Volume 1
Number 2

Studies on blood platelet phospholipids*

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[Received for publication June 9, 1959]

SUMMARY

Column chromatography on silicic acid was carried out on phospholipids from acetone-dried, chloroform-extracted human blood platelets. Fractions recovered had the following components: Phosphatidylethanolamine (PE) plus phosphatidylserine (PS) plus inositol phosphatide, PE plus PS, lecithin plus sphingomyelin, and PS alone. Eluates containing only phosphatidylethanolamine and phosphatidylserine were rechromatographed to obtain more complete separation. The resulting fractions appeared to contain one component, but on hydrolysis of these materials it was apparent that each was still contaminated with small amounts of the other. However, on high dilution of each of these mixtures, blood thromboplastin formation was promoted; this suggested that both PE and PS could act as platelet thromboplastic factor *in vitro*. Inhibitory activity was not noted. The presence of choline phosphatides in the fractions resulted in loss of activity. Plasmalogens were identified and estimated to comprise 23 per cent of total platelet cephalins. They were more closely associated with phosphatidylethanolamine than phosphatidylserine. Lysolecithin could not be detected in the mixed platelet extract or the separated fractions.

In previous work (1) it was shown that phospholipids from acetone-dried, chloroform-extracted human blood platelets could be separated by silicic acid paper and column chromatography. Phosphatidylethanolamine (PE), phosphatidylserine (PS), lecithin, sphingomyelin, and inositol phosphatide were resolved by a combination of these procedures. Small amounts of phosphatidylserine obtained by column chromatography could replace the entire platelet phospholipid extract in the thromboplastin generation and the prothrombin consumption tests. Phosphatidylethanolamine preparations had the same thromboplastic activity but they contained phosphatidylserine. An unidentified fraction was obtained which also possessed coagulation properties; this was tentatively identified as a phosphatidic acid.

In the present study an attempt was made to obtain further separation of phosphatidylethanolamine from phosphatidylserine by altering the column size, mode and temperature of elution, and by rechromatography of combined fractions. Paper chromatographic identification of phosphatides including plasmalogen and lysolecithin was supplemented by hydrolysis of the lipids and examination for ethanolamine and serine bases (2).

* This study was aided by a grant from the New York Heart Association.

PE and PS, isolated by repeated column chromatography, appeared pure by paper chromatography of the intact phosphatides. However, when these materials were hydrolyzed and their bases identified, it appeared that each component was still contaminated by the other. Other workers[1] (3) also have noted that PE and PS could not be obtained in chemically pure form from silicic acid columns. However, Marinetti *et al.* (4) recently reported recovery of pure PS by silicic acid chromatography of a relatively uncontaminated preparation of Folch fraction 3 from pig brain.

METHODS

Preparation of Mixed Phospholipid Extracts. Blood collection and processing was carried out by methods previously described (1). After venesection, however, all operations were performed in a cold room (8°C). Five hundred ml. of blood from a donor with a normal platelet count yielded 8 to 15 mg. of mixed platelet phospholipids.

Column Chromatography. The apparatus used has been described (1) and the procedure was basically that of Hirsch and Ahrens (5). Columns 37 cm. long and 1.2 cm. internal diameter were packed dry with

[1] D. J. Hanahan. Personal communication, 1958.

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18 g. of silicic acid.[2] Mixed platelet lipid (311 mg., 10.8 mg. phosphorus) was charged in 3.5 ml. of petroleum ether (b.p. 30°-60°C), and the first elution was made with 220 ml. of 1 per cent ethyl ether in petroleum ether, followed sequentially by 200 ml. 25 per cent ethyl ether in petroleum ether, and 350 ml. ethyl ether. These elutions were completed in 48 hours.

The phospholipids were then eluted with 450 ml. methanol in 2 ml. fractions. To preserve column integrity methanol was added very slowly to the ether at the head of the column. The phospholipid elution carried out at 20°C was completed in 24 hours but fractions were removed every half hour to storage at —20°C.

For study each fraction was taken to dryness under purified nitrogen and reconstituted in 4 ml. petroleum ether. Petroleum ether was selected because it was a more convenient solvent for paper chromatography and some of the phosphatides in the separated state were not completely soluble in methanol. Unless otherwise indicated, all calculations were based on the original 2 ml. fraction.

