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Title THE PHOSPHOLIPIDS IN HUMAN BLOOD FRACTIONS

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Although there is information available on the distribution of phospholipids in human serum and red cells, very limited data have been reported for white cells and platelets. To the knowledge of the author, no data have been reported on the distribution of phospholipids among the four blood fractions of individual subjects.

Due to the limited amounts of white cells and platelets present in blood, micromethods are essential for the analyses of these fractions in individual subjects. Although phospholipids have been separated from larger samples by others, their methods were not appropriate for micro amounts. Therefore, procedures were developed in this study which made it possible to isolate and to quantitate the individual components of samples of total phospholipid ranging from 20 to 40 μg.

The distributions of phospholipids were determined in serum, red cells, white cells and platelets isolated from the venous blood of four men and four women. The blood fractions were isolated and

lipid was extracted by methods previously developed in this laboratory. Total phospholipids were isolated by preparative thin-layer chromatography. Individual phospholipid components were separated and quantitated by the micromethod developed in this investigation. Components were eluted from microchromatoplates and quantitated by analysis of their phosphorus content. This method effectively separated phospholipid samples into lysophosphatidylcholine (LPhC), sphingomyelin (Sph), phosphatidylcholine + phosphatidylserine (PhC + PhS) and phosphatidylethanolamine (PhE).

Although all of the fractions contained higher proportional amounts of PhC + PhS than any other component, there were characteristic distributions in each of the blood fractions. Serum was characterized by high PhC + PhS and virtually no PhE. Red cells contained lower amounts of PhC + PhS and more Sph and PhE than any other blood fraction. The distributions of phospholipids in white cells and platelets were similar and resembled the general pattern found in red cells more than that of serum.

The marked differences in the distribution of phospholipids among the blood fractions emphasize the importance of concurrent analyses of all blood fractions in studies of human phospholipid metabolism.

THE PHOSPHOLIPIDS IN HUMAN BLOOD FRACTIONS

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THE PHOSPHOLIPIDS IN HUMAN BLOOD FRACTIONS

INTRODUCTION

Phospholipids are one of the important classes of lipids found in blood and other tissues. Most of the phospholipids are diacyl esters of a nitrogenous base derivative of L-α-glycerophosphoric acid. Higher percentages of unsaturated fatty acids are found in phospholipids than in triglycerides. In general, about 50 percent of the fatty acids of phospholipids are unsaturated (Ansell and Hawthorne, 1964).

The phospholipids are a heterogeneous class of compounds consisting of many specific phospholipids. The major individual phospholipids are: sphingomyelin (Sph), phosphatidylcholine (PhC), phosphatidylserine (PhS), phosphatidylethanolamine (PhE) and phosphatidylinositol (PhI), whereas some of the minor phospholipids are lysophosphatidylcholine (LPhC), lysophosphatidylethanolamine (LPhE), phosphatidic acid (PhA) and cardiolipin.

Phospholipids are present in serum, red cells, white cells and platelets in varying concentrations and distributions. The phospholipids comprise about 30 percent of the total lipid in serum, whereas they account for nearly two-thirds of the lipid content of red cells (Albritton, 1952; and Smith, 1965). The values reported for the concentration of phospholipids in white cells and platelets are limited and varied. The mean values cited for white cell phospholipids ranged

from 40 to 70 percent of total lipid (Malamos et al., 1962; and Smith, 1965), whereas platelet phospholipids comprised 65 to 80 percent of total lipid (Barkhan, Silver and O'Keefe, 1961; Lee and Erickson, 1938; Smith, 1965; and Woodside and Kocholaty, 1960).

Although isotope studies have shown that phospholipids were exchanged between plasma and red cells (Jones and Gardner, 1962; Marks, Gellhorn and Kidson, 1960; and Sakagami, Minari and Orii, 1960), the distribution of phospholipids within these two fractions varies markedly (Deuel, 1955; and Phillips and Roome, 1959). The phospholipid present in the highest proportions in both red cells and serum is PhC. The erythrocytes also contain considerable amounts of PhS and PhE (Phillips and Roome, 1959; Reed et al., 1960; and Ways and Hanahan, 1964). In contrast, serum phospholipids consist almost entirely of choline-containing components, i.e., LPhC, Sph, and PhC (Doizaki and Zieve, 1963; Hagopian and Robinson, 1965; and Williams, Kuchmak and Witter, 1966). There are limited data available on the distribution of phospholipids in human platelets; however, the patterns of distribution are reported to be more like those of red cells than serum (Barkhan et al., 1961; and Troup et al., 1960). To the knowledge of the author, there are no values reported in the literature for the distribution of individual phospholipids in normal human leucocytes.

The proportions of individual phospholipids in the blood fractions

are influenced by a number of diseases (Behrendt, 1957; Marinetti et al., 1959; and Phillips and Roome, 1962). Diet is more likely to influence the distribution of phospholipids in serum than in any other blood fraction (Antar, Ohlson and Hodges, 1964; and Nutrition Reviews, 1965).

The phospholipids in blood serve several functions: they are a part of cell membranes and contribute to the structure of the cells; they serve as a form of storage for fatty acids, phosphorus and nitrogenous bases; they function as intermediates in the transport, absorption and metabolism of fatty acids; and they are involved in the blood clotting mechanism (Hanahan, Gurd and Zabin, 1960). Phospholipids have been found to be one of the important factors involved in the process of thrombus formation (Monkhouse, 1960; and Troup et al., 1960). PhS and PhE seem to accelerate the clotting of blood (Kazal, 1965; and Kerr et al., 1965), whereas PhC has an inhibitory effect (Antar et al., 1964).

To the knowledge of the author, there are no data available on the interrelationships of individual phospholipids in the blood fractions of single individuals. The aim of this study was to develop a micromethod which would allow the isolation and quantitation of phospholipid components in the four fractions of blood. The method would then be used to analyze blood obtained from a limited group of healthy subjects. This investigation is part of a larger study in which the

concentrations of the amounts of total lipid, total phospholipid, and free and total cholesterol will also be determined.

REVIEW OF THE LITERATURE

Phospholipids in Blood Serum

Phospholipids in serum make up about 30 percent of the total lipid (Albritton, 1952; and Smith, 1965) and consist mainly of LPhC, PhC and Sph. One of the major functions of serum phospholipids is to stabilize the emulsion of lipids. The values reported for total phospholipid in normal subjects show some variation (Doizaki and Zieve, 1963; Goodwin, 1959; Jensen et al., 1965; Phillips, 1958; and Smith, 1965); however, the relative distributions of the individual phospholipids were rather constant (Doizaki and Zieve, 1963; Gjone, Berry and Turner, 1959; Hagopian and Robinson, 1965; and Williams et al., 1966).

Most of the mean values for total phospholipids reported within the past eight years were within the range of 180 to 220 mg per 100 ml of serum (Goodwin, 1959; Jensen et al., 1965; Phillips, 1958; and Smith, 1965). Higher values, however, such as 288 mg per 100 ml have been reported (Doizaki and Zieve, 1963).

