PHOSPHOLIPID STRUCTURE AND THROMBOPLASTIC ACTIVITY*

I. THE PHOSPHATIDE FRACTION ACTIVE IN RECALCIFIED NORMAL HUMAN PLASMA

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Many investigators have reported that phospholipids can accelerate the rate of coagulation of blood or blood plasma (see 1, 2 and 3 for early literature). Attempts to identify the active substance have led to confusing results and both lecithin and the "cephalin" fraction have been reported to be active. Recent reports have also been conflicting. POOLE[4] reported that both saturated and unsaturated higher fatty acids greatly accelerate the clotting time of recalcified plasma, while POOLE AND ROBINSON[5,6], ROBINSON[7], ROBINSON AND POOLE[8], and O'BRIEN[9,10], using older types of fractionation procedures, have presented evidence which indicates that phosphatidyl ethanolamine is the active phosphatide in lipid acceleration of blood clotting, a conclusion reached by previous investigators using similar techniques. RAPPORT[11] has concluded that phosphatidyl serine is the active phosphatide. BARKHAN *et al.*[12] found brain phosphatidyl serine to be inhibitory except at low concentration where it proved to accelerate clotting slightly. TURNER AND SILVER[13] and SILVER *et al.*[14] reported phosphatidyl serine to inhibit clotting, while in an earlier report CHARGAFF[1] concluded that phosphatidyl serine is inactive.

It is evident that the nature of the thromboplastic lipid has not been established. The possible activity of water-soluble impurities or phosphatide-hydrolysis products in clotting systems has not been absolutely excluded. Questions remained about the relationship between fatty acid composition, base requirement, and effect of plasmalogen structure on activity of phosphatides in clotting. In the present communication it will be shown that the phosphatidyl ethanolamine containing two unsaturated fatty acids is the active phosphatide, and in the following communication the relationship between fatty acid composition and phosphatidyl ethanolamine activity will be described. Definitive answers have been obtained by the use of the recently described column and paper chromatographic methods for the separation of phospholipids[15,16]. The conflicting reports in the literature appear to have been the result of inadequate procedures of isolation and characterization, differences in clotting test systems, and the failure to appreciate the labile nature of highly active phosphatide.

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MATERIALS AND METHODS

Sources and preparation of highly purified lipids

Synthetic L-α-distearoyllecithin, monopalmitoylglycollecithin, L-α-dimyristoylphosphatidyl ethanolamine, and L-α-distearoylphosphatidyl-L-serine were obtained from Dr. ERIC BAER. Other highly purified preparations used were beef-brain sphingomyelin prepared by Dr. G. V. MARINETTI, monophosphoinositide from horse liver isolated by Dr. J. M. MCKIBBIN[17], yeast dipalmitoleyllecithin[18] obtained through the courtesy of Dr. MARTIN ROBINSON, and cerebroside (cerebron) from Dr. N. RADIN[19]. Palmitic, stearic, oleic, lauric, myristic, octanoic, and hexanoic acids were purchased from Eastman Kodak Co., Rochester, N. Y. Arachidic, behenic, lignoceric, linoleic and linolenic acids were obtained from Delta Chemical Works, New York, N.Y. Surface-active agents BPE and TR were obtained through the courtesy of American Cyanamid Co., New York, N.Y. These agents are esters of sulfosuccinic acid. Agent BPE has two ester-linked 2-(*p-tert.*-butylphenoxy)-ethyl groups and agent TR two ester-linked $C_{13}H_{27}$ branched chains (mixed isomers).

Dipalmitoleylphosphatidic acid was prepared from dipalmitoleyllecithin by the enzymic method of KATES[20] and isolated from silicic acid columns using chloroform–methanol (4:1) for elution.

Lysolecithin was prepared by treating dipalmitoleyllecithin with moccassin venom. *Agkistrodon piscivorus* venom obtained from Ross Allen's Reptile Institute, Silver Springs, Fla., was incubated with the phosphatide in ether according to the method of HANAHAN *et al.*[21].

