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PLATELET PHOSPHATIDES: THEIR SEPARATION, IDENTIFICATION, AND CLOTTING ACTIVITY¹

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Agents capable of accelerating the conversion of prothrombin into thrombin in the presence of Ca^{++} may be said to have thromboplastic activity. Early studies of thromboplastic activity were carried out with materials derived from tissues. Wooldridge (1, 2), in 1886, produced intravascular clotting in animals by the intravenous administration of aqueous extracts of red blood cells, white blood cells, and tissues. He was of the opinion that the active factor in these extracts was a protein-lecithin compound. In 1912, Howell (3) also reported that a lipoprotein was responsible for the thromboplastic activity of aqueous tissues extracts. The lipid portion was a phosphatide which could be separated from the protein with fat solvents, and had the properties of "cephalin." He assumed that the thromboplastic activity of certain lecithins tested was due to contamination with cephalin. McLean (4, 5) extended these observations and showed that the potency of the cephalin preparation varied directly with the degree of unsaturation of the constituent fatty acids. Gratia and Levene (6) confirmed the work of Howell, using their own cephalin preparations. Wadsworth, Maltaner and Maltaner (7-9) prepared cephalin free of lecithin and showed that the lecithin had no thromboplastic activity. Cohen and Chargaff (10) and Chargaff (11) verified that the tissue thromboplastic factor was a lipoprotein and that the lipid portion was associated with the phosphatides.

It became of interest to determine whether plasma contained a thromboplastic factor analogous to that found in the tissues. Wooldridge (12) suggested that a thromboplastic substance existed in plasma ("Fibrinogen-B") which united

with tissue thromboplastin ("Fibrinogen-A") to cause clotting in shed blood. He believed that both were protein-lecithin compounds, and Mills and Guest (13) agreed with this concept. Howell (14) later suggested that there was a thromboplastic substance in plasma which was decreased in hemophilic patients. Chargaff and West (15) studied the effect of high speed centrifugation on human plasma and noted that the recalcified clotting time was prolonged. They were able to shorten the clotting time by adding tissue thromboplastin or a saline suspension of sedimentable "reddish-brown translucent pellets" obtained from plasma. These pellets were later shown by Flynn (16) and Tocantins (16) to contain platelets. Nevertheless this study stimulated interest in a "circulating thromboplastin." Conley, Hartmann and Morse (17) prepared "platelet-free" native plasma, using silicone-treated apparatus. Although this plasma did not clot in silicone, when transferred to untreated glass tubes at 37° C. the clotting time was relatively short. The addition of tissue thromboplastin caused prompt clotting in the siliconed as well as the nonsiliconed tubes. They concluded that plasma contained a thromboplastin precursor which was activated by contact with a foreign surface, and that this precursor alone or platelets alone could initiate blood coagulation. They postulated that hemophiliacs were deficient in the precursor. Shinowara (18, 19) studied a lipoprotein isolated from blood cells (thromboplastic cell component) and the nonclottable globulin of Cohn Fraction I (thromboplastic plasma component). Together, but not separately, these acted like tissue thromboplastin with respect to prothrombin activation. He regarded hemophilia as a disease in which the thromboplastic plasma component was deficient.

The concept of blood thromboplastin was clarified when it was shown by Biggs, Douglas and Macfarlane (20, 21) that normal blood could

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produce its own prothrombin converting activity from platelet and protein precursors. This idea resulted in the development of the thromboplastin generation test (22). It is now well known that blood can generate thromboplastic activity more potent than that of tissue extracts.

Early studies suggested the coagulant role of platelets. It was recognized by Bizzozero (23) and Hayem (24) that platelets disintegrated before fibrin formed in the process of clotting and that if blood was anticoagulated, the platelets remained intact. Howell (25) had no doubt that the disintegration of platelets furnished a material that would accelerate clotting and was essential in the changes leading to the activation of prothrombin to thrombin. Many of the early theories of blood coagulation, although differing in other points, did recognize that a phosphatide was released by the platelets during the process (25, 26). The importance of a platelet-plasma factor interaction was a concept later stressed by Quick and Epstein (27, 28), Brinkhous (29), and others (30-32).

Quantitative studies on platelet lipids were done by Erickson, Williams, Avrin and Lee (33), who found that 16 per cent of the dry weight of platelets was lipid, 73 per cent of the lipid was phospholipid and 68 per cent of the phospholipid was "cephalin." Chargaff, Bancroft and Stanley-Brown (34) reported that horse platelet "cephalin" shortened the recalcified clotting time of chicken plasma. Wolf (35) found that lipid extracts of whole human platelets were effective substitutes for platelets in clotting tests. It was soon noted that lipid extracts of brain (35, 36) and soy bean phosphatides (37) could substitute for platelets in the thromboplastin generation test. Klein and Farber (38) tested lipid preparations derived from a wide variety of natural sources (platelets, mammalian, avian, and plant tissues) and showed that all could contribute to thromboplastin generation.