Choline-containing phospholipids, i.e., LPhC, PhC and Sph, comprise more than 90 percent of the total (Marinetti et al., 1959; Phillips, 1958; Vogel, Zieve and Carleton, 1962; and Williams et al., 1966). Of these, PhC is present in the largest amount making up about 70 percent of the total. The remaining five percent or less

consists almost entirely of ethanolamine-containing phospholipids. Serine- and inositol-containing compounds are virtually absent. The values for individual phospholipids in serum, as reported by several investigators, are listed in Table I. The ranges of the mean values for the components were quite small. Of the studies summarized the mean values for individual phospholipids were within the following ranges: LPhC, 8 to 11 percent; Sph, 17 to 23 percent; PhC, 65 to 70 percent; and ethanolamine-containing phospholipids, 2 to 5 percent. The reported distributions of plasma phospholipids were very similar to those found in serum (Ansell and Hawthorne, 1964; Biezenski, 1964; Dawson, Hemington and Lindsay, 1960; and Marinetti et al., 1959).

Some variation in values of total phospholipid and phospholipid components have been reported as a result of high-sugar diets and various diseases. The PhE:total phospholipid ratio increased in the serum of subjects on diets high in simple sugars while the PhC:total phospholipid ratio decreased (Antar et al., 1964). In other studies with subjects on high sucrose diets, an increase in serum Sph was also observed along with increases in PhE (Nutrition Reviews, 1965). In patients with mild coronary disorders the distribution of phospholipids was quite similar to normal controls. However, in severe cases, the percentage of PhC was slightly less than the normal values (Marinetti et al., 1959).

TABLE I. DISTRIBUTION OF PHOSPHOLIPIDS IN HUMAN SERUM REPORTED IN THE LITERATURE.

	Phospholipids[1]									
	Sph	LPhC	PhC	C. Pl	PhS	S. Pl	LPhE	PhE	E. Pl	PhI
	Percent of Total Lipid Phosphorus									
Doizaki and Zieve, 1963	22	11	65					2		
Gjone et al., 1959	18	9	66							
Hagopian and Robinson, 1965	20	11	66					3		
Marinetti et al., 1959	21	9	67							
Phillips, 1958	19	7	69		trace			5		
Vogel et al., 1962	23	10	66					3		
Williams et al., 1966	17	8	70	1	trace		trace	1	1	1

[1] Sph: sphingomyelin
LPhC: lysophosphatidylcholine
PhC: phosphatidylcholine
C. Pl: choline plasmalogen
PhS: phosphatidylserine
S. Pl: serine plasmalogen
LPhE: lysophosphatidylethanolamine
PhE: phosphatidylethanolamine
E. Pl: ethanolamine plasmalogen
PhI: phosphatidylinositol

Phospholipids in Red Cells

The phospholipids of red cells differ in concentration, distribution and function from those in serum. Red cell phospholipids account for about 60 percent of the total lipid (Albritton, 1952; and Smith, 1965) and consist mainly of Sph, PhC, PhS and PhE. They act as structural components and are found almost exclusively in the membrane.

The mean values of total phospholipids in normal red cells are quite constant (Bradlow, Lee and Rubenstein, 1965; Erickson, 1940; Farquhar and Ahrens, 1963; Phillips, 1958; Reed et al., 1960; and Smith, 1965). The distribution of individual phospholipids, however, shows more variation (Axelrod, Reichenthal and Brodie, 1953; Dawson et al., 1960; Hanahan, Watts and Pappajohn, 1960; and Phillips and Roome, 1959). Most of the mean values reported for total phospholipids range from 290 to 350 mg per 100 ml of red cells (Albritton, 1952; Bradlow et al., 1965; Erickson, 1940; Farquhar and Ahrens, 1963; Reed et al., 1960; and Smith, 1965).

Although there is an active exchange between plasma and red cell phospholipids (Sakagami et al., 1965; and Farquhar and Ahrens, 1963), the distribution of individual phospholipids in these blood fractions is quite different. In contrast to the relatively simple composition of plasma phospholipids, those in red cells are a more complex

mixture. Erythrocytes contain more PhE and PhS and less PhC than do plasma phospholipids. The distribution of red cell phospholipids, as reported by several investigators, are presented in Table II. The choline-containing components make up about two-thirds of the phospholipids present. The remaining consist chiefly of ethanolamine-containing phospholipids with smaller amounts of serine- and inositol-containing components. Mean values for individual components, as percent of total phospholipid, have been reported within the following ranges: LPhC, 2 to 3 percent; Sph, 10 to 25 percent; PhC, 30 to 36 percent; PhS, 5 to 15 percent; PhE, 18 to 29 percent; PhI, 2 to 8 percent; and PhA, 1 percent or less.

A number of diseases influence the phospholipid content of erythrocytes (Behrendt, 1957; Bradlow et al., 1965; and Phillips and Roome, 1962). Total phospholipid content was reported to be above normal in sickle cell anemia (Behrendt, 1957; and Phillips and Roome, 1962), intermediate thalassemia (Phillips and Roome, 1962) and erythroblastic anemia (Behrendt, 1957), whereas values below normal were found in pernicious anemia (Behrendt, 1957; and Phillips and Roome, 1962), sprue (Phillips and Roome, 1962), hypochromic anemia, and hemolytic anemia (Behrendt, 1957).

Phospholipids in White Cells

There is little information in the literature on phospholipids in

TABLE II. DISTRIBUTION OF PHOSPHOLIPIDS IN HUMAN RED CELLS REPORTED IN THE LITERATURE.

	Phospholipids									
	Sph	LPhC	PhC	C. Pl	PhS	S. Pl	PhE	E. Pl	PhI	PhA[1]
	Percent of Total Lipid Phosphorus									
Axelrod et al., 1953			64			15		26		
Bradlow et al., 1965	25	3	31		13		28			
Dawson et al.; 1960	16		36	6	5	trace	18	6		1
Farquhar and Ahrens, 1963	21		36		10		29			
Hanahan, Watts and Pappajohn, 1960	10		45			2		30	8	
Phillips and Roome, 1959	23	2		33			43			
Reed et al., 1960	22	3	30		15		25		4	
Ways and Hanahan, 1964	24		30		15		26		2	

[1] PhA: phosphatidic acid

human leucocytes. The total phospholipid content of normal human white cells has been reported by some (Barkhan et al., 1960; Boyd, 1933; Boyd, 1936; and Smith, 1965). However, reports of the distribution of individual phospholipids in normal human leucocytes have not been found. The distribution of phospholipid components in leukemic cells of humans and polymorphonuclear leucocytes of animals, as reported in the literature, are presented in Table III.