Lysophosphatidyl ethanolamine was prepared from phosphatidyl ethanolamine isolated by silicic acid chromatography from soybean, rabbit appendix and human blood platelet phosphatides by moccasin-venom degradation. Mixtures of lysolecithin and lysophosphatidyl ethanolamine were prepared from phosphatide mixtures obtained from various sources by the same technique. In each case paper chromatographic procedures demonstrated the virtually complete hydrolysis of the phosphatides to the corresponding lyso compounds.

Isolation of phosphatidyl ethanolamine and other phosphatides from mixtures was accomplished by silicic acid column chromatography[15]. Initial attempts to isolate products from paper chromatograms showed this to be possible, but owing to rapid oxidative alteration of the phosphatides after chromatography this method was found less desirable for routine work.

Extraction of phospholipids

All extracts were prepared using the following general methods. A preliminary extraction with five volumes of 95% ethanol was followed by two chloroform–methanol (2:1) extractions. Solids were removed by filtration through a course-grade sintered glass filter and organic solvents were evaporated under reduced pressure and a stream of nitrogen (temperature 5–15°). Precipitation with acetone when used was accomplished by dissolving the lipids in the minimum quantity of diethyl ether followed by the addition of acetone until no further precipitation occurred. The phosphatide mixture was then dissolved in petroleum ether (30–60° boiling range). Where complete removal of water-soluble impurities was desired, a 1–3% dispersion of phosphatide was prepared in water and dialysis carried out for 1–5 days against frequent changes of 50-fold excess water. Final drying of preparations was accomplished in a desiccator over sodium hydroxide.

A mixed soybean phosphatide preparation (Asolectin) was obtained from Associate Concentrates, Inc., Woodside, N.Y. From 1–15 g wet weight of blood platelets were recovered on three occasions by centrifugation of normal human blood made available by Hyland Laboratories, Los Angeles, California. Human granulocytes from a case of granulocytic leukemia were also isolated by centrifugation techniques[22].

Chromatographic methods

All phospholipid preparations were checked for ninhydrin-positive contaminants by applying from ½ to 4 mg of lipid to Whatman No. 1 paper and followed by chromatography in phenol saturated with water (ammonia atmosphere) and lutidine saturated with water. The completed chromatogram was then sprayed with ninhydrin. Since lipids either fail to migrate in either solvent or migrate to the phenol or lutidine solvent fronts, the technique is ideal for the detection of amino acids or other ninhydrin-positive contaminants which migrate well behind the solvent fronts. Since very large quantities of lipid can be applied to the paper, it is possible to check for contaminants present in the range of 0.1%. The detection of phosphate contaminants is best done after chromatography in phenol followed by butanol–acetic acid–water (4:1:5) rather than in phenol–lutidine, since lutidine gives a blue background color with the phosphate spray reagent of HANES AND ISHERWOOD[23] used for localizing phosphate compounds.

The number of phospholipid constituents and the presence of other lipids in a preparation were determined by one-dimensional descending paper chromatography[15,16]. Two solvents not previously described have been used extensively. The mixture octanol–lutidine–H_2O (90:5:1.5)

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has been used in particular for the separation of inositol phosphatides and the mixture benzene–glacial acetic acid (9:1) has been used as a substitute for the octanol–acetic acid solvent and is applicable to both unimpregnated and silicic acid-impregnated papers. It has also been found that the octanol–lutidine–acetic acid solvent changes in 1 to 3 weeks in such a way that the order of migration of phosphatidyl ethanolamine and phosphatidyl serine is reversed. This solvent is referred to as the "aged" octanol solvent. The use of the above solvents allows the separation of all the known phosphatides including the glycerophosphatides, lyso-, acetal-, and inositol phosphatides and sphingomyelin. Very little difference in migration is noted with change in the degree of unsaturation of the fatty acids of a particular phosphatide and the plasmalogen forms of the glycerophosphatides are not separable from the fatty acid diester forms.

