Title

STUDIES ON HUMAN SERUM PHOSPHOLIPIDS AND PHOSPHOLIPID FATTY ACID COMPOSITION BY EXTENDED SILICIC ACID CHROMATO-GRAPHY

Author

Nelson, Gary J.

Publication Date

1961-02-01

UCRL-9571

UNIVERSITY OF CALIFORNIA

Ernest O. Lawrence

Radiation Laboratory

BERKELEY, CALIFORNIA

DISCLAIMER

UCRL-9571

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

STUDIES ON HUMAN SERUM PHOSPHOLIPIDS AND PHOSPHOLIPID FATTY ACID COMPOSITION BY EXTENDED SILICIC ACID CHROMATOGRAPHY

Gary J. Nelson

February 1961

By

Gary J. Nelson**

From the
Donner Laboratory
of
Biophysics and Medical Physics
University of California
Berkeley, California

Chromatographic Analysis of Serum Phospholipids

Introduction

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

An extended chromatographic analysis 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 were taken during the chromatographic run, a large initial amount of phospholipid was necessary to insure an adequate amount of phospholipid in the resulting fractions. This also provided better samples for gas chromatographic analysis of the fatty acid moiety of the individual phospholipids. Furthermore, 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) that the fatty acid moiety of complex lipids influences the migration rate of the lipids on silicic acid columns. Klein (7) has published a method for the separation of cholesterol esters based on this effect. Recently, Baer (8) has reported a method of separating various glycerophosphatides by their fatty acid components provided that 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 fatty acids occur in natural samples, including both saturated and unsaturated acids. Sphingomyelin and lysolecithin, each having only one fatty acid residue per molecule, would lend themselves more easily to this 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. Versene was added to the separated serum (5 mg/100ml. of serum) as an antioxidant. After the cells were removed, the serum was kept at $4^{\circ}C$. and under nitrogen whenever possible during all subsequent operations.

Ultracentrifugation

As only the total serum lipids 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/ml., the density used in the ultracentrifugal separation.

To 100 ml. of serum was added 26.1 g. of solid NaBr (dessicated, analytical reagent from Baker and Adams). The density of the resultant solution, (usually having a volume of 108 ml.) was checked by refractometry, and NaBr or water was added to adjust the solution to the required density, 1.210 g/ml. The solution was then transferred to 18 six ml. Spinco Lustron preparatory centrifuge tubes which were placed in a series 40.3 Spinco preparatory ultracentrifuge rotor and centrifuged for 48 hours at 40,000 rpm in a Spinco Model L preparative ultracentrifuge.

After centrifugation the top 1 ml. in the preparatory tube had a density of 1.20 g/ml. and contained all the major lipoproteins of the serum. This volume was removed by a special pipetting technique described elsewhere (9). The second ml., although clear of large molecules, was also removed and stored separately for later use as a blank in calculating the total concentration of lipoprotein obtained in the first ml. of the tube. The refractometric techniques used for this purpose were

developed by Dr. F. T. Lindgren. The lower 4 ml. were discarded. In this procedure there is a loss of 5-10% of the total lipoproteins with a density less than 1.20 g/ml. by adherence to the wall of preparative tube. It is assumed that this loss is uniformly distributed throughout the lipoprotein spectrum. Also the ultracentrifugal residue contains approximately 5% of the total serum lipid (10). Hence, the recovery by this procedure is approximately 85% of that obtained from extraction of unfractionated serum. Analytical ultracentrifuge runs were also performed on a portion of the top ml. for the purpose of determining the distribution of the lipoproteins in the various lipoprotein classes.

Extraction

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

Chromatography

The silicic acid was prepared as described (11) and contained 1/3 by weight Celite to increase the flow of solvent through the column. Columns consisted of 20 gm. of silicic acid - Celite mixture and had a 25 mm. diameter and were approximately

100 mm. in height. The column material was packed into the glass chromatographic column in a slurry of $CHCl_3$. The column was then washed with 500 ml. of $CHCl_3$ and then 500 ml. of methanol and finally with 500 ml. of acetone. The acetone was washed out with another 500 ml. of $CHCl_3$. The methanol wash tends to pack the column to a uniform height. If this is not done the column will undergo a noticeable contraction with time and especially when solvents are changed. However, methanol tends to deactivate the column and destroy its resolving power. The subsequent acetone wash restores the activity of the column to its initial conditions. Columns packed in this manner are reusable for several chromatographic runs if the same washing procedure is used before each run.

The lipid extract was dissolved in 10 ml. of $CHCl_3$ and added to the column. The non-phospholipids in the lipid extract are then removed by eluting with 1000 ml. of $CHCl_3$ at a flow rate of 1 ml/min. Generally, a column prepared in the manner described has a very slow flow rate under gravity alone, and a pressure system such as that described by Hirsch and Ahrens (12) is necessary to obtain a reasonable flow rate. After the $CHCl_3$ has eluted, 200 ml. of acetone were added to the column and a second fraction eluted. The $CHCl_3$ contains most of the non-phosphorus containing lipids of the lipid extract: cholesterol, cholesterol esters, triglycerides, and free fatty acids. However if there has been any oxidation of the lipids in this group, the oxidized material will be retained on the column during $CHCl_3$ elution. Acetone will remove such oxidation products as well as other, as yet unidentified, materials associated with phospholipids but not themselves phosphorus containing such as cerebrosides or sulfolipids.

The reservoir was then filled with 1000 ml. of 35% methanol in methylene chloride and the elution collector switched from large batch collection to an automatic fraction collector. An automatic valve, described elsewhere (13), cuts fractions of the desired volume, in this case 10 ml. After 100 fractions were obtained from this elution, approximately 20 additional 10 ml. fractions were obtained by elution with pure methanol. Figure 2 gives the elution curve obtained in such a chromatographic run. Phosphorus determinations were performed by the procedure given earlier (14).

Total recoveries as determined by phosphorus analysis varied from 95 to 102 % of the phosphorus added to the column.

The collecting tubes were screw cap vials (16 x 150 mm.) the caps of which were Teflon-lined. All tubes were capped as rapidly as possible after filling and stored at 4°C until further use. Samples for phosphorus determinations were usually taken as aliquots from each tube. Other aliquots were taken for infrared spectral analysis and gas chromatography. In cases where the amount of lipid material in the tube was small the entire sample was utilized, or it was pooled with its neighboring cuts.