The total lipid of normal human leucocytes has been reported to contain from 40 to 67 percent phospholipid (Malamos et al., 1962; and Smith, 1965). There is considerable variability among the mean values of total phospholipids which have been reported. Earlier studies found 804 to 870 mg of phospholipid per 100 gm with most of the values ranging from 400 to 1400 mg per 100 gm of white cells (Boyd, 1933; and Boyd, 1936). In a recent study on sixteen normal subjects Smith (1965) reported values ranging from 1570 to 2770 with a mean of 2050 mg per 100 gm of leucocytes.

The phospholipid content of white cells is influenced by leukemic conditions. Malamos et al. (1962) reported a higher phospholipid content in lymphatic leukemic leucocytes than in normal cells and associated the rise in phospholipid content with the increased metabolic activity of the leukemic cells.

TABLE III. DISTRIBUTION OF PHOSPHOLIPIDS IN WHITE CELLS OF HUMANS AND ANIMALS REPORTED IN THE LITERATURE.

Source	Phospholipids						Investigator
	Sph	PhC	PhS	PhE	PhI	PhA	
	Percent of Total Lipid Phosphorus						
Human							
leukemic leucocytes	10	42	9	29	6	4	Firkin and Williams, 1961
Guinea Pig							
polymorphonuclear leucocytes[1]	18	35	4	35	4	4	Karnovsky and Wallach, 1961
Rabbit							
polymorphonuclear leucocytes	29	32	39				Burt and Rossitter, 1950

[1] Figures represent "fractions" rather than pure phospholipids, i.e., PhC fraction.

Phospholipids in Blood Platelets

Since investigators have become interested in the thromboplastic activity of platelets as associated with their PhS and PhE content, data on platelet phospholipid distributions have been reported (Barkhan et al., 1961; Iacono, Zellmer and Malott, 1966; and Troup et al., 1960). Phospholipids have been found to comprise from 64 to 78 percent of the total lipid in platelets (Barkhan et al., 1961; Lee and Erickson, 1938; Smith, 1965; and Woodside and Kocholaty, 1960). The absolute amounts of total phospholipid have been reported to be 12 to 13 percent of the dry weight (Barkhan et al., 1961; and Lee and

Erickson, 1938) and 1655 mg per 100 gm of wet weight (Smith, 1965).

The phospholipids of platelets consist mainly of Sph, PhC and PhE with lesser amounts of PhS and PhI. The distributions of individual phospholipids which have been reported are summarized in Table IV. Iacono et al. (1966) presented their data as percent of total phospholipid. However, the values for the other studies included in Table IV were calculated on the basis of data given for the composition of total lipid.

TABLE IV. DISTRIBUTION OF PHOSPHOLIPIDS IN HUMAN PLATELETS REPORTED IN THE LITERATURE.

	Phospholipids				
	Sph	PhC	PhS	PhE	PhI
Investigator	Percent of Total Phospholipid				
Barkhan et al., 1961[1]	58		11[2]	28[2]	3
Iacono et al., 1966	19	32	14	20	10
Troup et al., 1960[1]	18	44	8	23	7

[1] Calculated from author's figures for average composition of total lipid.

[2] Barkhan et al., estimated PhS and PhE from values obtained for PhE + PhS (PhI).

METHODOLOGY

Review

Phospholipids have been separated into individual components by paper, thin-layer and column chromatography. Thin-layer chromotography is the method of choice of many workers in this area (Bradlow et al., 1965; Doizaki and Zieve, 1963; Parker and Peterson, 1965; and Phillips and Roome, 1962). Some of its advantages are speed, compactness of spots, and clear separations without "tailing", i.e., without elongation of spots in the direction of the point of origin.

The application of thin-layer chromatography to the separation of phospholipids has been reviewed by Ansell and Hawthorne (1964); Hanahan, Gurd and Zabin (1960); and Mangold (1965). The separations obtained are a result of the interrelationships of the adsorbent, the developing solvent, and the amount and kind of specific phospholipid being separated. The adsorbent commonly consists of a thin layer of Silica Gel G or Silica Gel H which is less than one millimeter in thickness. Although phospholipids may be separated by adsorption or partition chromatography, adsorption is more widely used. Phospholipids are most commonly resolved by relatively polar developing solvents consisting of combinations of chloroform, methanol, and water alone or in combination with acids or bases. Some of the developing solvents which have been used to effectively

separate phospholipids are summarized in Table V.

Some of the phospholipids which have been separated by thin-layer adsorption chromatography are lysophosphatidylcholine (LPhC), sphingomyelin (Sph), phosphatidylcholine (PhC), phosphatidylinositol (PhI), phosphatidylserine (PhS), phosphatidylethanolamine (PhE), and phosphatidic acid (PhA). Several investigators have separated most of these phospholipids (Abramson and Blecher, 1964; Biezenski, 1964; Parker and Peterson, 1965; Skidmore and Entenman, 1962; and Skipski, Peterson and Barclay, 1964), whereas others have separated only the major phospholipids (Bradlow et al., 1964; De Bohner, Soto and De Cohan, 1965; and Wood et al., 1964). Examples of the degree of isolation of individual phospholipids which investigators have achieved are presented in Table VI. The relative positions of the phospholipid components varied with the use of different developing solvents. The separations were also a reflection of the phospholipid content of the materials analyzed. PhS and PhI seldom have been isolated in studies on blood serum due to the small amounts present.

Development of the Micromethod

The aim in the development of this microprocedure was to achieve sufficient resolution of 25 to 40 μg of total phospholipid to facilitate the isolation of individual phospholipid components for quantitative analysis. The separation of this small amount of phospholipid would

TABLE V. SOME DEVELOPING SOLVENTS USED IN THIN-LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS.

Investigator	Developing Solvent	Proportions (v/v)
Wood et al., 1964	Chloroform-methanol-water	80:35:5
Stahl, 1965	Chloroform-methanol-water	65:25:4
Doizaki and Zieve, 1963	Chloroform-methanol-water	80:25:3
De Bohner et al., 1965	Chloroform-methanol-30% ammonia	14:6:1
Skipski et al., 1964	Chloroform-methanol-glacial acetic acid-water	25:15:4:2
Biezenski, 1964	Chloroform-methanol-glacial acetic acid-water	45:30:6:2
Duthie and Patton, 1965	Chloroform-methanol-glacial acetic acid-water	100:50:16:2
Skidmore and Entenman, 1962	Chloroform-methanol-7M ammonium hydroxide " " " " "	60:35:5 35:60:5
Abramson and Blecher, 1964	Chloroform-methanol-glacial acetic acid-water Chloroform-methanol-7M ammonium hydroxide	250:74:19:3 230:90:15

TABLE VI. PHOSPHOLIPIDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY.

