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Authors

Nelson, Gary J.
Freeman, Norman K.

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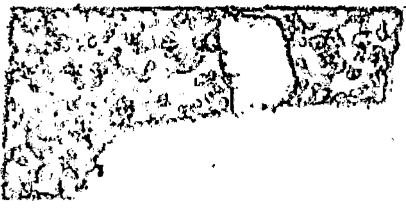

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Gary J. Nelson and Norman K. Freeman

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

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ABSTRACT

A semimicro method has been developed for the analysis of serum phospholipides by using chromatography and infrared spectrophotometry. The extracted lipides are separated into five fractions by successive elution from a silicic acid - celite column with methylene chloride, acetone, 35% methanol - 65% methylene chloride (two fractions), and methanol. The phospholipides are contained in the last three fractions. By suitable infrared absorption measurements of these latter three fractions--dissolved in appropriate solvents--the amounts of cephalin, lecithin, and sphingomyelin in the serum can be determined. The accuracy for a given component is somewhat dependent on the composition of the sample being analyzed. In general, the probable error is within 10% for lecithin and sphinogmyelin, and slightly greater for cephalin.

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INTRODUCTION

The classical methods for separating individual phospholipides rely mainly on solvent extraction[1-5] and various chemical complexing reactions[6,7,8] such as the $CdCl_2$ complex of phosphatidyl choline. Solvent purification of phospholipides frequently does not give adequate separation,[9,10] and while it is often possible to purify a phospholipide by these methods, they are not applicable to quantitative procedures. In view of these difficulties, the classical methods can be regarded as useful chiefly in nonquantitative preparatory procedures where large amounts of starting material are available.

Since 1940, adsorption chromatography[11-15] has been an increasingly important tool in the studies of lipides in general, and the phospholipides in particular. The phospholipides have received special attention since Borgstrom observed that they could be quantitatively separated from the non-phosphorus-containing lipides by the use of a silicic acid column.[15] This procedure, in which the nonphospholipides are eluted with chloroform and the phospholipides with methanol yields all of the phospholipides together in a single fraction. Investigations of silicic-acid-column chromatography of the phospholipides using chloroform-methanol mixtures showed that further fractionation was possible.[16-19] Lea and Rhodes were able to separate cephalin-lecithin mixtures obtained from egg yolk by eluting with 32%

MeOH–68% chloroform (v/v) on silicic acid.[20] In a more elaborate procedure involving rechromatography they have shown that additional separation can be obtained using silicic acid columns and the separations are quantitative.[21]

Hanahan et al. have also described the application of silicic acid chromatography to lipides of liver and yeast.[22] In a chromatogram of phospholipides from liver, it was possible to distinguish a peak corresponding to inositol phosphatide, in addition to those of the more abundant components. In the scheme given recently by Hirsch and Ahrens for total lipide chromatography, the separation of cephalin, lecithin, and sphingomyelin is partially achieved by elution with pure methanol rather than with chloroform-methanol mixtures.[23] Phillips has described a procedure for separation of serum phospholipides by silicic acid chromatography in which he reports the separation of a fraction composed mainly of lysolecithin.[24, 25] Further mention will be made of this work in the discussion section.

In general the methods mentioned above have used quantities of phospholipide in the range 0.1 to 1.0 gm. Many small fractions have been taken and characterized by weight, phosphorus content, and various other chemical analyses. The work in this laboratory has been directed toward developing simplified methods applicable to smaller quantities, using infrared spectrometry for both characterization and measurement. Analysis of serum for the principal lipide classes by such a method has been described in an earlier paper from this laboratory.[26] We now extend that method to provide for further fractionation and estimation of the principal serum phospholipides.

Because the method presented can be incorporated as an integral part of the total lipide analysis previously described,[26] it retains the advantages of

that method. These are (a) relatively small sample size; (b) simplicity and uniformity of techniques, and (c) nondestruction of material (insofar as handling losses and deleterious effects of exposure can be avoided). As given here, the method detects and analyzes cephalin, lecithin, and sphingomyelin in the ratios normally found in human serum. (The term cephalin is meant to include phosphatidyl ethanolamine and phosphatidyl serine together, since they are presumably eluted by the same solvent[22] and are virtually indistinguishable from one another by their infrared spectra.) The sample-size requirement is determined by the amount of the least abundant component being considered, as well as the limitation imposed by the available spectrophotometric equipment. The procedure will be described for a 10-ml serum sample, corresponding to 15 to 30 mg. of phospholipides. With the spectrophotometer used, this allows a fair estimate of the cephalin fraction, which usually amounts to 1 to 2 mg. Smaller samples could be used if only lecithins and sphingomyelins were of interest, or if a spectrometer capable of accommodating smaller quantities were used.

