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Research Article

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PLATELET PHOSPHATIDES. THEIR FATTY ACID AND ALDEHYDE COMPOSITION AND ACTIVITY IN DIFFERENT CLOTTING SYSTEMS *

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Previous studies from this laboratory revealed that phosphatidyl serine (PS) isolated from platelet phospholipid extracts by silicic-acid column chromatography could replace blood platelets in tests of thromboplastin generation and prothrombin consumption of platelet-poor "native" plasma (1, 2). When rigorous criteria of identification were employed, it was found that phosphatidyl ethanolamine (PE) fractions contained traces of PS (2); coagulant properties were therefore difficult to evaluate. Rouser, O'Brien, and Heller (3) recently accomplished the complete separation of PE from PS by means of a double column technique using silicic acid and ammoniated silicic acid. Their studies were carried out on lipids of beef brain, but the methods could be applied to human brain and platelet extracts.

In 1961, an earlier study of the fatty acids of platelet phosphatides with gas-liquid chromatography was reported (4). PE and PS were then, of necessity, studied as a combined fraction, and similarly the choline phosphatides were not adequately separated. In addition, the significance of oxidative changes consequent to prolonged separations and exposure of phospholipids to air was not appreciated, a factor which influenced the over-all results.

Zilversmit, Marcus, and Ullman (5) reported that 16 per cent (dry weight) of total platelet lipid extracts were in the plasmalogen form (phospholipids that liberate higher fatty aldehydes on hydrolysis). It could not, however, be determined how the plasmalogen was distributed among individual phosphoglycerides, although it was known (2) that the bulk of plasmalogen was associated with the combined PE–PS fractions. When methyl esters of fatty acids of phosphoglycerides are formed, the aldehydogenic chains of plasmalogens are simultaneously converted to dimethylacetals. These derivatives can be separated from methyl esters on polar and nonpolar gas-liquid chromatographic columns (4, 6, 7), thus affording an additional qualitative and quantitative measurement of plasmalogen to supplement other analytical methods (5).

In the present study, human platelet PE and PS were separated by techniques that minimized oxidative changes, which permitted an accurate evaluation of the activity of these lipids in different blood clotting systems. In addition, it was possible to carry out a detailed study of the fatty acid and fatty aldehyde composition of platelet phosphatides by gas-liquid chromatography.

MATERIALS AND METHODS

In order to standardize and insure reproducibility of the extractions, separations, and coagulation tests, 8 preliminary runs were performed with lipids of fresh, human brain tissue. The main data to be presented are the findings of 7 similar runs with human platelet material. Although details of the brain separations will not be presented, certain pertinent comparisons will be made with platelets.

Solvents were freshly redistilled in glass, and 1 per cent methanol was added to the chloroform after distillation. Procedures and transfers were carried out under highly purified nitrogen, less than 10 parts oxygen per million. Extracted lipids were not exposed to the atmosphere. "Deoxygenation" of solvents was carried out in a rotary evaporator connected to a water pump vacuum. Solvents were subjected to reduced pressure for 3 minutes after bubbling had ceased, and atmospheric pressure was restored with nitrogen. Absolute ethanol was rendered aldehyde-free by the addition of 3 g per L *m*-phenylenediamine dihydrochloride. After 24 hours, the ethanol was collected by distillation.

Preparation of platelet lipid extracts. Thirty-four units

* Investigation supported by a grant from the New York Heart Association. Presented in part at the meetings of the Federation of American Societies for Experimental Biology, Atlantic City N. J., April, 1962.

of blood from donors with normal or elevated platelet counts were collected and processed (2). A large heterogeneous pool was used to minimize the possibility of alterations in fatty acid composition due to dietary differences. Extraction was performed on approximately 13 g (wet weight) of platelet material at a time and yielded about 400 mg of lipid which was the starting material for each separation. The platelets were weighed, transferred to a Waring Blendor, and homogenized in 150 ml chloroform: methanol 2:1 for 40 seconds in a nitrogen atmosphere. The contents of the Blendor were transferred to a new sintered glass filter of coarse grade. Gentle suction was applied beneath the filter, which was covered with a funnel through which nitrogen was passed. When filtration was complete, the material on the surface of the filter was dried, and 150 ml of the same solvent was added. After 5 minutes, gentle suction was resumed and the re-extraction repeated. Finally, the filtrate was passed through fat-free sharkskin filter paper into a side-arm Erlenmeyer flask and was dried under reduced pressure and nitrogen in a 30° C water bath. During evaporation, deoxygenated absolute ethanol was added to the flask, thereby forming a water-ethanol azeotrope to aid in removal of last traces of water (8). The dry material was dissolved in chloroform: methanol 4:1, a sample removed for weight determination, and the remainder stored under nitrogen for a maximum of 12 hours at −20° before column chromatography. After storage, a precipitate was noted, which occasionally was removed by filtration without alteration of results. In most runs, the extract was placed on the columns intact.

Silicic acid chromatography. Mallinckrodt silicic acid, 100 mesh, was used. Sixty g of silicic acid was washed three times on a sintered glass filter with 180 ml of methanol, followed by 180 ml chloroform: methanol 1:1, 180 ml chloroform, and finally 60 ml methanol (3). The washing was facilitated by mild suction. The silicic acid was then placed in a 1,000-ml three-necked flask and heated 12 to 15 hours at 120° C under negative pressure and nitrogen. After cooling, 200 ml of chloroform: methanol 4:1 was added and the slurry poured into the glass column. The apparatus used was that of Hirsch and Ahrens (8) with one modification: column dimensions were altered to 2.5 × 40 cm so that 60 g of silicic acid extended to a height of 20 cm after application of nitrogen pressure (3). About 400 mg of platelet lipid was applied to the column in a small volume of chloroform: methanol 4:1, and the chromatography was done at 10° C. A stream of nitrogen was passed into each tube as it filled. Fractions were collected by hand (9), which permitted immediate capping and storage at −20° C. Samples of the 10-ml fractions were removed and examined by means of a rapid Ninhydrin[1] test (3), and approximately every tenth tube was studied in greater detail by thin-layer and silicic-acid paper chromatography. The flow rate was adjusted to 2 ml per minute by nitrogen pressure. The elution pattern was also followed by adding 1 ml of eluate to 1 ml 5 N sulfuric acid in a 30-ml Kjeldahl flask and heating at 320° C for 5 to 7 minutes, after which no further charring occurred. The intensity of charring was an index of the amount of organic material present. Eluting solvents for the first silicic-acid column separation (run A) were chloroform: methanol 4:1 until the fractions became negative to Ninhydrin. The run was completed with 990 ml chloroform: methanol 5:4 and 500 ml chloroform: methanol 1:9 (10). One-ml samples were removed for phosphorus determination, and before storage of fractions under nitrogen at −20° C, 0.1 ml concentrated ammonia was added to each tube in order to retard plasmalogen breakdown (3). The final concentration of ammonia in each tube was 0.15 M.

All Ninhydrin-positive fractions from run A were verified as solely a mixture of PE and PS by thin-layer and silicic-acid paper chromatography. These were pooled, evaporated to 5 ml, stored at −20° C, and applied 12 hours later to a deoxygenated ammoniated silicic acid column (3). This procedure, run B, separated PE from PS. The PE was eluted with chloroform: methanol 4:1, and the retained acidic PS with methanol. Fractions were monitored as for run A.

Phosphorus was analyzed by the method of Bartlett (11). The procedure was carried out in 25-ml, graduated, blood-sugar tubes (Nash, combination type) in a heating block. Standard curves were determined for the Coleman Jr. spectrophotometer at 700 mμ and the Beckman DU at 830 mμ; for convenience, measurements were made with the Coleman instrument.

Thin-layer chromatography. The identity and purity of lipids in individual fractions and pooled material was determined by this useful technique. Silica gel plates[2] were prepared, the lipids applied, and the chromatograms developed in a solvent system of chloroform: methanol: water: concentrated ammonia, 75:25:4:1 (3, 12). For identification, the air-dried plates were sprayed with 50 per cent sulfuric acid and heated in a convection oven at 250° C for 30 minutes. Less than 25 μg of phospholipid could be detected on the chromatograms. Although Rf values were not reproducible on thin-layer plates, the principal purpose of this chromatography was the detection of oxidation products and minor components. Large amounts, 0.5 to 1.5 mg, of some fractions were analyzed by thin-layer chromatography as a rigorous test of their purity: contamination with more than 0.01 mg of other lipids could be excluded.