The chemistry of coagulation-active lipids has received considerable attention. In 1942, Folch (39, 40) demonstrated that "cephalin" was not a single substance. With increasing amounts of alcohol added to a chloroform solution of crude brain cephalin, five fractions were obtained. Fraction I contained inositol phosphatide, Fraction III contained phosphatidylserine, and Fraction

V contained phosphatidylethanolamine. A number of laboratories carried out Folch fractionation procedures or modifications thereof, and used the fractions they obtained in tests of plasma and tissue thromboplastic activity. The results reported by different workers have not been in agreement. For example, using tissue thromboplastin systems Chargaff (11) found his preparations of phosphatidylserine to be inactive. Barkhan, Newlands and Wild (41) noted that phosphatidylserine was inhibitory in the thromboplastin generation test, whereas Rapport (42) reported that phosphatidylserine was the active phosphatide in his system. Garret (43) successfully used Folch Fraction V (phosphatidylethanolamine) in place of platelets in the thromboplastin generation test as did Barkhan, Newlands and Wild (41). Flute, Barkhan and Rhodes (44) found that brain phosphatidylethanolamine obtained by chromatography on Al_2O_3 columns had clot accelerating activity. O'Brien (45, 46) showed that egg phosphatidylethanolamine and inositol phosphatide were effective substitutes for platelets in blood coagulation tests. Robinson and Poole (47-49) found that chylomicra accelerated thrombin generation and demonstrated that they contained phosphatidylethanolamine.

The purpose of the present study was to separate and identify the phospholipid components of human platelets by means of paper and column chromatography (50), and to determine the clotting activity of each.

MATERIALS AND METHODS

Preparation of platelet phospholipids. Blood was collected by gravity flow into plastic bags which contained acid citrate dextrose (ACD) solution. The bags were centrifuged for 15 minutes at 4° C. and 1,400 rpm (500 G). The platelet rich plasma was expressed into siliconized test tubes and the packed cells usually returned to the donor. The platelets were washed four times with saline. Each wash was centrifuged for at least one hour at 3,000 rpm in an International Centrifuge, Clinical Model. The packed sediment was pooled and frozen at -20° C. overnight. The test tube was broken and any red or white cells which remained at the bottom were sliced away with a scalpel blade. This was done to insure a minimal degree of contamination with other cells. The packed platelets were thawed, suspended in four times their original volume of cold acetone, and placed at 4° C. for 30 minutes, after which the acetone was removed by centrifugation. This acetone extraction was repeated four to six times. The acetone-insoluble ma-

terial was extracted with 10 times its volume of chloroform at 4° C. The time of the initial extraction was two hours, the second was three, and the third was allowed to take place overnight. The extraction vessel was placed on an automatic rotator during these procedures to insure adequate mixing. The material was filtered after each extraction and the filtrates pooled. These were taken to dryness under a stream of nitrogen and stored at -20° C. under acetone in a dark bottle. Ordinarily, 500 ml. of blood from a patient with a normal platelet count, established by phase microscopy (51), yielded about 5 mg. of phospholipid.

Paper chromatography. Three solvent systems were used. The first was based on the method of Lea, Rhodes and Stoll (52). A round chromatography jar, 25 × 45 cm., was used.³ One hundred micrograms of platelet phospholipid in chloroform solution was applied to the starting line of silicic acid-impregnated Whatman No. 3 paper in 5 μ l. aliquots. The papers were allowed to equilibrate for 30 minutes in an atmosphere containing the solvent, 20 per cent methanol in chloroform, which was placed at the bottom of the jar. Developing solvent was slowly added to the reservoir through a small hole in the cover. The front was allowed to descend about 30 cm.; the duration of the run was four hours. The papers were removed and dried in air, the solvent front having been marked. Both sides were sprayed with a solution of 0.2 per cent ninhydrin in acetone and the color was allowed to develop at 100° C. in the dark. Substances containing free amino groups such as the cephalins appeared purple. The papers were then stained for lecithin and sphingomyelin by the method of Chargaff and co-workers (53, 54) for choline-containing lipids. The papers were immersed in a 2 per cent aqueous solution of phosphomolybdic acid for one minute. They were washed for five minutes in *n*-butanol and for 15 minutes in rapidly running tap water. This was followed by immersion in freshly prepared 0.4 per cent stannous chloride in 3 N HCl. A positive reaction was a blue spot on a white background. Occasionally the background turned blue to some extent, but this could be minimized by extra washing before the stannous chloride step.

The second system employed was similar to that of Dieckert and associates (55-57). The chromatography was carried out in a Reco "Chromatocab," Model A-300, using glass fiber filter paper.⁴ The solvent was composed of phenol, 200 Gm., dissolved in 400 ml. of ethyl ether, with the addition of 250 ml. of acetone and 50 ml. of distilled water. Two hundred μ g. of platelet phospholipid in chloroform was applied to silicated glass fiber filter paper, in 5 μ l. amounts. The chromatography cabinet was equilibrated with solvent for 30 minutes, and development occurred by the ascending method. The papers were removed after the front had ascended about 40 cm., which took two to three hours. Before staining, the pa-

³ Part No. C-4249-2X, Scientific Glass Co., Bloomfield, N. J.

