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The isolation and identification of lysolecithin from lipid extracts of normal human serum *

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SUMMARY

Chromatography on silicic acid was employed for the separation of phospholipid components of normal human serum. These components were identified as cephalins, lecithin, sphingomyelin, and lysolecithin by paper chromatography, chemical analyses, and infrared spectra. The quantities of the phospholipid component were determined.

A recent paper by Hirsch and Ahrens (1) has described the separation of the major classes of lipids in human serum by silicic acid chromatography. The major phospholipid constituents of serum were reported to be cephalins, lecithins, and sphingomyelin eluted from the column in that order.[1] Phillips (2, 3) has isolated a fourth phospholipid component of serum and presented evidence for its identification as lysolecithin, using the silicic acid chromatography technique of Lea *et al.* (4).

The present study offers conclusive confirmation of the presence of lysolecithin and identification of the major phospholipid components of normal human serum separated by the silicic acid chromatographic technique of Hirsch and Ahrens (1).

METHODS

Antecubital vein blood was drawn from apparently healthy 12-hour-fasted subjects of age 25 to 35 years. Blood samples were processed 2 hours after withdrawal.

Lipid Extraction. Serum (15 to 20 ml.) was added dropwise to 25 volumes of 2:1 (v/v) chloroform-methanol. After shaking, the extract was allowed to stand for 30 minutes and then filtered into a 1 l. separatory funnel. Flask and filter paper were washed twice with 20 ml. portions of chloroform-methanol. An equal volume of distilled water was carefully layered on top of the filtrate and allowed to equilibrate for at least 3 hours. Recoveries of lipid phosphorus were unaffected by the addition of salts ($CaCl_2$, $MgCl_2$) to the distilled water. The lower chloroform layer and the fluffy interphase were removed from the upper aqueous methanol layer. The extract was taken to dryness on a flash evaporator under vacuum and kept under nitrogen. The dried residue was taken up in 15 ml. of chloroform or petroleum ether (b.p. 35 to 70°C) and used immediately or stored in a stoppered flask at 4°C for not more than 2 days. All solvents used throughout extraction and chromatography were doubly redistilled from reagent grade material.

Silicic Acid Chromatography. Silicic acid columns were prepared as described by Hirsch and Ahrens (1) using 18 g. activated silicic acid and a water-jacketed assembly allowing maintenance of the column at a constant temperature of 25°C. Uniform packing of the dry column (1.8 × 12 cm.) was accomplished by gentle tapping with a glass rod, followed by firmer packing with an electric vibrator. Dehydrating washings with diethyl ether (10 ml.), 1:1 (v/v) acetone-diethyl ether (30 ml.), and diethyl ether (20 ml.) were followed by conditioning with petroleum ether for 6 to 10 hours before applying the sample.

Columns were re-used after complete stripping of all lipid components with 200 ml. methanol followed by the regular dehydrating washings and petroleum ether conditioning for 10 hours.

Elution was accomplished with 150 ml. diethyl

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[1] Throughout this article the term "cephalin fraction" refers to the corresponding fraction described by Hirsch and Ahrens (1).

ether followed by 500 ml. 1:4 (v/v) chloroform-methanol. Upon addition of chloroform-methanol, fractions of 360 drops were collected by means of an automatic fraction collector. The flow rate from the column was 12 to 20 ml. effluent per minute.

Analyses. Fractions were analyzed for phosphorus by the method of King (5). The samples within each peak were pooled for further analysis. Carboxylic acid ester groups were determined according to Rapport and Alonzo (6). The ferric perchlorate solution used in this method was prepared in n-butanol instead of in ethanol and only 4 ml. of this reagent was used. Amino groups were detected by a ninhydrin method (7). Triglycerides and glycerol were determined according to Van Handel and Zilversmit (8), although complete recovery and measurement of phospholipid glycerol was not possible by this method. Choline was estimated by the method of Glick (9) and cholesterol by the method of Sperry and Webb (10). Hemolytic activity was tested by 3 hours' incubation with 2 ml. of 1 per cent suspension of washed human erythrocytes in isotonic saline at room temperature.