The phenol–water (ammonia) and lutidine–water two-dimensional technique has been found to give useful phosphatide separations not obtainable with the above described phosphatide solvents. Saturated phosphatidyl serine migrates with an R_F of 0.25 in phenol, but fails to migrate in lutidine. Phosphatidyl serine containing unsaturated fatty acids migrates to the solvent fronts. All other phosphatides tested with the exception just noted migrate to the phenol front. In lutidine the fully saturated lecithins, phosphatidyl serine, phosphatidyl ethanolamine, and sphingomyelin fail to migrate whereas the corresponding compounds containing unsaturated fatty acids migrate to the lutidine front.

Techniques for the paper chromatography of phosphatides using phosphate buffer-impregnated paper have been developed during the course of this investigation and will be reported in detail later. Whatman No. 1 paper is impregnated with 0.1 *M* KH_2PO_4 or buffers prepared by mixing KH_2PO_4 and 0.1 *M* KOH. Buffers up to pH 8 are useful for the separation of inositol phosphatides, sphingomyelin, and lysophosphatides, using chloroform–methanol (4:1) as solvent.

Lipid materials (including fatty acids, glycerides, and phosphatides) were located on chromatograms with fluorescent dyes[16]. Other reagents were used as previously described[16] except that the ninhydrin spray was prepared as a 0.2% solution in *n*-butanol containing 10% lutidine (prepared fresh daily). Ninhydrin-positive materials were visualized after spraying by heating chromatograms to 95° for 5–10 minutes.

Silicic acid column chromatography was carried out by the method of LEA *et al.*[15]. A column, 1 cm in diameter containing 3 g dry weight of silicic acid was used. Not more than 40 mg of phospholipid dissolved in one ml or less of chloroform–methanol was applied to such columns.

Blood-clotting test systems

Low platelet human plasma was prepared fresh daily from citrated blood by centrifugation at 7000 × *g* using siliconed tubes and syringes. Recalcified plasma time determinations were carried out by mixing equal volumes of plasma and veronal buffer containing dispersed phosphatide followed by a 3-minute incubation at 37°. Another volume of 0.025 *M* calcium chloride was then added. The end point was taken as formation of the first definite fibrin threads and reported in seconds from the time the calcium was added. The results with this test system were compared with a similar system containing in addition one volume of a 1/10,000 dilution of Russell viper venom. The results from the two tests have been compared since both test systems have been used extensively in investigations of the role of phosphatides in coagulation.

Hydroxylation of phosphatides

Hydroxylation of phosphatides was carried out to replace the unsaturation of phosphatide fatty acids with hydroxyl groups. Performic acid was prepared as described by HIRS[24] and cooled to 5°. After incubation at 5° for four days the reaction appeared complete and the hydroxylated phosphatides had remarkably altered solubility properties. Such phosphatides were sparingly soluble in benzene, ether, and 95% ethanol, and were readily dispersed in water to give clear solutions. Chromatographic migration in all phospholipid solvents was greatly reduced, although migration in phenol–water and lutidine–water remained the same. The permanganate test was negative and the material gave a strongly positive periodate reaction. No significant free fatty acid release could be detected and no indication of the release of water-soluble phosphate compounds was observed.

Reaction with ninhydrin

Phosphatide preparations were treated with ninhydrin to remove the free amino group. A 25-mg sample was dissolved in 10 ml of benzene and 50 ml of *n*-butanol containing 50 mg of ninhydrin was added. The mixture was heated to 50°, one ml of 2,4-lutidine added, and the mixture evaporated to dryness under a stream of nitrogen at 50°. When the solution was concentrated to approximately one-half the original volume, 100 mg of solid ninhydrin was added. The dark blue colored solids were suspended in 10 ml of water and dialyzed against water until the blue color could no longer be detected. Paper chromatography showed that amino-containing phosphatide was no longer detectable on chromatograms. A large amount of a new phosphate-containing spot in

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the phosphatidic acid region (just behind the solvent front with most solvent systems) was detected. The lecithin and inositol phosphatide areas were not changed, and no significant release of free fatty acids or water-soluble hydrolysis products was observed.