Gas Chromatography

Aliquots chosen to yield approximately 2 mg. of phospholipid were subjected to transmethylation to obtain methyl esters. Two transmethylation procedures were used. The method of Stoffel, Chu, and Ahrens (15) was used to transmethylate the phosphatidyl ethanolamine and lecithin samples. It was found to be an excellent quantitative procedure with these compounds. Unfortunately it would not transmethylate sphingomyelin quantitatively and, in fact, yielded only 5 to 10% of expected methyl esters, when an identical procedure to that in the reference (15) was followed. Good recoveries were obtained when 5 or 10% H_2SO_4 in anhydrous methanol was used for transmethylation of sphingomyelin. The method of Stoffel *et al.* might be more satisfactory if the reaction is carried on in a sealed vial (17), rather than an open one as described. After transmethylation, half of the sample was subjected to a catalytic microhydrogenation as described by Farquhar *et al.* (16) using platinum dioxide[1] as the catalyst.

In each run 80 µg. of methyl ester were applied to the column as reported earlier, and all operating conditions were the same (2). The gas chromatographic unit was designed and built in this laboratory (18) and uses an ionization detector of the type designed by Lovelock (19) with a Sr^{90} source of beta particles.

Fatty Acid Calculation

The fatty acid composition of the methyl ester samples applied to the gas column was found by measuring the resultant areas of the elution peaks recorded on the readout recorder of the gas chromatographic unit. Two recorders were operated simultaneously, the second with a sensitivity 10 times the first. After the methyl linoleate peak had appeared, the first recorder was switched to a sensitivity 25 times its initial sensitivity. The unit was calibrated as described (2) with purified methyl esters obtained from the Hormel Foundation.

The method of calculating the percentage of each individual fatty acid ester involved the use of an IBM 704 computer. The program was designed to perform all the necessary calculations on the basis of 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 (20).

Results

In Figures 1 and 2 are shown column chromatographic runs performed under identical conditions except that in the first chromatogram, after 500 ml. of 35% methanol had eluted, 70% methanol in methylene chloride was used, while in the second instance 1000 ml. of 35% methanol was used, followed by pure methanol. There is no improvement in resolution in the first case and little reduction in the volume necessary to remove all the phospholipids from the column. Furthermore, when concentrations of methanol above 35% were used, no separated peak could be detected appearing after sphingomyelin. When concentrations of methanol below 35% were used, the sphingomyelin peak tended to be spread over a very broad region. Thus it appeared that 35% methanol in methylene chloride offerred the best solvent system for the elution of serum phospholipides.

On the other hand, no concentration of methanol used was capable of separating lecithin from sphingomyelin cleanly. In Figure 2 is also shown the distribution of the phospholipids in the elution curve as determined by infrared spectroscopy (shaded areas). In Figure 3 are shown the infrared spectra of representative samples from

the various elution peaks shown in Figure 2. 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. In Figure 4, three spectra from the lecithin-sphingomyelin overlap region are shown (tubes 27,30,33). Only the region from 5 to 7 μ is given in these curves as lecithin and sphingomyelin have similar absorptions 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. The varying composition of these fractions is obvious. Curve a calculates to more than 95% lecithin on this basis while Curve c is more than 75% sphingomyelin. Curve b is approximately an equal mixture.

Curve d in Figure 3 is probably lysolecithin on the basis of its infrared spectrum. It is almost identical spectrally to lecithin except for certain quantitative differences in absorption intensities at the major absorption bands. This, coupled with its elution characteristics, indicates that it is lysolecithin.

In the earlier study (2) a preliminary report on the fatty acid composition of the individual phospholipids of serum was given. Several elution peaks in gas chromatograms were observed that could not be identified at that time. Certain inferences could be made from the position of the unknowns on the gas chromatogram but identification was not possible in this manner alone. For this reason microhydrogenation of the samples has been carried out. Figure 5A is the gas chromatomatogram of the fatty acid methyl esters from normal human serum phosphatidyl ethanolamine. In Figure 5B the gas chromatogram of the same sample is shown after microhydrogenation.

The slow moving component with an elution time of 144 minutes has disappeared and been replaced by a new peak at 48 minutes. The arachidonate peak was, of course, removed also and replaced by the corresponding arachidate methyl ester peak. In addition, the minor components have disappeared but no corresponding new peaks have appeared in the chromatogram of hydrogenated esters indicating that the hydrogenation products have elution times identical to those of the major components. The elution

times of the new peaks present in the hydrogenated sample correspond exactly to those for standard samples of arachidic (C-20) and behenic (C-22) acid methyl esters on this column. Table I shows the percentages of the major groups of the chromatographs shown in Figure 5. Agreement is good for the conversion of twenty and twenty-two carbon unsaturated fatty acid esters to arachidic and behenic acid methyl esters. As these were the only peaks appearing after stearic acid ester in the hydrogenated sample, the minor as well as the major components present in the natural sample were assumed to be twenty and twenty-two carbon unsaturated fatty acid esters. In Figure 6 typical gas chromatographic runs on methyl esters from serum lecithin samples are shown which indicate the decreasing amounts of long chain unsaturated material present in these samples between the leading and trailing edge of the elution peak on the silicic acid column and a reduction of the short chain esters compared to phosphatidylethanolamine.

While the above evidence indicates that the previously unidentified components were largely twenty-two carbon polyunsaturated fatty acid esters, the degree of unsaturation could not be determined by hydrogenation. However, it was known from data obtained on standard compounds 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, 16, 21). Figure 7 is a semi-log plot of retention time versus number of double bonds present in the normal chain twenty and twenty-two carbon fatty acid methyl esters as obtained from a chromatogram of serum lecithin. The straight line obtained indicates the presence of hexenoic, pentenoic, tetraenoic, trienoic, and monoenoic twenty-two carbon polyunsaturated fatty acids. Dienoic and trienoic twenty carbon fatty acids are also present as well as arachidonic acid. Table II summarizes the data as to which fatty acids have been tentatively identified in normal human phospholipids by these techniques.