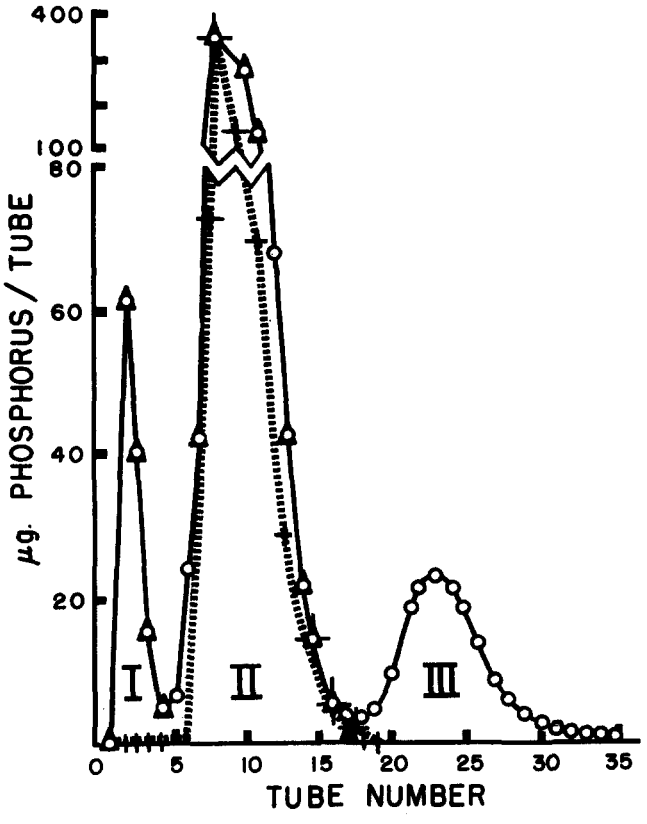

FIG. 1. Elution pattern of phospholipids from silicic acid with 500 ml. 1:4 (v/v) $CHCl_3$—MeOH. Extract from 15 ml. normal human serum: ○; rechromatography of Peaks I and II: ▲; beef spinal cord sphingomyelin (6 mg.) + L-α-dimyristoyl lecithin (6 mg.): + dotted line.

Paper Chromatography. Chromatography was carried out on unimpregnated paper according to Witter *et al.* (11), using 30:7 (v/v) 2,6-dimethyl-4-heptanone-acetic acid. Chromatography on silicic acid impregnated paper was carried out according to Marinetti and Stotz (12), using the n-butyl ether-acetic acid-chloroform-water system and on silicic acid impregnated glass fiber filter paper according to Dieckert *et al.* (13), using the ether-acetone-phenol-water system. Spots were detected with Rhodamine B (11), ninhydrin (11), and iodine vapor.

Infrared Spectra. Infrared spectra were obtained on a Perkin-Elmer Model 13 spectrophotometer using an NaCl prism. Samples were prepared by evaporating chloroform solutions onto an AgCl plate. Samples were run against air as a blank, using the constant energy program.

Materials. Sphingomyelin prepared by the method of Rapport and Lerner (14) was obtained from the Sylvana Chemical Company, Orange, New Jersey. Synthetic L-α-dimyristoyl lecithin and L-α-dimyristoyl phosphatidylethanolamine were obtained from LaMotte Chemical Company, Chestertown, Maryland. Lecithinase A was prepared from commercial pancreatin (Viobin Corporation) by the method of Gronchi (15). Lysolecithin was prepared by the action of lecithinase A on L-α-dimyristoyl lecithin and on beef spinal cord lecithin (Sylvana Chemical Company).

RESULTS

Lipid phosphorus was recovered in the initial extraction procedure to the extent of 88.2 to 97.2 per cent. Lipid phosphorus eluted from silicic acid columns was recovered to the extent of 90 to 101 per cent. Complete recovery of the triglycerides and free and esterified cholesterol was observed in the diethyl ether fraction initially eluted from columns. Only traces of phosphorus were found in this fraction and in the final methanol elution used for stripping the columns.