Analysis of tissue phospholipides, which frequently contain significant amounts of other components not included in the scheme for serum analysis, will require modifications to account for those components. Schwarz et al have studied some tissue lipides by infrared characterization of chromatographic fractions.[27] Lipides from virus preparations,[28] milk, and red blood cells have also been examined in this laboratory by these techniques.

The accuracy of the method is approximately plus or minus ten percent, somewhat poorer for the minor component cephalin, and somewhat better for the major components, lecithin and sphingomyelin. However, it should be stated that absolute accuracy in this field is extremely hard to determine, as pure reference compounds are not readily available and purity criteria are arbitrary. This problem will be discussed further in the appropriate section.

EXPERIMENTAL METHODS

Materials

All solvents are C.P. reagent grade. In general we have not found it necessary to redistill such solvents. Their purity can be checked by recording the infrared spectra of their nonvolatile residues. Complete solvent blanks may also be run through the entire procedure and the absorbances determined at the analytical wavelengths for each eluted fraction. Corrections may be applied if necessary, or further purification may be indicated.

The adsorbent is prepared by sifting and mixing together thoroughly two parts (by weight) of C.P. precipitated silicic acid (Fisher Scientific Co.) and one part celite (analytical filter aid, Johns Manville). The mixture is washed with methanol, dried, and finally heated to 120°C for 24 hr. It may be stored in a closed vessel, or preferably in a desiccator.

Lipide Extraction

Any method which extracts all the phospholipides would be acceptable provided it excludes all nonlipide components. Nonlipide material in the total extract gives a total weight that vitiates recovery figures. It may also interfere with the movement of the phospholipides on the column, and some contaminants appear to aid the spontaneous decomposition and air oxidation of the lipides.

The extraction method used is a modification of the procedure of Sperry and Brand[29] which was based mainly on the work of Folch.[30] A 10-ml serum sample is pipetted into a 250-ml volumetric flask containing 83 ml of methanol.

Then 83 ml of chloroform are added to the flask, and the flask is placed in a $60^{\circ}C$ water bath for 15 minutes. After warming, the flask is cooled in water until it returns to room temperature and is brought to volume with chloroform. The precipitated protein is removed by filtration through a fast filter paper into a 250-ml graduated cylinder. The volume recovered after the filtration is recorded and future calculations corrected for the amount lost in filtration. The filtered extract is again brought to 250 ml with chloroform and transferred to a 500-ml separatory funnel, which must be fitted with a Teflon stopcock, and 50 ml. of distilled water are added to the funnel. It is then shaken vigorously for 5 minutes, after which time it is allowed to stand until complete separation of both phases is achieved (this usually takes 1 or 2 hours).

The lower (organic) phase containing the lipides is drawn off; the aqueous phase is discarded. The organic phase is transferred to a 250-ml distillation flask, and the solvent removed with a rotary evaporator under reduced pressure. When most of the solvent has been removed, the residue is transferred, using chloroform, to a tared vial and the remaining solvent is removed by blowing nitrogen over the surface. The vial is placed in a vacuum desiccator for 24 hours and then weighed.

For other initial volumes of serum, proportional volumes of extraction solvents are required.

Chromatography

The columns are prepared on the basis of 10 to 15 mg of phospholipides to 1 gm of silicic acid–celite mixture. For serum lipides, this ratio is also satisfactory for the complete lipide analysis.

The column size for 10 ml of serum extract is 2 gm of silicic acid-celite. We have been using a commercially available column holder[31] containing a Teflon stopcock and a separable neck with a Teflon gasket. (Since the fritted glass disk in this column is of a coarse porosity two thicknesses of filter paper have been used in addition to prevent leakage of column material during elution.) For a 2-gm column, a 1-cm-diam tube has been used, giving a column height of approximately 3 cm. Other sizes are proportional.

The column material is added as a slurry in methylene chloride[32] and packed by air pressure from an air line or a syringe fitted to the top of the column. Gentle tamping with a glass rod is advisable to prevent channeling and to provide a level surface. The column is washed with approximately ten volumes of methylene chloride before adding the lipides to the column. (A column volume is approximately 2.5-ml per gm of silicic acid–celite).