Investigator	Source of Phospholipid	LPhC	Sph	PhC	PhI	PhS	PhE	PhA
One-Dimensional Chromatography								
Biezenski, 1964	Human plasma	X	X	X	X	X	X	X
Bradlow et al., 1965	Human red cells	X	X	X		X	X	
De Bohner et al., 1965	Rat liver		X	X	X		X	
Doizaki and Zieve, 1963	Human serum	X		X			X	
Parker and Peterson, 1965	Rat liver	X	X	X	X	X	X	
Skipski et al., 1964	Rat serum	X	X	X			X	
	Rat liver	X	X	X	X	X	X	X
Wood et al., 1964	Human plasma	X	X	X			X	
Two-Dimensional Chromatography								
Abramson and Blecher, 1964	Synthetic standards	X	X	X	X	X	X	X
Skidmore and Entenman, 1962	Rat liver	X	X	X	X	X	X	X

be appropriate for white blood cell and platelet samples. A phospholipid standard consisting of a solution of weighed amounts of purified Sph, PhC, PhS and PhE[1] was used to investigate the effectiveness of various procedures for separation. Resolutions were compared on both standard-sized plates, 20 x 20 cm, and microchromatoplates, i.e., microscope slides, 2.5 x 7.6 cm.

Separation of Phospholipids on Standard Thin-Layer Chromatography Plates

Although it was questionable whether the sample size could be reduced sufficiently, standard-sized plates were used to develop technique and observe the characteristics of the various developing solvents used by others. The patterns of separation varied when the standard was dissolved in different solvents, i.e., chloroform or petroleum ether. Therefore, all standards were dissolved in chloroform.

Various developing solvents, one- and two-dimensional chromatography, and alterations in the adsorbent composition were tried in an effort to achieve good resolution. Although separations were obtained when a number of developing solvents were used, the solvents of Doizaki and Zieve (1963) and Skipski et al. (1964) brought about the best resolutions. However, "tailing" was evident and neither PhS

[1] Applied Science Laboratories, Inc.

nor Sph were sufficiently resolved. Two-dimensional chromatography as described by Abramson and Blecher (1964) and Skidmore and Entenman (1962) brought about adequate separation for identification, but resolution was insufficient for isolation. "Basic" plates as described by several (Biezenski, 1964; Skipski et al., 1964; and Abramson and Blecher, 1964) were also investigated. There was no apparent improvement in separation on the "basic" plates as compared with the standard "neutral" plates.

Upon examining the results obtained and amount of sample applied, it became evident that 20 x 20 cm plates would be inappropriate for this study. At least 150 μg of phospholipid mixture had to be applied to bring about clear visualization of the individual components; the phospholipid present in the smallest quantity was then 20 μg. This amount of phospholipid sample was too large for the amounts of leucocyte and platelet fractions to be analyzed. Therefore, microchromatoplates were investigated.

Separation of Phospholipids on Microchromatoplates

With the use of microchromatoplates total lipid has been separated into as many as five or six components (Peifer, 1962). Microchromatoplates were tried in this study in an attempt to resolve small quantities of total phospholipid. The developing solvents for these plates were always freshly made and the atmosphere within the

developing chamber was saturated before the plates were introduced. Coplin staining dishes with covers were used as the developing chambers. The individual spots were detected by exposure to iodine vapor. Amounts ranging from 30 to 60 μg of phospholipid were separated by various developing solvents; this was comparable to one-fourth to two-fifths the amount needed for separations on the standard-sized plates.

Microchromatoplates Prepared by a Modification of the Method of Peifer. In this study microchromatoplates were initially prepared according to a modification of Peifer's (1962) original method. Peifer prepared the microchromatoplates with Silica Gel G and subjected them to steam prior to air-drying in order to decrease adsorbent fragility. Since Silica Gel H was used in this study, the steaming was omitted. Although it was difficult to maintain uniform thickness on the various plates, only those plates which appeared to be uniformly coated were used for analyses.

Of the developing solvents tried, the solvent of Doizaki and Zieve (1963), chloroform-methanol-water 80:25:3 (v/v), brought about the best resolution. However, it was impossible to obtain reproducible results, there was considerable "tailing" and PhS did not separate from the other phospholipid components.

Upon critically examining the microchromatoplates which

produced similar separations it was observed that the best reproducibility was achieved on those plates with adsorbent layers which were evenly coated and of similar thickness. Since adsorption is a concentration-dependent process and the first monolayers of the adsorbed material have higher heats of adsorption than the succeeding layers, the thickness of a plate determines the amount which can be separated effectively. Therefore, a sample may exceed the adsorptive capacity of a thinner plate which results in poor separation, "tailing" or smearing whereas it can be separated on a thicker plate.

Microchromatoplates Prepared by a Modification of the Method of Stahl. In an attempt to produce microchromatoplates with more uniform layers of adsorbent, the procedure according to Stahl (1965) was modified. The microchromatoplates were rinsed with acetone and placed on top of standard-sized plates which were in place on the aligning tray. A slurry of 20 gm of Silica Gel H and 72 ml of redistilled water was applied with the Desaga applicator. The microchromatoplates were then air-dried for one-half hour. The plates seemed to be evenly coated and to contain adsorbent layers of uniform thickness.

Although several developing solvents were investigated, adequate separations were obtained in only a few instances and these were, again, not reproducible. In none of the resolutions was PhS separated.

In general, the reproducibility was not greatly improved when microchromatoplates were prepared with Stahl's equipment. Although the plates appeared to contain uniform layers of adsorbent, this apparently was not the case.

Pre-Coated Microchromatoplates. In an attempt to obtain plates with adsorbent layers of uniform thickness, microchromatoplates which were pre-coated with Silica Gel H were obtained.[2] The adsorbent layer, which was 150 μ in thickness, was very uniformly coated. However, it was not as thick as the plates which were previously prepared, and developing solvents reacted somewhat differently.

With the comparatively polar developing solvent of Biezenski (1964), chloroform-methanol-glacial acetic acid-water 45:30:6:2 (v/v), very poor separation resulted; therefore, less polar solvents were investigated. Resolutions were improved with the solvent of Duthie and Patton, i. e., chloroform-methanol-glacial acetic acid-water 100:50:16:8 (v/v). Several less polar developing solvents were tried and separations became progressively better until the best resolution was obtained with a solvent consisting of chloroform-methanol-glacial acetic acid-water 75:25:8:4 (v/v). The reasons for selecting this developing solvent were the even distribution of components from

[2] Schaar Chemical Co., Chicago, Ill.

point of origin to solvent front, the lack of "tailing," and reproducibility.

One of the limiting factors of the developing solvent chosen for use in this study was that PhS was not separated. Although a number of investigators have separated PhS (Biezenski, 1964; Bradlow et al., 1965; Parker and Peterson, 1965; and Skipski et al., 1964), others have reported a PhS-PhI fraction (De Bohner et al., 1965; and Redman and Keenan, 1964) or have not separated or identified it (Mangold, 1965). Standard-sized plates were used in most of the studies reported in the literature. Since developing solvents react differently on microchromatoplates than on the standard plates, the conditions of this study were unique.