Figure 1 shows the typical elution pattern of the phospholipids from normal human serum obtained by elution with chloroform-methanol 1:4 (v/v). The three peaks, I, II, and III, were always obtained with the same efficiency of separation. No sign of column overloading was evident, even with the lipid extract from 100 ml. plasma (approximately 175 mg. phospholipid). Poor separation was noted in old columns, probably due to hydration, or when positive pressure was applied during elution, or when column temperature exceeded 25°C.

Table 1 shows the amount of lipid phosphorus in the three peaks expressed as mg. phosphorus per 100

ml. serum and as the percentage of total recovered lipid phosphorus from eight normal serum specimens.

Peak I. As shown in Table 2, the mean carboxylic acid ester bond to phosphorus ratio was 2.01 (1.90 to 2.08). This falls within the range of variation for both determinations when these were performed on standard lipids. Glycerol was present, choline was absent, and a positive ninhydrin reaction was obtained. No hemolytic activity could be demonstrated. This falls within the range of variation for both determinations when these were performed on standard lipids. The Peak I components were subdivided on silicic acid by using 200 ml. chloroform-methanol 7:1 (v/v), followed by 200 ml. chloroform-methanol 4:1 (v/v). These components were revealed by paper chromatography to be principally phosphatidylethanolamine and a trace of phosphatidylserine. Table 1 shows that these cephalins were present to the extent of 0.63 mg. phosphorus per 100 ml. serum and represent 6.4 per cent of the total lipid phosphorus. These values are in good agreement with those given by Phillips (3), Posborg Petersen (16), Sinclair (17), and Hack (18).

Peak II. Table 1 shows that Peak II contained 85 per cent of the total recovered lipid phosphorus and represents 8.3 mg. phosphorus per 100 ml. serum. Table 2 shows that the Peak II material had no hemolytic activity and was ninhydrin-negative when the reaction was carried out in solution. Glycerol was present and the mean ester bond to phosphorus ratio was 1.57 (1.46 to 1.83). The choline to phosphorus ratio was only 0.73. The discrepancy between these ratios suggested a mixture of phospholipids. Figure 1 shows a model experiment in which a mixture of synthetic L-α-dimyristoyl lecithin and beef spinal cord sphingomyelin failed to separate and showed identical elution characteristics to the Peak II material under the conditions used. The infrared spectrum of the Peak II material as compared to synthetic L-α-dimyristoyl lecithin and beef spinal cord sphingomyelin was compatible with a mixture of lecithin and sphingomyelin. Lecithin showed characteristic bands at 1740, 1470, and 1170 cm.$^{-1}$ Sphingomyelin showed principal bands at 1625, 1535, and 1470 cm.$^{-1}$ All five bands were present in the Peak II material.

Paper chromatography revealed the presence of both lecithin and sphingomyelin, and a trace of ninhydrin-reacting material. The method employed for carboxylic acid ester determination in these experiments does not detect the sphingomyelin fatty acid held through the amide linkage since it is apparently not freed by hydroxylamine under alkaline conditions. Under the conditions of 90-minute alkaline hydrolysis described by Glick (9), sphingomyelin choline was not released. The King procedure (5) estimates the phosphorus from both lecithin and sphingomyelin, whereas the Glick procedure (9) estimates the choline and the Rapport and Alonzo method (6) determines the fatty ester bonds from lecithin only. Therefore in a mixture of lecithin and sphingomyelin, both the choline to phosphorus and fatty ester to phosphorus ratios will be lower than the theoretical value for lecithin alone. The carboxylic acid ester to phosphorus ratio of 1.57 therefore allows estimation of these Peak II components on the assumption that the carboxylic acid ester to phosphorus ratio for lecithin is 2.00. This gives in Peak II a mean lecithin content of 78.5 per cent and sphingomyelin content of 21.5 per cent. Corresponding correction of the choline to phosphorus ratio gives a value of 0.93 for lecithin. Thus lecithin represents 66.4 per cent and sphingomyelin 18.4 per cent of the total recovered phosphorus, or 6.54 and 1.79 mg. phosphorus per 100 ml. serum respectively. These values are in good agreement with those reported by previous workers (3, 16, 17, 18).