Rechromatography of Phosphatidylethanolamine and Phosphatidylserine Fractions. In an attempt to separate PE and PS, fraction numbers 25 to 30 (Fig. 1) were combined, suspended in 1 ml. of 20 per cent methanol in chloroform and rechromatographed at 10°C on 8 g. of silicic acid.[2] The latter was dried overnight at 115°C and washed in the column with 20 per cent methanol in chloroform for 15 hours prior to charging in the usual manner. Successive elutions were performed on the same charge as follows: 1000 ml. methanol : chloroform 1 : 4; 240 ml. methanol : chloroform 2 : 3; and 200 ml. methanol : chloroform 4 : 1 (v/v). One-ml. fractions were collected, evaporated, and resuspended in 2 ml. petroleum ether. Calculations were based on the original 1 ml. collected unless otherwise stated. The tube contents were tested for ninhydrin reactivity (4 to 5 μl. aliquots).

The concentrations of eluting solvents were varied since it was not known which proportion would most effectively partition these two fractions. However, it was found that the methanol : chloroform 1 : 4 eluted all the PE and PS applied to the column. After the first few tubes containing the column wash were discarded, the next 42 fractions, ninhydrin positive, were taken for further study. Two successive tubes were pooled for hydrolysis and paper chromatography, while the third was divided into two equal portions for the thromboplastin generation tests and phosphorus determinations.

Paper Chromatography. Usually 10 μl. from each fraction were applied to silica impregnated Whatman No. 1 filter papers, 6.5 cm. wide and 35 cm. long. Large concentrations of phospholipid were used in many instances in order to avoid missing small amounts of contaminant. When abrupt changes in the chromatographic patterns were found (appearance or disappearance of components), the run was repeated twice. Other details were the same as previously described (1) for the system using diisobutyl ketone : acetic acid : water (40 : 25 : 5, v/v/v) (6). Wet chromatograms were promptly viewed under ultraviolet light. Stains for ninhydrin and choline were used for confirmation and further identification of spots (1).

For mixed extracts and the diisobutyl ketone : acetic acid : water system (with 0.001 per cent Rhodamine 6G dye as the stain) (6), a minimum of 1.2 μg. of lipid phosphorus was required for adequate chromatograms. For detection of a single spot, at least 0.2 μg. of lipid phosphorus was required.

Hydrolysis. Estimation of ethanolamine and serine was carried out by alkaline hydrolysis and the formation of a colored derivative with dinitrofluorobenzene (2). The sensitivity of the method for measuring ethanolamine was increased by the use of microcuvettes in a Beckman spectrophotometer. Phosphorus was analyzed in duplicate by the method of Dryer *et al.* (7).

Aldehyde was determined in duplicate on 0.5 ml. aliquots with Schiff's reagent (6) and palmitaldehyde[3] as the standard. For the detection of acetal phosphatides on paper chromatograms, 2,4-dinitrophenylhydrazine in 3N HCl was used (6).

Thromboplastin generation tests (8) were performed on 0.5 ml. aliquots transferred to a test tube and taken to dryness under nitrogen. A suspension in 0.3 ml. of imidazole buffer (pH 7.35, 270 milliosmols per liter) was made immediately. This was arbitrarily designated the "undiluted" tube. The lipid was dispersed by rapid agitation with a pointed glass rod. The emulsions were grossly turbid in direct proportion to the lipid content, and settling was not observed after standing for more than 6 hours. All fractions had the same dispersion characteristics regardless of clotting activity. In the late methanol fractions in which only small amounts of lipid were present, several successive tubes were pooled for adequate study. To determine whether any lipid with clotting activity remained on the silicic acid columns after completion of the elu-

[2] Bio-Rad Laboratories, Richmond, Calif. Batch SAB-8.

[3] K & K Laboratories, Long Island City 1, N.Y. The material was supplied as the sodium bisulfite salt. The free aldehyde was liberated by the addition of excess 3N HCl to 5 g. of the salt followed by filtration and repeated washing with distilled water.