Table III presents the fatty acid composition of 9 fractions taken from the

silicic acid chromatography shown in Figure 2. In the previous study (2) large amounts of polyunsaturated long chain fatty acid in phosphatidyl ethanolamine and lecithin were not observed. This was probably due to oxidative deterioration of the sample during some phase of the experiment. Much greater precautions to prevent this possibility were maintained in this present study. While the degree of unsaturated observed in the phosphatidyl ethanolamine is high, this still represents only a small amount of the total fatty acids present in the serum phospholipids. Lecithin, on the other hand, is decidedly more saturated. Table III shows that the more unsaturated lecithin molecules tend to elute off silicic acid more rapidly than their corresponding saturated analogues. Fraction 2 in Table III, representing tube No. 14 contains 65% unsaturated material and is the initial lecithin eluted from the column. Fraction 6 in Table III, representing tube No. 27 contains almost 50% saturated material and is near the end of lecithin elution. There does not appear to be a simple relationship between the amounts of unsaturated and saturated fatty acids present. In fraction 2 there is 15.3% twenty carbon hexenoic acid and 27.8% arachidonic acid while only 6.3 and 9.4 % respectively of oleic and linoleic acid. Yet in fraction 6 there is 17.3 and 28.1% oleic and linoleic respectively and only 0.9 and 5.5% respectively of the former acids. The migration rate on the column is apparently dependent on the number of double bonds present in the fatty acid moieties of the molecule rather than chain length.

Conversely, sphingomyelin shows little unsaturation beyond linoleic and less total unsaturation than either phosphatidyl ethanolamine or lecithin. Nevertheless, the same general elution property, is present in the sphingomyelin fraction. The leading edge has more unsaturated material than the trailing edge.

The lysolecithin sample, fraction 9 in Table III, is more highly saturated than any lecithin fraction, with over 78% of its fatty acid composition being made up of palmitic and stearic acid.

Discussion

The evidence obtained in extended silicic acid chromatography of the total serum phospholipids makes it appear unlikely that any single concentration of methanol will separate lecithin from sphingomyelin. Several authors (22-24) have nevertheless maintained this can be accomplished. In this latter instance, one must not rule out the possibility that what the author interpretated as a pure sample was rather a mixture of the two or more compounds. Whenever elution schemes are used that involve multiple changes in the concentration of eluting solvent, false peaks occur which can easily be interpreted as complete separation when they are actually mixtures of the same materials obtained in the previous fraction. Gradient elution chromatography may offer a method of obtaining true resolution of lecithin and sphingomyelin (25). A system of concave gradient elution which offers theoretical advantages over convex gradient elution (26) is currently being investigated in this laboratory.

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. From the infrared spectrum it appears as if phosphoinositol is eluted in this fraction, and in certain subfractions may account for 20 to 40% of the fraction. As the concentration of methanol is lowered, better separation of this phosphatidylethanolamine fraction is obtained, but with a corresponding lag in the elution of lecithin and other slowly eluting compounds. Again this situation could be greatly facilitated by the use of concave gradient elution.

There is evidence for the presence of lysolecithin. In three extended chromatographic runs the range of lysolecithin found was 2.1 to 4.3 percent with an average of 3.0%. This value agrees with the average of 4.7% found by Phillips in his original report (27) for pooled serum, although he later reported rather higher values in serum lipoproteins and the serum proteins with $\rho > 1.21$ g/ml. (10) 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 subjects consisting of normals and coronary cases, representing a spread of 7.9 to 13.3%. However, they included phosphoinosital in their lysolecithin value with indications that the former contributed the major portion. These authors also stated that an aqueous wash during extraction resulted in loss of lysolecithin. Gjone, Berry and Turner (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.

There remains the possibility that spontaneous degradation may be the cause of some of the confusion in this matter. To test this hypothesis, a sample of lecithin in tube No. 20 in Figure 2 was exposed to oxygen for 24 hours and then rechromatographed. Approximately, 15% of sample was obtained in the 100% methanol elution and, when the fatty acids of this portion of the sample were analyzed, the composition resembled that originally obtained in fraction 9 given in Table III, primarily saturated fatty acids. This evidence suggests the possibility that the lysolecithin reported in serum is primarily an artifact.

Generally the results of this extended chromatographic runs have given results in agreement with the previous studies. The average values in three runs analyzed in detail were: 5.6% non-choline containing phospholipids (phosphatidyl ethanolamine), 71.2% lecithin, 20.2% sphingomyelin, and 3.0% lysolecithin. The low value for non-choline containing phospholipids is in agreement with most other recent reports (2,3,4).

While there had been several recent reports (28-30) on the fatty acid composition of the total phospholipid fraction of human serum, no detailed study on the fatty acid composition of the individual phospholipids has been previously presented. As there are significant differences in fatty acid composition between the individual phospholipid, total serum phospholipid analysis should be evaluated carefully in this respect. Lecithin, which is the major phospholipid of human serum, contributes

85% of the fatty acid to the total fatty acid composition of serum. Generally, studies (28-30) on the total serum phospholipid fatty acid composition reflect the lecithin fatty acid composition and obscure the contributions of phosphatidyl ethanolamine and sphingomyelin. For the latter, this is particularly true when a transmethylation procedure is used which fails to yield the fatty acid associated with sphingomyelin.

While the fatty acid composition of lysolecithin was essentially palmitic and stearic with some oleic and traces of other saturated and unsaturated fatty acid, some of the long chain saturated fatty acids were present in small amounts, although less than the amounts found in sphingomyelin. If lysolecithin is derived from lecithin, this would indicate that these fatty acids are probably present in lecithin are perhaps phosphatidyl ethanolamine but in extremely small quantities.

Hanahan originally proposed (31) the concept that the unsaturated fatty acid residues occupy the α' position preferentially while the saturated fatty acid residues are always found in the β position in naturally occurring lecithins. Recently, this view was contradicted by Marinetti et al (32-34) who maintained that saturated and unsaturated fatty acids were found on both positions in natural lecithins. Hanahan et al., however, have published a new study (35) on this subject in which they maintained that saturated fatty acid moieties are found on the α' position and unsaturated on the β position in egg lecithin. It would seem from the data presented here 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 analyzed were made up by well over 50% unsaturated fatty acid residues. It is not possible from this information to say whether all the saturated fatty acids occupy the same position although the composition of the lysolecithin might be considered to support this view.