Peak III. Table 2 shows that this material was ninhydrin-negative and contained glycerol. The mean carboxylic acid ester to phosphorus ratio was 1.11 (1.02 to 1.26) and the choline to phosphorus ratio was 1.17. Hemolytic activity was clearly present with 0.015 μmole of material and detectable with 0.010 μmole. The infrared spectrum differed from that of sphingomyelin as well as from that of the Peak II material but was similar to that of the lecithin standard. Bands were observed at 1740 and 1170 cm.$^{-1}$ but not at 1625 or 1535 cm.$^{-1}$ This indicates that the Peak III material was not contaminated with sphingomyelin. The only differences from the spectrum of lecithin were quantitative rather than qualitative. Nelson and Freeman (19) did not report the presence of lysolecithin in their studies on serum. However, lysolecithin would probably have eluted with the mixed lecithin and sphingomyelin fraction in their solvent systems and the lysolecithin spectrum, which is qualitatively similar to that of lecithin, would have been masked by lecithin. Paper chromatography of the Peak III material yielded a spot having the same mobility as the product of pancreatic lecithinase A action on L-α-dimyristoyl lecithin, beef spinal cord lecithin, or on the Peak II material. The Peak III substance was resistant to the action of pancreatic lecithinase A. It was concluded from the evidence that the Peak III material was lysolecithin, which Table 1 shows to compose 8.9 per cent of the total lipid phosphorus or 0.88 mg. phosphorus per 100 ml. serum.

Figure 1 shows the results of rechromatography of

TABLE 1. PHOSPHORUS CONTENT OF CHROMATOGRAPHICALLY SEPARATED PHOSPHOLIPID COMPONENTS OF NORMAL HUMAN SERUM

Exp. No.	Sex	Mg. lipid P per 100 ml. serum				Percentage of total lipid P		
		I *	II †	III ‡	Total	I *	II †	III ‡
23	M	0.55	7.12	1.03	8.70	6.4	82.1	11.5
27	F	0.63	9.57	0.96	11.16	5.6	85.7	8.6
28	F	0.98	7.35	0.85	9.18	10.7	80.1	9.2
29	F	0.85	10.95	1.07	12.87	6.6	85.1	8.3
40	M	0.64	8.23	1.00	9.87	6.5	83.4	10.1
49	M	0.59	9.44	0.71	10.74	5.5	87.9	6.6
57	F	0.34	6.02	0.60	6.96	4.9	86.5	8.6
62	M	0.42	7.94	0.82	9.18	4.6	86.5	8.9
Mean		0.63	8.33	0.88	9.84	6.4	84.7	8.9
S.D.		±0.21	±1.58	±0.16	±1.78	±1.9	±2.6	±1.4

* Cephalins.
† Lecithin plus sphingomyelin.
‡ Lysolecithin.

TABLE 2. PROPERTIES OF COMPONENT PEAKS OF PHOSPHOLIPIDS FROM SERUM

	Carboxylic acid ester/P	Choline/P	Glycerol	Ninhydrin	Hemolytic Activity	R_f *		
						Solvent A †	Solvent B †	Solvent C †
Peak I	2.01	0	+	+	0	0.47 ± 0.07 0.10 ± 0.10	0.57 ± 0.14 0.66 ± 0.06	0.80 ± 0.17
L-α-dimyristoyl phosphatidyl ethanolamine	–	–	–	–	–	0.55 ± 0.11	0.56 ± 0.12	0.80 ± 0.17
Peak II	1.57	0.73	+	0	0	0.69 ± 0.09 0.40 ± 0.10 0.56 ± 0.04 ‡	0.60 ± 0.09 0.30 ± 0.10 –	0.67 ± 0.13 0.18 ± 0.04 ‡
L-α-dimyristoyl lecithin	–	–	–	–	–	0.70 ± 0.07	0.53 ± 0.11	0.75 ± 0.16
Sphingomyelin	–	–	–	–	–	0.39 ± 0.10	0.36 ± 0.11	0.67 ± 0.18
Peak III	1.11	1.17	+	0	+++	0.41 ± 0.09	0.18 ± 0.10	0.38 ± 0.11
L-α-dimyristoyl lecithin plus lecithinase A	–	–	–	–	–	0.35 ± 0.12	0.16 ± 0.09	0.29 ± 0.14
Peak II plus lecithinase A	–	–	–	–	–	0.25 ± 0.10	0.20 ± 0.10	0.27 ± 0.09