The lipide extract is added to the column in 2 ml of methylene chloride. Care must be taken not to disturb the level surface of the column and to see that there is no channeling present. The lipides are washed on to the column with another 3 ml of methylene chloride, and then the liquid level is brought to the surface of the silicic acid. Again, care must be taken not to allow the liquid level to fall below the silicic acid level at this time or at any time during the chromatography. After addition of the sample to the column, the elution is carried out according to the scheme shown in Table I.

Since a few broad cuts are being taken rather than a large number of small ones, care must be taken in changing collection vessels to ensure that the leading edge of each new solvent is retained in the proper fraction. The flow rate is adjusted by the application of low pressure and is not critical as long as it does not exceed 4 or 5 ml per minute. Under gravity alone the flow rate is usually far too slow for practical purposes.

Table I

Silicic acid chromatographic scheme for a 2-gm column

Silicic Acid - Celite Mixture [2/1(W/W)]

Fraction	Solvent	Volume (ml)	Components
I	CH_2Cl_2	100	Nonphospholipides (cholesterol, cholesteryl esters, glycerides, fatty acids)
II	Acetone	20	Principally non-phospholipides(oxidized?), pigments and trace of phospholipides
III	35% methanol 65% CH_2Cl_2	10	Cephalins(phosphatidyl ethanolamine, +phosphatidyl serine) and small amounts of non-phospholipides
IV	35% methanol 65% CH_2Cl_2	25	Lecithins
V	95% methanol 5% H_2O	50	Lecithins and sphingomyelins.

Fractions II, III, and IV are small enough to collect in 25-ml screw-cap vials which have been previously tared. These fractions are evaporated to dryness under nitrogen on a hot plate at 50°C, and then desiccated for not less than 6 hours before weighing. Fractions I and V are collected in 150-ml beakers, evaporated with nitrogen until the volumes are sufficiently reduced, and then transferred to a vial of the same type as described for fractions II, III, and IV. After desiccation, weights are obtained on each fraction, mainly for correlation with the infrared data.

Fraction I contains virtually all of the nonphospholipides, or 70 to 80% of the total serum lipides. Fraction II is very small in amount, serving mainly to remove residual nonphospholipides. It is particularly helpful in cases where unavoidable oxidation has taken place, with acetone acting as a scavenger for oxidized materials, etc. Traces of phospholipides amounting to less than 1% of the total, are usually found in Fraction II. For our purposes the first two fractions are only weighed for inclusion in total recovery figures.

Fraction III consists mainly of cephalin (phosphatidyl ethanolamine plus phosphatidyl serine), with small amounts of nonphospholipides still persisting.

Fraction IV contains 60 to 80% of the lecithin present in the total extract. It is usually quite colorless and free from contamination.

The remaining lecithin is eluted together with sphingomyelin in Fraction V. It has not been possible in our experience to separate sphingomyelin free of lecithin. However, it is a virtue of our procedure that by employing infrared analysis it is unnecessary to do so. Indeed Fractions IV and V could be analyzed as a single mixture, but it is desirable to remove enough of the lecithin so that the concentrations of the two components are more nearly comparable.

INFRARED MEASUREMENTS

All our measurements have been made with a Baird Associates double-beam recording infrared spectrophotometer equipped with a sodium chloride prism. The absorption cell is of our own design and has an optical path length of 0.9mm and a volume of 0.15 ml.

Fractions I and II are not included in the infrared determinations, normally.

Fraction III usually contains between 1.0 and 2.5 mg of material and is dissolved in 0.20 ml of carbon disulfide[33] for recording its spectrum. This is done by transferring the sample in a minimum volume to a small, graduated centrifuge tube--the graduations are 0.20, 0.375, 0.50, 0.675, 1.00 ml. The volume is then brought to the desired level and 0.15 ml is transferred to the absorption cell. A preliminary gravimetric weight on each fraction is useful here in that the optimum concentration for a 0.9-mm cell is between 5 and 7.5 mg/ml, so that weights of from 1.0 to 1.5 mg are diluted to 0.20 ml and weights from 1.5 to 2.5 mg are diluted to 0.375 ml.

Fraction IV usually contains between 10 and 15 mg of lecithin and is transferred into a 2 ml. volumetric flask with carbon disulfide and made up to volume. If the weight is between 5 and 10 mg, a 1.0-ml volumetric flask is used.

Fraction V is usually dissolved in 1.0 ml of chloroform, because carbon disulfide does not transmit in the necessary spectral regions and sphingomyelin is relatively insoluble in carbon disulfide.

