J. Lipid Research, January, 1962
Volume 3, Number 1

Studies on human serum lipoprotein phospholipids and phospholipid fatty acid composition by silicic acid chromatography*

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[Received for publication February 6, 1961]

SUMMARY

Normal human serum phospholipids were fractionated by silicic acid column chromatography. A large number of fractions was obtained with which it was possible to study the effect of fatty acid composition on elution rate. Phospholipids containing unsaturated fatty acids eluted from the silicic acid column more rapidly than their saturated analogues. It was found that lecithin could not be cleanly separated from sphingomyelin by any concentration of methanol or multiple-solvent elution scheme. The fatty acid compositions of the individual phospholipids were studied. Phosphatidyl ethanolamine was the most unsaturated phospholipid of serum; lecithin, sphingomyelin, and lysolecithin showed less unsaturation, in that order. In all phospholipids, 20 and 22 carbon unsaturated fatty acids were present. Long-chain saturated, as well as odd-chain saturated, fatty acids were present and were particularly prominent in the sphingomyelin fraction.

In previous studies reported by this laboratory (1, 2), data on the phospholipid and phospholipid fatty acid composition of human serum and serum lipoprotein fractions were presented. Other authors (3, 4, 5) have published data from similar studies. Most of these latter studies have presented evidence for the presence of lysolecithin as well as the phosphatidyl ethanolamine, lecithin, and sphingomyelin fractions reported in the work from this laboratory. The procedure previously used here did not show the presence of lysolecithin; if any were present in the samples it would have been included in the lecithin fraction.

A more extensive chromatographic fractionation of the total serum phospholipid was undertaken to improve the resolution of the individual phospholipids and attempt to establish the presence or absence of lysolecithin as well as other minor components. As a large number of fractions was taken during the chromatographic run, a large initial amount of phospholipid was necessary to permit subsequent analysis of the fatty acid moiety of the individual phospholipids. The subfractionation of the major elution peaks provided a means of studying the effect of fatty acid composition of a phospholipid on the elution rate from the column when methanol is the eluting solvent. It has been recognized previously (6, 7) that the fatty acid moiety of complex lipids influences the migration rate of the lipids on silicic acid columns. Rhodes and Lea (6) showed that hydrogenating natural lecithin mixtures did not affect the shape of the elution curve but that an individual fraction taken from any region of the hydrogenated elution pattern produced a more homogeneous curve than the original. Although they did show that the iodine value was higher at the leading edge in the natural samples, they were inclined to believe that chain length was the predominant factor affecting migration rate.

Klein (8) has published a method for the separation of cholesterol esters based on this effect. Recently, Baer (9) has reported a method of separating various glycerophosphatides by their fatty acid components when both fatty acid residues are identical. In practice, this procedure has little application to most natural phospholipid mixtures because different fatty acid residues occur in the same molecule, and a great variety of both saturated and unsaturated fatty acids occurs in natural samples. Sphingomyelin and lysolecithin, each having only one fatty acid residue per molecule, might lend themselves more easily to this

* This work was supported in part by the United States Atomic Energy Commission.

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method of separation.

EXPERIMENTAL PROCEDURE

Blood was drawn in 250 ml quantities from healthy donors as described previously (2). Again subjects were chosen with above-average serum lipid levels. Ethylenediaminetetraacetic acid was added to the separated serum (5 mg per 100 ml of serum) to inhibit oxidation of lipids by sequestering polyvalent cations in the serum. After the cells were removed, the serum was kept at 4° and under nitrogen whenever possible during all subsequent operations.

Ultracentrifugation. As only the unfractionated serum lipoproteins were being studied in this experiment, a multiple ultracentrifugal separation as previously performed (2) was unnecessary. Nevertheless, it was decided to concentrate the lipoproteins of the serum before extracting the lipids. This offered an advantage during subsequent extraction operations by reducing the volume of solvents needed in the modified Sperry extraction procedure. It also meant that the lipid extract was not a total lipid extract of whole serum but an extract of the lipoproteins of serum with a density less than 1.20 g per ml.