Rowe in recent studies (36) also analyzed the fatty acid composition of serum phospholipids by their elution rate off a silicic acid column. However, a multiple solvent elution scheme was used with the result that there was considerably more overlapping of constituents in the various fractions analyzed. He also did not report

any fatty acid with chain length longer than twenty carbons and no odd chain acids. Nevertheless, the same general elution characteristics were observed. The more unsaturated material was eluted more rapidly, and the degree of unsaturation was found to be highest in phosphatidyl ethanolamine and lowest in lysolecithin. In general, however, the degree of unsaturation was lower than that reported in any of the fractions analyzed in this study.

The phosphatidyl ethanolamine and lecithin fatty acid composition were similar quantitatively and differed only qualitative, which could be indicative of similar metabolic processes in the formation of these compounds. Indeed, some recent studies (37,38) have indicated that phosphatidyl ethanolamine is transformed directly to lecithin by methylation of the ethanolamine to choline. Perhaps the most noticeable difference between phosphatidyl ethanolamine and lecithin fatty acid composition is the significant amount of fatty acids with chain lengths of thirteen, fourteen, and fifteen carbons. In phosphatidyl ethanolamine they contribute approximately 10% of the total fatty acids, yet in lecithin they are only 0.7% of the total. As the phosphatidylethanolamine, but not the lecithin, fraction contains phosphatidyl serine and perhaps phosphoinositol, these relatively uncommon fatty acids could be contributed by these compounds rather than the major component, phosphatidylethanolamine.

In all column chromatographic studies and, perhaps, paper chromatography as well, there is a dependence of the elution characteristics on the fatty acid composition of the molecule. Natural mixtures contain a wide variety of different isomorphs and it is unlikely that clean and distinct separation can easily be obtained in many cases. Unsaturated compounds of one class may migrate with saturated molecules in another. It may be wise to consider other criterion of purity than simply the chromatographic homogeneity of the sample when using chromatographic isolation procedures in dealing with the individual phospholipides.

The identification of the unsaturated fatty acids in this work is not intended to be absolute. A definite identification requires larger amounts of material than could be obtained. Even such elegant micro procedures as those outlined by Stoffel and Ahrens (39) require many times the amount of available sample. There is usually not enough of any single fatty acid in human serum phospholipide, except for the most common components, to characterize it using the standard oxidative and U-V isomerization techniques unless large pooled volumes of serum are utilized. On the other hand, tentative identification and reporting of fatty acid components as characterized by gas chromatographic analyses is felt to be significant and worthy of report until more definitive methods of microanalysis are developed.

Summary

Normal human serum phospholipids were separated by silicic acid column chromatography. A large number of fractions were obtained with which it was possible to study the effect of fatty acid composition on the elution rate of the column. Phospholipids containing unsaturated fatty acid molecules 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. Small amounts of lysolecithin were found, but evidence is presented that suggests lysolecithin is an artifact of lecithin.

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. Twenty and twenty-two carbon unsaturated fatty acids were present in all phospholipids. Long chain saturated, as well as odd chain saturated fatty acids, were present, and were particularly prominent in the sphingomyelin fraction.

Acknowledgement

The authors 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.

Footnotes

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

** Postdoctoral Fellow of the National Heart Institute, United States Public Health Service.

1 Obtained from Engelhard Industries, Inc., 113 Astor Street, Newark 2, New Jersey

References

1. Nelson, G.J., and Freeman, N.K., J. Biol. Chem., 234, 1375 (1959).

2. Nelson, G.J., and Freeman, N.K. J. Biol. Chem., 235, 578 (1960).

3. Phillips, G.B., Biochim. et Biophys. Acta, 29, 594 (1958).

4. Marinetti, G.V., Albrect, M., Ford, T., and Stotz, E., Biochim. et. Biophys. Acta. 36, 4 (1959).

5. Gjone, E., Berry, J.F., and Turner, D.A., J. Lipid Research, 1, 66 (1959).

6. Freeman, N.K., Annals N.Y. Acad. Sci., 69, 131 (1957).

7. Klein, P.D., and Janssen, E.T., J. Biol. Chem., 234, 1417 (1959).

8. Baer, E., Buchnea, D., and Grof, T., Canad. J. Biochem. Physiol., 38, 853 (1960).

9. deLalla, O.F., and Gofman, J.W., in D. Glick (Editor), Methods of Biochemical Analysis, Vol. I. Interscience Publishers, Inc., New York, 1954, p. 459.

10. Phillips, G.B., Proc. Soc. Exptl. Biol. Med., 100, 19 (1959).

11. Freeman, N.K., Lindgren, F.T., Ng, Y.C., and Nichols, A.V., J. Biol. Chem., 227, 449 (1957).

12. Hirsch, J., and Ahrens, E.H., Jr., J. Biol. Chem., 233, 311 (1958).

13. Nelson, G.J., Anal. Chem., 32 (1960).

14. Griswold, B.L., Humbler, F.L., and McIntyre, A.R., Anal. Chem., 23, 192 (1951).

15. Stoffel, W., Chu, F., and Ahrens, E.H., Jr., Anal. Chem., 31, 307 (1959).

16. Farquhar, J.W., Insull, W., Jr., Stoffel, W., and Ahrens, E.H., Jr., Nutrition Reviews, 17, (supplement) (1959).

17. Rowe, C.E., Biochem. J., 73, 438 (1959).

18. Upham, F.T., Lindgren, F.T., and Nichols, A.V. Lawrence Radiation Laboratory Report, No. 9039, (1960).

19. Lovelock, J., J. Chromatog., 1, 35 (1958).

20. Tandy, R., Lindgren, F.T., Martin, W., Wills, R., Lawrence Radiation Laboratory Report, No. 9472, (1960).

21. Lipsky, S.R., Landowe, R.A., and Godet, M.R., Biochim. et. Biophys. Acta, 31, 336 (1959).

22. Lovern, J.A., Olley, J., Hartree, E.F., and Mann, T., Biochem. J., 67, 630 (1957).

23. Hanahan, D.J., Dittmar, J.C., and Warashina, E., J. Biol. Chem., 228, 685 (1957).

24. Gray, G.M., and MacFarlane, M.G., Biochem. J., 70, 409 (1958).

25. Wren, J.J., Nature, 811, 816 (1959).

26. Lakshmanan, J.K., and Lieberman, S., Arch. Biochem. Biophys., 53, 258 (1954).

27. Phillips, G.B., Proc. Natl. Acad. Sci., U.S., 43, 566 (1957).

28. Ahrens, E.H., Jr., Insull, W., Jr., Hirsch, J., Stoffel, W., Peterson, M.L., and Thomasson, H.J., Lancet, 1, 115 (1959).

