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Fractionation of the Lipids of Eye Lenses by Gradient Elution on Activated Silicic Acid Columns

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Dried and powdered eye lenses were extracted with chloroform and the extract was fractionated by gradient elution from activated silicic acid columns. The elution of the phospholipids was studied and several peaks with different properties were obtained. Some figures are given for the composition of the lipids from a sample of cattle eyes obtained from a Swedish abattoir.

Chloroform extracts of lens tissue contain fluorescent substances and during the present investigation attempts have been made to fractionate these. The methods previously available were not suitable for the requirements of this separation. The usefulness of silicic acid columns was demonstrated by Borgström [1] and it has been used successfully in lipid chromatography by Lea and Rhodes [2]. The theory and application of gradient elution has been described by Tiselius and coworkers (for references see Lederer and Lederer [3]).

The chemical composition of the lipids of the eye lens has been described in detail and a survey of the literature has been given by Bellows [4]. However as in other branches of lipid research the application of chromatography should provide further information on the separation and determination of the various components. The fluorescent material in the different chromatographic fractions will be described in a later paper.

MATERIAL AND METHODS

Preparation of the lipid sample. The lenses from 3 500 cattle eyes were smeared on glass plates and dried at 37°. This material was scraped off, ground in a porcelain ball mill and then extracted with chloroform in a Soxhlet for 72 h. After evaporation under reduced pressure the lipid sample was redissolved in chloroform and filtered again.

Chromatographic procedure. The columns were prepared mainly following the directions of Borgström [1]. Silicic acid (Baker) was washed with chloroform and ethanol until no further fluorescent matter was removed and then activated at 120°C for 24 h. 20 mm columns were packed with a mixture of this silicic acid powder (10 g) and filter aid (Hyflo supercel, 5 g) suspended in chloroform. A solution of the lipid extract (300 mg) in chloroform (50 ml) was poured onto each column and the column was then washed

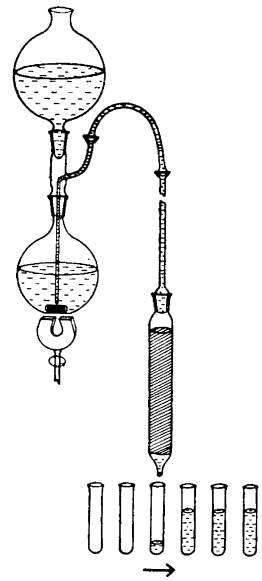

Fig. 1. The gradient elution apparatus. The solvent in the mixing chamber is mixed with a magnetic stirrer. Gradient elution is started by replacing the top vessel containing chloroform with one containing the chloroform-methanol mixture.

with pure chloroform; about 225 ml seemed to be the optimum quantity. The column was then eluted with chloroform-methanol using a gradient elution method as shown in Fig. 1. A mixture of methanol and chloroform (1:2) flowed continuously into the mixing vessel which contained about 100 ml of chloroform. Fractions were collected over 20 min intervals and the elution curves were therefore plotted against the time.

Paper chromatography. Each fraction was hydrolysed with sulphuric acid and then neutralised with baryta. The paper (Whatman No. 1) was equilibrated over the solvent in the development tank; ascending development was used; solvent composition is given in Table 3.

Spot tests on paper. Spot tests were made by dropping 0.2 ml from each fraction onto paper placed over a hot air stream. Ethanolamine and serine were made visible by spraying with 0.1 % ninhydrin in *n*-butanol and then heating at 95° for 10 min. Choline was tested for by the method of Bevan, Gregory, Malkin and Poole [5].

Quantitative determination. Phospholipids were calculated as 25 P following the method of Brante [6] which is a modification of the methods of Teorell [7] and Fiske-Subbarow [8].

Cerebrosides and gangliosides were estimated by the methods elaborated by Svennerholm [9].

Cholesterol was estimated by a routine photometric method. The sample was dissolved in chloroform (5 ml), and then acetic anhydride (2 ml) and concentrated sulphuric acid (0.2 ml) were added, the mixture was shaken mechanically in the dark for 5 min and the absorption was measured at 630 mμ.

Glycerol was determined by the method of Blix [10].

The distribution of plasmalogens in different fractions was estimated by the method described by Feulgen and Grünberg [11]. The absorption was measured directly without extraction by amyl alcohol.