⁴ No. X-934-AH, 30 × 37.5 cm., H. Reeve Angel & Co., Inc., 52 Duane St., New York, N. Y.

pers were heated to 90° C. for 10 minutes in order to evaporate the phenol, as it might have interfered with the spot tests. The same staining techniques as above were utilized.

It should be mentioned that the 20 per cent methanol in chloroform system also worked well using the silicated glass fiber filter paper and the ascending method. The staining reactions are more clear and sensitive on glass fiber paper.

The third system used was devised by Marinetti and associates (58-60). The solvents were diisobutyl ketone, acetic acid, and water, (40: 25: 5, v/v/v). Chromatography was carried out by the ascending method, using Whatman No. 1 paper impregnated with silicic acid. A rectangular chromatography jar, 30 × 60 cm., was used.⁵ One hundred fifty μ g. of platelet phospholipid, also in 5 μ l. amounts, was applied and the solvent front was allowed to travel 38 to 40 cm. The time of the run was about 15 hours. The solvent front was marked, and the papers dried in air for no longer than 20 minutes. They were then immersed in a 0.001 per cent aqueous solution of Rhodamine G (color index 752) for five minutes, followed by washing in tap water for another five minutes. The wet chromatogram was examined under ultraviolet light. Phosphatidylserine, inositol phosphatide and phosphatidic acids appeared blue or purple while phosphatidylethanolamine, lecithin and sphingomyelin appeared yellow. The papers were dried and stained with ninhydrin. Since the cephalin group could be separated in a single unidimensional run, this method proved to be the most useful for the identification of platelet phosphatides. It is important to perform the staining reactions within the stated time sequence because the blue spots disappear after a short time (as little as 30 minutes), and hence their presence can be missed.

Preparation of silica impregnated papers. All grades of paper were treated the same way. Pyrex trays, 37.5 × 25 cm., were used. The papers were immersed individually in a solution of sodium silicate-distilled water (1: 1, v/v) for three to five minutes. They were removed, held in a vertical position, and stroked with a glass rod until the surface was smooth and no solution dripped from the bottom. Extreme care was taken in handling the papers at this point to prevent tearing. They were placed in 6 N HCl for exactly 30 minutes. This was followed by washing in rapidly running tap water until no chloride could be detected in the wash with 1 per cent AgNO₃. Chloride remaining on the paper interfered with the chromatography, but too much washing resulted in loss of the silica. The tap water wash was followed by a rinse in distilled water. Occasionally the papers were given a final rinse in methyl alcohol and then in ethyl ether, but this step was found to be unnecessary. The washed papers were dried in air and finally in a mechanical convection oven at 100° C. for one hour. The papers were stored between two sheets of plate glass, which kept them straight.

Technique of spot elution. Large numbers of chromatograms were run. The outside lanes were stained, and the

⁵ No. B-3816 X, Scientific Glass Co., Bloomfield, N. J.

corresponding central areas were cut out and placed in 150 ml. of chloroform. In this manner 1 to 2 mg. of phospholipid was eluted from the paper. The chloroform was taken to dryness under nitrogen. The remaining phospholipid was suspended in saline or imidazole buffer, pH 7.35, for testing.

Authentic phospholipids for comparison of chromatographic mobility in staining reactions. Egg phosphatidylethanolamine was the gift of Dr. C. H. Lea, Low Temperature Research Station, Cambridge, England. Synthetic phosphatidylethanolamine [L-alpha- (dimyristoyl) cephalin] was kindly provided by Dr. Erich Baer, Banting Institute, Toronto, Canada. Egg sphingomyelin was donated by Dr. H. E. Carter, Noyes Laboratory, University of Illinois, Urbana. Egg lecithin was purchased from Bios Laboratories, New York, N. Y.

Column chromatography. The first method used was similar to that of Hanahan and co-workers (61-63) and Lea, Rhodes and Stoll (52). Chloroform-methanol (4:1) was employed as solvent for the entire procedure. The size of the column was 30×1.5 cm.⁶ It was packed with a mixture of 12 Gm. silicic acid (100 mesh, Mallinckrodt) and 6 Gm. Hyflo-supercel. Seventy-five mg. of platelet phospholipid in 8 ml. of chloroform was applied to the column, which was attached to an automatic fraction collector (Technicon, supplied with a drop counter). One thousand ml. of solvent was allowed to pass down the column at a flow rate of 1 to 2 ml. per minute. Four ml. fractions were collected. These were taken to dryness under a stream of nitrogen and tested for thromboplastic activity. Aliquots were also taken for identification by means of paper chromatography.