Silicic-acid paper chromatography. This was used to supplement and confirm the findings with thin-layer chromatography. The methods were the same as previously reported (1, 2).

Oxidation studies. The pooled PE and PS fractions were examined for the presence of auto-oxidative changes as follows. Samples were dissolved in cyclohexane at a concentration of 1 mg per ml and transferred to 1-cm cuvettes. The molar extinction coefficients were determined at 235 and 275 mμ (3) in a Beckman DU spectro-

[1] 1,2,3-Indantrione hydrate.

[2] Brinkman Instruments, Inc., Great Neck, N. Y.

photometer. In addition, a continuous scan was made with a Beckman DB recording spectrophotometer.

Blood coagulation studies. Less than 24 hours after the separated lipids were identified and oxidation studies carried out, a sample was weighed. Amounts of 1 to 10 mg were removed, evaporated under reduced pressure and nitrogen to a small volume, and transferred under nitrogen to a Potter-Elvehjem 5A tissue grinder, where they were dried. Imidazole buffered saline (2) was added, the lipid was rapidly emulsified, and transferred to a cork-stoppered test tube. The suspension produced was considered the "undiluted specimen," from which a sample was removed for phosphorus determination. Fractions of PS, as well as of lecithin, sphingomyelin, and phosphatidyl inositol (PI), formed stable emulsions, but PE could only be partially suspended. Other procedures were carried out in an attempt to emulsify PE. These included the following. *a*) Sonication with two types of apparatus (Raytheon model DS-101 and Branson model S-75). Satisfactory emulsions were produced with these instruments, but slight settling was noted after 40 to 60 minutes. *b*) Admixture of PE with sodium desoxycholate.[3] It was previously determined that sodium desoxycholate at a concentration of 0.75 mg per ml did not interfere with the thromboplastin generation test (TGT). Therefore, the undiluted specimen of PE was prepared in imidazole buffered saline containing 0.75 mg sodium desoxycholate per ml, and a stable emulsion was formed. *c*) Suspension of PE with other materials: oleic acid, cholesterol, lecithin, and polysorbate 80 (Tween 80). All these emulsions showed some settling after 1 hour. The lipids to be tested were substituted for platelet reagent in the TGT immediately after suspension in buffer (1, 2). The activity of the fractionated material was compared to that obtained with total platelet lipid or crude brain "cephalin." In addition, the effect of various phosphatides on the recalcified clotting time of platelet-poor plasma was studied. Fresh citrated plasma from a fasting donor was used for each group of experiments. Blood was collected into silicone-treated test tubes through plastic tubing (Cutter "saftidonor") by gravity flow. During the test, 0.1 ml of freshly prepared lipid in imidazole buffered saline was added to 0.1 ml of plasma in uncoated glass tubes, and after a 3-minute incubation period at 37° C, 0.1 ml of 0.025 M calcium chloride was added (13).

Plasmalogen determinations. The plasmalogen content of PE and the choline phosphoglycerides was determined by iodine addition (5, 14). A 3-ml sample was removed from a total PE pool and the determinations were carried out in quadruplicate. These samples contained 1.08 μmoles phosphorus per ml. PS was not analyzed by the technique because of the small amounts recovered. Calculations of dimethylacetal peak areas by gas-liquid chromatography were correlated with the results of iodine addition and the combined information was used as a measure of plasmalogen.

[3] Nutritional Biochemicals Corp.

Gas-liquid chromatography. A Barber-Colman model 10 chromatographic apparatus equipped with two columns and two strontium^{90} detector cells was employed. U-shaped pyrex glass columns, 4 or 6 feet in over-all length with an internal diameter of 4 to 5 mm, were used. Column performance and linearity of detector response to mass were checked at least twice a week with standards obtained from the Metabolism Study Section of the National Institutes of Health. The range of error was 0 to 3 per cent. Gas-liquid chromatographic analyses on individual methyl ester samples were repeated many times with varying quantities before conclusions were drawn as to column performance. Initially, quantification of dimethylacetals gave results that were low. This was found to be a function of the pH of the stationary support. Traces of acid resulted in distorted peaks of reduced size—probably aldehydes—that separated poorly from methyl esters.

Methyl esters (and dimethylacetals) were formed from column eluates as previously reported (4). In the lecithin and sphingomyelin fractions, methyl esters were also produced by the method of Nelson and Freeman (15), as well as by sealed tube hydrolysis with 5 per cent hydrogen chloride in superdry methanol (4).

Nonpolar columns. The stationary supports used were Chromosorb W, Gas-Chrom P, Gas-Chrom CL, Gas-Chrom Z (siliconized),[4] Anakrom AB, Anakrom ABS (siliconized), and Celite 545.[5] Initially, all of these 80–100 mesh supports were washed with acid and alkali. The pH was 6.4, as determined by suspending a small amount of powder in distilled water. When the stationary supports were directly coated with stationary phase, Apiezon L or M, the dimethylacetals did not appear. It was found that *the stationary supports must not be brought back to neutrality after the alkali wash* (16, 17). They were treated thereafter in the following manner. The material was washed with 1 per cent methanolic potassium hydroxide, which was removed by filtration through a Buchner funnel. The support was dried in a convection oven for 12 hours at 120° C, and then at a concentration of 1 mg per ml distilled water, the pH of the powder was 7.4. The alkaline stationary support was coated with 10 to 15 per cent Apiezon L or M (18). The columns were conditioned for 12 to 24 hours at 225° to 250° C under argon pressure slightly above that used for normal operation. During the conditioning period, the columns were "soaked" with loads of 75 to 100 mg mixed methyl esters (19). The column efficiency was 2,700 theoretical plates at methyl stearate. Detector cells were operated at 750 to 1,250 v, depending on the amount giving optimal linearity, and the amplifier setting was 3×10^{-6} a. The column temperature was 197° C, the detector was maintained at 220°, and flash heater at 197° C. Argon pressure was varied to achieve optimal flow rates. For comparison, methyl esters of platelet PE and PS were simultaneously analyzed on an Apiezon column at the

[4] Applied Science Laboratories, State College, Pa.

[5] Analabs, Hamden, Conn.

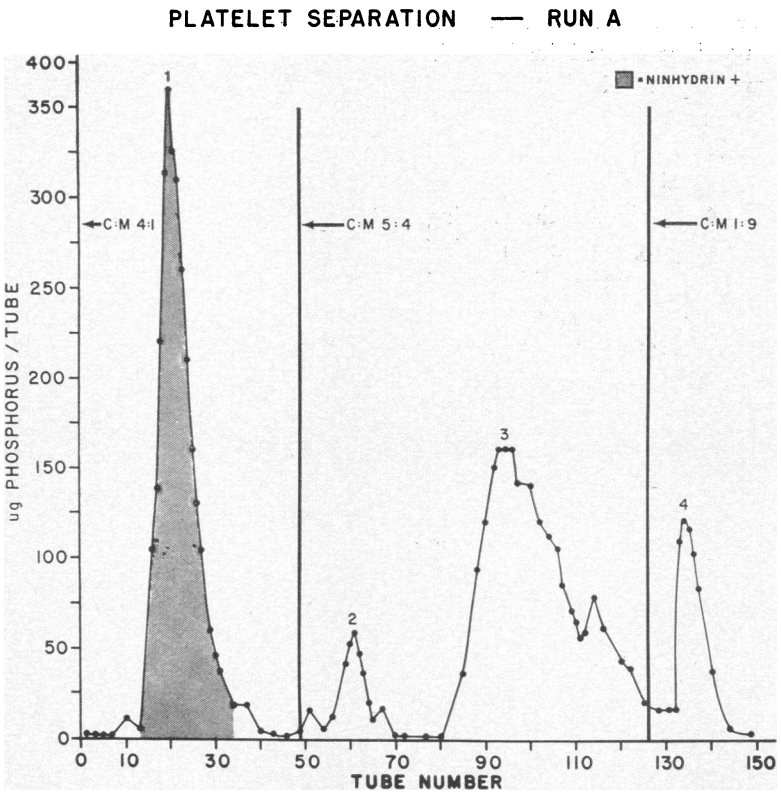

FIG. 1. FRACTIONATION OF THE MAIN PHOSPHOLIPID CLASSES IN BLOOD PLATELETS ON A SILICIC ACID COLUMN. Phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) were separated from the other platelet phosphoglycerides. All the PE and most of the PS was in peak 1. Peak 2 was mainly phosphatidyl inositol (PI), but contained small amounts of PS. Lecithin (peak 3) was well separated, but overlapped with sphingomyelin (peak 4).