To 100 ml of serum was added 26.1 g of NaBr. The density of the resultant solution (usually having a volume of 108 ml) was checked by refractometry and adjusted to 1.210 g per ml. The solution was then transferred to 18 6-ml centrifuge tubes and centrifuged for 48 hours at 40,000 rpm in a 40.3 Spinco ultracentrifuge rotor. After centrifugation, the top 1 ml, containing all the major lipoproteins of the serum, was removed by a pipetting technique described elsewhere (10). The second milliliter, although clear of large molecules, was also removed and stored separately for later use as a blank in calculating the total concentration of lipoprotein in the first milliliter of the tube by a refractometric technique developed by Dr. F. T. Lindgren. The lower 4 ml were discarded. In this procedure, there is a loss of 5% to 10% of the total lipoproteins with a density less than 1.20 g per ml caused by adherence to the wall of the preparative tube. This loss is assumed to be uniformly distributed throughout the lipoprotein spectrum. In addition, the ultracentrifugal residue contains approximately 5% of the total serum lipid (11). Hence, the recovery by this procedure is approximately 85% of that obtained by extraction of unfractionated serum. Analytical ultracentrifugation was also performed on a portion of the top milliliter for the purpose of determining the distribution of the lipoproteins in the various lipoprotein classes.

Extraction. The extraction procedure was altered from that previously described (1). The pooled top 1-ml fractions (total volume 18 ml) were added to 166 ml of methanol[1] in a 500-ml volumetric flask; to this was added 166 ml of $CHCl_3$ and the mixture was heated at 60° for 15 minutes. After cooling, the solution was brought to volume with $CHCl_3$ and filtered into a 1000 ml separatory funnel fitted with a Teflon stopcock. To the funnel was then added 100 ml of H_2O and it was vigorously shaken for 5 minutes. The two phases were allowed to separate overnight at 4°. The lower phase was drawn off, transferred to a 500 ml round-bottom flask, and evaporated to dryness in a rotary evaporator. The total lipids were transferred to a tared vial with a minimum volume of $CHCl_3$. The solvent was removed by blowing nitrogen over the surface and the lipids were placed *in vacuo* for 24 hours. They were then weighed and prepared for chromatographic analysis or stored at −20° until chromatography.

Chromatography. The silicic acid was prepared as described (12) and contained one-third by weight of Celite[2] to increase the flow of solvent. Columns consisted of 20 g of silicic acid-Celite mixture, had a 25-mm diameter, and were approximately 100 mm in height. The column material was packed with a slurry of $CHCl_3$ and washed with 500 ml of $CHCl_3$, 500 ml of methanol, 500 ml of acetone, and finally with 500 ml of $CHCl_3$. The methanol packs the column to a uniform height and prevents contraction with time or when solvents are changed. Methanol, however, deactivates the column and destroys its resolving power; the subsequent acetone wash restores the activity. The columns are reusable for several chromatographic runs if the same washing procedure is used before each run. A pressure system is necessary to obtain a reasonable flow rate (13).

The lipid residue, dissolved in 10 ml of $CHCl_3$, was added to the column and the nonphospholipids removed by eluting with 1000 ml of $CHCl_3$ at a flow rate of 1 ml per minute. The efficacy of this procedure was determined with standard mixtures of cholesterol esters, triglycerides, cholesterol, and free fatty acids prepared in the ratios expected in normal serum and by checking the weight recoveries. In addition, the weight and phosphorus content of each sample was determined before it was chromatographed, and from these data the expected weight of the nonphosphorus-containing

[1] All solvents were reagent grade commercial solvents. The $CHCl_3$ contains 0.5% to 1% ethanol as a preservative. In addition, the purity of solvents was checked by recording infrared spectra of their nonvolatile residues. The solvents used in the gas-liquid chromatography operations were all redistilled, and solvent blanks were carried through the entire operation.

[2] Obtained from the Johns-Manville Company, 22 East 40th Street, New York 16, New York.

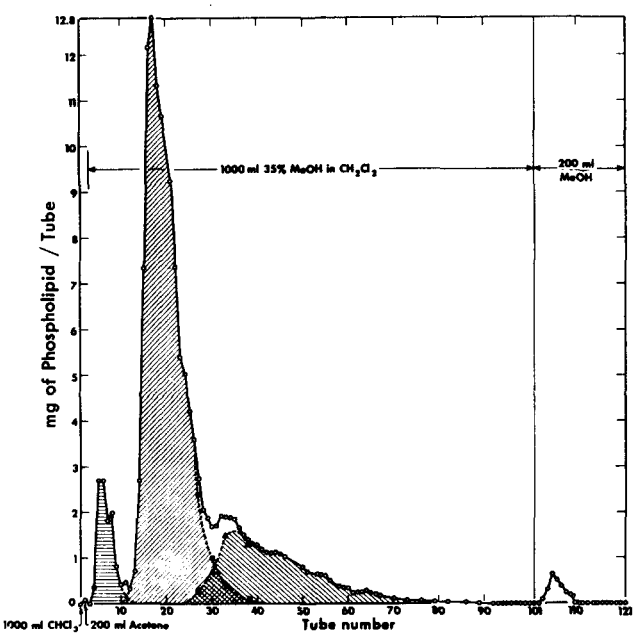

FIG. 1. Elution curve of serum phospholipids from silicic acid column. Weight added to column: 6.02 mg phosphorus; recovered: 5.80 mg phosphorus. The shaded area distinguishes the regions occupied by the various phospholipids as they were eluted from the column, determined by infrared spectrophotometry.

lipids in the sample was compared to that recovered in the $CHCl_3$ fraction.