The spectrum of each eluted fraction is recorded from 5 to 11 microns (μ). Over the bands whose absorbances are to be measured, the scanning rate

should be slow enough to allow full response of the recorder. A cell containing pure solvent is used in the reference beam, and for the desired precision it is necessary to run background curves of solvent contained in the sample cell. Absorbancies at the specified absorption maxima are calculated in the customary way as $\text{Log}_{10}(T_o/T_s)$ where T_o is the transmittance of the solvent and T_s is the transmittance of the sample solution.

Typical spectra obtained from the chromatographic eluates of a serum sample are shown in Fig. 1. The region from 6.0 to 7.2μ is obscured in carbon disulfide solutions, and the regions from 6.4 to 6.7, 7.8 to 8.7, and 9.3 to 9.9μ are obscured in chloroform. The dotted line is the solvent transmission.

Fraction III (Figure 1A) is the cephalin fraction. The concentration is determined by calculating the absorbancy at 5.8μ and using the experimentally determined absorption coefficient (which includes a cell constant, as all the absorption coefficients stated in this paper do) to convert absorbancy into mg/ml. The rest of the curve is used primarily to indicate the purity of the sample. The absence of a 10.3μ band shows that no lecithin is present. The 9.3μ band can also be calibrated, but this has considerably more interference from contaminants than the 5.7-μ band.

Fraction IV (Figure 1B) is calculated as lecithin, and usually the 9.2 or the 10.3-μ bands are used to calculate the concentration of the lecithin present. The 5.8-μ band is less reliable but can also be used.

Fraction V(Figure 1C) is a two component mixture containing both lecithin and sphingomyelin. The absorption curves of lecithin and sphingomyelin are very similar beyond 7μ (see Fig. 2) but they differ significantly in the 5.8 to 6.2 micron regions as seen in the chloroform-solution spectrum. By

using these absorption bands at 5.8 and 6.1μ and two standard simultaneous equations, it is possible to calculate the amounts of lecithin and sphingomyelin present. The absorption of each compound at 10.3μ is almost identical, and this band provides a convenient check on the total weight of material in the fraction. The weight calculated at 10.3μ should agree with the sum of the weights given by the two simultaneous equations.

Briefly, the mathematics of the simultaneous equations are given in this manner.[34] An equation for the absorption at 5.8μ and a similar equation for the absorption at 6.1μ are set up thus:

$$A_{5.8} = \alpha_L^{5.8} C_L + \alpha_S^{5.8} C_S \quad (1)$$

$$A_{6.1} = \alpha_L^{6.1} C_L + \alpha_S^{6.1} C_S \quad . \quad (2)$$

If the values of alpha are known for a particular absorption cell, these two equations can be rearranged to yield the concentrations in terms of the absorbancies and four new constants:

$$C_L = k_1 A_{5.8} + k_2 A_{6.1} \quad (3)$$

$$C_S = k_3 A_{5.8} + k_4 A_{6.1} \quad . \quad (4)$$

Substituting typical values for the constants, k, the equations become

$$C_L = 11.25\ A_{5.8} - 1.5\ A_{6.1} \quad (5)$$

$$C_S = -\ 2.25\ A_{5.8} + 22.5\ A_{6.1} \quad . \quad (6)$$

Since the volume of the solution is known, concentrations are converted to weights by simple multiplication. The weight of lecithin determined in

Fraction V by Eq. 5 is then added to that determined in Fraction IV to give the total amount of lecithin present. The total amount of sphingomyelin is simply calculated by the use of Eq. 6.

The amounts of individual phospholipides can be calculated as percentage either of the total phospholipide fraction, or of the total lipide extract.

CALIBRATION

The infrared spectra of the reference compounds are shown in Fig. 2. Figures 2A and B are cephalin and lecithin, respectively, in carbon disulfide, and Fig. 2C is sphingomyelin in chloroform. Figure 2D is sphingomyelin on NaCl plates, showing the full spectrum unobscured by solvent absorption.

The materials used as standards have been natural products, purified as fas as possible by chromatography. As further criteria of purity we have relied mainly on their infrared spectra and chemically determined phosphorus values. Cephalin was prepared from the acetone-insoluble lipides of egg yolk by rechromatographing twice according to the procedure of Lea and Rhodes.[20] Lecithin obtained from serum in our chromatographic procedure (Fraction IV) was further rechromatographed three times for use as a standard. Cephalin and lecithin fractions so prepared have been considered acceptable if their phosphorus content is $4.0 \pm 0.1\%$, and their infrared absorptions at 10.3μ are minimal and maximal, respectively.