Rockefeller Institute, New York, by Dr. John W. Farquhar (18).

Polar columns. The stationary supports were the same as those used for nonpolar columns. Pretreatment of the stationary supports with alkali was also helpful. The optimal pH was 6.8. It was found advantageous to "soak" the columns with methyl ester mixtures during the conditioning period. If apparent hydrolysis of dimethylacetals occurred, "resoaking" the column restored their analysis to the expected full response. The supports were coated with 10 to 15 per cent ethylene glycol adipate [6, 7] (EGA). Operating conditions were: cell voltage, 1,000 to 1,100; amplifier setting, 3×10^{-8} a; column temperature, 173° C; detector, 220° C; and flash heater, 173° C. Column efficiency was approximately 2,900 theoretical plates at methyl stearate. Ethylene glycol succinate was also used as stationary phase and gave results similar to those for EGA (4).

[6] Applied Science Laboratories, State College, Pa.

[7] Several batches of catalyst-free EGA of high molecular weight (7) that provided columns of lower "bleed" rates were furnished by Dr. J. W. Farquhar.

RESULTS

Column chromatography

With accumulated experience, the qualitative and quantitative aspect of the fractionations became more precise. When the starting material consisted of 10 to 16 g (wet weight) of platelets, the weight of the lipid obtained after extraction was 400 to 420 mg, or approximately 32 mg per g platelets. At the conclusion of run A, about 56 mg of PE and PS were obtained. After their separation in run B, the yield of PE was 25 to 35 mg and of PS, 13 to 16 mg. The final amounts varied with the number of tubes acceptable as Ninhydrin-positive, since samples that gave trace Ninhydrin reactions were arbitrarily rejected. Although all the PE finally recovered was confined to one peak (Figure 3), there were variable amounts of PS in the inositol fractions (Figure

1); this probably represented the sodium salt of PS (20), but was not investigated further. The results of run A are depicted in Figure 1. All the Ninhydrin-positive material was found in peak 1. Peak 2 was mainly PI, but showed small amounts of PS. Analysis of peak 3 showed lecithin only, and peak 4 contained mainly sphingomyelin with small overlapping amounts of lecithin. No attempt was made to purify the sphingomyelin material. Figure 2 is a photograph of a thin-layer plate showing different concentrations of samples from peak 1. The lipid was applied in varying amounts to insure that only PE and PS were present. Figure 3 indicates the phosphorus analyses on eluates from the ammoniated silicic acid column, run B. Although the ascending limb of the PE peak was always sharp, a certain amount of tailing was noted in the descending portion. It was important to allow at least 50 ml of Ninhydrin-negative eluate to appear before adding the methanol. The PS elution consistently showed a sharp configuration. Figure 4 is a photograph of a thin-layer plate examined after the PE–PS fraction was separated, pooled, and concentrated. Oxidation and breakdown products, which appear as extra spots (3), were absent. Identification was further confirmed by silicic acid paper chromatography and the pattern shown by the fatty acids and aldehydes on gas-liquid chromatography.

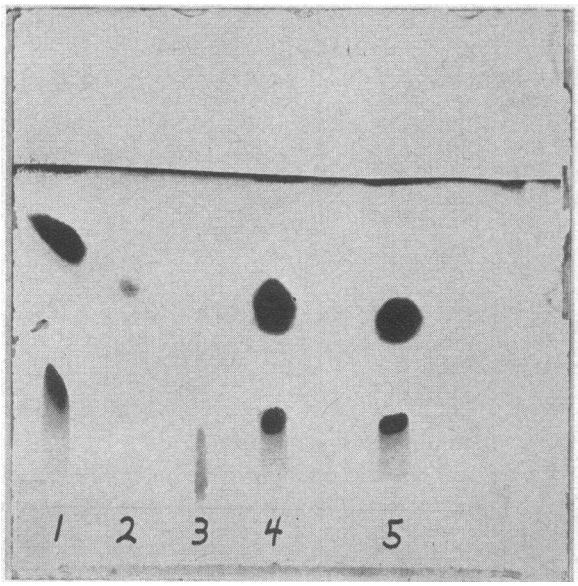

FIG. 2. THIN-LAYER CHROMATOGRAM ON SILICA GEL OF PEAK 1, RUN A. Before the combined phosphatidyl ethanolamine-phosphatidyl serine (PE–PS) fraction was rechromatographed, it was evaporated to a small volume and varying concentrations were applied to a thin-layer plate, to verify that other lipid material was not present. Lanes 1, 4, and 5 represent the PE–PS fraction, lanes 2 and 3 are PE and PS markers, respectively.

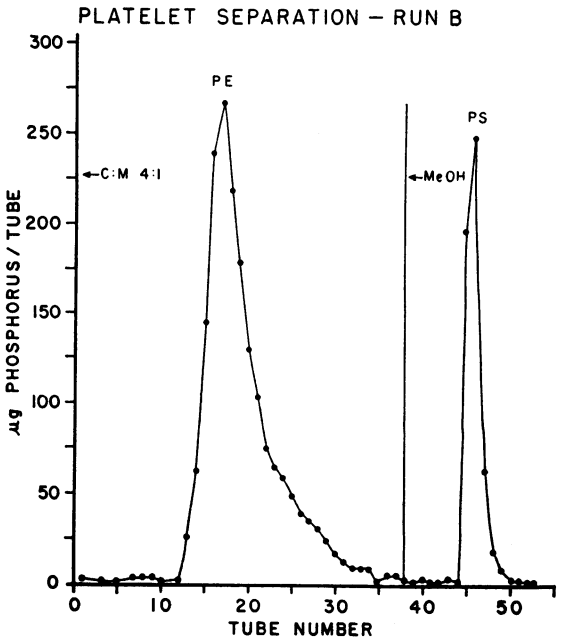

FIG. 3. SEPARATION OF PHOSPHATIDYL ETHANOLAMINE (PE) FROM PHOSPHATIDYL SERINE (PS) ON AN AMMONIATED SILICIC ACID COLUMN. The column was made alkaline by passage of 70 ml ammoniated chloroform: methanol 4:1 before application of PE–PS (3). Consequently PE was eluted first, but PS was retained and recovered later with methanol.

Figure 5 represents the absorption spectra of platelet PE and PS, respectively, as well as of the oxidized forms of these phosphatides. The lipids formed clear solutions in cyclohexane, which was a prerequisite for spectrophotometry (3). Molar extinction coefficients at 235 and 275 mμ averaged 279 and 47 for PE, while the values for PS were 618 and 179. These findings indicated that minimal oxidative changes took place (21). Figure 6 depicts a thin-layer plate of the oxidized PE and PS samples shown in Figure 5.

Blood coagulation studies

A. *Thromboplastin generation test.* Table I shows results obtained with the various phospholipids studied and with controls. The shortest

substrate clotting times obtained after 6 minutes of incubation in the TGT are presented, and the amount of lipid phosphorus introduced into the generating mixture is indicated. Separate TGT experiments were carried out 6 times with similar results.

PE had no activity in the TGT in any concentration. The PE preparations in which emulsification was enhanced by ancillary means were also inert in this test. Clotting times obtained with PS were invariably within 2 to 3 seconds of control values. The PI fractions (run A, peak 2) containing PS were also highly active. Clotting times with this combination of phospholipids were not more than 1 second from control times at optimal concentration. Lecithin and sphingomyelin had no clotting activity. In general, optimal clotting times were noted with PS at a 1 : 10 dilution. When active materials were more concentrated, a typical anticoagulant effect was observed

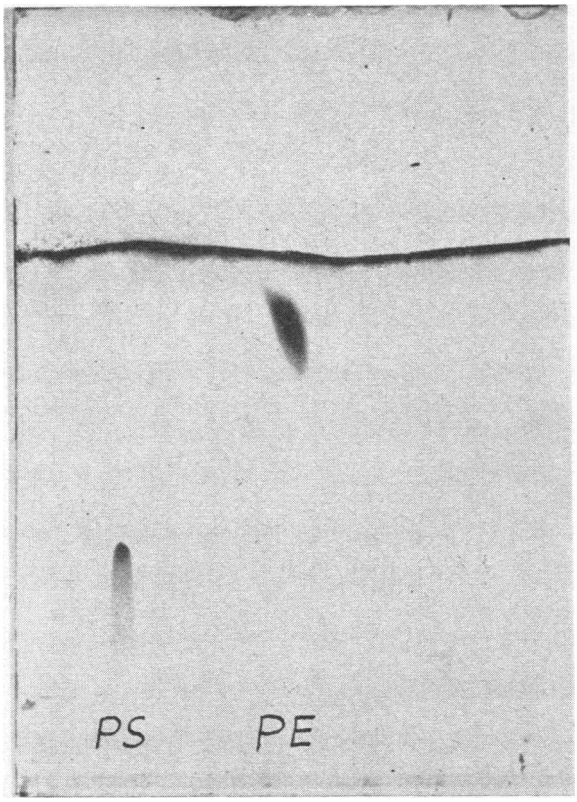

FIG. 4. THIN-LAYER SILICA GEL CHROMATOGRAPHY OF PHOSPHATIDYL ETHANOLAMINE (PE) AND PHOSPHATIDYL SERINE (PS) AFTER RUN B. PS always shows a trail in this solvent system. Silicic acid paper chromatography confirmed these results.