The pattern of movement of PhS was investigated when phospholipid mixtures containing PhS were chromatographed with the developing solvent used in this study. The R_f value, i.e., the distance a substance migrates from the point of origin divided by the distance the solvent front migrates from the origin, of PhS was determined. A standard of PhS was applied on the same plate with a standard phospholipid mixture. The pattern of migration of PhS was almost identical with that of PhC.

In an attempt to reaffirm the location of PhS, ninhydrin spray was used for detection. Although the area in which PhC was located reacted positively to ninhydrin, it is recognized that small amounts

of amino-acid containing phospholipids other than PhS, i.e., certain degradation products, might have had a slight influence on this reaction. However, the R_f value as compared to the phospholipid standard mixture and the positive ninhydrin reaction were taken as evidence that PhS had an R_f value comparable to PhC under the conditions of this study.

A small amount of phosphorus-containing compounds nearly always remained at the point of origin when phospholipids were chromatographed. This has been observed by others using different developing solvents (Abramson and Blecher, 1964; Doizaki and Zieve, 1963; Skidmore and Entenman, 1962; and Skipski et al., 1964). Although most of the investigators considered this to be lipid phosphorus (Abramson and Blecher, 1964; Doizaki and Zieve, 1963; and Skidmore and Entenman, 1962), Skipski et al. (1964) considered this phosphorus as nonlipid in nature. In this study it was termed lipid phosphorus and calculated as a percent of the total lipid phosphorus.

Although this method had limitations, it separated phospholipid mixtures which were approximately one-fourth as large as those required for standard-sized plates. Acceptable separations were obtained when 25 to 40 μg of total phospholipid was applied. However, amounts approximating 80 μg exceeded the adsorptive capacity of the adsorbent layer and poor separations resulted.

Elution

Phospholipids are commonly quantitated by analysis of their phosphorus content and subsequent calculation by the use of an appropriate factor. Phosphorus determinations have been carried out on phospholipids which have been eluted from silica gel (Abramson and Blecher, 1964; Biezenski, 1964; and De Bohner et al., 1965) or digested with it (Doizaki and Zieve, 1963; Parker and Peterson, 1965; and Shen and Dyroff, 1962). The presence of silica gel in digested samples has been reported to cause interference (Doizaki and Zieve, 1963; and Shen and Dyroff, 1962). Recoveries of 95 to 108 percent of the lipid phosphorus have been cited when phospholipids ranging in amounts from 100 to 625 μg were eluted (Abramson and Blecher, 1964; Biezenski, 1964; De Bohner et al., 1965; and Skipski et al., 1964).

Many different methods of elution have been used. Because of the simplicity of the procedure and the high degree of recovery reported by Biezenski (1964), his method was used in this study. The method uses ethanol-chloroform-glacial acetic acid-water 100:30:20:2 (v/v) as the eluting solvent. The samples were extracted twice, using 2 ml and 1 ml, respectively, of eluting solvent. Extractions were facilitated by heating for 30 minutes in a 40° water bath.

For good phospholipid recovereis, it is recommended that the plates be exposed to iodine for a minimum of time, that silicic acid be removed from the plates rapidly, and that the eluting be done as quickly as possible (Skipski et al., 1964; and Wood et al., 1964). These suggestions were adapted as procedures in this study.

Micromethod for the Isolation and Quantiation of Phospholipid Classes of Blood Fractions

Equipment

1. Pipets, graduated, 1, 2, 5 and 10 ml.
2. Transfer pipet.
3. Graduated cylinder, 25 ml.
4. Volumetric flasks, 2 ml.
5. Centrifuge tubes, 35 ml, glass-stoppered.
6. Funnels, 3 cm upper edge diameter.
7. Microchromatoplates, pre-coated with Silica Gel H, 150 μ thick (Schaar Scientific Co., Chicago, Ill.).
8. Forceps.
9. Razor blades, single edge.
10. Coplin staining dishes (Central Scientific Co. #48050).
11. Iodine chamber, small jar with cover, corrugated polyethylene on bottom to prevent slippage.
12. 40° water bath.

13. Centrifuge.

14. Mettler micro balance, Model M5.

Reagents

1. Acetic acid, glacial (A. C. S.).

2. Chloroform, reagent grade, redistilled.

3. Ethanol, absolute, redistilled.

4. Methanol, absolute. Methanol was redistilled over potassium hydroxide.

5. Water, redistilled.

6. Developing solvent; chloroform-methanol-glacial acetic acid-water 75:25:8:4 (v/v).

7. Eluting solvent according to Biezenski (1964); ethanol-chloroform-water-glacial acetic acid 100:30:20:2 (v/v).

8. Iodine crystals, 'Baker Analyzed' reagent.

9. Nitrogen gas.

10. Phospholipid standards, purified: LPhC, lot #589-11; Sph (beef brain), lot #656-17; PhC (egg), lot #656-11; PhS, (beef), lot #656-41; and PhE (plant), lot #656-39 (Applied Science Laboratories, Inc., State College, Penn.)

11. Standards adjusted to contain the approximate proportions of the major phospholipids found in serum (Standard I), red cells (Standard II), and white cells and platelets (Standard III).

Individual Phospholipid Standards	Percent by Weight: Standard I (Serum)	Standard II (Red Cells)	Standard III (White Cells and Platelets)
Sph	23	13	12
PhC	76	43	51
PhS	0	16	6
PhE	2	28	31

Procedure

Any previously isolated phospholipid sample of known phosphorus content may be separated into its phospholipid components by this method. A standard which was adjusted to contain approximately the proportional amounts of individual phospholipids commonly found in the sample was spotted concurrently with each sample. The standard phospholipid mixtures were made by combining weighed amounts of individual phospholipid standards and dissolving them in a given volume of chloroform.

Application of the Sample. Microchromatoplates, 2.5 x 7.6 cm, which were pre-coated with Silica Gel H were used; they were not activated prior to use. Two or three microchromatoplates were treated identically. The plates were divided into two vertical lanes approximately 1.2 cm wide by scraping the silica gel from an area near the center with a pair of forceps. Both the sample and the

standard were applied to the microchromatoplate under a continuous stream of nitrogen. They were applied approximately one centimeter from the lower edge of the plate with a calibrated micropipet. Amounts ranging from 15 to 40 μg of sample were chromatographed in the left lane and 30 to 50 μg of standard were separated in the right lane.

Development of the Microchromatoplates. The freshly made developing solvent which consisted of chloroform-methanol-glacial acetic acid-water 75:25:8:4 (v/v) was thoroughly mixed immediately prior to use. The atmosphere of the developing chamber was equilibrated 15 minutes before the plates were introduced. The solvent front was allowed to ascend to approximately 6.5 cm from the lower edge of the plate.