The second column chromatographic procedure was devised by Hirsch and Ahrens (64). Dr. Hirsch designed the column which was used,⁷ and he kindly provided us with silicic acid prepared by his method (64). Eighteen Gm. of silicic acid was packed in the column, and it was washed with 50 ml. of ethyl ether. Two hundred forty mg. of platelet phospholipid in 5 ml. of ether was applied to the column when the wash had almost descended to the level of the silicic acid. Five hundred ml. of ether was then allowed to pass down the column over a period of 16 hours. Fractions of 10 ml. were collected. The ether was followed by the addition of 400 ml. of redistilled methanol which eluted the phospholipids. One hundred forty fractions of 3 ml. each were collected over a period of 18 hours. The column was kept at a temperature of 8° C. by means of a constant flow of tap water through the jacket surrounding it. Fractions obtained were dried under a stream of pure nitrogen.

Phosphorus determinations were carried out by the method of Dryer, Tammes and Routh (65). Nitrogen determinations were done by the microdiffusion method of Conway (66).

2, 4 Dinitrophenylhydrazine in 3 N HCl was used as a spray to detect acetal phosphatides (59).

Fractions were prepared for testing by suspending

approximately 0.5 mg. in 0.3 ml. of saline or the imidazole buffer. This was referred to as the "undiluted" fraction.

The thromboplastin generation tests were performed using Spaet's (67) modification of the method described by Biggs and Douglas (22).

Platelet-poor native plasma was prepared as described by Spaet, Aggeler and Kinsell (68), with the following exceptions. Blood was drawn into iced siliconed tubes by means of a siliconed needle connected to polyethylene tubing. Nine-tenths ml. of the prepared native plasma was added to 0.1 ml. of the test fraction diluted 1:5. After two hours of incubation at 37° C., the prothrombin content of the serum which resulted was determined by the method of Ware and Stragnell (69). Before any phospholipid preparation was used for chromatography, an aliquot was tested for activity in the thromboplastin generation test.

RESULTS

Paper chromatography

With the solvent system containing phenol, ether, acetone, and water, two spots were noted in an average obtained from over 20 runs. The leading spot had an Rf value of 0.9. This fraction was strongly positive for ninhydrin and always ran with egg phosphatidylethanolamine as well as the synthetic compound. The second spot was more elongated, with an Rf value of 0.7. It was negative for ninhydrin and positive for choline. Figure 1 is a photograph of a typical chromatogram with both the ninhydrin and choline stains present.

When the solvent system composed of 20 per cent methanol in chloroform was used, the second spot in the previous system could be resolved. The leading spot was again comparable to control markers with phosphatidylethanolamine, and this was followed by two fractions with Rf values of 0.3 and 0.4, both positive for choline. The spot with the Rf value of 0.4 ran consistently with purified lecithin, and that with the Rf value of 0.3 ran with sphingomyelin. Figure 2 is a diagram of a chromatogram obtained with this system.

The solvent system containing diisobutyl ketone: acetic acid: water (40: 25: 5, v/v/v) gave the best resolution and five spots were obtained. Figure 3 is a diagrammatic drawing of the chromatogram. The leading spot noted in the previous systems was resolved into two components: phosphatidylethanolamine and phosphatidylserine. Another spot, not previously noted, had an Rf value of 0.2. It was thought to be inositol phos-

⁶ No. 5-723, Fisher Scientific Co., New York, N. Y.

⁷ No. 572824, Scientific Glass Co., Bloomfield, N. J.

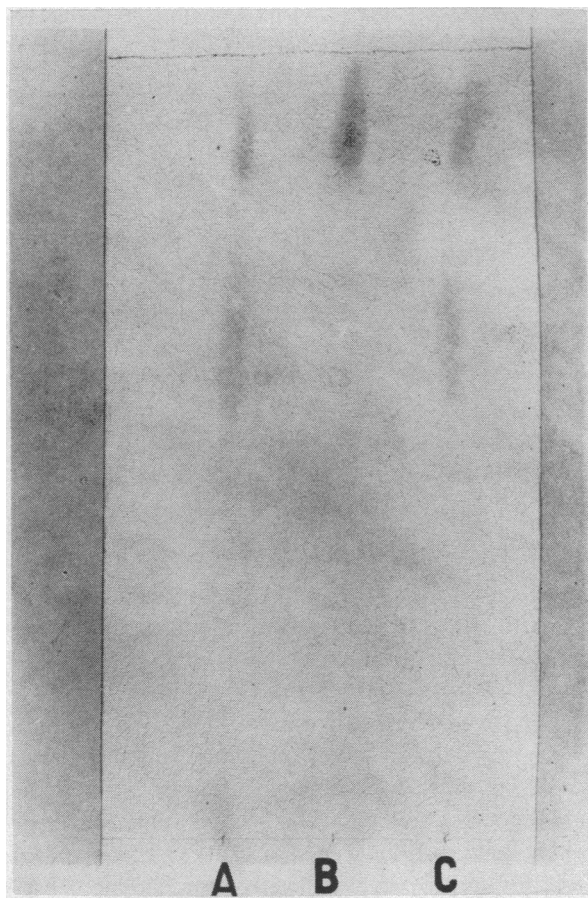


FIG. 1. CHROMATOGRAM OBTAINED WITH SOLVENT SYSTEM COMPOSED OF PHENOL, ETHER, ACETONE AND WATER

A and C, platelets; B, synthetic phosphatidylethanolamine.

phatide because of its staining reaction and R_f value (59).