When 1000 ml of $CHCl_3$ had been collected, as a single fraction, 200 ml of acetone was added to the column and a second fraction eluted. The $CHCl_3$ contains most of the nonphosphorus containing lipids of the extract: cholesterol, cholesterol esters, triglycerides, and free fatty acids. However, if there is any oxidation of these lipids, the oxidized material will be retained on the column during $CHCl_3$ elution. Acetone will remove such oxidation products as well as other materials, as yet unidentified, associated with phospholipids but not themselves phosphorus-containing.

The reservoir was then filled with 1000 ml of 35% methanol in methylene chloride and the elution switched from batch collection to an automatic fraction collector. An automatic valve, described elsewhere (14), cut 10-ml fractions. After 100 fractions were obtained, approximately 20 additional 10-ml fractions were obtained with 100% methanol. Total recoveries by phosphorus analysis varied from 95% to 102% of the phosphorus added to the column. Phosphorus determinations were performed by the procedure given earlier (15).

The collecting tubes were screw-cap vials (16 × 150 mm) with Teflon liners. All tubes were capped as rapidly as possible after filling and stored at 4° to await phosphorus determinations, infrared spectral analysis, and gas-liquid chromatography. If the amount of lipid in the tube was small, the entire sample was utilized or pooled with neighboring cuts.

Gas-Liquid Chromatography. Two transmethylation procedures were used. The method of Stoffel *et al.* (16) was an excellent quantitative procedure for use with phosphatidyl ethanolamine and lecithin samples. Unfortunately, this technique would usually yield only 5% to 10% of the expected methyl esters of sphingomyelin. Quantitative recoveries were obtained when 5% or 10% H_2SO_4 in anhydrous methanol was used to transmethylate sphingomyelin. The method of Stoffel *et al.* might be more satisfactory if the reaction were carried out in a sealed instead of an open vial (17). After transmethylation, half of the sample was catalytically hydrogenated as described by Farquhar *et al.* (18) using platinum dioxide[3] as the catalyst.

Sphingomyelin samples containing lecithin were subjected to partial basic hydrolysis as described by Hack (19). Subsequent extraction yielded sphingomyelin substantially free of lecithin or its fatty acids. The sphingomyelin was then transmethylated in methanolic H_2SO_4. In a few cases where a sample of lecithin was transmethylated in methanolic HCl, such as tube No. 27 (Fig. 1), the sphingomyelin present was not removed because this methylation procedure yields only methyl esters derived from the lecithin molecule.

Eighty micrograms of methyl ester was applied to the column operated as reported earlier (2). The gas-liquid chromatographic unit was designed and built in this laboratory (20) and uses an ionization detector of the type designed by Lovelock (21) with a Sr^{90} source of beta particles. Two recorders were operated simultaneously, the second with a sensitivity 10 times that of the first. After the methyl linoleate peak appeared, the first recorder was switched to a scale 25 times its initial sensitivity. The unit was calibrated as described (20) with purified methyl esters obtained from the Hormel Foundation.

The percentage of each individual fatty acid ester was calculated by an IBM 650 computer. The program was designed to perform all the necessary calculations from the absolute elution times of the individual components in minutes and the peak heights of the components in arbitrary units (in this case, inches) above the base line of the recorder chart. The method is published in greater detail elsewhere (22).

[3] Obtained from Engelhard Industries, Inc., 113 Astor Street, Newark 2, N. J.

RESULTS

Figure 1 shows a chromatographic run using 35% methanol in methylene chloride. When concentrations of methanol above 35% were used, no separate peak appeared after sphingomyelin. When lower concentrations were used, the sphingomyelin peak spread over a very broad region. Apparently 35% methanol in methylene chloride offered the best solvent system for the elution of serum phospholipids, although no concentration of methanol was capable of cleanly separating lecithin and sphingomyelin.