Fifth fractions from scaled-up serum-phospholipide chromatography, or pooled fifth fractions from routine serum-phospholipide chromatography have served as a source of sphingomyelin. For purification, they have been subjected to mild basic hydrolysis as described by Hack;[35] then the extract from the neutralized hydrolysis mixture has been rechromatographed by the usual procedure.

Fraction V is taken as "pure" sphingomyelin if its phosphorus content is satisfactory and no ester carbonyl absorption at 5.8μ in the infrared can be detected.

While synthetic compounds of rigorous purity would be desirable as standards, most of those have been prepared so far (by Prof. Erich Baer, Toronto) have been cephalins and lecithins containing saturated fatty acids. These have limited solubility in carbon disulfide when the fatty-acid chain lengths correspond to those expected to occur in natural phospholipides. On one occasion we were fortunate in being able to compare the spectral absorption data for purified serum lecithin with those for synthetic dioleyl lecithin (courtesy of Prof. Baer), which is much more soluble than the saturated lecithins. (Egg lecithins are generally similar to those from serum.) Absorptivities at the major peaks agreed within 2 to 3% except at 5.8μ, where the coefficient for serum lecithin was about 10% lower than the synthetic. This presumably reflects a lower content of ester groups per gram, and could arise from a difference in fatty-acid size, or possibly from the presence of lysolecithin or some unknown contaminant in the natural material. In the absence of more definite information about the fatty-acid composition of serum phospholipides and the fluctuations thereof, we have used the empirical data for the natural substances, subject to the criteria stated above.

Table II lists a set of typical absorption coefficients of the measured phospholipides at their principal absorption bands. The absorption coefficients, designated a, are absorbances per unit concentration (mg/ml) in an absorption cell of 0.9 mm optical path length. The cephalin and lecithin samples are dissolved in carbon disulfide, and the sphingomyelin sample is dissolved in

Table II

Absorption coefficients, α, for the principle absorption bands of the major serum phospholipides

Wavelength (μ)	Absorption coefficients, α, in absorbancy per mg/ml		
	Cephalin	Lecithin	Sphingomyelin
3.4	0.126	0.122	0.139
5.8	0.077	0.080	0.003
6.1		0.011[a]	0.047
6.8			0.038
8.1	0.060	0.073	
9.2		0.074	0.063
9.3	0.073	0.070	
10.3	0.020	0.044	0.045

[a]This value is obtained in chloroform; this region is obscured in carbon disulfide. These absorption coefficients apply to concentrations within the range 5.0 to 7.5 mg/ml over which the calibration curves are quite linear. For concentrations above this range the validity of these α's is questionable.

chloroform. It should be emphasized that these values are not transferable to other instruments. Independent calibration must be carried out for the same spectrometer and absorption cells that are to be used in the analysis.

Very high absorbancies as well as very low ones are to be avoided as less accurate on general spectrophotometric principles. This may sometimes necessitate repeating the infrared measurement at a different concentration in cases where the estimate of the volume of dilution fails to give a concentration in the right range.

The calibration values should be checked periodically. We have found them to remain stable within a few percent over periods of several months, although they are subject to variation from changes in cell characteristics or instrumental conditions if these are not well controlled.

RESULTS AND DISCUSSION

The validity of the method was determined by running duplicate samples completely through the analyses and then running standard samples through the chromatography. This gives ample evidence that the method is reproducible. Data on the duplicate runs are presented in Table III. All samples were chromatographed by identical procedures on a 2-gm column, and all weights were determined from infrared measurements. Table IV compares the gravimetric weights, weights determined from the infrared measurements, and the weights calculated from lipide phosphorus (phospholipide = lipide phosphorus $\times$ 25).[36] The gravimetric values in Fraction III are considerably higher than corresponding infrared values, presumably because of non-phospholipide impurities. Weights calculated from phosphorus are slightly lower than corresponding infrared values. This may be because part of the

Table III

Duplicate analyses of serum phospholipides

Run number	Cephalin (mg)	Lecithin (mg)	Sphingomyelin (mg)
1A	1.23	19.9	4.55
1B	1.20	18.9	5.32
2A	0.59	12.8	5.42
2B	0.59	13.5	4.73
3A	0.88	16.7	2.46
3B	0.84	17.2	2.30
4A	1.33	15.4	3.70
4B	1.26	14.5	3.45
5A	1.21	17.4	5.96
5B	1.12	16.7	6.02

Table IV

Comparison of the chromatographic fractions in terms of gravimetric weight, infrared calculated weight, and weight as phospholipide from chemical phosphorus analyses. Five separate runs are shown.