After these separations were accomplished, large numbers of chromatograms were run and the spots eluted. The phospholipids could not be emulsified adequately in saline or the imidazole buffer. The poor suspensions obtained were inert in the thromboplastin generation test.

Column chromatography

Using the methods of Hanahan and associates (61-63) and Lea, Rhodes and Stoll (52), with chloroform:methanol (4:1), aliquots were taken from alternate tubes for paper chromatography and testing for thromboplastic activity. In the early fractions, phosphatidylethanolamine predominated.

Phosphatidylserine was not separated as a single fraction, and was probably mixed with phosphatidylethanolamine. After about 400 ml. of eluate had been tested, the lecithin and sphingomyelin fractions were encountered. The fractions to be used for clotting tests were taken to dryness, but these suspended very poorly. As suggested by Rapport (42), some fractions were mixed with a small amount of lecithin in a chloroform solution, dried and were resuspended. Although the emulsion was finer, there was no improvement in the thromboplastin generation test. The addition of 0.5 mg. of sodium desoxycholate (Matheson, Coleman & Bell) emulsified the fractions, but this compound was found to be anticoagulant in the thromboplastin generation test when tested alone in concentrations of 0.5 mg. per ml.

The original mixture of crude phospholipid was reconstituted and dried by pooling aliquots from each of the fractions. The thromboplastic activity which it showed before chromatography was lost, and no further work was done with these fractions.

It was speculated that the chloroform might

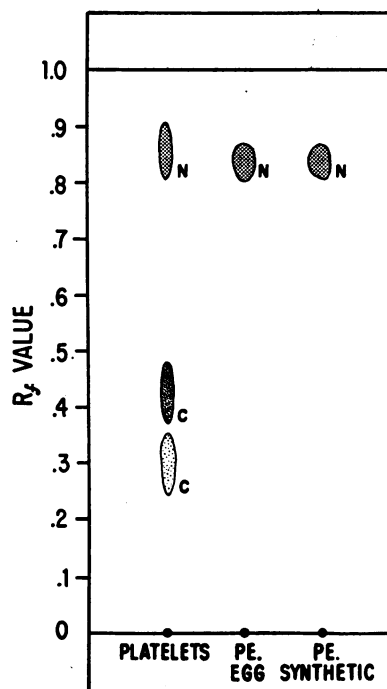


FIG. 2. DIAGRAM OF CHROMATOGRAM OBTAINED WITH SOLVENT SYSTEM COMPOSED OF 20 PER CENT METHANOL IN CHLOROFORM

N, ninhydrin positive (cephalins); C, choline positive (lecithin, sphingomyelin).

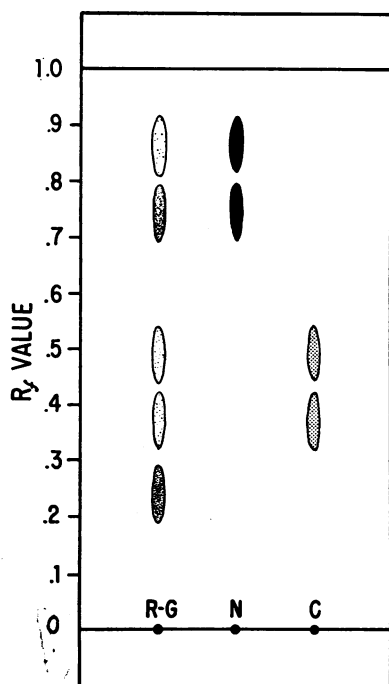


FIG. 3. DIAGRAM OF CHROMATOGRAM OBTAINED WITH SOLVENT SYSTEM COMPOSED OF DIISOBUTYL KETONE, ACETIC ACID AND WATER

Only the platelet lipids are shown. R-G, spots seen with Rhodamine-G dye and ultraviolet light; N, ninhydrin positive; C, choline positive.

have been responsible for denaturation of the phospholipids during passage down the column, since changes in properties of phospholipids have been noted when they are stored in this solvent (59). The method of Hirsch was undertaken since it eliminated the use of chloroform, and was capable of separating phospholipids. The ether was passed down the column first in order to remove any remaining glycerides, sterols, and other nonphospholipids (64). Phosphorus determinations performed on a number of ether fractions at random showed none to be present. The ether was followed by methanol, which eluted the phospholipids.

Five procedures were carried out on the methanol eluates: 1) identification by means of paper chromatography, 2) substitution for whole platelets in the thromboplastin generation test, 3) effect on prothrombin consumption of platelet poor native plasma, 4) determination of phosphorus content, 5) microdiffusion nitrogen determinations on some of the late fractions.

