obtained. When the chloroplast pigments were applied to columns equilibrated with 2% by vol. of diethyl ether in petroleum ether (b.p. 40–60°), β-carotene, the chlorophylls and lutein were eluted as a large peak (V_e, 24 ml) near the void volume. On continued washing with this solvent, violaxanthin gradually leached off the column (V_e, 70 to 96 ml) followed by neoxanthin (V_e, 104 ml).

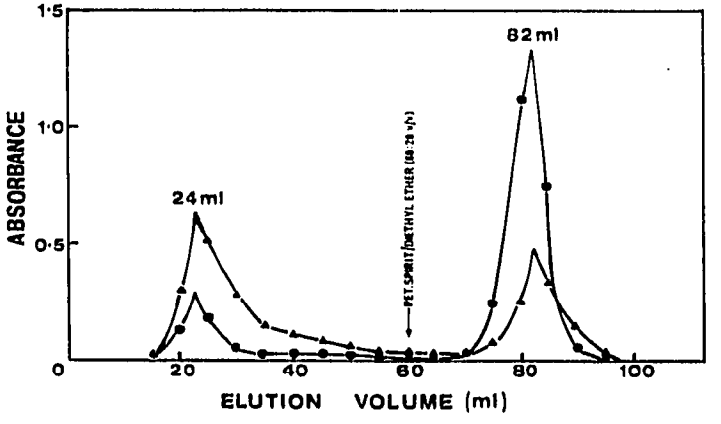

Fig. 2. Fractionation of chloroplast pigments from *Poa trivialis* (1 g) on Sephadex LH-20 columns (25 cm × 2.4 cm I.D.) equilibrated with petroleum ether (b.p. 40–60°) and eluted with petroleum ether–diethyl ether (80:20). ●—●, absorbance due to chlorophylls at $\lambda_{max.}$ = 640–660 mμ. ▲—▲, absorbance due to carotenoids read at 470 mμ to avoid contribution from absorbance due to chlorophylls. Further details are given in the text.

Discussion

The separation of lipid-soluble substances on Sephadex LH-20 is influenced by several chromatographic mechanisms[2,3,5,6,8,22,23] including molecular sieving, interaction with the gel matrix and liquid–liquid partition chromatography. The pronounced changes in the elution volumes of the fatty acids, phospholipids and chloroplast pigments as a result of increasing the polarity of the column eluents indicate that the adsorptive characteristics of the gel[3,5,6,8] played a major role in the resolution of the polar lipids. However, the elution of the fatty acids in order of decreasing molecular weight, from Sephadex LH-20 columns equilibrated with solvent of high polarity, suggests that molecular sieving also contributed to these fractionations.

The separation (Fig. 1) of the individual fatty acids (C_2, C_4, C_{10} and and C_{18}) is considerably better than that obtained in concurrent studies by ADDISON AND ACKMAN[6]. These investigators report that lauric (C_{12}) and behenic (C_{22}) acids were eluted, from Sephadex LH-20 columns equilibrated with chloroform, as a single band; behenic acid was located at the beginning of the band and most of the lauric acid at the end. While the improved separation now described may be partly attributed to the use of larger columns, the two reports are anomalous. The impaired resolution of the fatty acids, as a result of increasing the polarity of the column eluent, indicates that the chloroform used by ADDISON AND ACKMAN[6], which was relatively free of polar constituents (0.0027% by vol. of water), should have yielded better separation of individual fatty acids than that obtained with the chloroform (2% by vol. of ethanol and 0.05% by vol. of water) employed in the present study.

The behavior both of phospholipids and fatty acids on Sephadex LH-20 appears to be similar to that reported by NYSTRÖM AND SJÖVALL[24] for methylated Sephadex. However, better separation of the polar lipids appears to have been obtained in the present study.

In agreement with the results of MAXWELL AND WILLIAMS[7] little or no fractionation of chloroplast pigments or phospholipids was obtained on columns equilibrated with chloroform containing over 20% by vol. of methanol. However, milk phospholipids were separated into three fractions on columns equilibrated with chloroform. Further reduction in the polarity of solvent resulted in fractionation of chloroplast pigments.

The separations described suggest that chromatography on Sephadex LH-20 may be used as a preliminary step in the purification of individual phospholipids and chloroplast pigments. Furthermore, Sephadex LH-20 may prove useful for structural studies of lipids by providing a convenient approach to the separation of residual triglycerides, diglycerides[5], monoglycerides and fatty acids produced during controlled enzymic hydrolysis of lipids (*cf.* GARTON[25]).

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