Detection of the Phospholipid Components. The microchromatoplates were removed from the developing chamber and air-dried for 30 seconds. The components were revealed by exposure to iodine vapor. The plates were exposed to the iodine vapor only until the components were just visible. Each individual phospholipid was outlined with a razor blade. The individual spots were identified by comparing their R_f values with those of the mixed standard on the same plate.

Elution. Extraneous silica gel below the point of origin, above the solvent front, and along the vertical sides of the microchromatoplate was removed and discarded. When the iodine had evaporated each spot was individually scraped with a razor blade into a separate 35 ml glass-stoppered centrifuge tube. The use of funnels facilitated a complete transfer. Those areas devoid of spots were discarded except for an area free of phospholipid approximating the size of the spots which was used for analysis as the silica gel blank.

The eluting solvent consisted of ethanol-chloroform-water-glacial acetic acid 100:30:20:2 (v/v). The area on the microchromatoplate corresponding to each spot was separately washed with one-half milliliter of eluting solvent which was collected in a 35 ml glass-stoppered centrifuge tube. Each funnel was also carefully washed with approximately 1 1/2 ml of the eluting solvent so that each individual sample was suspended in a total of 2 ml of solvent. The samples were mixed thoroughly for one minute by a mechanical mixer prior to being placed in a 40° water bath for 30 minutes. Due to expansion of the eluting solvent while the samples were in the water bath, the ground glass stoppers were replaced by marbles. Each sample was again thoroughly mixed for one minute with the use of a mechanical mixer near the midpoint of the 30-minute time interval.

After the extraction process in the water bath was complete, the samples were centrifuged at 2000 rpm for 15 minutes. The

supernatant solution was aspirated and transferred to a 2 ml volumetric flask using a constricted transfer pipet. The eluting process was repeated a second time using 1 ml of the eluting solvent. The contents of the volumetric flask were evaporated to approximately 1 ml with nitrogen, the 1 ml from the second extraction was added, and the volume was made to exactly 2 ml with eluting solvent.

Phosphorus Determination. Triplicate aliquots in amounts to contain approximately 0.05 micrograms of phosphorus were measured, transferred into 7 x 50 mm tubes and evaporated to dryness with nitrogen. Phosphorus was determined by an adaptation of the micromethod of Lowry et al. (1954) as adapted by Hawthorne, Smith and Pescador (1963). The percent of total lipid phosphorus was calculated for each spot. The recovery of lipid phosphorus was calculated by totaling the phosphorus content of each of the spots and comparing this sum to a direct phosphorus analysis of an aliquot of the phospholipid sample prior to chromatography.

Reliability of the Method

Individual phospholipid standards and standards adjusted to contain the major phospholipids in serum (Standard I), red cells (Standard II) and white cells and platelets (Standard III) in amounts reported in the literature were used to evaluate the method just described.

The percent recovery of total lipid phosphorus was based upon a direct analysis of phosphorus in the standard. The mean recoveries (and ranges) of individual phospholipid standards in replications of four to eight were: Sph, 93 percent (87 to 106); PhC, 99 percent (87 to 111); PhS, 92 percent (89 to 94); and PhE, 96 percent (88 to 105). The recoveries obtained on the adjusted phospholipid standards are summarized in Table VII. The mean recoveries (and ranges) of total lipid phosphorus were: Standard I, 94 percent (91 to 97); Standard II, 97 percent (91 to 103); and Standard III, 103 percent (99 to 106).

The recoveries of the individual phospholipids from the mixed standards were acceptable, with the exception of PhS. It appeared to separate somewhat when it comprised about 15 percent of the standard; however, no separation of PhS was obtained when it made up only six percent of the standard. The separation of PhS, within the conditions of this study, was dependent upon a very sensitive interrelationship of the factors which influence resolution.

Although all standards were stored in a nitrogen atmosphere at 0^{o}, partial degradation may explain some of the slight differences between the theoretical and analyzed values. Some degradation products, e. g., lysophospholipids, have chromatographic properties which are different from those of the original di-acylated phospholipids.

One of the limitations of a method such as this is that plasmalogens are not separated from their corresponding di-acylated

TABLE VII. RECOVERY STUDIES ON PHOSPHOLIPID STANDARDS.

Phospholipid Standards	Number of Replications	Actual Phospholipid Composition	Determined Phospholipid Composition Mean (Ranges)	Recovery of Total Lipid P.[1] Mean (Ranges)
		%	%	%
Standard I[2] (Serum)	2			94 (91-97)
Point of Origin		0	6 (3-9)	
Sph		23	23 (20-25)	
PhC		76	67 (64-70)	
PhE		2	4 (2-6)	
Standard II[3] (Red Cells)	3			97 (91-103)
Sph		13	12 (10-14)	
PhC		43	46 (44-47)	
PhS		16	11 (9-13)	
PhE		28	31 (29-32)	
Standard III[4] (White Cells and Platelets)	4			103 (99-106)
Sph		12	14 (13-14)	
PhC		51	52 (50-53)	
PhS		6	0 -	
PhE		31	34 (32-35)	

[1] P. represents phosphorus.

[2] Standard contains phospholipids in amounts commonly found in serum.

[3] Standard contains phospholipids in amounts commonly found in red cells.

[4] Standard contains phospholipids in amounts found in leukemic white cells and platelets as reported by limited investigations.

phospholipids, e.g., choline plasmalogen from phosphatidylcholine. Since plasmalogens are only slightly less polar than the diester phospholipids, they are usually concentrated at the upper edge of the spots of their corresponding di-acylated phospholipids (Bradlow et al., 1965; and Mangold, 1965).

EXPERIMENTAL PROCEDURE

Description of Subjects

Four men and four women ranging in ages from 24 to 53 years were used as experimental subjects. They were all in generally good health and active professionally. However, two subjects reported organic disorders of sufficient magnitude to require daily hormone therapy; subject TB had an hypoactive thyroid and took thyroxin daily, and subject MR took estrogenic hormones daily.

Data on the sex, age, height and weight for the subjects are given in Table VIII. Red and white cell counts, differential counts, hemoglobin values and sedimentation rates are presented in Table IX; these data were obtained to provide supporting evidence that the blood used for analyses was normal. Most of the values cited in Table IX were within the normal ranges reported in the literature (Albritton, 1952; and Wintrobe, 1961).

Procedure

Blood was collected from the antecubital vein of subjects who had fasted overnight for at least 12 hours. About 100 ml of blood was taken for the isolation of serum, erythrocytes, leucocytes and platelets.[3] Standard clinical procedures were used for red and white cell,

[3]Blood was collected by Adelle Anderson, Oregon State University Student Health Service.

TABLE VIII. SEX, AGE, HEIGHT AND WEIGHT OF EIGHT SUBJECTS.

Subject	Sex	Age Years	Height cm	Weight Kg
TB[1]	F	26	168	55
SP	F	30	163	52
MR[2]	F	43	168	73
BH	F	45	175	71
DC	M	24	183	68
JR	M	25	184	75
RD	M	26	173	59
PW	M	53	178	85

[1]Subject reported hypoactive thyroid and took thyroxin daily.