Changing the height of the column did not seem to improve the resolution appreciably because elution peaks spread out with the longer elution time. An improvement in resolution was obtained with a smaller lipid load, but the amounts of phosphatidyl ethanol-

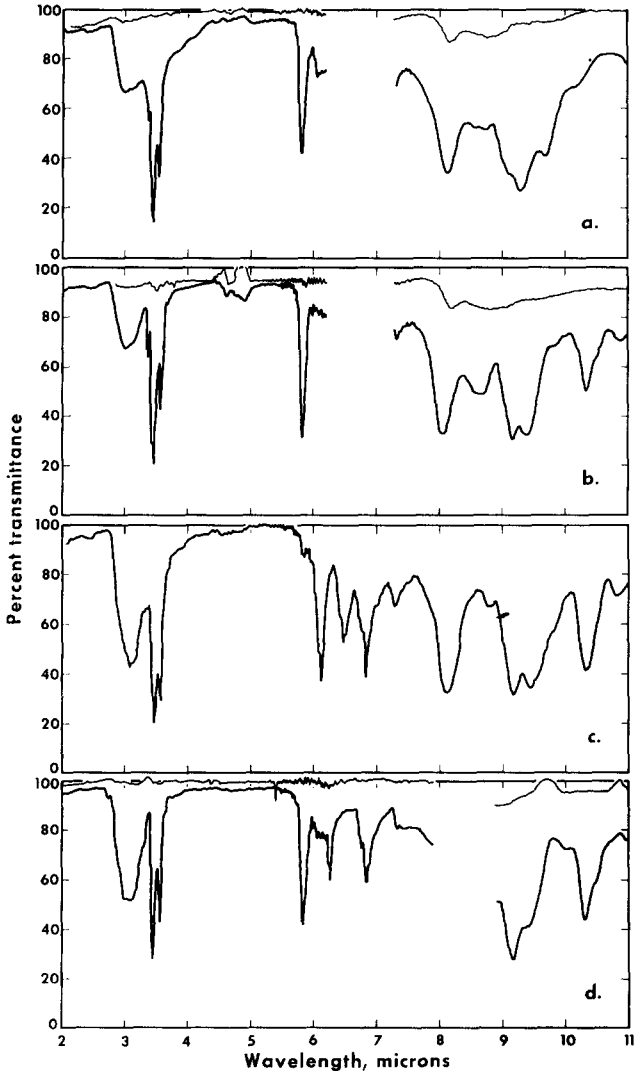

FIG. 2. Infrared spectra of samples from tubes in the various elution regions shown in Figure 1. Curve *a* (from tube 6): phosphatidyl ethanolamine slightly contaminated (probably with phosphoinositol). This spectrum was run in CS_2, 7.5 mg/ml. The region from 6.2 to 7.2 μ is obscured by the solvent. The upper curve is a CS_2 versus CS_2 background. Curve *b* (from tube 20): spectroscopically pure lecithin, in CS_2 solution, 6.9 mg/ml. Curve *c* (from pooled tubes 48 and 49): sphingomyelin with a trace contamination of ester-containing material as indicated by the 5.8-μ absorption. This film was run on NaCl plates. Curve *d* (from pooled tubes 105 to 108): probably lysolecithin. Run in $CHCl_3$ solution, 6.2 mg/ml. The region from 7.8 to 8.8 μ is obscured by the solvent.

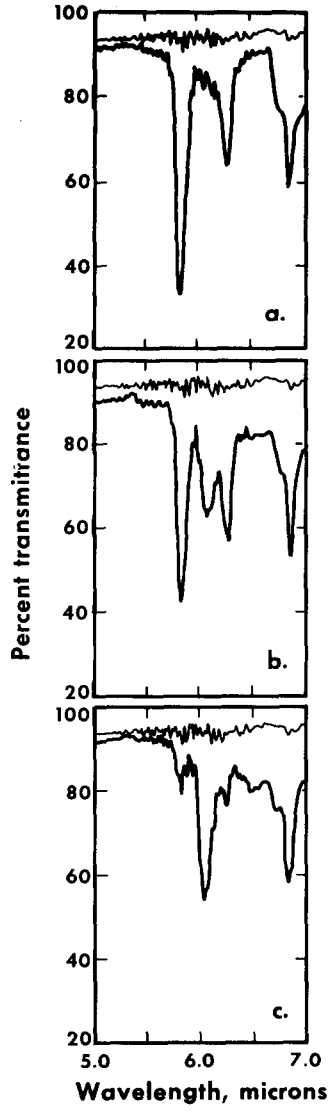

FIG. 3. Infrared spectra of samples showing the overlapping of lecithin and sphingomyelin in the elution curve shown in Figure 1. Curve *a*, a sample from tube 27; *b*, tube 30; *c*, tube 33. All samples were run in $CHCl_3$ solution at 7.5 mg/ml. The upper curve is $CHCl_3$ versus $CHCl_3$ background. The absorption band at 6.25 μ is caused by the solvent, chloroform, and should not be confused with the sphingomyelin absorption band 6.1 μ.

amine and lysolecithin were reduced below the minimum that could be handled conveniently.