Run number	Fraction number	Weight (mg)		
		Infrared	Chemical	Gravimetric
1A	I		0.00	53.4
	II		0.28	0.75
	III	3.6	3.4	4.2
	IV	14.9	15.0	14.9
	V	7.2	6.7	7.2
2B	I		0.00	49.4
	II		0.28	0.84
	III	0.59	0.52	1.6
	IV	11.7	10.6	11.7
	V	6.5	6.5	5.6
3A	I		0.00	22.9
	II		0.28	1.3
	III	0.84	0.81	2.9
	IV	13.5	11.5	12.6
	V	5.8	5.7	6.4
4B	I		0.00	72.8
	II		0.26	1.1
	III	1.3	1.1	2.1
	IV	11.7	12.0	12.6
	V	6.3	6.5	7.3
9A	I		0.00	72.4
	II		0.16	6.0
	III	2.4	2.1	3.8
	IV	16.1	16.3	16.0
	V	6.8	6.9	6.9

nonphospholipide impurities are esters and are measured at 5.8μ in the infrared, or because of handling losses in recovering the material for phosphorus analysis.

Table V gives a detail of a complete chromatographic run, showing the comparison of all fractions by weight, infrared, and chemical phosphorus analyses from initial extraction to final separations.

Table VI presents the results obtained for the analysis of an artifical mixture of cephalin, lecithin, and sphingomyelin chromatographed by the standard precedure. The phospholipides used were prepared from egg yolk and human serum as described for calibration standards. Notice the tailing of the lecithin into the fifth fraction. This lecithin, although triply chromatographed with only the fourth fraction being retained each time, still associated strongly with the sphingomyelin when chromatographed in the mixture. In this run there was no indication of phosphorus in Fraction I and approximately 2% of the total phosphorus was recovered in Fraction II. There was no indication of any lecithin in Fraction III.

The procedure is perfectly adaptable to scaling up as desired. Initial extracts weighing over 400 mg have been analyzed using 10-gm columns with equally satisfactory results. A 5-ml serum extract and a 1-gm column can be used also, but there is further loss in accuracy in the cephalin determination. In order to use smaller volumes and still include cephalin in the analysis, it will be necessary to use a spectrophotometer that is equipped to handle smaller absorption cells. This seems entirely feasible, and will be explored.

It is also possible to combine this procedure with the method of Freeman et al.[26] for the analysis of the other lipide components without loss of accuracy or any changes in the phospholipide elution characteristics. Thus with one procedure that is nondestructive to the material analyzed, seven different

Table V

Analytical results for a 10-ml of serum-lipide extract showing recoveries of total lipide and phospholipide

Chromatography:	Amount added to column	86.0 mg
	Total weight of all fractions	80.1 mg
	Recovery	94.3%

Breakdown of chromatographic fractions:

Fraction No.	Weight (mg)		
	Infrared	Chemical	Gravimetric
I		0.00	54.3
II		0.33	1.1
III	1.2	1.0	1.8
IV	14.8	13.3	15.0
V	9.4	8.8	8.9
Totals of			
III, IV, V	25.4	23.1	25.7

Phospholipides in initial extract, by phosphorous determination: 25.4 mg

Distribution of the Phospholipides:

Cephalin	1.2 mg	4.7%
Lecithin	18.9 mg	74.4%
Sphingomyelin	5.3 mg	20.9%

Table VI

Results of the chromatographic infrared analysis of a prepared known mixture of phospholipides

Constituent	Amount present (mg)	Anount found (mg)	% Error
Cephalin	2.9	2.5	-12.2
Lecithin	13.7	12.5	- 8.6
Sphingomyelin	6.2	6.3	+ 1.5
Totals	22.8	21.3	- 6.3

Chromatography

Fraction	Weight (mg)		
	Infrared	Chemical	Gravimetric
I		0.0	0.3
II		0.3	0.4
III	2.5	1.9	2.6
IV	7.7	6.8	8.1
V	10.1	9.0	10.1
Totals	21.3	18.0	22.5

lipide components can be determined quantitatively on a single serum sample. The infrared analysis could of course be combined with any other suitable chromatographic scheme which performs substantially the same separation.