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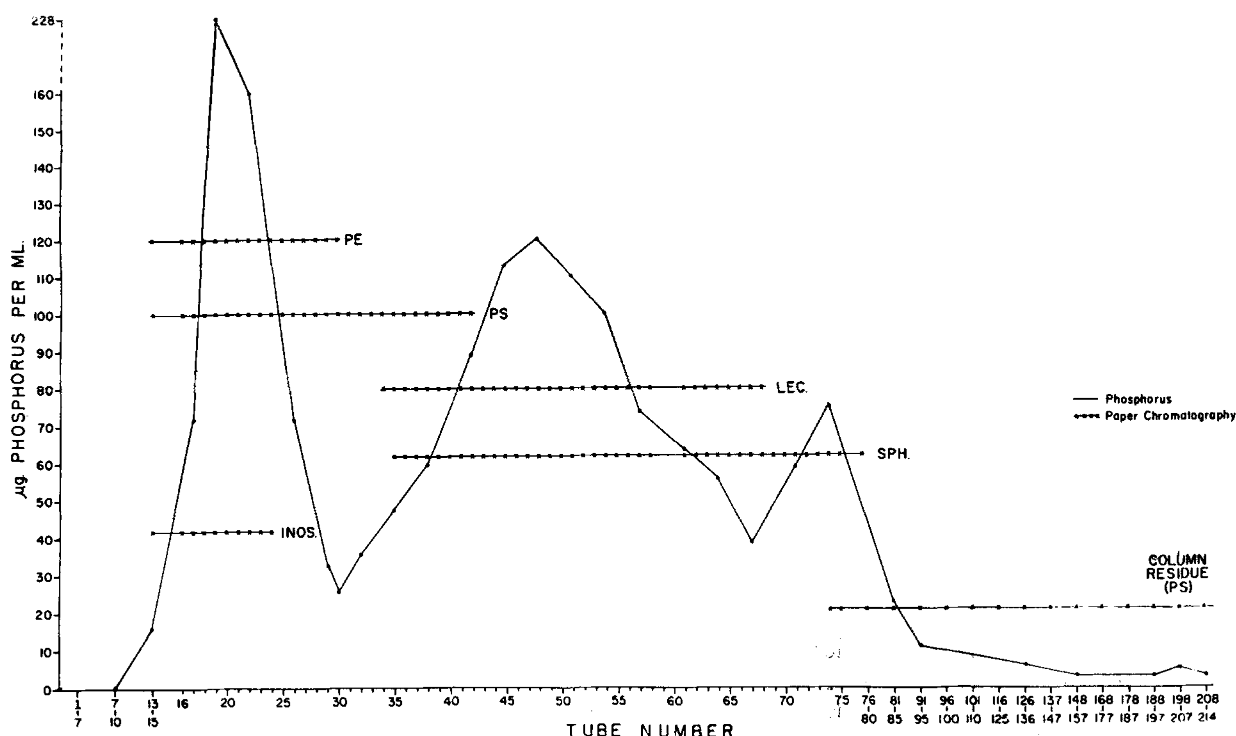

FIG. 1. Phosphorus analysis and paper chromatographic identification of original fractions eluted with methanol. Material under the first peak was ninhydrin and Schiff positive beginning with tubes 13 to 15, and had thromboplastic activity. The second peak was choline positive, ninhydrin negative, and contained Schiff reacting material in progressively decreasing quantities. These fractions did not promote thromboplastic activity. In the region of tube 75 the choline reaction became negative and clotting activity reappeared. In the paper chromatographic study, it is to be noted that the early appearance of lecithin and sphingomyelin interfered with recovery of pure phosphatidylserine. The "column residue" was almost pure phosphatidylserine. Fractions 25 to 30 were taken for rechromatography.

tions, the columns were unpacked, the silicic acid suspended in a large volume of methanol, and filtered. The filtrate was evaporated under nitrogen and tested in the thromboplastin generation test.

The prothrombin consumption tests were performed in duplicate, using plasma from the same donor throughout the study. A 12-hour fast preceded each blood collection. Platelet-free "native" plasma (no anticoagulant) was prepared as previously described (1), except that the entire procedure was carried out in a cold room. Buffer blanks and plasma alone in duplicate were used as controls. Serum prothrombin times were determined after 2 hours' incubation by the method of Ware and Stragnell (9).

The late fractions from the original methanol elution were investigated for hemolytic activity in the following manner: Approximately 50 µg. of phospholipid was evaporated to dryness and resuspended in 0.3 ml. of imidazole buffer. Dilutions were carried out in a rack of five Kahn tubes. Finally, 0.1 ml. of 1 per cent suspension of washed sheep red blood cells (10) was placed in each tube. Gross examination for hemolysis was carried out after 2 hours' incubation at 37°C.

Upon completion of coagulation tests, phosphorus analyses, hydrolysis studies, and hemolysis tests, the last 75 methanol fractions were pooled and evaporated to dryness. One mg. of material was obtained, which was analyzed for ninhydrin positive material and phosphorus.

RESULTS

Column Chromatography. The fractions eluted with ethyl ether were ninhydrin negative and gave no Liebermann-Burchard reaction.[4] A faint trace of phosphorus was found in an occasional tube.