Figure 1 also shows the distribution of the phospholipids in the elution curve determined by infrared spectroscopy (shaded areas). Figure 2 shows the infrared spectra of representative samples from the various elution peaks shown in Figure 1. A small contamination of phosphatidyl ethanolamine in the lecithin fraction (<2%) could not have been detected in the infrared spectrum of lecithin. Curve *a* is from the phosphatidyl ethanolamine peak, curve *b* from the phosphatidyl choline peak, curve *c* from the sphingomyelin region, and curve *d* from the final peak eluted with 100% methanol. Figure 3 shows three spectra from the lecithin-sphingomyelin overlap region (tubes 27, 30, 33). Only the region from 5 to 7 μ is given, as the curves are similar in the other regions of the infrared spectrum. The 5.8-μ absorption is characteristic of an ester bond and is absent in sphingomyelin. The 6.1-μ absorption is characteristic of an amide link and is absent in lecithin. Curve *a* calculates to 96.4% lecithin on this basis, while curve *c* is 77.8% sphingomyelin. Curve *b* is 44.3% lecithin and 55.7% sphingomyelin.

The material in curve *d* of Figure 2 is almost identical spectrally to lecithin except for certain quantitative differences in absorption intensities of the major bands and certain qualitative differences in the shape of the spectrum in the region from 8 to 9 μ that are consistent with the structural differences between lecithin and lysolecithin. The spectral appearance and the elution characteristics provide a strong indication that this material is lysolecithin.

In the earlier study (2), several elution peaks in the gas-liquid chromatograms could not be identified. In the present work, hydrogenation has been used to characterize these peaks. Figure 4A and 4B show the gas-liquid chromatograms of methyl esters from serum phosphatidyl ethanolamine before and after hydrogenation. The major peak at 144 minutes has disappeared, and a new peak at 48 minutes corresponding to methyl behenate (22:0) is present. The arachidonate peak was, of course, removed also and replaced by the corresponding methyl arachidate peak. In addition, the minor components have disappeared but no new peaks appeared, indicating that their hydrogenation products have the same elution times as the major

TABLE 1. COMPOSITION OF THE MAJOR FATTY ACID FRACTIONS, GROUPED BY CHAIN LENGTH, SHOWN IN FIGURE 4 (MASS PER CENT OF TOTAL METHYL ESTER SAMPLE)

Fatty Acid Fraction	Before Hydrogenation	After Hydrogenation
C_{16}	13.6	14.2
C_{18}	27.6	29.0
C_{20}	26.6	25.8
C_{22}	20.4	19.9
Totals	88.2	88.9