29. Hallgren, B., Stenhagen, S., Svanborg, A., and Svennerholm, L., J. Clin. Invest., 39, 1424 (1960).

30. Nichols, A.V., Wills, R.D., and Lindgren, F.T., J. Clin. Nutrition, In press.

31. Hanahan, D.J., J. Biol. Chem., 211, 313 (1954).

32. Marinetti, G.V., Erbland, J., and Stotz, E., Biochim. et Biophys. Acta, 33, 403 (1959).

33. Marinetti, G.V., Erbland, J., Temple, K., and Stotz, E., Biochim. et Biophys. Acta, 38, 524 (1960).

34. Marinetti, G.V., Erbland, J., and Stotz, E., Biochim. et Biophys. Acta, 38, 534 (1960).

35. Hanahan, D.J., Brockerhoff, H., and Barron, E.J., J. Biol. Chem., 235, 1917 (1960).

36. Rowe, C.E., Biochem. J., 76, 471 (1960).

37. Bremen, J., Figard, P.H., and Greenberg, D.M., Biochim. et Biophys. Acta, 43, 477, (1960).

38. Artom, C., and Hofland, H.B., Biochem. Biophys. Research Communications, 3, 244 (1960).

39. Stoffel, W., and Ahrens, E.H., Jr., J. Lipid Research, 1, 139 (1960).

Figure Captions

Fig. 1. Elution curve of serum phospholipids from a silicic acid column. Weight added to column 151.3 mg., recovered 149.0 mg. on the basis of phosphorus analysis.

Fig. 2. Elution curve of serum phospholipids from silicic acid column. Weight added to column 175.4 mg., recovered 170.1 mg. on the basis of phosphorus analysis. The shaded area distinguish the region occupied by the various phospholipid as they eluted from the column as determined by infrared spectrophotometry.

Fig. 3. Infrared spectra from the various elution region shown in Figure 2. Curve a is a sample from tube 6 and is phosphatidylethanolamine 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 is a sample from tube 20 and is spectroscopically pure lecithin, in CS_2 solution, 6.9 mg/ml: Curve c is a sample from pooled tubes 48-49, and is sphingomyelin with a trace contamination of ester containing material as indicated by the 5.8 μ absorption. A film run on NaCl plates: Curve d is probably lysolecithin and is taken from tubes 105 to 108, pooled, and run in $CHCl_3$ solution 6.2 mg/ml. The region from 7.8 to 8.8 μ is obscured by the solvent.

Fig. 4. Infrared spectra of samples showing the overlapping of lecithin and sphingomyelin in the elution curve shown in Figure 2. Curve a is sample from tube 27; b, tube 30; c, tube 33. All samples were run in $CHCl_3$ solution at 7.5 mg/ml. Upper curve is $CHCl_3$ versus $CHCl_3$ background.

Fig. 5. A typical gas chromatogram obtained from phosphatidyl ethanolamine of human serum lipoproteins with $\rho > 1.20$ gm/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/min. Column temperature 195°C. Stationary phase, polyester of diethylene glycol succinate. Samples were injected in a solution of hexane which produced initial solvent peak.

Fig. 6. Gas chromatograms from lecithin samples obtained from chromatography shown in Figure 2. A is a sample from tube 20. B is a sample from tube 26. The decrease between the twenty and twenty two carbon unsaturated fatty acids in Curve A and B is

obvious. Operating conditions as described in Figure 5.

Figure 7. A semi log plot of the data obtained from chromatograms of lecithin fatty acid esters of retention time as a function of number of double bonds present in the molecule for the methyl esters of the twenty and twenty two carbon unsaturated fatty acids.

Table I

Composition of the Major Fatty Acid Groups Shown in Figure 1 by Carbon Chain Length

(Mass % of Total Methyl Ester Sample)

Fatty Acid	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

Table II

Fatty Acid Composition of Human Serum Phospholipids

(Mass % 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	3.8	
22:0			0.2	0.5
20:4	25.2	12.2	6.7	
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	57.6	85.5
Unsaturated	62.9	58.4	42.4	14.5

* Contains phosphatidylserine and phosphoinositide

Table III

Fatty Acid Composition of Chromatographic Fractions

(Mass % 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	0.3	0.4	
2.93	20:3	2.1	3.6	4.8	4.5	3.5	2.3	6.1	2.3	
3.01	22:0									0.5
3.33	20:4	27.8	22.8	19.8	15.7	8.1	5.5	8.1	10.1	
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

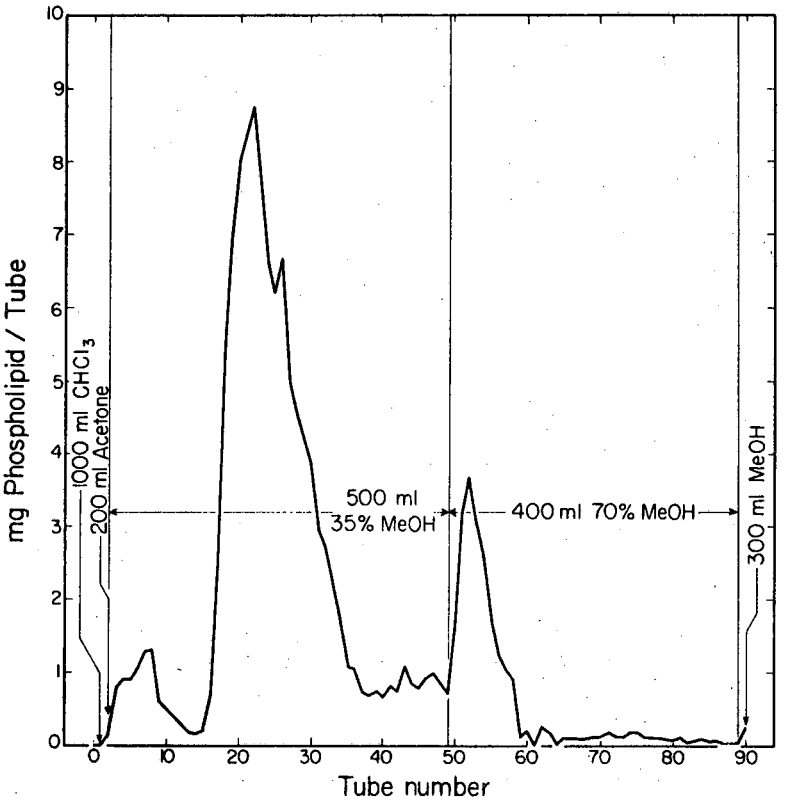

mg Phospholipid / Tube
Tube number
1000 ml $CHCl_3$
200 ml Acetone
500 ml
35% MeOH
400 ml 70% MeOH
300 ml MeOH
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
70
80
90