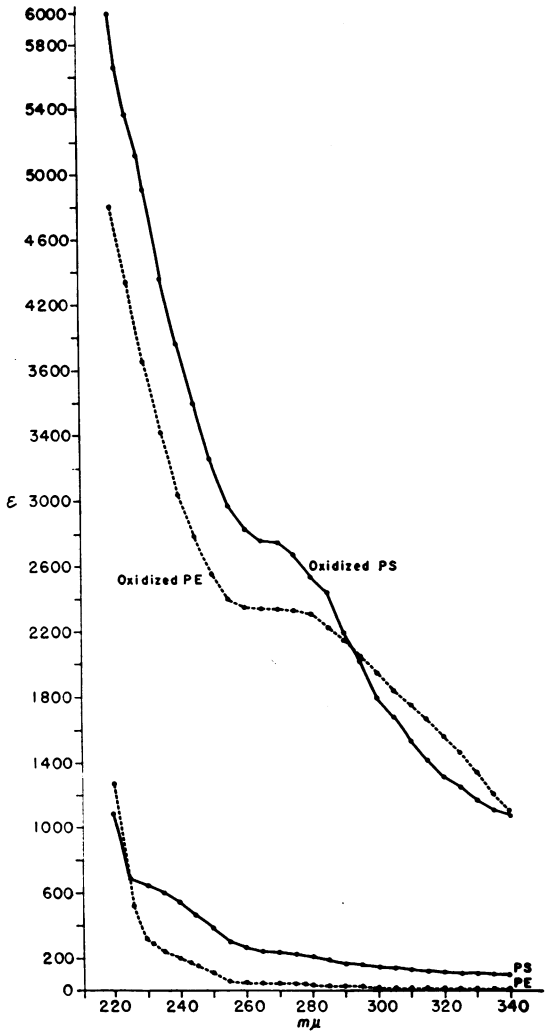

FIG. 5. ABSORPTION SPECTRA OF PHOSPHATIDYL ETHANOLAMINE (PE), PHOSPHATIDYL SERINE (PS), OXIDIZED PE, AND OXIDIZED PS. The lower curves represent spectra obtained with PE and PS after separation and maintenance in a nitrogen atmosphere. For comparison, the upper curves illustrate spectra of the same lipids after storage and handling by conventional techniques.

(1, 2). Table I also shows that 2.5 μg lipid phosphorus in the generating mixture gave optimal clotting times with PS, whereas 11.7 μg were required for the total platelet lipid extract. On the other hand, when PS was compared with brain "cephalin," the amounts needed for maximal activity were similar.

After varying periods of storage in buffer at − 20° C, the PS fractions showed considerable loss of activity in the TGT. After storage in cyclohexane under nitrogen at − 20° C, however,

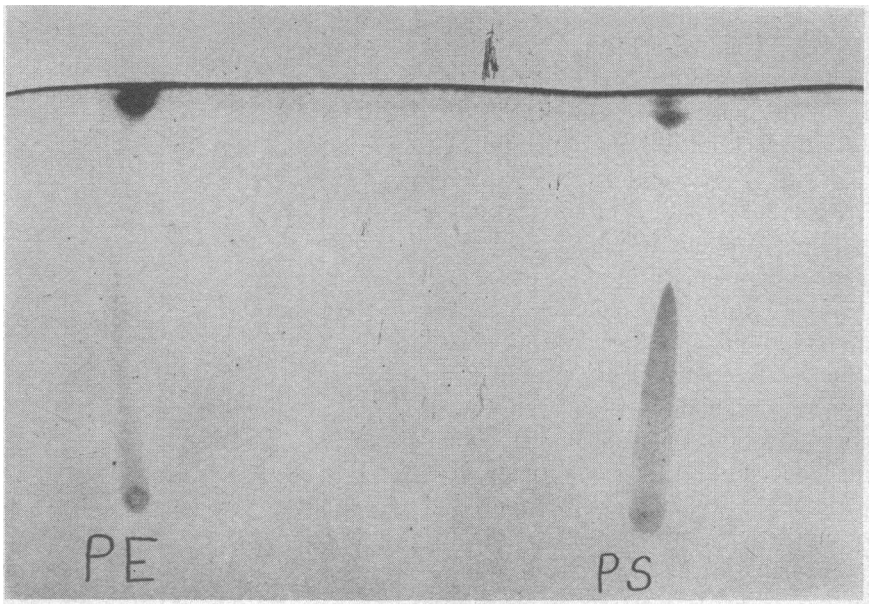

FIG. 6. THIN-LAYER SILICA GEL CHROMATOGRAM OF OXIDIZED PHOSPHATIDYL ETHANOLAMINE (PE) AND PHOSPHATIDYL SERINE (PS). The absorption spectra of these samples are shown in the upper portion of Figure 5. Oxidized PE migrates in the solvent front, shows streaking, and considerable material remains at the point of application. Oxidized PS develops marked streaking and shows material behind the solvent front and at the origin. Compare with Figure 4.

activity was preserved for at least 3 months. This correlated with spectrophotometric evidence of oxidation.

B. *Recalcified clotting time.* Figure 7 depicts the results obtained when serial dilutions of PE, PS, and total platelet lipid extracts were studied in the recalcified clotting time of platelet-poor plasma. In marked contrast to results with the TGT, PE showed good activity in this system. PS was slightly more active than PE at all concentrations. Coagulation times with total platelet lipid extracts were consistently shorter than the purified phosphoglycerides. All concentrations of lecithin and sphingomyelin were inert. PI, which contained PS, gave the same values as PS. This combination of lipids had good clotting activity. The series of recalcified clotting times were carried out on 5 separate occasions with the same results. All clotting studies carried out with brain phospholipids gave results similar to those obtained with platelet phosphatides.

TABLE I

Separated platelet phosphatides in the thromboplastin generation test *

Platelet phosphatide	Optimal clotting time after 6-minute incubation in test	P per lipid sample in generating mixture
	seconds	*μg*
PE	>90	All concentrations
PS	10	2.5
PI(+PS)	8	2.2
Lecithin	>90	All concentrations
Sphingomyelin	>90	All concentrations
Total platelet lipid (chloroform: methanol extract)	9	11.7
Brain "cephalin" control (acetone-dried, chloroform extract)	9	2.4

* Abbreviations: PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, PI = phosphatidyl inositol. Results are compared with appropriate controls. PS and PI(+PS) were active, while PE, lecithin, and sphingomyelin were inert.

Gas-liquid chromatography

Table II lists the molar distribution of the fatty acids and aldehydes in separated platelet phosphatides on EGA columns. Table III lists the equivalent information provided by Apiezon columns, and also lists the confirmatory findings with our PE and PS preparations analyzed independently by Dr. John W. Farquhar. Figures 8 and 9 show