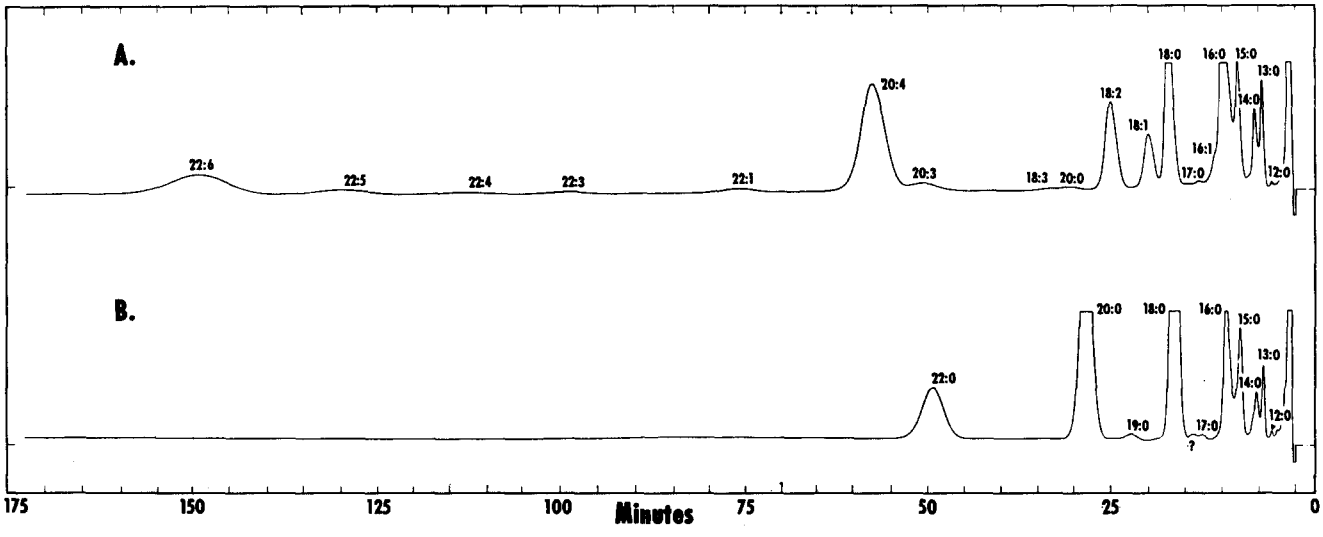

FIG. 4. A typical gas-liquid chromatogram obtained for the fatty acids from phosphatidyl ethanolamine of human serum lipoproteins with d < 1.20 g/ml. A: natural sample; B: hydrogenated sample. Fatty acids are designated by chain length and number of double bonds in the molecule. Full scale deflection 10^{-9} amp. Sample injected, 80 μg. Flow rate, 75 ml/minute. Column temperature, 195°. Stationary phase, polyester of diethylene glycol succinate. Samples were injected in a solution of hexane, which produced initial solvent peak.

TABLE 2. FATTY ACID COMPOSITION OF HUMAN SERUM PHOSPHOLIPIDS (MASS PER CENT OF TOTAL METHYL ESTER SAMPLE)

Tentative Identification by Chain Length and No. of Double Bonds	Phosphatidyl Ethanolamine*	Lecithin	Sphingomyelin	Lysolecithin
12:0	0.2		0.1	0.2
13:0	2.4	0.2	0.1	0.3
14:0	2.8	0.3	1.0	1.1
15:0	4.3	0.2	0.5	0.7
16:0	11.7	26.7	36.9	42.9
16:1	1.9	1.2	1.7	1.3
17:0	0.8	0.5	1.1	2.3
18:0	14.2	13.5	8.5	34.9
18:1	5.3	9.5	5.8	11.5
18:2	8.3	23.0	14.5	1.4
20:0	0.7	0.2	0.8	0.4
18:3	0.4	0.6	0.6	0.3
20:2	0.1	0.4	0.4	
20:3	1.3	3.7	0.2	
22:0			6.7	0.5
20:4	25.2	12.2	3.8	
23:0			5.4	0.8
22:1	3.5	0.7	1.6	
24:0			3.0	1.4
22:3	0.7	0.6		
24:1			3.1	
22:4	0.8	0.4		
22:5	2.7	1.4	1.7	
22:6	12.7	4.7	2.5	
Saturated	37.1	41.6	64.1	85.5
Unsaturated	62.9	58.4	35.9	14.5

* Contains less than 10% phosphatidylserine and some unidentified compounds.

components. Table 1 shows the percentages of the major fatty acid fractions, grouped by chain length, obtained from the chromatograms shown in Figure 4 before and after hydrogenation. As the arachidic and behenic ester peaks were the only ones appearing after methyl stearate in the hydrogenated samples, the minor as well as the major components present in the natural sample were assumed to be 20 and 22 carbon unsaturated fatty acid esters.

While this indicates that the previously unidentified components were largely 22 carbon polyunsaturated fatty acid esters, the degree of unsaturation could not be determined by hydrogenation. It is known, however, that the curve of the log of retention time versus number of double bonds per molecule will be a straight line for any homologous fatty acid methyl ester series differing only by the number of double bonds present in the molecule (2, 17, 23). Thus, when the chain length of an homologous series is known, the degree of unsaturation of each member is easily determined. Table 2 summarizes the data for the fatty acids tentatively identified in normal human phospholipids by these techniques.

Table 3 presents the fatty acid composition of nine fractions of the silicic acid chromatogram shown in Figure 1. In the previous study (2), large amounts of polyunsaturated long-chain fatty acid in phosphatidyl ethanolamine and lecithin were not observed. This was probably caused by oxidative deterioration of the sample during some phase of the experiment. Greater precautions were taken to prevent oxidation in the present study. The percentage of unsaturated acids observed in the phosphatidyl ethanolamine, although high, still represents only a small amount of the total fatty acids in the serum phospholipids. Lecithin is decidedly more saturated. Table 3 shows that unsaturated lecithin molecules tend to elute off silicic acid more rapidly than their saturated analogues. Fraction 2 in Table 3 contains 65% unsaturated material and is the initial lecithin eluted from the column. Fraction 6 in Table 3 contains almost 50% saturated material and is near the end of lecithin elution. No simple relationship between the amounts of unsaturated and saturated fatty acids present is apparent. Fraction 2 has 15.3% 22 carbon hexenoic acid and 22.8% arachidonic acid, while only 6.3% and 9.4%, respectively, of oleic and linoleic acid. Yet fraction 6 has 12.3% and 28.6% oleic and linoleic, respectively, and only 0.9% and 5.5%, respectively, of the former acids. The migration of lecithin is apparently dependent on the number of double bonds in the molecule as well as the fatty acid chain length.