Results of phosphorus analysis on fractions initially eluted with methanol are given in Figure 1. As shown on the abcissa, the contents of certain tubes were pooled to facilitate their study. The first peak represents the cephalin fraction and the second peak lecithin and sphingomyelin. The "column residue" refers to material eluted after the second peak began to approach baseline levels. Recovery of phosphorus from

[4] The early supernatants obtained when the whole platelets were washed in acetone gave a positive Lieberman-Burchard reaction.

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the column in the original methanol elution was 92.5 per cent (10 out of 10.8 mg.).

The milligram of lipid in the combined last 75 methanol fractions was found to contain 3.8 per cent phosphorus. Forty per cent was ninhydrin reacting material, a finding that invalidates the previous tentative identification as phosphatidic acid (1).

Paper Chromatography. Results of paper chromatograms on the individual original methanol fractions are given in Figure 1. The "column residue" had the following characteristics: It migrated as a well-defined single spot, and the staining reaction with Rhodamine G was bright blue. The R_f value was 0.6 to 0.8, the ninhydrin reaction was positive, choline was not detected, and there was a faint positive reaction with 2,4-dinitrophenylhydrazine. These properties resembled phosphatidylserine, but not phosphatidic acid or lysolecithin. The material also possessed thromboplastic activity.

The pooled original methanol fractions 195 to 207 and 210 to 214 did not exhibit hemolytic activity. Thus lysolecithin could not be found by the methods employed in this study, although this lipid has been described in serum and red cells (11).

TABLE 1. Phosphorus Analyses and Paper Chromatography of Rechromatographed Fractions

Tube Number	*µg. Phosphorus per ml.*	*Paper Chromatography*	
		PE	*PS*
18	—	+	
24	9.3	+	
27	5.4	+	
30	3.3	+	+
33	2.6	+	+
36	2.0	+	+
39	1.6	+	+
40-41	—		+
42-51	7.5		+

In Table 1 the results of rechromatography of the combined PE and PS fractions are shown. The solvent pair, methanol : chloroform 1 : 4 eluted all the ninhydrin positive material. Initially, phosphatidylethanolamine was eluted as a pure fraction. This was followed by a mixture, and finally by pure phosphatidylserine.

Thromboplastin Generation Test. (See Fig. 2.) A fraction was considered active if the thromboplastin time was within 10 seconds of control. All the fractions could be diluted about 75 times and still remain within 10 seconds of control. The results through tube 24 represent the activity of the cephalin group. Tubes 25 to 30 show the activity of phosphatidylethanolamine and phosphatidylserine. The thromboplastin time in tubes 31 to 33 was accounted for by phosphatidylserine. The appearance of lecithin and sphingomyelin in fraction 34 coincided with an abrupt loss of thromboplastic activity which persisted through fraction 76, when sphingomyelin became undetectable. At this point clotting activity returned. The thromboplastin times of the column residue were not as consistently close to control as the early fractions, but most values approximated that range of activity.

The effect of purification was reflected in the phosphorus values of the active fractions; that is, when the fractions were used, much less phospholipid was required to give the same thromboplastic activity as when mixed platelet lipid was employed. When whole platelet phospholipid extract was used in the thromboplastin generation test, it was found that optimal activity was obtained when the concentration of phosphorus in the reagent tube was 30 µg. per ml. In the original methanol fractions (tubes 31 to 33, for example) phosphorus values of 0.5 µg. per ml. were calculated at optimal clotting ranges. The same situation was noted in the rechromatographed fractions which were identified as PE. Phosphorus concentration averaged 0.2 µg. per ml. as compared to 30 µg. per ml. of mixed material.

When the rechromatographed chloroform : methanol fractions were tested in the thromboplastin generation test, activity within control range was obtained uniformly with undiluted and 1 : 1 diluted materials. The tubes containing primarily PE and primarily PS had the same order of activity as those which contained mixtures of the two lipids.

Thromboplastin generation tests were repeated on six fractions after 5 months' storage in petroleum ether at —20°C. There was no change in the clotting activity as measured by this test. Fractions stored in buffer at —20°C were also stable after 5 months.

The ethyl ether eluates of the original column separation were tested for thromboplastic activity and were found to be inert. The material removed from the silicic acid packing after chromatography also had no clotting activity.