Figure 4 is a composite of the resulting data. Phosphorus analyses showed the following: Of the 240 mg. total phospholipid applied to the column, 7.85 mg. represented phosphorus. Nearly 90 per cent of the phosphorus was recovered (7.1 mg.). The resolution, Figure 4A, showed two main peaks, representing the cephalin and lecithin-sphingomyelin fractions, respectively. The phospholipid in the first peak was ninhydrin positive and choline negative, whereas the second peak was choline positive and ninhydrin negative.

Further identification was accomplished by paper chromatography. As seen in Figure 4B, six fractions were obtained. The early tubes showed a mixture of phosphatidylethanolamine,

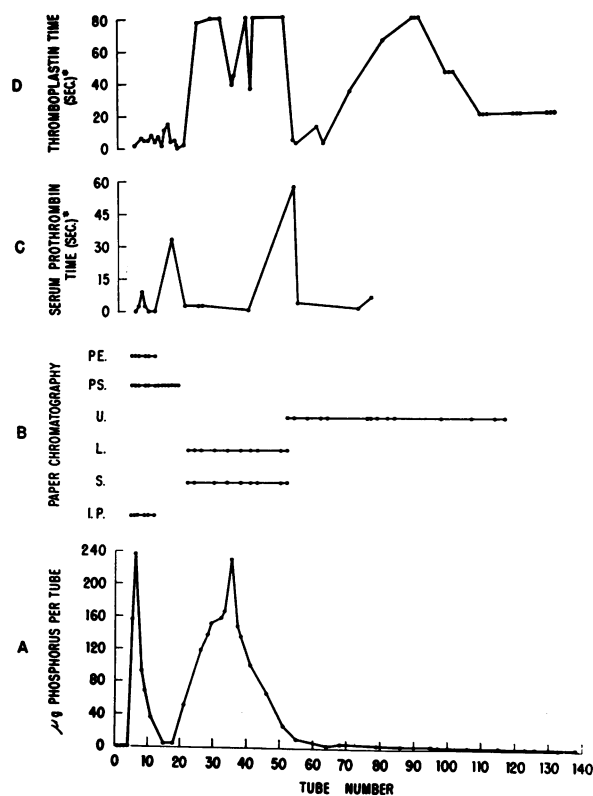


FIG. 4. PHOSPHORUS CONTENT, PAPER CHROMATOGRAPHY AND CLOTTING ACTIVITY OF COLUMN CHROMATOGRAPHY COMPONENTS

PE, phosphatidylethanolamine; PS, phosphatidylserine; U, unidentified component (phosphatidic acid?); L, lecithin; S, sphingomyelin; I.P., inositol phosphatide. Asterisk indicates difference in time from control values. In the thromboplastin times, low figures indicate activity; in the serum prothrombin times, high figures indicate activity.

phosphatidylserine, and inositol phosphatide. Tubes 13 through 19 contained phosphatidylserine only. Lecithin and sphingomyelin were present in Tubes 22 through 52 and were always together on the same chromatogram. This fraction was followed by another containing a single component which could not be positively identified. The average Rf value was 0.5. It stained a dense blue color with Rhodamine G, and the ninhydrin and choline reactions were negative. It also failed to stain with 2, 4 dinitrophenylhydrazine. This spot persisted from Tubes 52 through 117. Chemical analysis revealed that these fractions contained phosphorus, but no nitrogen (66). It is possible that this material was a phosphatidic acid.

The results of the serum prothrombin times, Figure 4C, are charted as seconds greater than control. The control times were obtained by adding 0.9 ml. of plasma to 0.1 of saline instead of the test substance. The results noted are those obtained using plasma from the same donor, although they represent a composite of several different runs. Comparable results were obtained with plasma from a different donor. Improvement of prothrombin consumption was produced by the early fractions containing the mixture of phosphatidylserine, phosphatidylethanolamine, and inositol phosphatide. The fraction containing pure phosphatidylserine as well as the early part of the unidentified component also produced an improvement in prothrombin consumption. The activity of the fractions in the prothrombin consumption tests was compared to that obtained from crude brain cephalin (36). Brain material was used as control because of its abundance and its predictable behavior in the tests. One hundred per cent activity was taken as that in the concentration of crude cephalin optimal in the thromboplastin generation test. This preparation had a phosphorus content of 60 mg. per ml., whereas the phosphatidylserine fraction of similar activity had a phosphorus content of 0.3 mg. per ml. This represents an increase in activity of approximately 200-fold in the phosphatidylserine fraction.