* R_f computed to center of spot ± ½ spot length ÷ distance to solvent front.
† Solvent A = Diisobutylketone/acetic acid (30:7) (11); Solvent B = n-Butyl ether/acetic acid/$CHCl_3$/H_2O (40:35:6:5) (12); Solvent C = Ether/acetone/phenol/H_2O (13).
‡ Ninhydrin positive.

the pooled material in Peaks I and II after taking to dryness and redissolving in chloroform. It may be seen that no Peak III material appeared, which indicates that lysolecithin was not formed during chromatography.

The occurrence and quantity of lysolecithin was unchanged when water washing of the lipid extract was omitted. No significant differences in elution pattern or peak quantities were observed when equal portions of the same dried serum lipid extract were dissolved in chloroform or in petroleum ether. The quantity and elution pattern of the serum phospholipids was unchanged whether serum or the lipid extracts were processed within 2 hours or stored for 1 week at 4°C. No quantitative differences were observed in the peak materials when serum was compared with heparinized plasma from the same subject and using siliconized glassware to prevent breakdown of formed elements.

DISCUSSION

Well-defined separation of three major phospholipid components from human serum was afforded by the use of 1:4 (v/v) chloroform-methanol elution from silicic acid columns. Identification of these peak components revealed them to be different from the three major peaks obtained by Hirsch and Ahrens (1) by methanol elution from a silicic acid column. These workers reported cephalin, lecithin, and sphingomyelin to be eluted, in that order. The present study indicates that the peaks are cephalin, lecithin and sphingomyelin, and lysolecithin, in that order. The finding of lysolecithin in human serum offers confirmation to similar reports by Phillips (2, 3). In earlier experiments some separations of lecithin and sphingomyelin were accomplished with chloroform-methanol 3:2 (v/v), although this separation was more effective when followed with chloroform-methanol 1:1 (v/v). No other combination of chloroform and methanol, nor methanol alone, effected separation between lecithin and sphingomyelin.

Paper chromatography revealed the presence of minor amounts of a ninhydrin-reacting substance in the Peak II material, which appeared only when the spot was concentrated on filter paper but which did not react with ninhydrin in solution. This material did not give positive reactions with Rhodamine B or iodine vapor. This may be similar to the unidentified ninhydrin reacting substance noted by Phillips (3) and may represent as yet undescribed lipid.

The origin of lysolecithin in serum is not clear at present. Control experiments render it unlikely that it is formed during extraction, purification, or chromatography of the extract. It may be possible that it is formed shortly after the blood is drawn, although there is no evidence to favor this possibility.

It is of interest that Gray and Macfarlane (20) reported the production of lysophosphatides from the corresponding plasmalogens when chloroform-methanol extracts of ox heart were chromatographed on silicic acid without prior removal of the neutral fat fraction. In the studies reported here, acetone extraction of neutral lipids, and silicic acid chromatography of the acetone-insoluble phospholipids showed the same three peaks in the same relative amounts.

Hajdu *et al.* (21) reported the chemical isolation and purification of β-palmitoyl lysolecithin from plasma and also from liver, heart, and adrenal medulla. These workers found that 50 per cent of the lysolecithin occurred with the α-position bound in a hemiacetal linkage with a fatty aldehyde. This was converted to β-lysolecithin on allowing the extract to stand at pH 2.0 for 24 to 48 hours. It was also noted that lysolecithin was decomposed on silicic acid only in the presence of aqueous solvents.

Studies [2] on a number of patients suffering from various disorders showed a marked elevation both in serum lysolecithin and serum amylase in acute pancreatitis without a simultaneous increase in total phospholipid concentration. This suggests that lysolecithin is indeed a normal constituent of serum.

It is not certain whether plasma lysolecithin may be a precursor or a breakdown product of lecithin or neither. Borgström (22) has reported the presence of lysolecithin in intestinal contents but it is not known whether this is absorbed as such.

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