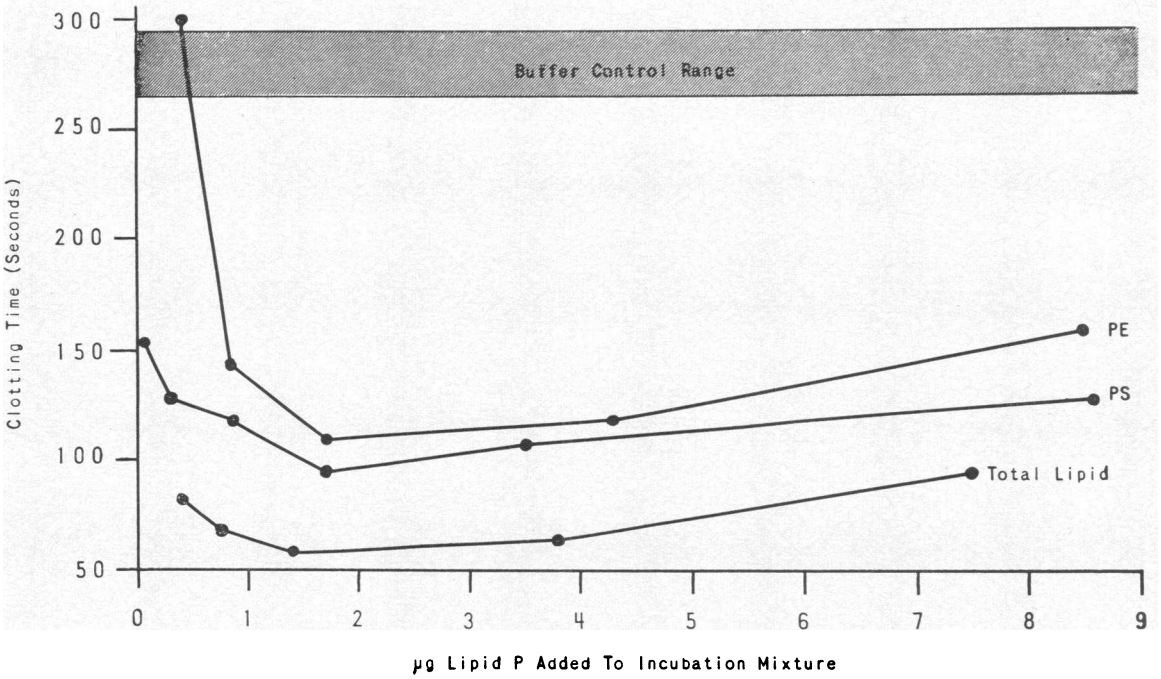

FIG. 7. EFFECT OF PLATELET LIPIDS ON THE RECALCIFIED CLOTTING TIME OF PLATELET-POOR PLASMA. Both phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) were capable of shortening the recalcified clotting time of platelet-poor plasma. Phosphatidyl inositol (PI)–PS gave the same results, but lecithin and sphingomyelin (not shown) had no activity. The total platelet lipid extract always showed the shortest clotting times.

TABLE II

Fatty acids and aldehydes of platelet phosphatides on ethylene glycol adipate polyester at 173° C *

Familiar name	Shorthand designation†	PE	PS	PI(+PS)	Lecithin
		moles %	*moles %*	*moles %*	*moles %*
Lauric	12:0		0.2	trace	0.1
	14:0 DMA		0.2		
Myristic	14:0	trace	0.6	trace	0.1
	15:0	0.1	0.5		0.3
	16:0 DMA	8.8	0.9	0.4	0.2
Palmitic	16:0	3.1	0.9	1.9	35.0
Palmitoleic	16:1	0.4	1.4	0.5	1.3
	?	0.3			
	17:0	0.1		0.6	0.7
	?	0.2	trace		
	18:0 DMA	19.9	0.2	0.5	
Stearic	18:0	18.2	46.9	46.1	13.7
Oleic	18:1	7.2	20.1	11.7	31.2
Linoleic	18:2	0.8	1.7	1.4	5.2
Linolenic	18:3	1.3			
Arachidic	20:0	0.2	1.3	trace	0.8
	20:1		1.3		1.1
	20:3			1.5	0.5
Arachidonic	20:4	36.2	23.9	35.4	9.8
	20:5	0.3			
	22:un‡	1.9			
	22:5	0.2			
	22:5	0.1			
	22:6	0.8			

* Abbreviations as in Table I, and DMA = dimethylacetals.
† No. of carbon atoms:no. of double bonds.
‡ un = unsaturated acid.

TABLE III

Fatty acids and aldehydes of platelet phosphatides on Apiezon L at 197° C *

Familiar name	Shorthand designation†	PE	PE ‡	PS	PS ‡	PI(+PS)	Lecithin
		moles %	*moles %*	*moles %*	*moles %*	*moles %*	*moles %*
Lauric	12:0			trace		0.2	
Myristic	14:0	trace	trace	trace		0.3	trace
	15:0	0.1	0.2			0.3	0.2
	16:0 DMA	10.5	11.1	0.3	1.8	1.2	0.8
Palmitic	16:0	3.3	3.5	0.7	1.8	2.7	33.4
Palmitoleic	16:1	0.3	0.3	0.3	0.3	0.7	0.9
	17:0br§ DMA	0.3					
	17:0			0.3	0.2	0.4	trace
	18:0 DMA	19.0	21.1	0.2		0.8	
Stearic	18:0	13.8	16.1	47.2	44.4	45.6	16.5
	18:1 DMA	3.8	2.1				
Oleic	18:1	8.4	6.9	22.6	22.4	11.7	31.3
Linoleic and linolenic	18:2 + 18:3	0.9	1.0	0.9	1.7	0.6	5.2
Arachidic	20:0			1.3			
	20:1			0.6			
	20:3	1.1	0.9	0.9	1.4	1.1	
Arachidonic	20:4(+20:5)	37.5	36.8	24.9	26.2	34.6	11.9
	22:5	0.5					
	22:6	0.6					

* Abbreviations as in Tables I and II.
† No. of carbon atoms: no. of double bonds.
‡ Results obtained by Dr. J. W. Farquhar (18).
§ br = branched-chain acid.

tracings of chromatograms of PE on EGA and Apiezon columns, and Figures 10 and 11 show them of PS on similar columns.

Noteworthy features of each phosphatide are as follows. *a*) *PE*. The main saturated fatty acid was stearic, 15 moles per cent, and the main unsaturated acid was arachidonic, 36 moles per cent. The fatty aldehydes of platelet phospholipids were confined mainly to PE and almost all were of the saturated straight-chain 16:0 and 18:0 type. The 18:0 aldehyde was twice the amount of 16:0 aldehyde. Twenty- and 22-carbon aldehydes were not found, but one type of branched 17-carbon saturated aldehyde (7) was noted on Apiezon. It is significant that the molar ratio of saturated fatty acids plus fatty aldehydes to unsaturated fatty acids approached unity in the ethanolamine phospholipid class (12) (Table IV). *b*) *PS*. The main saturated fatty acid was stearic, 46 moles per cent, and the main unsaturated fatty acids were oleic, 21 moles per cent, and arachidonic, 25 moles per cent. The aldehydes found were principally of the saturated straight-chain 16:0 and 18:0 type, but were present in very small amounts. As in PE, the ratio of saturated fatty acids plus fatty aldehydes to unsaturated fatty acids approached unity (Table IV). *c*) *PI (with PS)*. The qualitative fatty acid and aldehyde composition of these fractions was like PS in that stearate, oleate, and arachidonate predominated, but there was more arachidonate and less oleate. Although small amounts of PS were present in these fractions, the

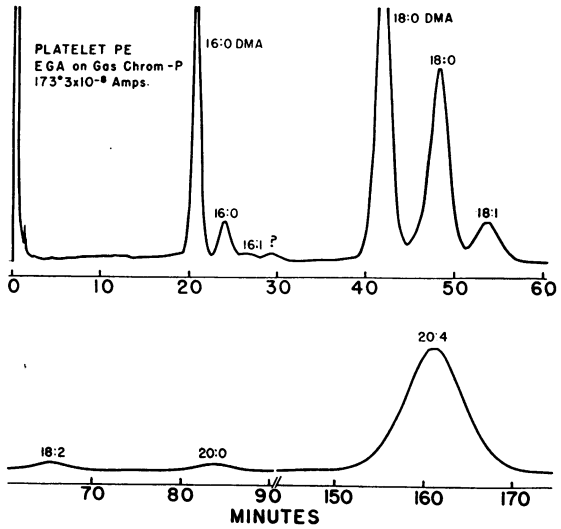

FIG. 8. TRACING OF GAS-LIQUID CHROMATOGRAM SHOWING FATTY ACIDS AND ALDEHYDES OF PLATELET PHOSPHATIDYL ETHANOLAMINE ON ETHYLENE GLYCOL ADIPATE (EGA) POLYESTER (POLAR). Note the large dimethylacetal (DMA) peaks preceding the 16:0 and 18:0 fatty acids.