In the thromboplastin generation tests (Figure 4D) crude brain cephalin was again used as control in place of platelet reagent. The "thromboplastin time" was the difference between the

TABLE I
Tube dilution of optimal activity in active fractions

Tube no.	Dilution
	%
5	10
6	20
7	50
8	50
9	50
10	20
11	50
12	10
13	20
14	20
15	10
16	50
17	50
18	20
19	20
20	20
53	50
54	50
62	50

substrate clotting time of the crude cephalin control and that of the test substance after six minutes of incubation. The most active fractions in the prothrombin consumption technique all showed activity in the thromboplastin generation test. However, some fractions gave activity in the thromboplastin generation test alone (*i.e.*, Fractions 5 through 10). The reason for these differences is not clear, but may represent different concentration requirements in the two tests. The fractions containing only phosphatidylserine reached control values, and the first part of the unidentified fraction also showed good activity. For each fraction, serial dilutions were tested to determine optimal activity. This was usually ob-

TABLE II
Thromboplastin times (seconds) given by phosphatidylserine in the thromboplastin generation test

Tube no.	Aliquot No. 1			Aliquot No. 2		
	Incubation time (min.)			Incubation time (min.)		
	2	4	6	2	4	6
13, undiluted	33	26	25			
13, 1:1	19	18	23			
13, 1:4	20	16	12	24	16	12
13, 1:9	21	16	17	21	15	16
13, 1:50	27	23	22	25	21	22
13, 1:100	30	26	24			
Control	11	10	11	16	10	11
18, 1:1	27	12	11	14	11	11
18, 1:4				12	12	13
18, 1:9				16	14	14
Control	21	9	9	12	10	12

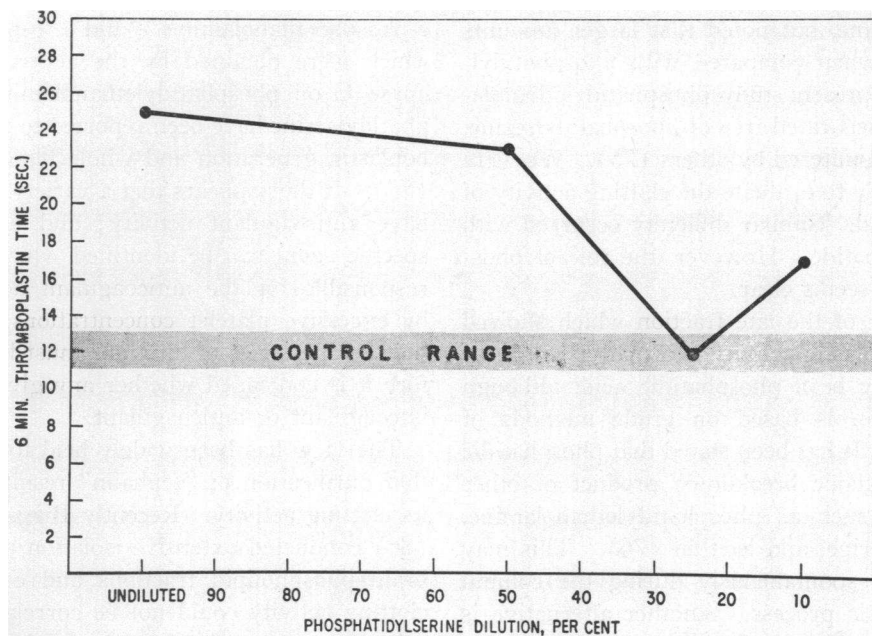


FIG. 5. SERIAL DILUTION OF PURE PHOSPHATIDYLSERINE IN THE THROMBOPLASTIN GENERATION TEST

tained with the original suspension diluted between 10 and 50 per cent (Table I). As shown in Table II, repetition of tests gave good agreement in active fractions; and even in fractions with less activity, six minute clotting times agreed within 10 seconds. The data presented in Figure 4D are typical values obtained using that concentration of phospholipid which gave the shortest clotting times. Some of the fractions gave definite activity even when diluted to 1 per cent.

It was also noted that if the concentration of phospholipids was above the optimal range there was impaired thromboplastin generation. This is shown in Figure 5 with phosphatidylserine. Optimal activity was found with the original preparation diluted to 25 per cent, and changes in concentration in either direction resulted in definite reduction of activity. At optimal activity the phosphorus content of the reagent was about 0.4 $\mu\text{g.}$ per ml. The crude platelet phospholipid mixture used for chromatographic separation was also tested in the thromboplastin generation test. On the basis of rough estimates, its optimal activity was at concentrations of at least 12 $\mu\text{g.}$ per ml. of phosphorus in the reagent. Thus there appeared to be at least a 40-fold increase of activity in the phosphatidylserine fraction.