Acylation

The preparation of *N*-acetyl and *N*-succinyl derivatives of the amino-containing phosphatides was as follows: 20 mg of phosphatide was dissolved in 6 ml of pyridine and 2 ml of acetic anhydride or 0.5 g of succinic anhydride was added. The mixture was heated at 60° for 30 min, evaporated to dryness under the same heat and a stream of nitrogen, emulsified in water, dialyzed, and dried. Paper chromatography showed that no change had been produced in lecithin, but that phosphatidyl ethanolamine was no longer detectable on chromatograms. A new spot migrating to the solvent front was observed in each case.

RESULTS AND DISCUSSION

Lack of thromboplastic activity of neutral lipids

It was first confirmed that the thromboplastic activity resided in the phosphatide fraction and that other lipids were relatively inactive. The activity was found to be localized almost exclusively in the acetone-precipitable fraction from various tissues. In agreement with these findings the first fraction to emerge from silicic acid columns containing cholesterol and triglycerides was inactive. A preparation of mixed cerebrosides and cerebron were also inactive. Saturated fatty acids from C_6 to C_{24} and oleic, linoleic, and lenolenic acids were tested, and it was observed that acids containing at least 16 carbon atoms would shorten the clotting time to a small extent. When compared to phosphatides the fatty acids were relatively inactive. The shortest clotting times with fatty acids at 25–100 mg % concentration could be obtained with soybean phosphatidyl ethanolamine at a concentration of 0.5 mg % or less. Phosphatides, but not fatty acids, were found to give increased prothrombin utilization in the prothrombin-utilization test.

Lack of influence of water-soluble non-lipid substances

It was next established that the thromboplastic activity associated with the phosphatide fraction was not attributable to water-soluble impurities. Phospholipid mixtures were dialyzed against water until paper chromatographic examination indicated absence of impurities and then tested. By this procedure variable losses of activity were observed. This loss of activity was attributable to oxidation of phosphatides during dialysis rather than to the loss of active water-soluble impurities. This was evident since phosphatide fractions from silicic acid columns were free of non-lipid impurities (that remained at the top of the column) and retained full activity, while the non-lipid impurities which were eluted with aqueous solvents after elution of phosphatides were inactive. A variety of authentic samples of sugars, water-soluble phosphate esters, amino acids, and purines and pyrimidines were inactive at concentrations far in excess of those that could be present in the phosphatide mixtures.

Thromboplastic activity of hydrolysis products of phosphatides

The possibility that hydrolysis products of phosphatide were responsible for the observed activity was then investigated. Samples of glycerol, glycerophosphate, glycerylphosphoryl choline, glycerophosphoryl ethanolamine, phosphoryl choline, phosphoryl ethanolamine, phosphoserine, ethanolamine, choline, and serine were all

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found to be inactive. The phosphatide mixtures were inactivated by moccasin venom. The lyso glycerophosphatides, shown to be present by paper chromatography, were inactive as were the fatty acids split from the phosphatides. In contrast to these findings, enzymically produced phosphatidic acid was found to be quite active, although not as active as the original phosphatide mixture. Only traces of phosphatidic acid have been found in our samples and hence this phosphatide could not be the active substance from tissues. These preliminary data thus demonstrated that the activity was indeed a property of a phosphatide and not of impurities or hydrolysis products of phosphatides. The nature of the active phosphatide was then investigated.