Prothrombin Consumption Tests. (See Fig. 2.) The results with the original methanol column fractions indicated that as the cephalin fraction was purified the prothrombin consumption increased. The early pooled "column residue" tubes were able to increase prothrombin consumption, but the later fractions did not produce as marked an increase despite the fact

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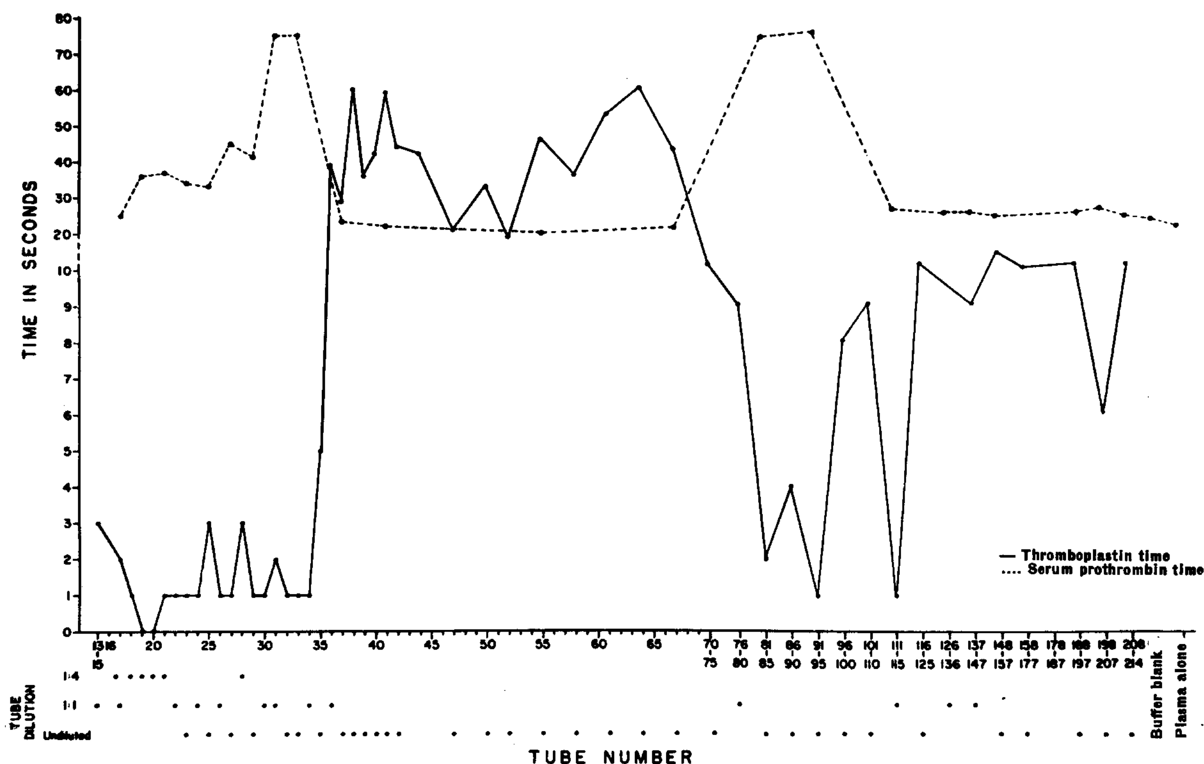

Fig. 2. Results of thromboplastin generation and prothrombin consumption tests on original methanol fractions. The points connecting the solid lines represent the results when the indicated fraction was used as platelet reagent in the thromboplastin generation test. The difference between the clotting time of the fraction and the brain cephalin control at the end of 6 minutes' incubation is designated as the thromboplastin time. Low numbers on the ordinate represent activity. The figures beneath the abcissa scale represent the dilution at which the tube showed the maximal activity indicated in the chart. The points between the broken lines represent the serum prothrombin time (prothrombin consumption) of the original methanol fractions. A high value on the ordinate scale indicates ability to increase the prothrombin consumption of platelet-free native plasma.

that they were active in the thromboplastin generation test. (It is possible that the 1 : 4 dilution of the small amount of lipid in these tubes was insufficient to increase prothrombin consumption.) In these studies, as in the thromboplastin generation test, the amount of purified material which gave the same prothrombin consumption as mixed platelet lipid was quite small. After dilution, the 0.1 ml. of mixed lipid added to the plasma contained a total of 382 μg. of phospholipid, whereas the phosphatidylserine fractions contained an average of 9 μg. and active column residue fractions contained 0.13 μg. of phospholipid.

Plasmalogen. Quantitative aldehyde studies are shown in Figure 3. The bulk of the plasmalogen was in the cephalin fraction. Approximately 23 per cent of the phosphorus in tubes 16 to 31 was present as plasmalogen[5] (12). As reported by others (13), the ethanolamine plasmalogens predominated; indeed, the drop in plasmalogen value coincided with the disappearance of phosphatidylethanolamine. In the rechromatographed methanol : chloroform fractions plasmalogen was noted only in the tubes where PE predominated. Tube 21 contained a total of 1.2 μg. In the other rechromatographed fractions sufficient material for quantitative study was not available, but they were qualitatively Schiff positive through tube 33.