The lipid fractions were weighed after evaporation of the solvent under reduced pressure.

Purification of solvents. Only analytical grade reagents were used. It was necessary to remove small amounts of fluorescent impurities because of the experimental method to be used. Distillation under water pump vacuum from a large flask immersed in a water bath at 45° gave satisfactory purification. The separation was poor unless the condenser joint was heated strongly with an electrical heating tape. No rubber connections were used.

RESULTS

General composition of the lipids. The lens powder was extracted only with chloroform but the denaturation of the proteins by drying and the long extraction period should permit sufficient release of the lipids.

It is possible to outline the composition of the lipid sample without considering the effect of the gradient elution. As stated by Borgström [1], chloroform elution will give a good separation of the phospholipids from the cholesterol esters and glycerides and the free fatty acids. Phosphorus determination on the chloroform eluate confirmed the retention of the phospolipids on the column provided the recommended volume of chloroform was not exceeded by too much. This separation is of assistance in several analytical determinations.

The amounts of glycerol and cholesterol in the lipids eluted by chloroform were determined on the material from four columns and gave mean values of 0.53 % of the original sample for glycerol and 27.5 % for cholesterol. The chloroform eluate may contain various esters but for the sake of simplicity can be regarded as containing glycerides, cholesterol and cholesterol esters. The possibility of free fatty acids being present is neglected. If the glycerides have a molecular weight as high as that of tristearin, 891, they will amount to 5.1 % of the original sample and the corresponding mean value for cholesterol and its esters will be 34.9 %. A molecular weight as low as 700 will give values of 3.6 and 36, respectively. The percentage figures are thus not influenced to any great extent by errors in the molecular weight assumed for the glycerides. A mean molecular weight lower than that of tristearin was assumed in estimating the approximate values given in Table 1.

Table 1. Constituents of the lipids.

	%
Glycerides	4.5
Cholesterol and its esters	35.5
Phospholipids	48.0
Cerebrosides	0.8
Gangliosides	(0 < 0.5)
Undefined material	11.0

Before chromatography the sample contained 48 % phospholipids. This could also be determined from the amount of phospholipid fraction obtained by chromatography. The results from 8 columns are given in Table 2 with the percentage of phospholipid worked out in three different ways. In each case they are higher than the values determined from the original sample. The 25 × P formula thus does not apply to some of the lipids remaining on the column after elution with chloroform. The gradient elution was intended to concentrate these lipids into definite fractions.

Gradient elution. The material in the fractions eluted by chloroform methanol was weighed and the phospholipids present were estimated. These values when plotted gave a reproducible pattern. There were some variations but the characteristic peaks of the curves were always recognisable. There

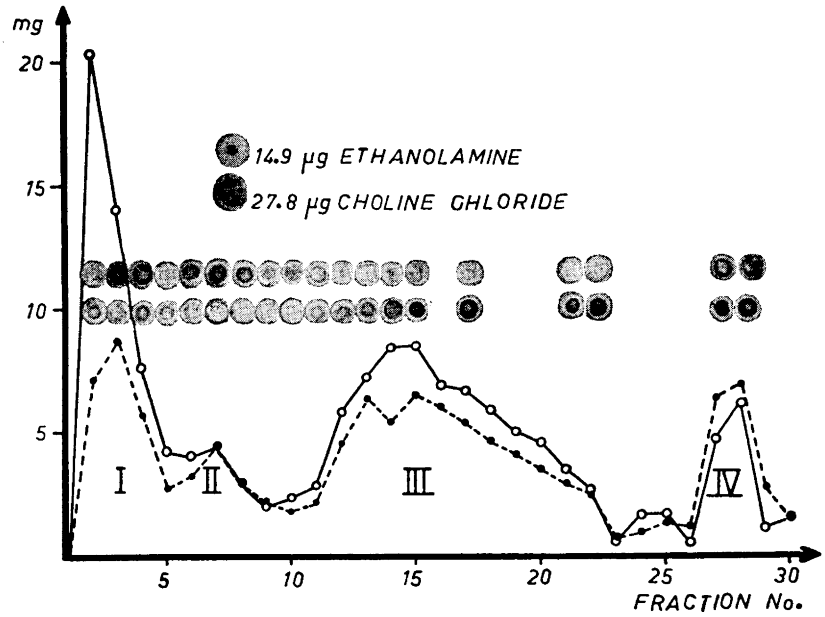

Fig. 2. Fractions obtained by elution with chloroform methanol. The unbroken line represents the lipid amount of each fraction and the dotted line the calculated weight of phospholipids. The spots show ninhydrin reactions and choline tests made on paper with material from the corresponding fractions. The four phospholipid peaks are marked I—IV.

were three phospholipid peaks designated I, II and III in Figs. 2, 3 and 4 with a further peak, IV, in the material stripped from the column with methanol. There were sometimes signs of a slightly smaller peak in front of peak I, see Figs. 3 and 4.