DISCUSSION

The phosphatidylserine fraction obtained from whole platelet lipid extracts was able to replace the entire crude mixture in the thromboplastin generation and prothrombin consumption tests. Our results are in agreement with those of Troup and Reed (70), who reported that phosphatidylserine derived from platelets and red cells gave maximal thromboplastic activity at very small concentrations. O'Brien (45) reported activity in three phosphatidylserine preparations which he tested. Working with brain fractions, other laboratories have reported thromboplastic activity with phosphatidylserine. Therriault and Nichols (71) found that brain phosphatidylserine was inactive alone, but when combined with brain lecithin in chloroform solution, the product had marked thromboplastic activity. Gobbi and Stefanini (72) reported that Folch Fractions II and III (phosphatidylserine) were the most active but neither was as potent as the entire cephalin preparation. On the other hand, Rouser, White and Schloredt (73, 74) have stated that phosphatidylethanolamine, containing two unsaturated fatty acids, is the active phosphatide in platelets and tissues. Troup and Reed (70) found activity in phospho-

tidylethanolamine, but noted that larger amounts were needed when compared with phosphatidylserine. In the present study, phosphatidylethanolamine was not separated free of phosphatidylserine, a difficulty encountered by others (75). We were therefore unable to evaluate the clotting activity of the former lipid. Similar difficulty occurred with inositol phosphatide. However, the role of phosphatidylserine seems clear.

The identity of the late fraction which showed good thromboplastic activity is a matter of speculation. It may be a phosphatidic acid, although this assumption is based on crude methods of identification. It has been stated that phosphatidic acid is a metabolic breakdown product of other phospholipids such as phosphatidylethanolamine, phosphatidylserine, and lecithin (76). This may have occurred spontaneously during the column chromatographic process. Another alternative is that this is a true part of the platelet phosphatide complex which was not separated on paper. Clotting activity has been found in preparations containing phosphatidic acid (46, 73, 77).

Lecithin and sphingomyelin could not be tested separately since they were eluted together. This is possibly due to poor separation of these phosphatides at the low temperatures used in the present studies (78). The combined fractions had only negligible thromboplastic activity.

The data suggest that the phosphatides may also act as anticoagulants as previously reported (34, 79, 80). At high concentrations the thromboplastic potency was low compared with the results achieved when each test substance was appropriately diluted. The anticoagulant nature of lipids is under investigation in other laboratories. Turner, Silver and Tocantins (81), using Folch fractionation of hog brain, have found antithromboplastic activity in Fraction III (phosphatidylserine). Holburn, Silver, Turner and Tocantins (82) reported that phosphatidylserine interfered with the formation of thromboplastin and the action of formed thromboplastin. They could produce a prolongation of the whole blood clotting time for 24 to 48 hours *in vivo*, by injecting solubilized phosphatidylserine. Sphingosine, a component of sphingomyelin and the cerebrosides (83), has been found by Hecht, Landaburu and Seegers (84) to possess inhibitory properties in early stages of blood coagulation. The compounds,

o-phosphoethanolamine and *o*-phosphoserine, which were obtained by the action of phospholipase C on phosphatidylethanolamine and phosphatidylserine have been reported to inhibit thromboplastin generation and whole blood coagulation (85). It thus appears that a variety of lipids may have anticoagulant activity; and at present no specific agent can be identified which is uniquely responsible for the anticoagulant activity shown by excessive platelet concentrations. It is clear that a wide series of dilutions must be studied before it is concluded whether any given compound is coagulant or anticoagulant.

The view has been widely held for many years that purification of "cephalin" resulted in loss of its clotting activity. Recently Biggs and Bidwell (86) conducted extensive isolation procedures on brain phospholipid fractions and concluded that clotting activity could not be correlated with any substance concentrated by the methods they used. It is apparent that there are discrepancies in the findings of the various laboratories investigating the problem. Some of these have a possible explanation. First, the methods of purifying the phospholipids are not identical in each laboratory. The complete separation and purification of the components of a crude phosphatide mixture is extremely difficult, and techniques such as Folch fractionation do not give pure reagents. Changes during the isolation procedure alone are enough to alter the final results. If the fatty acids become saturated, the components emulsify poorly and cannot be tested. Some phospholipids undergo changes even when stored under solvents in the cold. Secondly, the coagulation systems employed for measuring activity are not similar in the laboratories studying the phosphatides. Finally, each investigator uses a different quantity of starting material and in different concentrations. It is clear that the phosphatides will not lose clotting activity as they are further purified, if precautions are taken to avoid denaturation. The present data show that comparatively small amounts of purified phosphatidylserine had the same activity as crude phospholipid preparations. This was evidenced by the finding that our purified phosphatidylserine gave 200 and 40-fold more activity in the prothrombin consumption and thromboplastin generation tests, respectively, as estimated on the basis of relative phosphorus content.

SUMMARY AND CONCLUSIONS

1. Crude phospholipid extracts of human blood platelets have been subjected to paper and column chromatography on silicic acid.

2. The components resolved were: phosphatidylethanolamine, phosphatidylserine, lecithin, sphingomyelin and inositol phosphatide.

3. Phosphatidylserine obtained by column chromatography could replace platelets in thromboplastin generation and prothrombin consumption tests.

4. An unidentified fraction (phosphatidic acid ?) showed thromboplastic activity.

5. The fraction containing phosphatidylethanolamine was active, but phosphatidylserine and inositol phosphatide were present. Thus, the role of this phosphatide could not be clarified by this study.

6. Lecithin and sphingomyelin, obtained as a single fraction, were inactive.

7. High concentrations of the various phosphatides appeared to act as anticoagulants.

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