Fig. 1

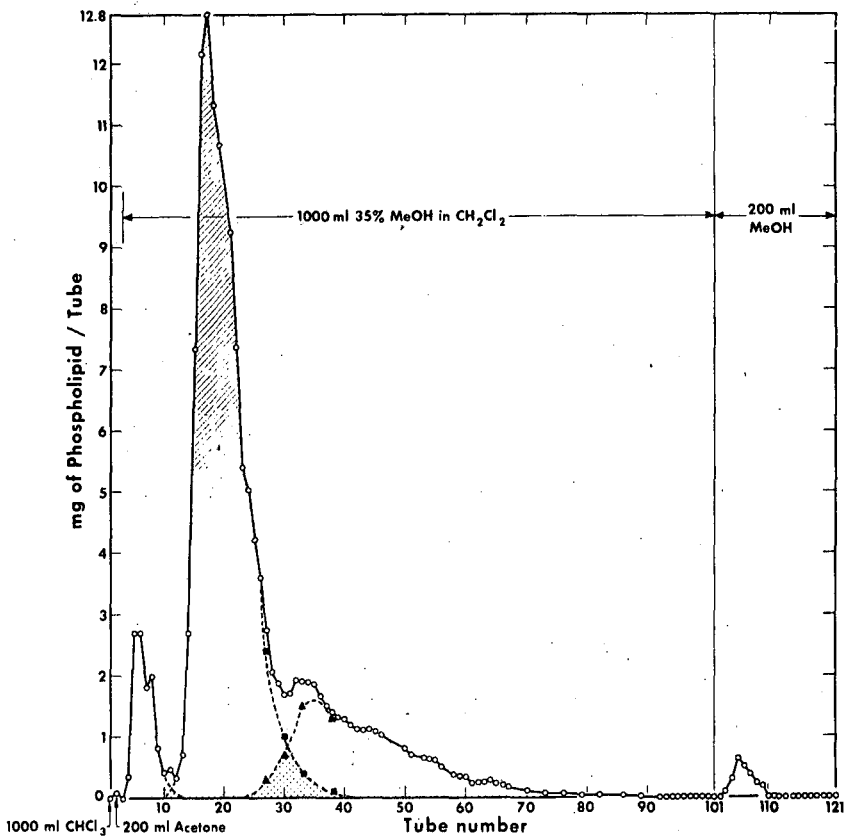

12.8
12
11
10
9
8
7
6
5
4
3
2
1
0
mg of Phospholipid / Tube
1000 ml 35% MeOH in CH_2Cl_2
200 ml MeOH
1000 ml $CHCl_3$
200 ml Acetone
10
20
30
40
50
60
70
80
90
101
110
121
Tube number