Spot tests for cephalins and lecithin were made on paper using the ninhydrin and choline reactions. The ninhydrin reaction was strong for peaks I and II but very weak for peak III, while the lecithin choline reaction was strong only for peak III. The cephalins should therefore be eluted in front of the lecithin (see Fig. 2). Since the cephalins can be divided into phosphatide ethanolamine and phosphatide serine peaks I and II were investigated to see whether they differed in this way. After hydrolysis the nitrogeneous constituents were separated by chromatography on paper as shown in Fig. 3. The results are shown in Table 3.

The elution curves showing also the spot tests and the paper chromatographic results indicate that the phosphatidyl ethanolamine, the phosphatidyl serine and the phosphatidyl choline are eluted in that order and correspond to the peaks I, II and III. It is also evident that the chromatogram does not give a complete separation especially for peaks I and II. The ninhydrin reaction showed only a single weak spot with an R_F value different from those of ethanolamine and serine. There can be only very little of other strongly reacting amino groups.

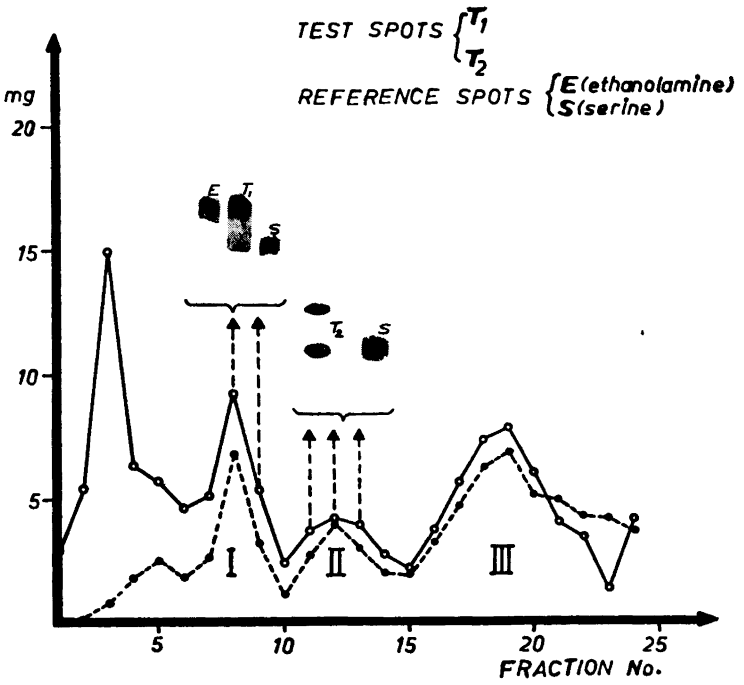

Fig. 3. Fractions obtained by gradient elution with chloroform methanol; same scale as in Fig. 2. The paper chromatograms shown were run on hydrolysed material.

Table 2. Phospholipid fractions from a series of columns.

Initial elution with chloroform ml	a Eluted by chloro-form mg	300—a	b Eluted by chloro-form/ methanol mg	a + b Total wt. of eluates mg	Number of fractions	300—a as % of 300	b as % of 300	b as % of a + b
135	119	181	171	290	50	60	57	59
135	121	179	176	297	60	60	59	59
135	130	170	178	307	60	57	59	58
135	108	193	200	308	60	64	67	65
450	147	153	165	312	80	51	55	53
270	106	194	223	329	89	65	74	68
150	128	172	154	282	55	57	51	55
225	125	175	135	261	44	58	45	52
	122.9	177.1	175.3	298.6		59.0	58.4	58.6