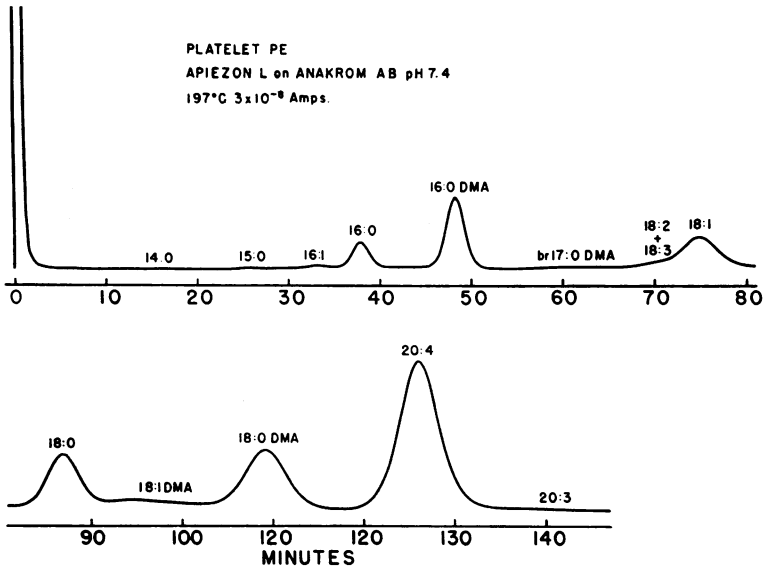

FIG. 9. FATTY ACIDS AND ALDEHYDES OF PLATELET PHOSPHATIDYL ETHANOLAMINE (PE) ON APIEZON L (NONPOLAR). Arachidonic was the main fatty acid of PE.

fatty acid pattern was felt to be representative of PI. *d) Lecithin.* Large amounts of palmitic acid, 34 moles per cent, were found in lecithin. The major unsaturated fatty acid was oleic, 31 moles per cent. Aldehydes of type 18:0 were not found and there was less than 1 mole per cent 16:0 aldehyde. Lecithin differed from PE in containing more palmitic and oleic but less arachidonic acid. There were marked quantitative differences between PS and lecithin with regard to palmitate, stearate, and arachidonate. Results with samples of PE and PS methyl esters chromatographed on Apiezon columns at the Rockefeller Institute were qualitatively and quantitatively similar to those reported here (Table III).

The dimethylacetals in PE comprised about 33 moles per cent of the total fatty chains. If the aldehydogenic group is linked to the α' (C-1)

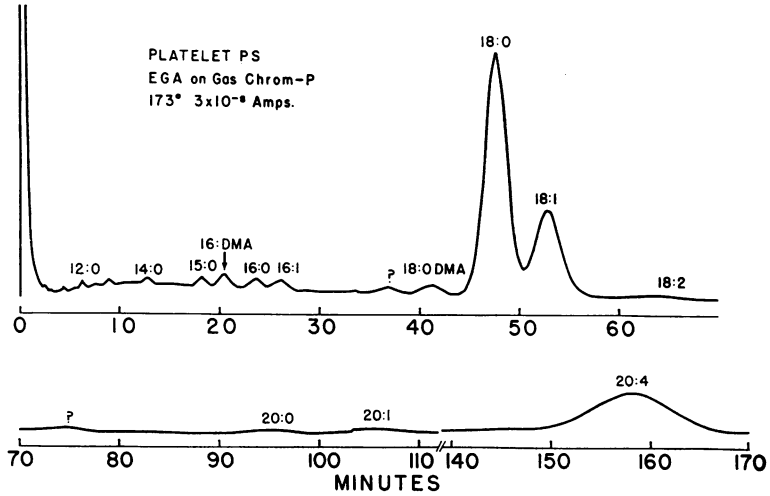

FIG. 10. TRACING OF GAS-LIQUID CHROMATOGRAM OF FATTY ACIDS AND ALDEHYDES OF PLATELET PHOSPHATIDYL SERINE (PS) ON ETHYLENE GLYCOL ADIPATE (EGA) POLYESTER. Very little plasmalogen (DMA) was present in the PS fractions. PS contained large amounts of stearate.

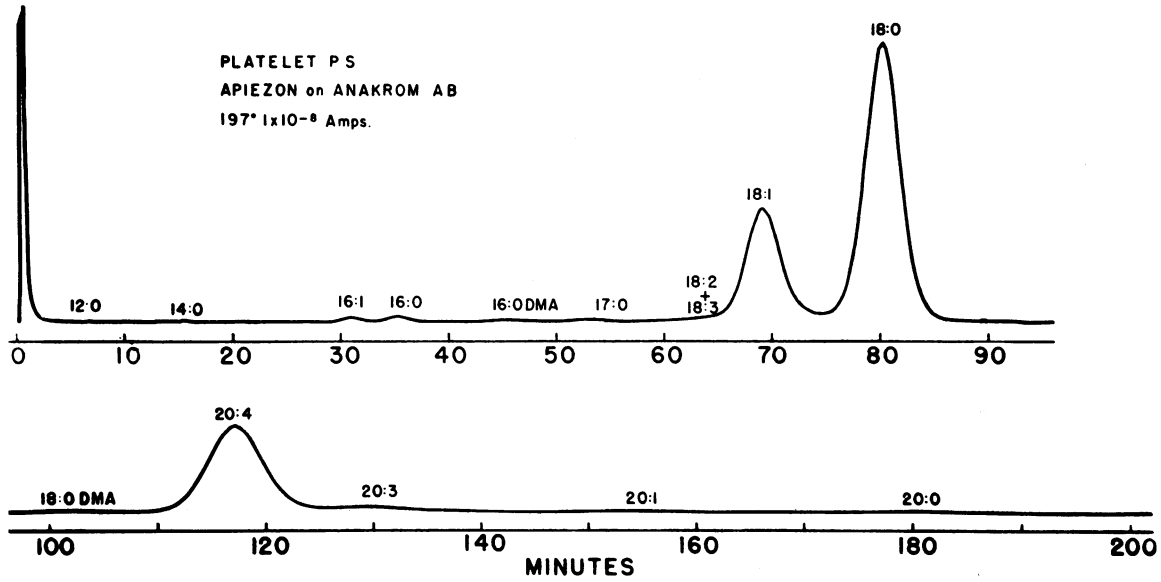

FIG. 11. TRACING OF GAS-LIQUID CHROMATOGRAM OF PLATELET PHOSPHATIDYL SERINE (PS) ON APIEZON L. As in phosphatidyl ethanolamine, arachidonic was the main unsaturated fatty acid.

carbon atom of glycerol and an unsaturated fatty acid is linked to the β (C-2) carbon as an acyl ester (see Discussion), then PE is 66 per cent in the plasmalogen form. This agrees with results obtained by iodine addition, in which PE was found to contain 65 per cent plasmalogen. Iodine addition showed 5 per cent of the choline phosphatides to be plasmalogen.

DISCUSSION

An important advance in lipid chemistry is the technique of quantitative isolation of PE separately from PS in a high degree of purity (3, 9). It was possible to recover these lipids in relatively substantial amounts from platelets. Molecular extinction coefficients were determined at 235 mμ, the region of conjugated diene hydroperoxide absorption, and at 275 mμ, a region which shows other products of auto-oxidation (21). The PE and PS preparations were essentially free of oxidation products, and findings were similar to those previously reported by Rouser and associates (3). In our experience, other techniques for the separation of PE from PS such as Folch fractionations or single silicic-acid column runs invariably resulted in mixtures of lipid classes as well as oxidation products.

PI was eluted as a sharp peak, but thin-layer and paper chromatography of concentrated samples showed the presence of traces of PS. Similar identification procedures revealed lecithin fractions to be pure. The sphingomyelin peak contained traces of lecithin. Although lecithin and sphingomyelin can now be separated (9), the procedure was not used in this study. The over-all pattern of run A was similar to that obtained with red cell

TABLE IV

*Molar composition of fatty chains of platelet phosphatides**

Platelet phosphatide	A Saturated fatty acids		B Fatty aldehydes		C Unsaturated fatty acids		A/C		A+B/C	
	EGA	Apiezon	EGA	Apiezon	EGA	Apiezon	EGA	Apiezon	EGA	Apiezon
PE	21.7 †	17.2	28.7 †	33.6	49.2	49.3	0.44	0.35	1.02	1.03
PS	50.4	49.5	1.3	0.5	48.4	50.2	1.04	0.99	1.07	1.00
Lecithin	50.7	50.1	0.2	0.8	49.1	49.3	1.03	1.02	1.04	1.03

* Abbreviations as in Table I, and EGA =ethylene glycol adipate.
† The figure for saturated fatty acids is probably too high and that for fatty aldehydes too low, since 18:1 dimethylacetal has a retention time similar to that of stearate on EGA.

phospholipids and phosphatides of human plasma by Hanahan, Watts, and Pappajohn (10). Thin-layer chromatography was valuable for rapid detection of contaminating lipids and oxidation products. This procedure, in combination with silicic-acid paper chromatography and the fatty acid patterns on gas-liquid chromatography was sufficient for evaluation of completeness and purity of the separations.