Sphingomyelin shows only small percentages of unsaturated fatty acids beyond linoleic and less total unsaturation than either phosphatidyl ethanolamine or lecithin. Nevertheless, the same dependence of migration rate on fatty acid composition of sphingomyelin is observed.

The lysolecithin sample, fraction 9 in Table 3, is more highly saturated than any lecithin fraction, being over 78% palmitic and stearic acids, and 12% oleic - the main unsaturated acid.

DISCUSSION

The elution of the phosphatidyl ethanolamine is accomplished easily and quickly with this elution scheme although the resulting material is again a mixture, with phosphatidyl ethanolamine being the primary component. With lower methanol concentrations, better separation of this phosphatidyl ethanol-

TABLE 3. FATTY ACID COMPOSITION OF CHROMATOGRAPHIC FRACTIONS
(MASS PER CENT OF TOTAL METHYL ESTER SAMPLE)

Retention Time Relative to Methyl Stearate on DEGS*	Tentative Identification by Chain Length and No. of Double Bonds	Fraction No. 1 Tube 5†	Fraction No. 2 Tube 14†	Fraction No. 3 Tube 16†	Fraction No. 4 Tube 18†	Fraction No. 5 Tube 22†	Fraction No. 6 Tube 27†	Fraction No. 7 Tube 43†	Fraction No. 8 Tube 65	Fraction No. 9 Tube 105 to 108†
0.20	12:0	0.2						0.3	0.2	0.2
0.26	13:0	2.4	0.1	0.2	0.2	0.2	0.2	0.5	0.2	0.3
0.33	14:0	2.1	0.2	0.2	0.3	0.3	0.4	1.8	2.8	1.1
0.45	15:0	4.8	0.2	0.2	0.2	0.3	0.3	0.3	0.5	0.7
0.58	16:0	7.9	16.8	20.3	22.6	29.4	35.1	19.9	44.4	42.9
0.68	16:1	1.8	0.6	0.6	0.7	0.9	1.0	1.5	0.7	1.3
0.77	17:0	0.7	0.4	0.4	0.5	0.5	0.5		0.3	2.3
1.00	18:0	12.7	16.2	15.9	14.5	11.7	10.5	4.4	6.1	34.9
1.17	18:1	5.3	6.3	7.1	8.3	10.5	12.3	5.6	5.8	11.5
1.46	18:2	10.1	9.4	15.8	21.5	28.3	28.6	20.5	16.8	1.4
1.73	20:0	0.7	0.2	0.3	0.2	0.2	0.2	1.2	0.5	0.4
1.94	18:3	0.5	0.4	0.5	0.6	0.8	0.6	0.7	0.5	0.3
2.48	20:2			0.4	0.5	0.6	0.4			
2.93	20:3	2.1	3.6	4.8	4.5	3.5	2.3	0.3	0.4	
3.01	22:0							8.1	10.1	0.5
3.33	20:4	27.8	22.8	19.8	15.7	8.1	5.5	6.1	2.3	
3.96	23:0							7.1	1.1	0.8
4.40	22:1	1.7	1.7	1.3	1.1	0.5	0.4	2.2	1.2	
5.30	24:0							5.8	0.9	1.4
5.73	22:3	1.1	1.3	0.9	0.9	0.4	0.3			
5.90	24:1							5.5		
6.59	22:4	1.1	1.3	0.8	1.0	0.3	0.2			
7.55	22:5	3.5	3.3	2.1	1.6	1.1	0.4	1.4	1.8	
8.67	22:6	14.3	15.3	8.4	5.2	2.3	0.9	4.2	3.6	

* Diethylene glycol succinate.
† Tube numbers refer to Figure 1.

amine fraction is obtained, but with a corresponding lag in the elution of lecithin and other slowly eluting compounds.

While it was not possible to separate lecithin from sphingomyelin in this work, several authors (24, 25, 26) have reported that this can be accomplished with sharp changes in the methanol concentration. But when elution schemes are used that involve discontinuous changes in the concentration of the eluting solvent, false peaks may occur that can easily be interpreted as being indicative of complete separation although they actually contain mixtures of compounds obtained in the earlier fraction.