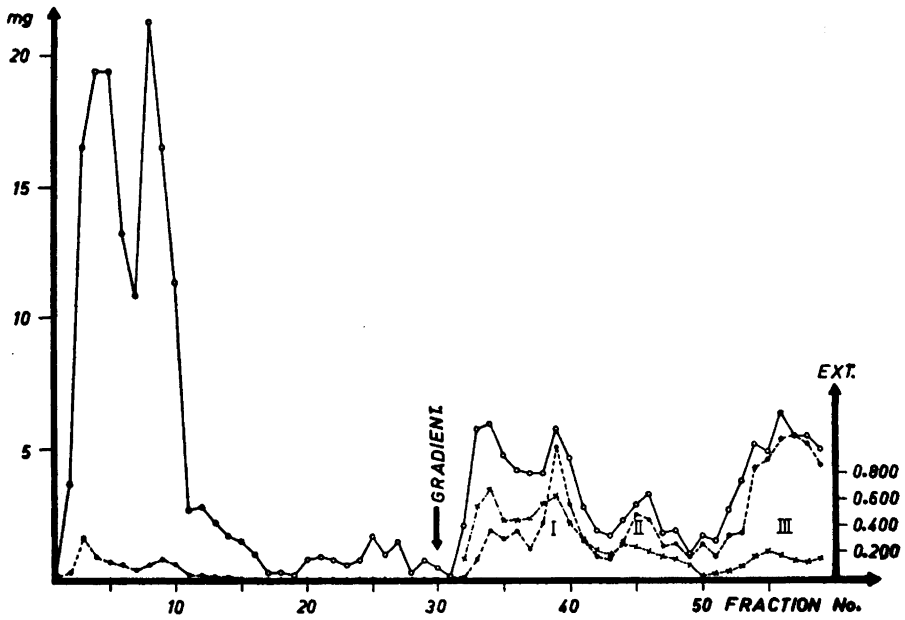

Fig. 4. Chromatogram including the fractions eluted by chloroform; scale as in Fig. 2. The distribution of Feulgen material positive is shown by the curve with crosses; scale as extinction, on the right. In this chromatogram the chloroform eluate had a higher phosphorus content than usual.

Solubility tests permit only rough conclusions to be drawn if the fractions do not contain pure compounds but seemed to accord with the elution sequence given above. The solubility in ethanol was higher for the lecithin fractions than for the first fractions while the material of peak IV was almost insoluble in ether and was preceded by fractions with low ether solubility.

The residue stripped from the column with methanol was designated peak IV and was almost solely phospholipids calculated as 25 × P. To ascertain

Table 3. Paper chromatography of hydrolysed fractions. The R_F values in brackets indicate weaker spots.

	Time of development	Solvent (% v/v) *n*-Butanol:acetic acid:water	R_F values of reference spots		R_F values of hydrolysed compounds
			Serine	Ethanolamine	
PEAK I	9 h	40:10:50	—	0.31	(0.18), 0.31, (0.63)
PEAK II	6 h 20 min	40:10:50	0.17, (0.29)	0.27	0.27, (0.60)
	9 h 35 min	66:17:17	0.16	0.33	(0.16), 0.33
PEAK III	8 h 15 min	66:17:17	0.13	—	0.13, 0.29
	10 h	40:10:50	0.19	0.28	0.16, 0.29, (0.60)