Fig. 2

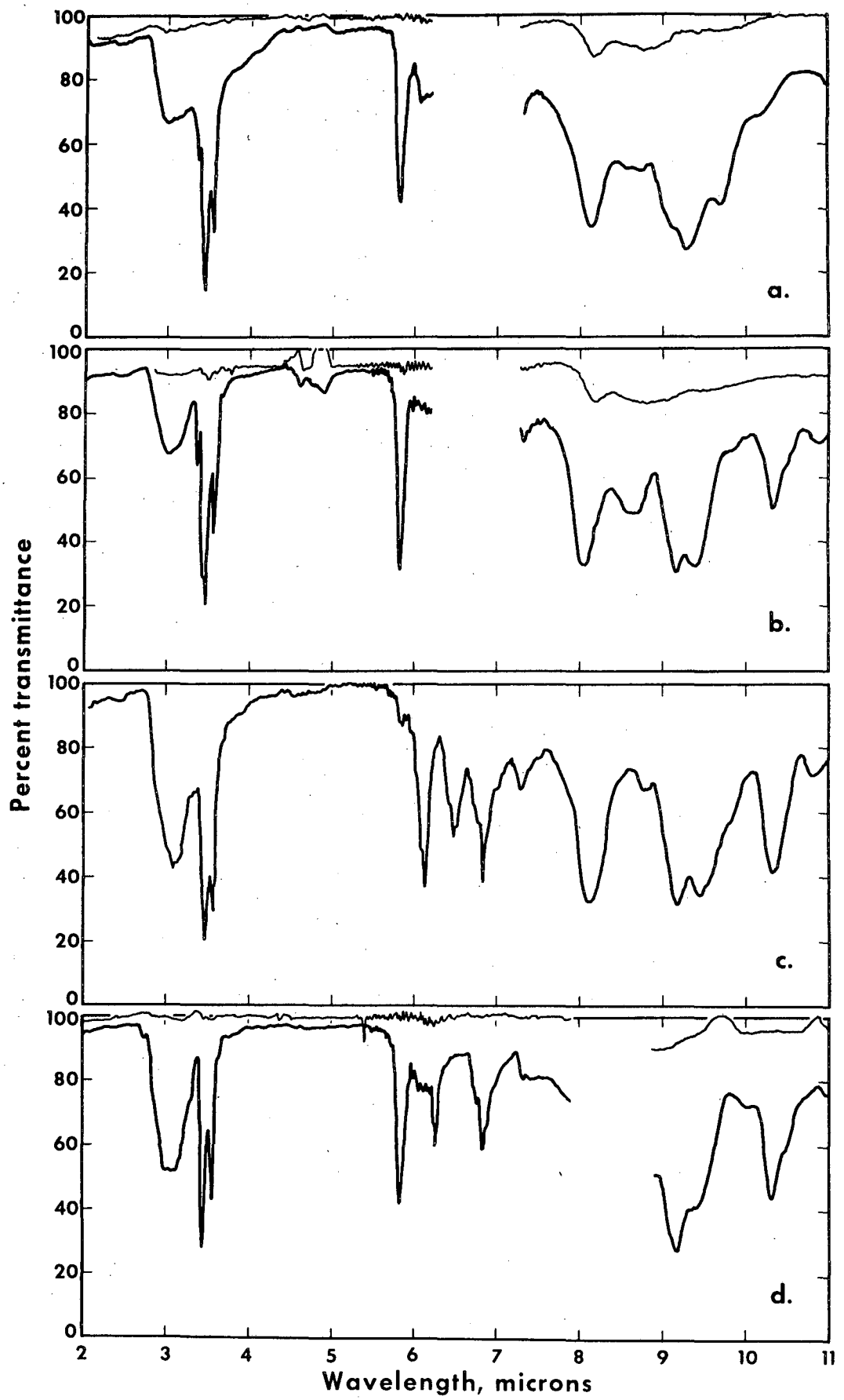
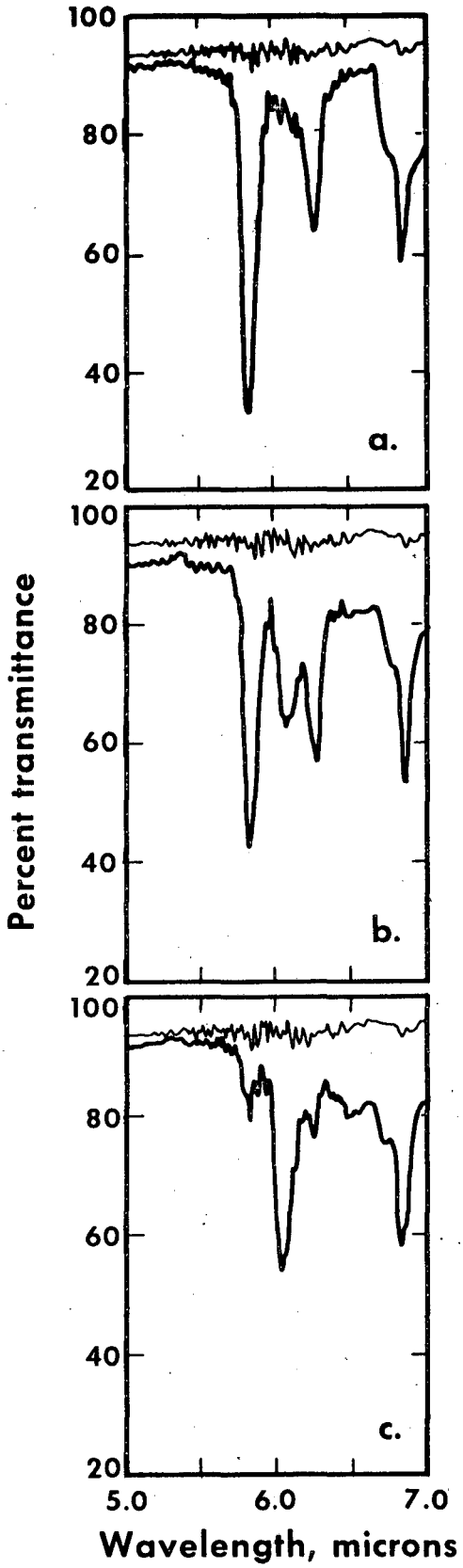

Percent transmittance
100
80
60
40
20
0
a.
b.
c.
d.
2
3
4
5
6
7
8
9
10
11
Wavelength, microns

Fig. 3


100
80
60
40
20
a.
b.
c.
Percent transmittance
5.0
6.0
7.0
Wavelength, microns

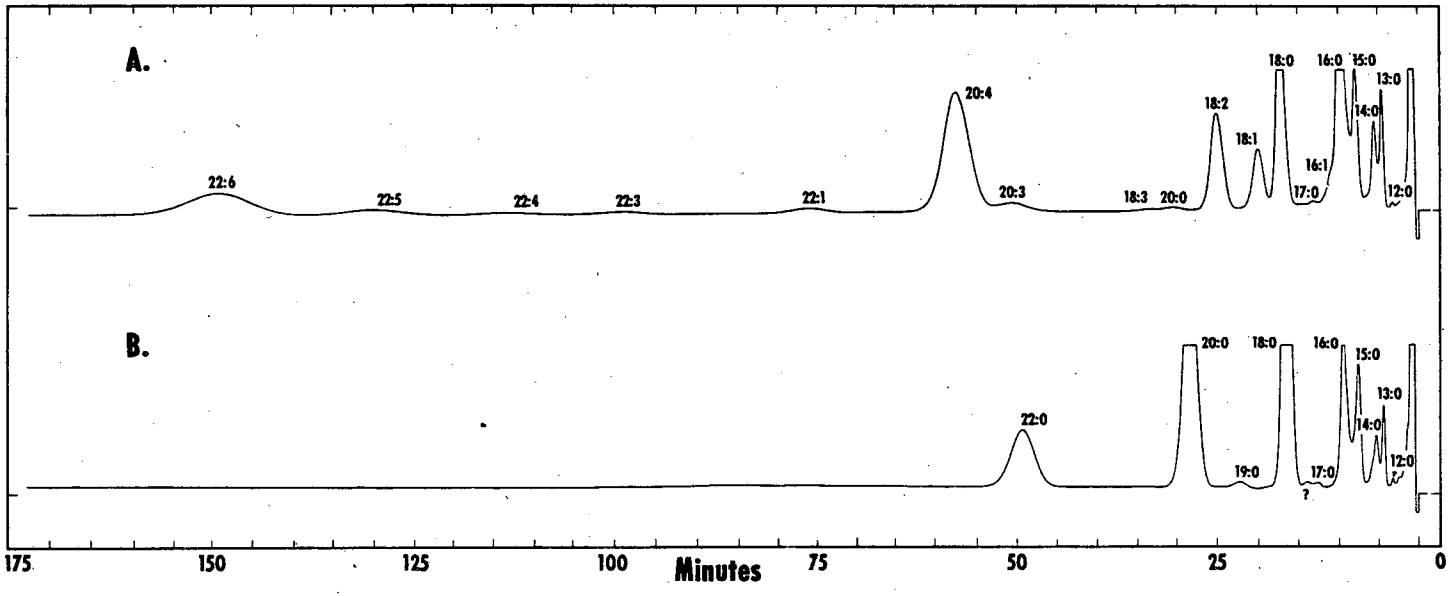

A.
22:6
22:5
22:4
22:3
22:1
20:4
20:3
18:3
20:0
18:2
18:1
18:0
16:1
17:0
16:0
15:0
14:0
13:0
12:0
B.
22:0
20:0
19:0
18:0
17:0
?
16:0
15:0
14:0
13:0
12:0
175
150
125
100
Minutes
75
50
25
0