There is evidence for the presence of lysolecithin; in three extended chromatographic runs the range of lysolecithin concentrations found was 2.1% to 4.3%, with an average of 3.0%. This agrees with the average value of 4.7% found by Phillips in his original report (27) for pooled serum, although he later reported higher values in serum lipoprotein fractions with d < 1.21 g per ml (28) and also an average value of 7.1% with a spread of 5.8% to 8.3% in normal subjects' total serum phospholipids (3). Marinetti *et al.* (4) reported much higher values of lysolecithin in 17 normal subjects and coronary cases, representing a spread of 7.9% to 13.3%. They included phosphoinositol in their lysolecithin values, however, and indicated that the former contributed the major portion. These authors also stated that an aqueous wash during extraction resulted in loss of lysolecithin. Gjone *et al.* (5) also reported higher values of lysolecithin. In eight normals they found an average of 8.9% and a spread of 6.6% to 11.5%. An aqueous wash was used in their extraction procedure. It should be pointed out, however, that these authors were generally working with a total lipid extract of serum that may well contain a higher percentage of lysolecithin than the lipids of lipoproteins with d < 1.20 g per ml. Indeed, Phillips' data (11) indicate that about half of the serum lysolecithin is contained in the high-density residue with d > 1.20 g per ml.

In general, the results of these chromatographic runs have agreed with those of previous studies. The average values in three runs analyzed in detail were: 5.6% noncholine-containing phospholipids (phosphatidyl ethanolamine), 71.2% lecithin, 20.2% sphingomyelin, and 3.0% lysolecithin, given as mole per cent determined by phosphorus analyses. The low value for noncholine-containing phospholipids agrees with most other recent reports (2, 3, 4).

Although there have been several recent reports (29, 30, 31) on the fatty acid composition of the total phospholipid fraction of human serum, only a few studies have been done on the fatty acid composition of the individual phospholipids. Recently, Hanahan *et al.* (32) presented data on the fatty acid composition of separated serum phospholipids in the case of lecithin and sphingomyelin. The authors only reported on six major fatty acids for lecithin and 11 for sphingomyelin. In general, their results are in agreement with the data obtained in this study. They found, as in the present study, that sphingomyelin contained less unsaturated fatty acids than lecithin, although their value for sphingomyelin unsaturation, 9%, differs considerably from the 35.9% reported here. The values for the individual fatty acids agree well for lecithin and somewhat less well for sphingomyelin.

Hanahan originally proposed (33) the concept that unsaturated fatty acid residues occupy the α' position preferentially while saturated fatty acid residues are always found in the β position of naturally occurring lecithins. Recently, this view was contradicted by Marinetti *et al.* (34, 35, 36), who maintained that saturated and unsaturated fatty acids were found in both positions. Hanahan *et al.*, however, have published a new study (37) on this subject in which they maintain that in egg lecithin, saturated fatty acid moieties are found in the α' position and unsaturated acids in the β position. The data presented here indicate that human serum lecithin cannot have all its unsaturated fatty acid residues attached to a single position in the molecule, as many of the fractions contained more than 50% unsaturated fatty acid residues.

In recent studies, Rowe (38) also analyzed the fatty acid composition of serum phospholipids by their elution rate off a silicic acid column. A multiple solvent elution scheme was used, however, causing considerably more overlapping of constituents in the various fractions. He did not report any fatty acid with a chain length longer than 20 carbons or any odd-chain acids. Nevertheless, the same general elution characteristics were observed. The more unsaturated material was eluted earlier, and the degree of unsaturation was highest in phosphatidyl ethanolamine and lowest in lysolecithin. In general, the unsaturation was lower than in any of the fractions analyzed in this study.

Generally, the same fatty acids were found in the phosphatidyl ethanolamine and lecithin fractions, but the relative amounts of the individual fatty acids varied greatly between the two compounds. For example, the fatty acids with chain lengths less than 16 carbons contributed approximately 10% of the total fatty acids in the phosphatidyl ethanolamine fraction and only 0.7% in the lecithin fraction. As the phosphatidyl ethanolamine fraction, but not the lecithin fraction, contains phosphatidyl serine and other impurities, these relatively uncommon fatty acids could be contributed by these compounds rather than phosphatidyl ethanolamine. Significant differences were observed also in the palmitic, linoleic, arachidonic, and docosahexenoic fatty acid percentages in these two compounds.

The author wishes to thank Drs. Frank Lindgren and Norman K. Freeman for their advice and criticism of this work, and Dr. John W. Gofman for his continued interest and support.

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