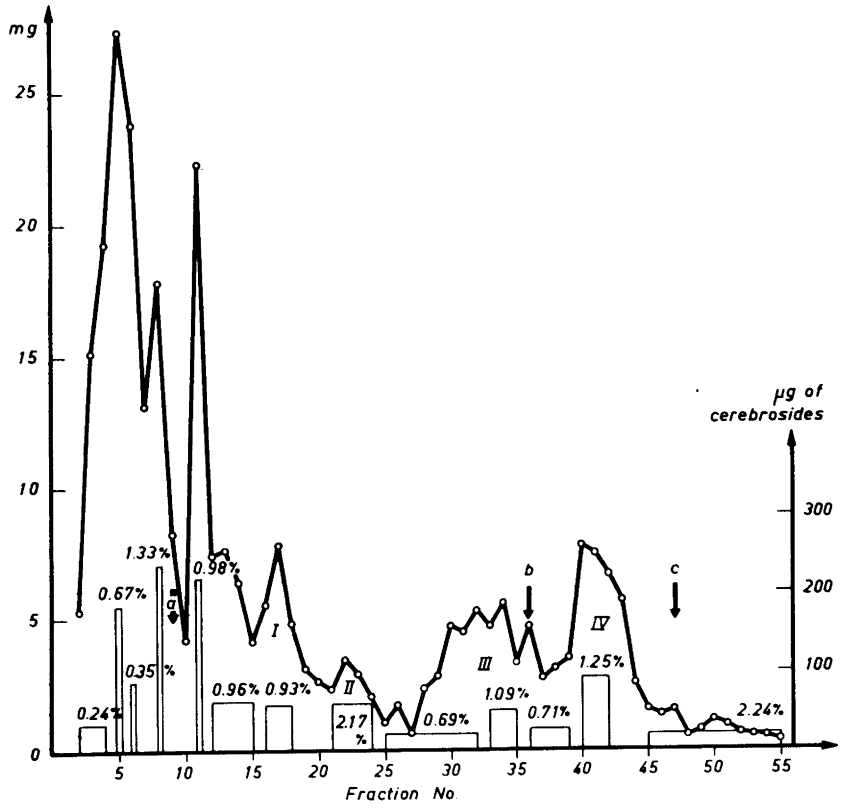

Fig. 5. Elution of the cerebrosides. The curve shows the weight eluted. The height of the columns shows the total cerebroside content of the corresponding fractions; scale on the right. The figures on the columns give the cerebroside contents of each combined fraction as a percentage of total material eluted. Phosphorus determinations were not made in this case but the position of the peaks can be deduced from the shape of the curve. The arrows indicate: a) the start of chloroform methanol elution, b) methanol in the top vessel, c) stripping with methanol. A more prolonged elution with chloroform would have given a better separation of the first fractions eluted by chloroform methanol.

whether sphingomyelin or other glycerol-free phospholipids were present the phosphatide content was calculated from the result of glycerol determinations. The mean value of four determinations was 6.3 % corresponding to 53 % glycerophosphatides using the factor 8.4. This factor should possibly be reduced to allow for the presence of compounds such as lysolecithin; small amounts of cerebrosides may also cause a slight increase. It can however be concluded that sphingomyelin or some similar compound is eluted in the peak IV region.

Lea and Rhodes [2] separated the lipids of egg yolk on silicic acid columns and found lysolecithin in the strongly adsorbed residue on the column. The possibility of this being present in the peak IV material does not invalidate the conclusions above since different phosphatidyl compounds have the same

P:glycerol ratio. The high value given by calculation with 25 × P may however obscure the presence of undefined material. Lea and Rhodes rejected the material insoluble in cold ether before chromatography and therefore would not have found sphingomyelin.

No attempts were made to follow the elution of the plasmalogens quantitatively. The Feulgen reaction was used in one case and the densities were plotted on the weight curve; see Fig. 4. High values were recorded at the beginning of the curve but further along the reaction was weaker. It might be expected that the plasmalogens and the cephalins would be eluted together. This assumption was not entirely refuted by the experimental results but was incompatible with the first part of the curve. The initial hump of peak I corresponded to a peak in the plasmalogen curve. It is therefore possible that a plasmalogen fraction was eluted just in front of the cephalins but was incompletely separated from them.

The cerebrosides amount to 0.8 % of the original lipid sample. The chromatographic fractions were grouped to give sufficient material for study, see Fig. 5. The largest amounts of cerebrosides were eluted by chloroform although some of the later fractions contained slightly higher proportions of cerebrosides. Because of the small quantities present, no attempt was made to find whether the differences in chemical composition of the cerebrosides influenced the order of elution.

The fractions eluted by chloroform methanol gave more material than that shown by phosphorus determinations on the original material. The phospholipid content in the first part of the gradient elution curve is apparently low as can be seen in Figs. 2 and 3. Cholesterol determinations were made to check the separation, but only low and insignificant values were obtained even when the preceding elution was brief.

The first fractions eluted by chloroform methanol had a low phosphorus content even after prolonged elution with pure chloroform, see Fig. 4. Taurog, Enteman, Fries and Chaikoff [12] found that magnesium oxide retains cephalins and a preliminary test showed that the undefined material eluted with the cephalins could be separated using this adsorbent.

Work on further purification of the fractions is in progress and will be published later.

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