Fig. 5

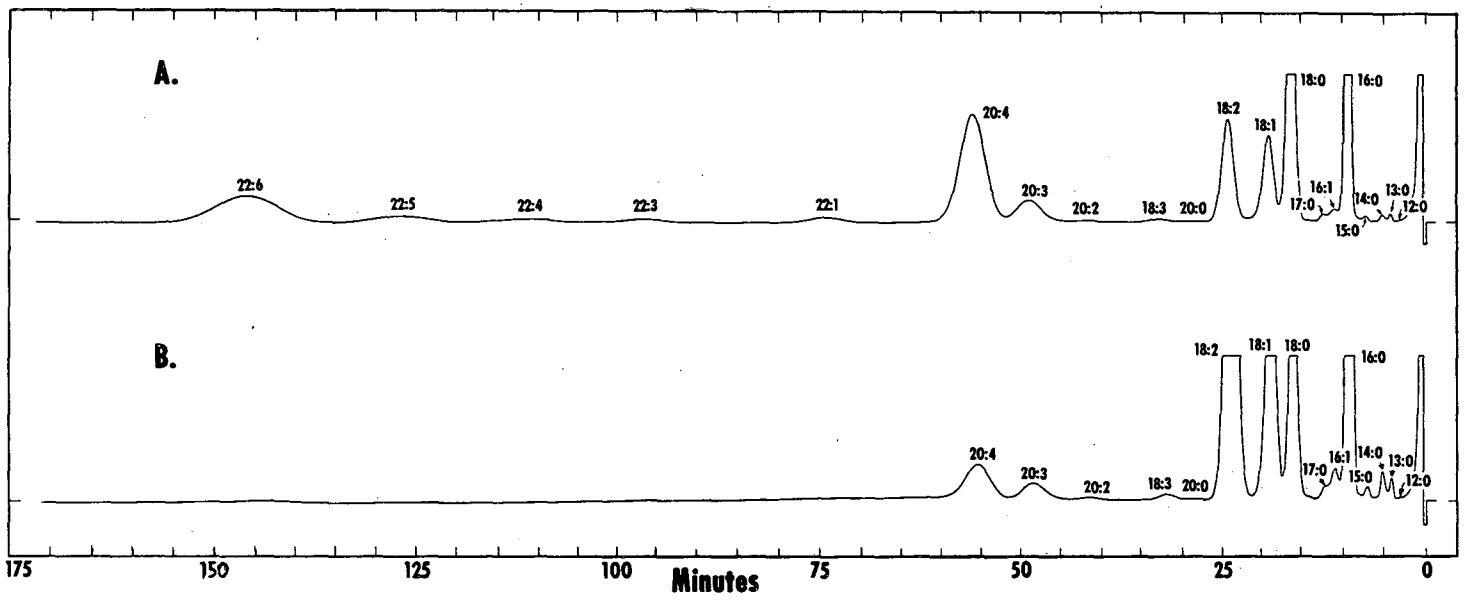

A.
22:6
22:5
22:4
22:3
22:1
20:4
20:3
20:2
18:3
20:0
18:2
18:1
18:0
16:0
16:1
17:0
14:0
15:0
13:0
12:0
B.
20:4
20:3
20:2
18:3
20:0
18:2
18:1
18:0
16:0
16:1
17:0
15:0
14:0
13:0
12:0
175
150
125
100
Minutes
75
50
25
0

Fig. 6

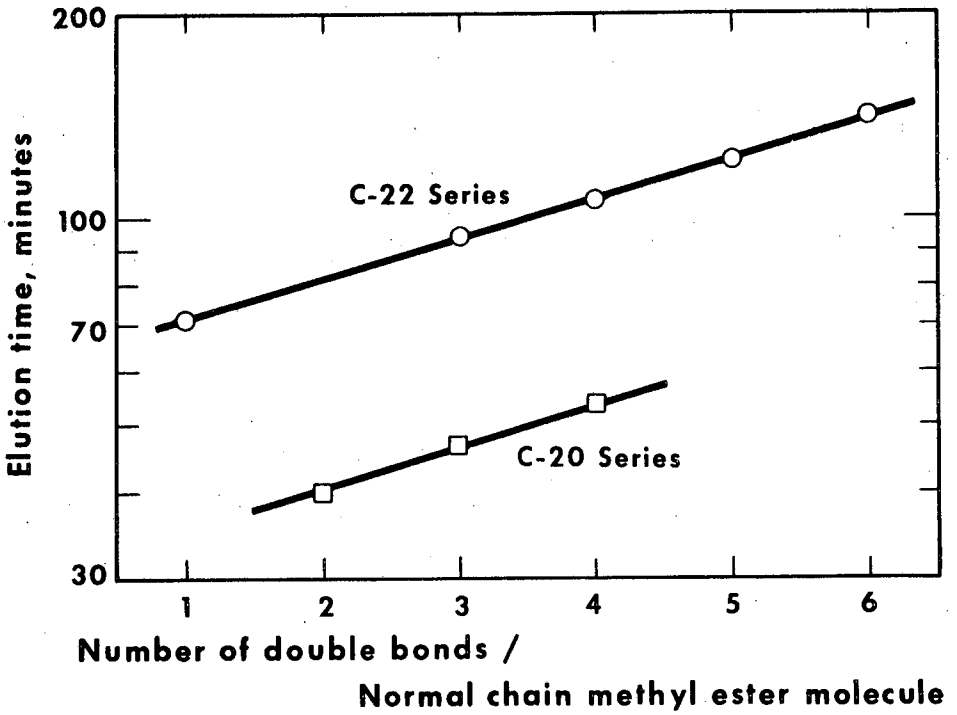

200
100
70
30
Elution time, minutes
C-22 Series
C-20 Series
1
2
3
4
5
6
Number of double bonds /
Normal chain methyl ester molecule

Fig. 7