[2]Subject took estrogenic hormones daily.

and differential counts, hemoglobin determinations and sedimentation rates.[4]

Blood fractions were isolated according to the procedure of Smith (1965).[5] About 3 ml each of serum and red cells and 70 mg wet weight each of white cells and platelets were isolated.

Total lipid was extracted from each blood fraction according to

[4]Red and white cell counts, differential counts, hemoglobin determinations and sedimentation rates were done by technologists at Oregon State University Student Health Service.

[5]Isolation of the blood fractions was done by Sookja Park.

TABLE IX. RED AND WHITE CELL COUNTS, DIFFERENTIAL COUNTS, HEMOGLOBIN CONTENT AND SEDIMENTATION RATES OF EIGHT SUBJECTS.

			Leucocytes					Sedimentation Rate (minutes)	
Subject	Red Cells per cmm	White Cells per cmm	Polymorphonuclear Neutrophils %	Lymphocytes %	Monocytes %	Eosinophils %	Hemoglobin gm/100 ml	15	60
TB	3.5×10^6	5.2×10^3	59	35	4	1	11.6	1	7
SP	3.9	5.6	62	32	0	5	12.0	1/2	4
MR	4.4	7.2	69	26	4	1	14.4	1/2	8
BH	4.1	6.4	75	21	2	1	13.2	1/2	3
DC	--[1]	5.2	70	29	0	1	13.6	1/2	1
JR	4.3	6.0	66	31	1	2	14.0	1/2	1
RD	4.0	7.5	67	31	1	0	13.2	1/2	2
PW	4.7	5.5	69	30	0	1	13.0	1/2	4

[1] Red cell count was not taken.

Smith's (1965) modification of the method of Folch, Lees and Sloane-Stanley (1957).[6] The total lipid was dissolved in exactly one milliliter of solvent and measured portions were used for the isolation of total phospholipid and for the determination of total lipid phosphorus. The total phospholipids were isolated by preparative thin-layer chromatography (Maier and Mangold, 1964) on standard-sized plates and eluted according to the method of Biezenski (1964) as previously described. Aliquots which were estimated to contain from 25 to 40 μg of total phospholipid were separated into their individual phospholipid components and quantitated using the method developed in this laboratory as described previously. The percent of each phospholipid class was calculated as a percent of total lipid phosphorus. The percent recovery of each sample was also calculated.

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[6]Total lipid extractions were done by Sookja Park and Gail Woosley.

RESULTS AND DISCUSSION

The distributions of individual phospholipids in the blood fractions of the eight subjects are presented in Table X. The phospholipid components are cited from left to right in the order in which they were resolved on the microchromatoplates from point of origin to solvent front. The values of the individual phospholipids, expressed as percent of total lipid phosphorus, showed characteristic patterns for each blood fraction. However, some variation did exist among individual subjects.

The major phospholipid component of all blood fractions was PhC + PhS. Nearly two-thirds of the serum phospholipids consisted of PhC + PhS, whereas there was virtually no PhE present. The phospholipids of red cells contained higher proportions of Sph and PhE and a lesser amount of PhC + PhS than any other blood fraction. The distributions of phospholipids in white cells and platelets were similar. In both of these fractions PhC + PhS made up slightly over one-half of the total lipid phosphorus; Sph and PhE each made up about one-fifth. In most of the subjects PhC + PhS was present in slightly larger amounts in the white cell phospholipids, whereas platelet phospholipids contained slightly higher proportions of PhE.

The mean values (and ranges) for the phospholipid components in the serum of the eight subjects were: LPhC, 3 percent (1 to 5);

TABLE X. DISTRIBUTION OF PHOSPHOLIPIDS IN THE BLOOD FRACTIONS OF EIGHT SUBJECTS.

Blood Fraction	Subject	Percent of Total Lipid Phosphorus					
		Point of Origin	Unidentified LPhC Component		Sph	PhC + PhS	PhE
Serum	TB	5	3	13	19	61	-
	SP	2	2	13	17	68	-
	MR	4	5	21	19	51	-
	BH	4	4	12	20	61	-
	DC	-	1	9	20	66	4
	JR	4	2	12	15	68	-
	RD	1	1	14	23	61	-
	PW	4	2	10	19	65	-
	Mean	3±1.2[1]	3±1.4	13±3.4	19±2.3	63±5.2	trace
Red Cell	TB	5	2	-	26	42	25
	SP	9	3	-	30	39	19
	MR	5	5	-	30	40	20
	BH	3	3	-	34	29	31
	DC	3	1	-	34	39	23
	JR	2	2	-	29	34	33
	RD	3	4	-	32	40	22
	PW	2	1	-	33	40	25
	Mean	4±2.1	3±1.4	-	31±2.4	38±3.9	25±4.7
White Cell	TB	-	5	-	21	60	15
	SP	4	2	-	14	60	20
	MR	-	4	-	17	53	26
	BH	15	4	-	18	41	22
	DC	5	4	-	20	57	15
	JR	6	9	-	21	55	10
	RD	5	5	-	15	53	23
	PW	6	3	-	16	61	14
	Mean	5±4.3	5±1.9	-	18±2.7	55±5.9	18±5.3
Platelet	TB	-	6	-	21	52	22
	SP[2]	-	-	-	-	-	-
	MR	4	4	-	16	52	24
	BH	8	2	-	16	51	23

TABLE X. (cont.)

Blood Fraction	Subject	Percent of Total Lipid Phosphorus: Point of Origin	LPhC	Unidentified Component	Sph	PhC + PhS	PhE
	DC	5	4	-	17	53	21
	JR	5	8	-	16	50	22
	RD	5	8	-	18	54	14
	PW	4	5	-	16	54	22
	Mean	5±2.2	5±2.1	-	17±1.6	52±1.5	21±3.1

[1]Standard deviation from the mean.

[2]No platelet sample.

Sph, 19 percent (15 to 23); PhC + PhS, 63 percent (51to 68); and unidentified component, 13 percent (9 to 21). These values were similar to those reported in the literature (Table I). Some of the values reported for LPhC by others were higher. This might be explained by variations in handling and storage procedures; LPhC values have been found to nearly double during storage for one month at 0° (Vogel et al., 1962). All of the phospholipid samples in this study were analyzed within less than two weeks after the samples were obtained. There was an unidentified component present in all serum phospholipid samples which separated between LPhC and Sph. It was not possible to identify this component with any of the individual phospholipid standards used in this study.

The mean percentages (and ranges) of red cell phospholipid distribution were: LPhC, 3 percent (1 to 5); Sph, 31 percent (26 to 34);

PhC + PhS, 38 percent (29 to 42); and PhE, 25 percent (19 to 33). The values for LPhC and Sph were similar to values reported in the literature (Table II). However, the PhC + PhS component was about two-fifths of total lipid phosphorus in this study as compared to a range of two-fifths to three-fifths reported by others. Also, PhE was higher in this study comprising about one-fourth of the total lipid phosphorus, whereas others reported values of about one-sixth.