Hydrolysis. Results obtained with the original methanol fractions are given in Figure 4. In the early tubes large amounts of PE and PS were found, with PS predominating after fraction 31. In every subsequent tube PS was found along with smaller amounts of PE, even in the column residue.

The same general pattern was obtained with the rechromatographed eluates (Fig. 5), that is, PE was the major component at first, followed by a mixture of PE and PS and then persistence of PS. Pooled fractions 42 to 51, which were not hydrolyzed, were identified on paper as PS. It should be mentioned that the over-all error of the method of hydrolysis would make the figures obtained for serine in the rechromatographed

[5] Since the molar ratio of phosphorus to aldehyde is 1, the following formula was used:

$$\frac{\text{moles of aldehyde}}{\text{moles of phosphorus}} \times 100 = \text{phosphorus present as plasmalogen as a percentage of the total phosphorus.}$$

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TABLE 2. Correlation of Phosphorus Content with DNP Ethanolamine and DNP Serine in Rechromatographed Fractions *

Fraction	A DNP Serine (analyzed)	B P Content of Phosphatidylserine (calculated)	C DNP Ethanolamine (analyzed)	D P Content of Phosphatidylethanolamine (calculated)	E Sum of Columns B & D	F Total P (analyzed)
	μg./ml.	μg./ml.	μg./ml.	μg./ml.	μg./ml.	μg./ml.
22 & 23	5.1	1.5	12.6	6.4	7.9	
24						9.2
25 & 26	5.1	1.5	10.6	5.4	6.9	
27						5.4
28 & 29	5.0	1.5	5.2	2.6	4.1	
30						3.2
31 & 32	4.5	1.3	3.1	1.6	2.9	
33						2.6
34 & 35	3.3	1.0	1.9	1.0	2.0	
36						2.0
37 & 38	1.8	0.5	1.5	0.7	1.2	
39						1.6
40 & 41	2.1	0.6	0.7	0.4	1.0	

* Figures in column B are obtained by multiplying the result in column A by 31/105. Similarly, the values in column D are derived by multiplying the result in column C by 31/61. The totals in column E represent the values that should be found if all the phosphorus is derived from PE and PS. Column F is the actual amount of phosphorus in the adjacent tube.

fractions 10 to 15 and 16 to 17 doubtful, since the readings were quite close to the blank. However, the low ethanolamine figures are significant because of more sensitive methods of measurement.

Correlation of Phosphorus Content with Dinitrophenylhydrazine Ethanolamine and Dinitrophenylhydrazine Serine Analysis.[6] In Table 2 evidence is presented that the phosphorus measured was derived from phospholipid. A shortcoming of these calculations is that the phosphorus figure was taken from the adjacent tube because of insufficient material for determinations on every fraction. Column E represents the sum of the calculated amount of phosphorus if it were derived only from PE and PS. In column F the total amount of phosphorus in the adjacent fraction is given. The close correspondence of values in columns E and F suggests that all phosphorus could be accounted for as PE and PS phosphorus.

[6] Calculating on the basis of a nitrogen to phosphorus ratio of 1:1, one mole of PS contains one atomic weight of phosphorus and will yield after hydrolysis one mole of serine. Similarly, one mole of PE contains one atomic weight of phosphorus and will yield after hydrolysis one mole of ethanolamine. Therefore:

$$\mu g./ml.\ P = \frac{at.\ wt.\ P}{m.\ wt.\ serine} \times \mu g./ml.\ serine$$

and

$$\mu g./ml.\ P = \frac{at.\ wt.\ P}{m.\ wt.\ ethanolamine} \times \mu g./ml.\ ethanolamine$$

DISCUSSION

When mixed platelet phospholipid extracts were eluted from silicic acid columns with methanol, the first fractions possessed thromboplastic activity. This early component was a mixture containing phosphatidylethanolamine, phosphatidylserine, and inositol