Inactivity of lecithins, sphingomyelin, inositol phosphatide and synthetic phosphatides

Tests with well characterized phosphatide preparations at 1–100 mg % concentrations showed that distearoyllecithin, dipalmitoleyllecithin, monopalmitoylglycollecithin, palmitoleyllysolecithin, sphingomyelin, and the surface-active agents BPE and TR that have certain structural similarities to phosphatides were completely inactive in the recalcified plasma test system. It is interesting to note that the surface-active agents showed some activity in the Russell viper-venom system. The synthetic phosphatides, distearoylphosphatidyl serine and dimyristoyl- and distearoylphosphatidyl ethanolamine, were not well dispersed in aqueous media and the best dispersions obtained were found to settle out in 20–60 min. Neither of the samples showed any marked activity, although the sample of phosphatidyl serine tended to prolong the clotting time very slightly with some plasma samples. A slight shortening of the clotting time was obtained with the synthetic phosphatidyl ethanolamines. However, these synthetic samples were even less effective than saturated fatty acids in shortening the clotting time. Both synthetic phosphatidyl serine and phosphatidyl ethanolamine shortened the clotting time significantly in the Russell viper-venom test system. In this system they were somewhat more active than higher fatty acids. The liver monophosphoinositide preparation inhibited the recalcified plasma test system, but shortened the clotting times with the venom system. The fact that a phosphatide can be an inhibitor in recalcified plasma and yet can decrease clotting times with the venom system shows that the latter system cannot be used as a substitute for recalcified plasma. It is interesting that the inhibition noted with monophosphoinositide could be abolished readily by mixing aqueous dispersions of this phosphatide and dipalmitoleyllecithin.

The tests with well characterized phosphatide preparations demonstrated that lecithin containing saturated or unsaturated fatty acids was inactive, as was sphingomyelin. The highly active phosphatides in tissues are evidently not structurally similar to the synthetic samples of phosphatidyl serine and phosphatidyl ethanolamine.

Quantitative recovery of activity in the amino phosphatide fraction

The activity of phosphatide preparations was found to reside entirely in the amino phosphatide fraction by silicic acid column chromatography. The following fractions were obtained and tested routinely:

(1) The first fraction eluted with chloroform–methanol (4:1) containing neutral lipids.

(2) The amino-containing phosphatides appearing after fraction 1 with chloroform–methanol.

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(3) The remaining phosphatides (lecithin, sphingomyelin, inositol phosphatides, etc.) eluted with methanol.

The total phosphatide recovery from such columns averaged 97%. Fraction 2 was found to contain all of the clot-accelerating activity.

It was found that oxidative changes must be prevented in order to recover all of the thromboplastic activity applied to the column. Oxidative changes are minimized by evaporation of solvents under nitrogen at low temperature (5–10°) and storage under nitrogen. Fig. 1 shows the recovery of thromboplastic activity from silicic acid columns. The per cent of amino phosphatide in the mixture was determined by weighing fraction 2 from the column so that the actual amount of amino phosphatide in the mixture is plotted. It can be seen that virtually 100% recovery of thromboplastic activity was obtained in this case. Somewhat more than 100% recovery was usually obtained with soybean phosphatide indicating some inhibition by other phosphatides (probably inositol phosphatides). The best recovery of platelet activity was about 60%. This is probably due to failure to prevent oxidative changes of the highly unsaturated phosphatides present.

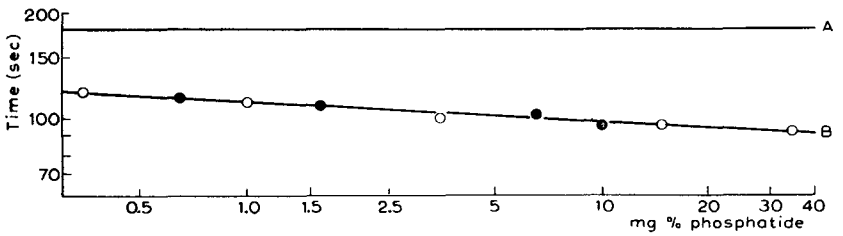

Fig. 1. Recovery of thromboplastic activity from silicic acid columns: A. control clotting time; B. phosphatide added; ○, rabbit appendix phosphatidyl ethanolamine fraction; and ●, mixed appendix phosphatide applied to column.