The mean values (and ranges) of phospholipid distribution in white cells were: LPhC, 5 percent (2 to 9); Sph, 18 percent (14 to 21); PhC + PhS, 55 percent (41 to 61); and PhE, 18 percent (10 to 26). The values obtained in this study for white cells agreed with the general patterns reported by others for leukemic and polymorphonuclear leucocytes.

The phospholipid distribution of platelets was in close agreement with those found in the literature (Table III). The mean percentages (and ranges) of the individual phospholipids were: LPhC, 5 percent (2 to 8); Sph, 17 percent (16 to 21); PhC + PhS, 52 percent (50 to 54); and PhE, 21 percent (14 to 24).

In the separations of all blood fractions from 0 to 15 percent of the total lipid phosphorus remained at the point of origin. In agreement with others who have reported this phenomenon (Abramson and Blecher, 1964; and Skidmore and Entenman, 1962), it was assumed that this phosphorus represented a uniform distribution of the

phospholipid sample applied to the microchromatoplate.

The distribution of phospholipid components in individual blood fractions showed definite patterns of consistency among the various subjects; however, some variability was evident. The degree of variability of the values for phospholipid components obtained in this study is expressed as standard deviation in Table X. The ranges of values obtained for an individual phospholipid within serum and red cells of different individuals were comparable to the ranges cited in the literature (Phillips, 1958; Phillips and Roome, 1959; and Ways and Hanahan, 1964). The ranges of values for white cells and platelets could not be compared to other reported ranges because there are no comparable data in the literature.

There were no consistent relationships between the values of phospholipid components and the age or sex of this limited group of subjects. However, there seemed to be some relationship among the values of PhC + PhS in the several blood fractions of an individual subject. For example, if the value for PhC + PhS was below the mean in one of the blood fractions of a subject, it was likely to be below the mean in the other fractions also, and vice versa. No consistent relationships existed between the increase in certain phospholipids and the decrease in others within an individual fraction of a given subject. For example, values for LPhC were not consistently above mean values when the values for PhC were below the mean.

The validity of the method developed was supported by the close agreement of the values obtained from duplicate microchromatoplates and the percent recovery of total lipid phosphorus. All of the corresponding phospholipids on duplicate plates agreed within ± 10 percent of their mean values, with the exception of those components which made up less than 10 percent of total lipid phosphorus. More than 80 percent of the duplicate values of phospholipids present in amounts greater than 10 percent of the total lipid phosphorus agreed within ± 5 percent of their mean.

The percent recoveries of total lipid phosphorus are presented in Table XI. The mean recoveries (and ranges) for the various blood fractions were: serum, 98 percent (93 to 102); red cells, 96 percent (93 to 102); white cells, 93 percent, (73 to 104); and platelets, 95 percent (88 to 103). In the one instance when recovery was less than 88 percent, the amount of phospholipid samples was limited; only 13 μg of total phospholipid was applied to each microchromatoplate. Recovery seemed to be adversely affected when amounts less than 15 μg of phospholipid were separated.

The data obtained in this study of eight subjects indicate that consistent differences in the patterns of phospholipids do occur among the four blood fractions. It would be desirable to expand this type of study to a greater number of subjects. Also, the method which was developed might be refined to differentiate more phospholipid

TABLE XI. RECOVERY OF TOTAL LIPID PHOSPHORUS OF SAMPLES. (expressed as percent)

Subject	Serum	Red Cells	White Cells	Platelets
TB	95	98	91	88
SP	93	96	95	--[1]
MR	100	93	92	91
BH	94	94	104	100
DC	100	97	100	103
JR	102	93	99	88
RD	97	102	92	92
PW	101	93	73[2]	100
Mean	98	96	93	95

[1]No platelet sample.

[2]Due to limited sample only 13 μg applied to each plate.

classes. At the present time, the significance of differences in phospholipid composition is not fully understood. As more becomes known of the biochemical roles of individual phospholipids, the ability to determine phospholipids concurrently in all blood fractions of subjects under various conditions of health and disease, and under various dietary regimes, may be of increasing importance.

SUMMARY AND CONCLUSIONS

The distributions of phospholipids were determined in serum, red cells, white cells and platelets of four men and four women ranging in ages from 24 to 53 years. The blood fractions were isolated and the lipid was extracted by methods previously developed in this laboratory. The total phospholipids were isolated from the lipid extract by preparative thin-layer chromatography. Individual phospholipids of each blood fraction were isolated on microchromatoplates and quantitated by the micromethod developed in this study. This method permits analysis of samples of 20 to 40 μg total phospholipid. The method was verified by analyses of phospholipid standards. The recovery and reproducibility of this method, when applied to samples, also substantiated its validity.

There were some consistencies in the distribution of phospholipids in all of the blood fractions. Small amounts of phosphorus-containing compounds remained at the point of origin in nearly all of the separations. Also, PhC + PhS was the phospholipid component consistently present in the largest proportions in the four blood fractions.

There were certain characteristic phospholipid distributions in each blood fraction. Serum phospholipids contained higher proportions of PhC + PhS than any of the other blood fractions, whereas

PhE was virtually absent in nearly all of the serum samples. The phospholipids of serum were unique among the blood fractions in consistently containing an unidentified component. Red cell phospholipids were characterized by the lowest percentages of PhC + PhS and the highest amounts of Sph and PhE among the blood fractions. The proportional amounts of phospholipids in white cells and platelets were very similar. Their PhC + PhS content was intermediate between that of serum and red cells, whereas their Sph content was quite similar to serum.

The values obtained for serum and platelet phospholipid distributions were comparable to those reported in the literature. The proportional amounts of LPhC and Sph in red cell phospholipids were similar to values cited by others; however, the values obtained in this study were slightly higher for PhE and lower for PhC + PhS than those reported by others. There were no comparable data reported for phospholipid distribution in normal human white cells; however, the values obtained in this study were of the same general pattern as those cited for leukemic and polymorphonuclear leucocytes in the literature.

Variations of values among individual subjects were comparable to those reported in the literature for serum and red cells. The reported data were insufficient to compare the variability of values obtained for white cells and platelets.

There were no consistent relationships between the distribution of phospholipids within the fractions and the age or sex of this limited group of subjects. In most of the subjects, however, there was a pattern of consistently high or consistently low proportions of PhC + PhS in all blood fractions.

Concurrent analyses of the blood fractions of subjects provide valuable information on the interrelationships of phospholipid distributions within an individual subject. This type of information may be useful in gaining a more complete understanding of human phospholipid metabolism. The data obtained in this study would be more complete if PhS had been isolated and the unidentified component in serum had been identified. However, this micromethod was reliable and reproducible for those components isolated.

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