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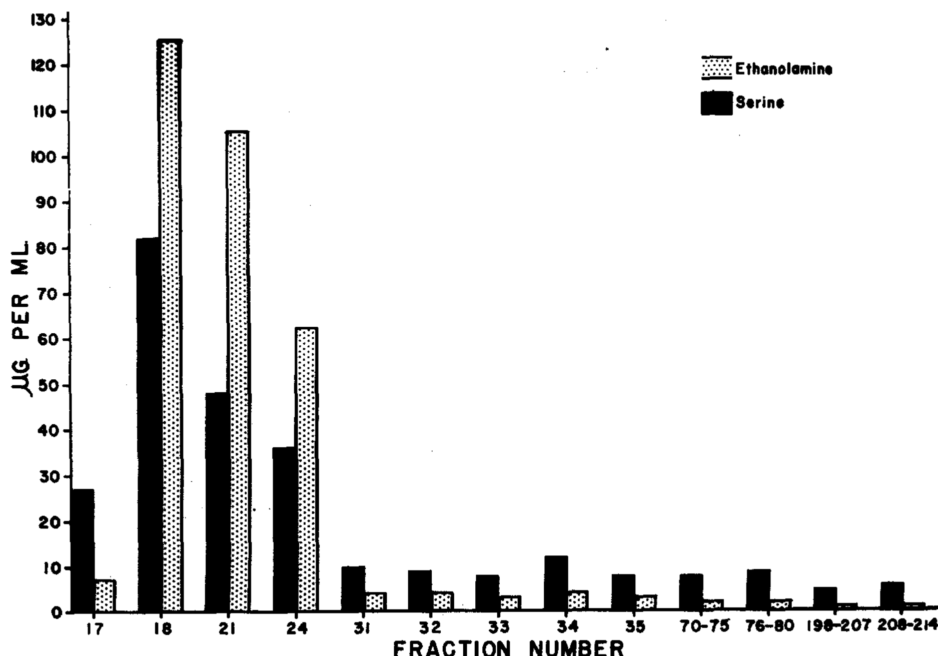

FIG. 4. Hydrolysis studies on original silicic acid column methanol eluates. Fractions 31 to 33, 198 to 207, and 208 to 214 appeared on paper chromatography to be phosphatidylserine; however, as seen above, PE was present in these fractions.

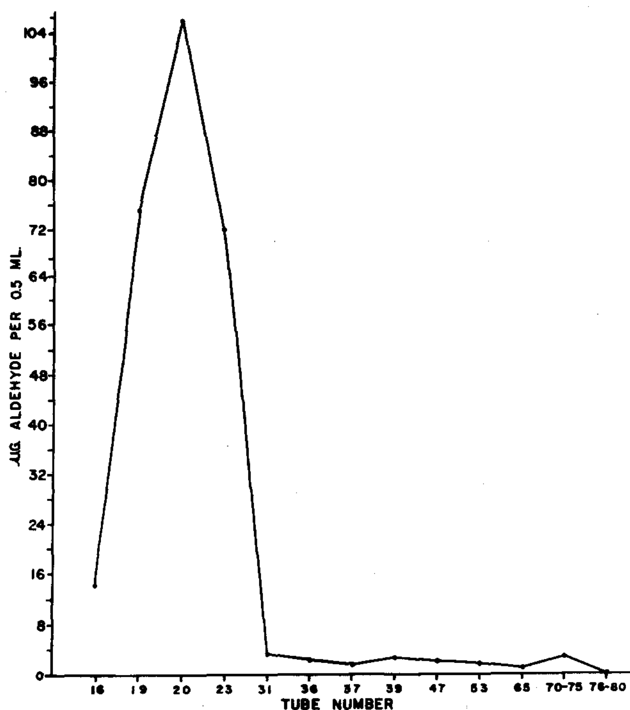

FIG. 3. Aldehyde determinations with Schiff's reagent on original silicic acid column methanol eluates. High values follow the distribution of PE rather than PS.

phosphatide. As the elution progressed, inositol phosphatide became undetectable and its disappearance was followed by that of phosphatidylethanolamine, leaving PS almost free of other phosphatides. The rapidity with which the choline phosphatides appeared (Fig. 1) may have been a function of temperature, since a higher yield of PS was obtained (1) by chromatography at 8°C than at 20°C. The appearance of lecithin and sphingomyelin coincided with a loss of coagulation activity. Elution beyond the point of complete recovery of lecithin and sphingomyelin again gave PS, which promoted thromboplastin formation. Thus there were two sources of phosphatidylserine in this initial separation. A second chromatographic procedure of the eluates containing PE and PS with methanol : chloroform 1 : 4 produced chromatographically pure phosphatidylethanolamine (Table 1).

Fractions which appeared to be homogeneous, judged by paper chromatography of the intact phosphatides, were found to be mixtures when hydrolyzed. Thus greater sensitivity is afforded by evaluation of hydrolysis products.