The application of this general scheme to other lipide extracts should only be done after preliminary studies to determine the identities of the materials obtained in the chromatographic fractions. There is reason to believe that other phospholipides not found in serum are eluted from silicic acid columns by the solvents used in this procedure.[22] Also tailing and interactions of the phospholipides with each other often change the elution characteristics of a phospholipide so as to make it unrecognizable on an elution basis alone. Fortunately, the infrared spectrum provides an indication of purity as well as quantity. For other systems, solvents and volumes will need to be changed as conditions indicate. Some preliminary studies on red-blood-cell phospholipides indicate that this method is suitable to a wide variation in the ratios of the components as long as no new component is added to the present three.

SOURCES OF ERROR

A possible source of error is the overlapping and tailing of the components into other fractions than the one in which they are supposed to be collected. In Fraction III the presence of any material from the later fractions can be detected by the presence of an infrared absorption band at 10.3μ. If as much as 5% lecithin or sphingomyelin are present in this fraction it will be detectable in the 10.3μ region. Since this band is ordinarily not seen in Fraction III, the error resulting from this cross contamination is assumed to be less than 5%.

Fraction III is more likely to be contaminated by nonphospholipide material that should have preceded it in elution. This is inferred from comparison of

weights with infrared and phosphorus determinations (Table III). In our experience these contaminants have not contributed any substantial error to the infrared determination.

In Fraction IV, it is impossible to detect the presence of less than 5% cephalin with any reliability. In this case the intensity of the 10.3μ band is used as the indication of purity. If the fourth fraction should contain 5% of cephalin, however, it could represent a 20 to 30% deficiency in the amount of cephalin that should have been found in Fraction III. This is part of the reason why reliability of the method for cephalin is lower than for the other components.

Contamination of Fraction IV by sphingomyelin in significant amounts can be detected by excessive absorption at 6.1μ, but only if the spectrum is taken in chloroform solution. In checking Fraction IV in that manner, measurable contamination has not been found.

Since Fraction V is expected to contain both lecithin and sphingomyelin, this overlap is not a matter of concern.

Deterioration of the sample by oxidation or decomposition as a result of excessive exposure is also a possible source of error. This can be minimized by carrying out all operations as soon and as quickly as possible, and by the use of nitrogen and/or vacuum for the evaporation steps. Extended storage periods should be avoided, and for such storage as may be necessary the samples or fractions should be kept under nitrogen or vacuum at low temperatures. Air oxidation of cholesteryl esters is especially troublesome, since the oxidation products tend to be retarded in their elution and may appear in various subsequent fractions.

The photometric error in infrared absorption measurements with our instrument is estimated as ± 1%, leading to concentration errors of ±3 to 4%.

This error can be made smaller in spectrophotometers with greater flexibility in the control of slit widths and other operating variables.

Phospholipide species other than cephalin, lecithin, and sphingomyelin have been assumed to be present in small enough amounts that they can be neglected. This is not strictly true, of course, and the analytical results, can be affected to the extent that such other species (plasmalogens, for example) are present.

Recently, Phillips has announced the isolation and identification of lysolecithin from normal human blood serum in the order of 5% of the total phospholipides.[24,25] If this is correct, this material would appear in this elution scheme in Fraction V and be included in the lecithin determination. However, in some extended fractionations of Fraction V, we have been unable to separate a discrete fraction following sphingomyelin. The discrepancy between our average values for lecithin (75%) and those reported by Phillips (69%) is approximately accounted for by the percentage he gives as lysolecithin.

It is impossible to assess the magnitude of the errors of calibration until better calibration compounds are available and the variability of composition of the individual phospholipide classes is studied more thoroughly. This source of error can not be reduced at present, but it is unlikely that it compares in magnitude to the errors resulting from various other considerations, as given above.