Since adequate amounts of platelet PE separated under nitrogen were available to us for the first time, an intensive effort was made to study its properties in the TGT, a system that has been found sensitive and specific for evaluating clot-promoting properties of phospholipids. This phosphatide is inert in the TGT, even if adequately suspended. Indeed, it is probable that activity in this test with PE preparations is an indication that they contain trace amounts of PS (2). This finding has also been reported by Slotta and Powers, who studied brain "cephalin" fractions, separated by the Folch technique (22, 23). In contrast to its inert behavior in the TGT, PE was effective in shortening the recalcified clotting time and active in other test systems (13, 24).

Wallach, Maurice, Steele, and Surgenor (25) separated PE from egg yolk, and in the unoxidized state it showed good clot-promoting activity in the thrombin generation test. These authors stressed that coagulation properties of PE were associated with micelles of a limited size and surface configuration, dependent upon the presence of a high percentage of polyunsaturated fatty acids. In this important study, the investigators have pointed out that information derived from colloid chemistry of soaps seems to be applicable to blood coagulation. Although these findings have added a great deal to our knowledge, it remains for future research to determine whether the early phases of blood coagulation, involving phospholipids and plasma proteins, can be attributed mainly to colloidal properties of the phosphatides. It is important to mention that human platelet PE is not comparable to egg PE with special reference to two points: *a*) platelet PE is 66 per cent in the plasmalogen form, whereas egg yolk contains no plasmalogen, and *b*) on the basis of the fatty acid composition given for egg yolk PE (25), a substantial degree of diunsaturation must be postulated, but from present evidence, the existence of such molecules appears unlikely in platelet PE.

PS fractions were active in the TGT to the same extent as previously noted (1, 2). Our observations and those of other investigators were confirmed (22, 26–28). It would have been of interest to study the clotting activity of PI, but contamination with PS was unavoidable. In TGT experiments with PI isolated from peas,[8] the material was inactive. The results with platelet and brain PI, containing small amounts of PS, were striking in that these showed excellent activity in both the TGT and recalcified clotting times.

Lecithin was inactive in the TGT and recalcified clotting time. Gas-liquid chromatographic studies indicated that it contained approximately a 1 : 1 molar ratio of saturated and unsaturated fatty acids, and traces of aldehyde. This may indicate that the presence of a 50 per cent molar content of unsaturated fatty acids is not the only requisite for coagulant activity of phospholipids.

PE and PS consistently and uniformly shortened the recalcified clotting time of platelet-poor plasma. The results with PE are in excellent agreement with those of Rouser, White, and Schloredt (13) as well as with those of experiments performed with these phosphatides in a collaborative study by Ferguson, Marcus, and Robinson (24). In our hands, the PS was more active than PE. On the basis of weight, however, the total platelet lipid extract and brain "cephalin" preparations were even more active in the recalcified clotting time.

In earlier experiments with the TGT (1, 2), PS fractions were many times more active than total lipid extracts on the basis of weight. It was surprising that PS, present as about 4 per cent of the total platelet lipid, showed only a fivefold increase in activity over total platelet lipid controls. This result was less than expected if all the activity in platelets were due to PS. There are two possible explanations: *a*) purification procedures may exert some unknown effect to diminish the clotting activity of PS, and *b*) the other platelet lipids, in themselves inactive, may enhance the PS activity. Such findings may also indicate that under physiological conditions, all the platelet phospholipids are responsible collectively for thromboplastic activity.

[8] Provided by Dr. A. C. Wagenknecht.

When the purified phospholipids were stored in cyclohexane under nitrogen, their clotting activity was retained for long periods. Even under these circumstances, however, they eventually showed spectrophotometric evidence of oxidation, and activity in the TGT was markedly diminished. In the Ferguson two-stage clotting test (24), the activity of PS seemed to improve after oxidative changes took place, and a similar phenomenon has been observed in other coagulation systems (25).

When difficulty was encountered in gas-liquid chromatographic analysis of dimethylacetals on nonpolar columns, the initial impression was that "acid sites" in the inert supports were responsible for hydrolytic changes, despite the fact that they were in a neutral pH range after acid and alkali washings. A systematic study of available gas-liquid chromatographic supports showed that uncoated packings that were made alkaline to a pH of 7.5 before application of the stationary phase gave good results. Pretreatment of stationary supports with alkali was first suggested by James (19), and this procedure has been in common use for chromatography of methyl esters and fatty aldehyde dimethylacetals on Apiezon columns (6). We also found that alkali washing of stationary supports improved the results with EGA columns. It has become apparent from gas-liquid chromatographic studies on aliphatic diamines (29) that treatment with alkali is not merely neutralization of "acid sites," but a more complicated phenomenon, not yet understood. It has been emphasized that gas-liquid chromatographic analyses, especially of plasmalogens, ought to be carried out on two types of columns, polar and nonpolar (7), and it seems desirable to confirm the accuracy of this method of plasmalogen calculation with other techniques such as iodine addition (14), or the *p*-nitrophenylhydrazone reaction (5). It was advantageous to evaluate column performance and detector response by comparison of results with those obtained on the same sample with another gas-liquid chromatographic apparatus of established linearity (18). Our quantification of platelet PE plasmalogen is 4 to 5 times higher than results reported in a recent publication by Blomstrand, Nakayama, and Nilsson (30). These authors used the hydrolysis technique of Dawson (31), who had previously reported values for human erythrocyte plasmalogens considerably lower than those found by Farquhar (12). These differences increase the need for confirmatory methods of determining plasmalogen.

Certain characteristics of the fatty acids and aldehydes that are distinctive for the various types of platelet phosphatides are worthy of comment, but their relevance to the biological properties of these phospholipids is not yet known. The main unsaturated fatty acid in PE and PS was arachidonic, as previously reported (4) when the "cephalins" were studied as a group, but now the 20:4 distribution is better defined. Although stearic was the main saturated fatty acid in PE, it accounted for less than 20 moles per cent of the fatty chains, whereas about half of the fatty acid components of PS were stearate. The fatty acids of lecithin showed substantial quantitative differences from PE and PS. Palmitic was the main saturated and oleic the main unsaturated fatty acid. The amount of oleate was increased over its level in the PE and PS fractions. Although arachidonic acid was low in the choline phosphoglycerides, more was present than originally reported (4) when separations were carried out under less stringent conditions with regard to oxidative changes.

Recently, important studies on the structure of phosphoglycerides have been reported by Marinetti, Erbland, and Stotz (32), Debuch (33), Tattrie (34), and Hanahan, Brockerhoff, and Barron (35). They indicated that in the plasmalogen and diacyl forms of PE and lecithin, the α' (C-1) carbon of glycerol was linked to either a saturated fatty acid or an aldehyde, and the β (C-2) carbon, to an unsaturated fatty acid. In platelet PE, the molar ratio of saturated fatty acids plus aldehydes to unsaturated fatty acids was unity (Table IV), as also for PS and lecithin. Farquhar has reported similar ratios in the fatty chains of erythrocyte phospholipids (12). These findings furnish indirect evidence that the fatty chains of human platelet and erythrocyte phospholipids are arranged as in other tissues (32–35). In general, there are many similarities between red cells (12) and platelets with regard to plasmalogen content and fatty acid composition. Farquhar found erythrocyte PE to be 67 per cent plasmalogen and 66 per cent of platelet PE is in this form. The main red cell fatty acids were similar to those of platelets in the separated "cephalin" and choline classes.

Recently, the large percentage of plasmalogen in human erythrocytes (12) and human myelin (36) led to speculation that these lipids might share common functions in cell plasma membranes. Since platelet phospholipids are similar to red cells and myelin, they may also be considered as largely derived from the plasma membrane. It will be important, however, to know the lipid composition of platelet organelles such as mitochondria before definite conclusions can be drawn. Further investigation will also be required to determine whether analogies of structure and function are possible in tissues showing the same membrane lipid constituents. Generalizations about the relationship of biological activity to lipid composition must be reconciled with differences in phosphatide make-up between the same tissues of various animal species (37, 38). As Gray and Macfarlane have demonstrated, investigations in comparative biochemistry indicate the need to avoid premature correlations of this nature (37, 38).

Although the function of plasmalogens is not known, speculations have been made (12, 39). Participation in blood coagulation is possible, but earlier investigations (5, 13) have given evidence against such activity.