Positive statements cannot be made concerning the individual role of platelet PE and PS in blood thromboplastin formation. However, the following observations are mentioned: In the original methanol fractions (31 to 33 and column residue, Fig. 1) in which

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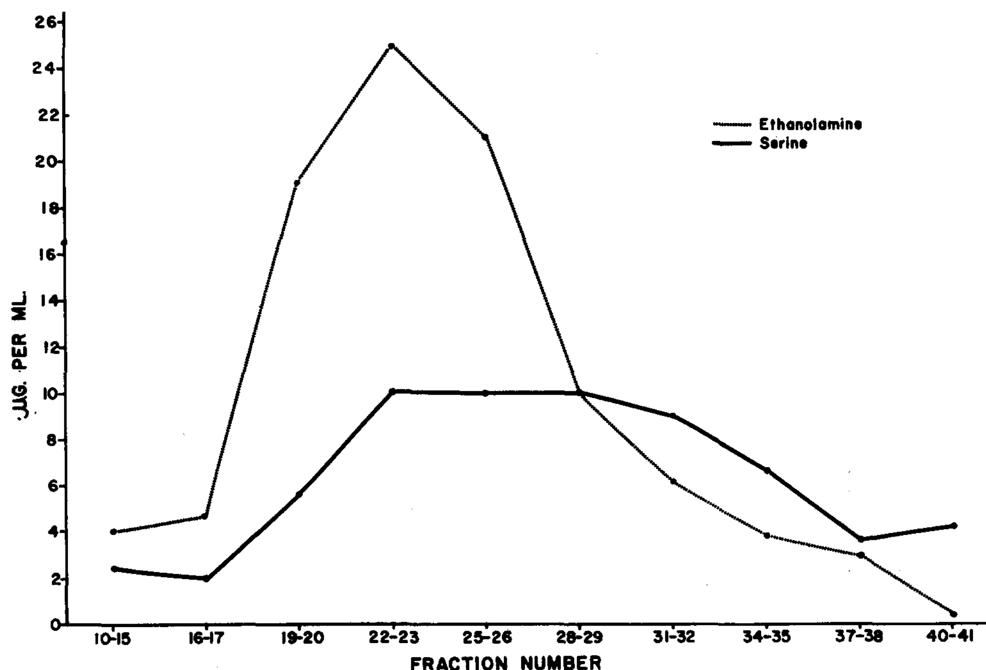

FIG. 5. Hydrolysis results on the rechromatographed fractions (methanol: chloroform 1:4). Although there was a good yield of PE, it could not be freed of PS. Again, paper chromatography revealed only the predominating phosphatide (see Table 1).

PS existed in highly purified form, clotting activity was noted at dilutions at which PE contamination was undetectable. The same phenomenon was noted in rechromatographed fraction number 18 (Fig. 5), which contained PE contaminated with trace amounts of PS. It is possible that PE and PS are interchangeable as platelet thromboplastic factors *in vitro*. However, evidence is insufficient to be conclusive.

Thromboplastic activity has been noted with PE in other laboratories (14 to 17) but PS was found to be inhibitory (17[7]). On the other hand, activity with PS has been noted at small concentrations (18) or under special circumstances (17, 19, 20). In our hands inhibitory activity occurred only at high concentrations (1), and the presence of lecithin (obtained from columns or purified commercial lecithin) caused a loss of thromboplastic activity. Possible explanations for some of these discrepancies are: (*a*) materials considered to be homogeneous actually were contaminated, (*b*) in the course of isolation the fatty acid component of the phospholipid molecule was oxidized, resulting in a reduction or loss of thromboplastic activity, (*c*) different systems were employed to study thromboplastic activity, and (*d*) under certain circumstances PE and PS might both be capable of promoting thromboplastin formation.

[7] Barkhan and Silver recently reported (American Society of Hematology Meeting, November 24, 1959, St. Louis, Mo.) acceleratory activity with low concentrations of PS fractions from brain.

Plasmalogens are phospholipids which will release higher fatty aldehydes when treated with mercuric salts or weak mineral acids (21). They are known constituents of nervous and muscular tissue and sperm (22). In this study plasmalogens were demonstrated in blood platelets and composed 23 per cent of the cephalin fraction. Most of the platelet plasmalogen was apparently of the ethanolamine type. Small amounts were also detected in the choline fractions. The presence of these phosphatides in fractions with thromboplastic activity is of interest, since the role of plasmalogens in lipid metabolism is not known (23). It is reported that brain plasmalogens do not have anticoagulant activity (24).

The authors wish to thank Dr. Theodore H. Spaet for help in preparation of the manuscript, and Dr. M. M. Rapport for helpful suggestions during the course of this study.

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