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FOOTNOTES

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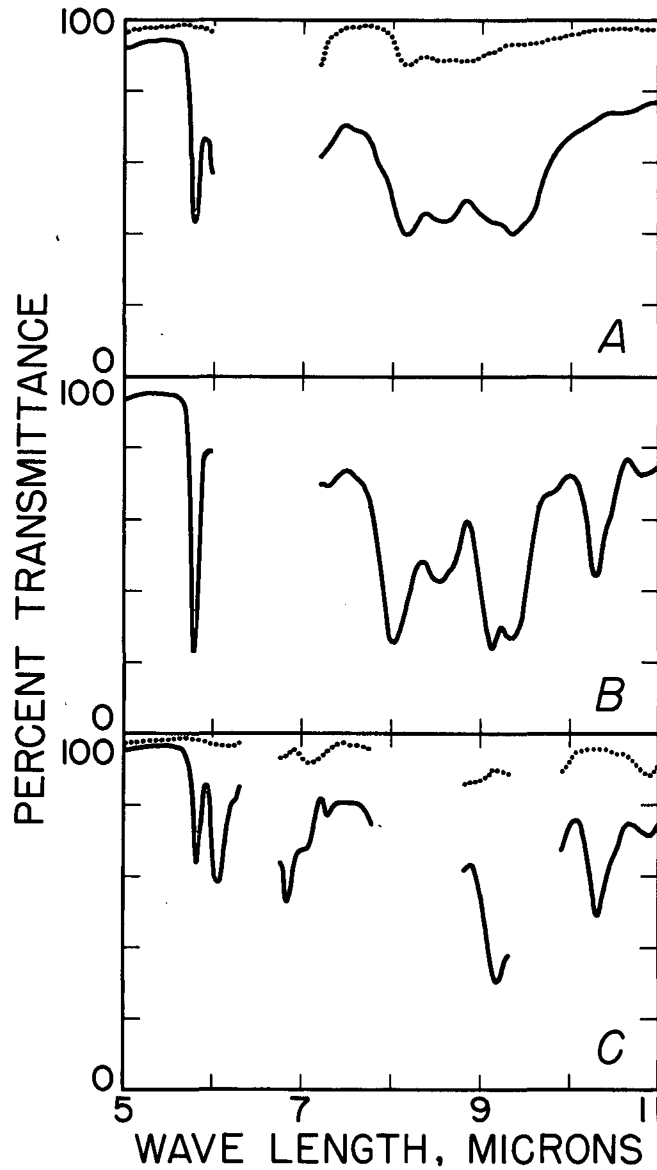

Fig. 1. Typical infrared spectra obtained from chromatographic eluates of a serum sample. The regions from 6.0 to 7.2μ is obscured in CS_2 solution and from 6.4 to 6.7, 7.8 to 8.7, and 9.3 to 9.9μ in chloroform. The dotted line is the solvent transmission. (A) Fraction III, cephalin, 3.79mg in 0.5ml of carbon disulfide, (B) Fraction IV, lecithin, 8.00 mg/ml in carbon disulfide and (C) Fraction V, lecithin and sphingomyelin mixture, 5.63 mg/ml in chloroform.

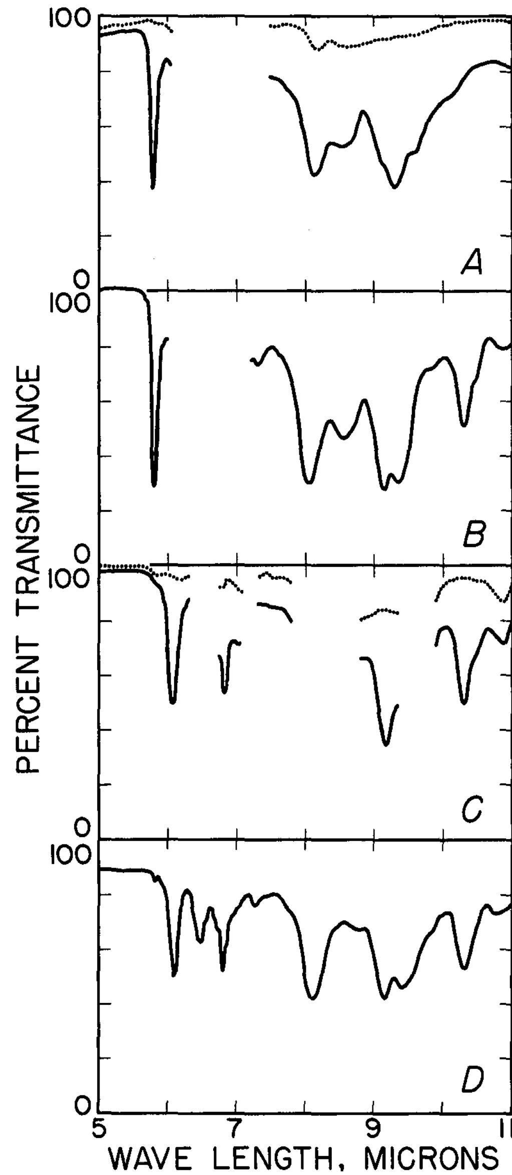

Fig. 2. The infrared spectra of reference compounds.
(A) Cephalin, 5.06 mg/ml in carbon disulfide,
(B) lecithin, 6.55 mg/ml in carbon disulfide,
(C) sphingomyelin, 6.06 mg/ml in chloroform, and
(D) film of sphingomyelin on NaCl plates. All reference compounds in this figure were prepared as indicated in the text.

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