SUMMARY

1. Two types of column chromatography, silicic-acid and ammoniated silicic-acid, were used to fractionate the phospholipids of human blood platelets and obtain highly purified phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). All separation procedures were carried out under nitrogen, thus minimizing oxidative changes. Fatty acid and plasmalogen concentrations were measured by gas-liquid chromatography on polar and nonpolar columns.

2. A total lipid extract of 400 mg contained 25 to 35 mg PE and 13 to 16 mg PS.

3. There were distinct differences in the fatty acid and fatty aldehyde composition among the separated platelet phosphatides. Stearate, oleate, and arachidonate predominated in the "cephalin" class, while the main fatty acids of lecithin were palmitate and oleate. Plasmalogen was found mainly in the ethanolamine phospholipid group. Sixty-six per cent of platelet PE was in the plasmalogen form.

4. Both PE and PS shortened the clotting time of recalcified platelet-poor plasma, but only PS was active in the thromboplastin generation test. Phosphatidyl inositol fractions contained small amounts of PS and showed marked thromboplastin-promoting properties in both tests.

5. Similar studies carried out on human brain phospholipids gave comparable results.

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REFERENCES

1. Marcus, A. J., and Spaet, T. H. Platelet phosphatides: their separation, identification, and clotting activity. J. clin. Invest. 1958, **37**, 1836.
2. Marcus, A. J., Ullman, H. L., and Wolfman, M. Studies on blood platelet phospholipids. J. Lipid Res. 1960, **1**, 179.
3. Rouser, G., O'Brien, J., and Heller, D. The separation of phosphatidyl ethanolamine and phosphatidyl serine by column chromatography. J. Amer. Oil chem. Soc. 1961, **38**, 14.
4. Marcus, A. J., Ullman, H. L., and Ballard, H. S. Fatty acids of human platelet phosphatides. Proc. Soc. exp. Biol. (N. Y.) 1961, **107**, 483.
5. Zilversmit, R. D., Marcus, A. J., and Ullman, H. L. Plasmalogen in human blood platelets. J. biol. Chem. 1961, **236**, 47.
6. Gray, G. M. The separation of the long chain fatty aldehydes by gas-liquid chromatography. J. Chromat. 1960, **4**, 52.
7. Farquhar, J. W. Identification and gas-liquid chromatographic behavior of plasmalogen aldehydes and their acetal, alcohol, and acetylated alcohol derivatives. J. Lipid Res. 1962, **3**, 21.
8. Hirsch, J., and Ahrens, E. H., Jr. The separation of complex lipide mixtures by the use of silicic acid chromatography. J. biol. Chem. 1958, **233**, 311.
9. Rouser, G., Bauman, A. J., Kritchevsky, G., Heller, D., and O'Brien, J. S. Quantitative chromatographic fractionation of complex lipid mixtures: brain lipids. J. Amer. Oil chem. Soc. 1961, **38**, 544.
10. Hanahan, D. J., Watts, R. M., and Pappajohn, D. Some chemical characteristics of the lipids of human and bovine erythrocytes and plasma. J. Lipid Res. 1960, **1**, 421.
11. Bartlett, G. R. Phosphorus assay in column chromatography. J. biol. Chem. 1959, **234**, 466.
12. Farquhar, J. W. Human erythrocyte phosphoglycerides. I. Quantification of plasmalogens, fatty acids, and fatty aldehydes. Biochim. biophys. Acta (Amst.) 1962, **60**, 80.

13. Rouser, G., White, S. G., and Schloredt, D. Phospholipid structure and thromboplastic activity. I. The phosphatide fraction active in recalcified normal human plasma. Biochim. biophys. Acta (Amst.) 1958, **28**, 71.
14. Rapport, M. M., and Alonzo, N. F. The structure of plasmalogens. V. Lipids of marine invertebrates. J. biol. Chem. 1960, **235**, 1953.
15. Nelson, G. J., and Freeman, N. K. The phospholipid and phospholipid fatty acid composition of human serum lipoprotein fractions. J. biol. Chem. 1960, **235**, 578.
16. James, A. T. Personal communication.
17. Gray, G. M. Personal communication.
18. Farquhar, J. W., Insull, W., Jr., Rosen, P., Stoffel, W., and Ahrens, E. H., Jr. The analysis of fatty acid mixtures by gas-liquid chromatography: construction and operation of an ionization chamber instrument. Nutr. Rev. 1959, **17**, suppl., 1.
19. James, A. T. Qualitative and quantitative determination of the fatty acids by gas-liquid chromatography *in* Methods of Biochemical Analysis, D. Glick, Ed. New York, Interscience, 1960, vol. 8, p. 1.
20. Marinetti, G. V., Erbland, J., and Stotz, E. The separation of ionic forms of phosphatidylserine by column chromatography. Biochim. biophys. Acta (Amst.) 1958, **30**, 41.
21. Lea, C. H. Some observations on the preparation and properties of phosphatidylethanolamine *in* Biochemical Problems of Lipids, G. Popják and E. LeBreton, Eds. New York, Interscience, 1956, p. 81.
22. Slotta, K. H. Thromboplastin. I. Phospholipid moiety of thromboplastin. Proc. Soc. exp. Biol. (N. Y.) 1960, **103**, 53.
23. Slotta, K. H., and Powers, J. K. Determination of ethanolamine and serine in phospholipids. Fed. Proc. 1962, **21**, 293.
24. Ferguson, J. H., Marcus, A. J., and Robinson, A. J. Purified platelet phospholipids and blood coagulation. In preparation.
25. Wallach, D. F. H., Maurice, P. A., Steele, B. B., and Surgenor, D. M. Studies on the relationship between the colloidal state and clot-promoting activity of pure phosphatidylethanolamines. J. biol. Chem. 1959, **234**, 2829.
26. Therriault, D., Nichols, T., and Jensen, H. Purification and identification of brain phospholipides associated with thromboplastic activity. J. biol. Chem. 1958, **233**, 1061.
27. Troup, S. B., Reed, C. F., Marinetti, G. V., and Swisher, S. N. Thromboplastic factors in platelets and red blood cells: observations on their chemical nature and function in *in vitro* coagulation. J. clin. Invest. 1960, **39**, 342.
28. Barkhan, P., Silver, M. J., and O'Keefe, L. The lipids of human erythrocytes and platelets and their effects on thromboplastin formation *in* Blood Platelets, S. A. Johnson, R. W. Monto, J. W. Rebuck, R. C. Horn, Jr., Eds. Boston, Little, Brown, 1961, p. 303.
29. Smith, E. D., and Radford, R. D. Modification of gas chromatographic substrates for the separation of aliphatic diamines. Anal. Chem. 1961, **33**, 1160.
30. Blomstrand, R., Nakayama, F., and Nilsson, I. M. Identification of phospholipids in human thrombocytes and erythrocytes. J. Lab. clin. Med. 1962, **59**, 771.
31. Dawson, R. M. C. A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem. J. 1960, **75**, 45.
32. Marinetti, G. V., Erbland, J., and Stotz, E. The structure of beef heart plasmalogens. J. Amer. chem. Soc. 1959, **81**, 861.
33. Debuch, H. Über die Stellung des Aldehyds im Colaminplasmalogen aus Gehirn. Hoppe-Seylers Z. physiol. Chem. 1959, **314**, 49.
34. Tattrie, N. H. Positional distribution of saturated and unsaturated fatty acids on egg lecithin. J. Lipid Res. 1959, **1**, 60.
35. Hanahan, D. J., Brockerhoff, H., and Barron, E. J. The site of attack of phospholipase (lecithinase) A on lecithin: a re-evaluation. Position of fatty acids on lecithins and triglycerides. J. biol. Chem. 1960, **235**, 1917.
36. Webster, G. R. Studies on the plasmalogens of nervous tissue. Biochim. biophys. Acta (Amst.) 1960, **44**, 109.
37. Gray, G. M. The phospholipids of ox spleen with special reference to the fatty acid and fatty aldehyde compositions of the lecithin and kephalin fractions. Biochem. J. 1960, **77**, 82.
38. Gray, G. M., and Macfarlane, M. G. Composition of phospholipids of rabbit, pigeon and trout muscle and various pig tissues. Biochem. J. 1961, **81**, 480.
39. Rapport, M. M. Plasmalogen structure and the chemical reactivity of α,β-unsaturated ethers *in* The Biology of Myelin, S. R. Korey, Ed. New York, Paul B. Hoeber, 1959, p. 282.