Relative activity of various mixtures and amino phosphatide content

Initial tests showed that preparations of mixed phosphatides from various sources had greatly different activities. It was also found that the method of preparation of the phosphatide mixtures from a given source determined the activity of the mixture. The use of heat and the exposure to air, particularly during prolonged dialysis, were found to be the major causes of loss of activity. Fig. 2 shows the clotting times obtained with phosphatide mixtures from various sources. The "oxidized" appendix and granulocyte preparations were mixtures that had lost most of their

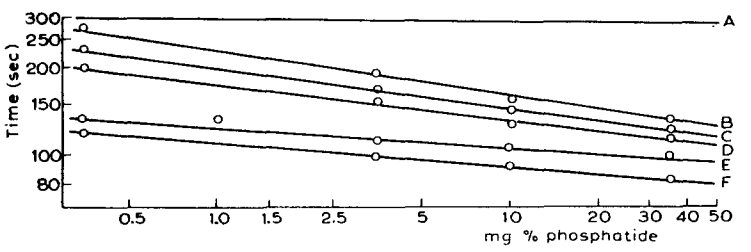

Fig. 2. Recalcified plasma clotting times of phosphatide mixtures. A. Control time, B. "oxidized" rabbit appendix, C. "oxidized" granulocyte, D. soybean, E. rabbit appendix, and F. human blood platelets.

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activity after prolonged dialysis and standing. The logarithm of the final concentration of phosphatide is plotted against the logarithm of the clotting time in seconds. Each point plotted is an average value of at least five separate determinations. It has been found that if the temperature is carefully maintained at 37° and air oxidation of dilute phosphatide dispersions is avoided, a straight line is obtained by this method of plotting the data.

Table I shows the percentage of amino phosphatide in the preparations shown in Fig. 2. The values were determined by weighing fraction 2 from silicic acid columns. These values show that preparations low in amino phosphatide are also low in thromboplastic activity. The fact that preparations containing approximately the same amount of amino phosphatide may differ markedly in activity indicates structural heterogeneity of the amino phosphatide fraction.

TABLE I

PERCENTAGE OF AMINO PHOSPHATIDE IN PHOSPHATIDE MIXTURES

	*Platelet**	*Appendix**	*Soybean***	*Oxidized appendix****	*Oxidized Granulocyte****
% Amino phosphatide	24.2	25.0	24.3	3.6	2.7

* phosphatidyl ethanolamine + phosphatidyl serine.
** contains only phosphatidyl ethanolamine.
*** principally phosphatidyl serine.

Phosphatidyl ethanolamine as active phosphatide

The structural heterogeneity of the amino phosphatide fraction was then investigated. The possibility that the differences in activity resulted from variations in the content of phosphatidyl serine was ruled out readily. Phosphatidyl ethanolamine and phosphatidyl serine are readily separated on paper chromatograms using fresh octanol–lutidine–acetic acid or the "aged" octanol solvents. Examination of the amino phosphatide fraction from silicic acid columns in these solvents showed that neither the egg nor soybean fractions contained appreciable amounts of phosphatidyl serine. Thus a true structural heterogeneity of phosphatidyl ethanolamine was indicated. Phosphatidyl ethanolamine of rabbit appendix and human granulocytes is more readily destroyed by oxidation than is phosphatidyl serine with the result that the "oxidized" samples from these sources contain principally phosphatidyl serine and these are also relatively inactive.

In our hands the amino phosphatide fraction from silicic acid columns has yielded two incompletely separated peaks corresponding to phosphatidyl serine and phosphatidyl ethanolamine. We have, however, obtained each of these phosphatides almost completely free of the other from such columns by applying the phosphatide to the column in a very small volume of solvent and collecting only the initial and final portions of the two peaks. Phosphatidyl ethanolamine was found to have activity equal to that of the original mixture of the two glycerophosphatides. This demonstrated that phosphatidyl ethanolamine is the active phosphatide. Phosphatidyl serine was only slightly active and the activity may have resided in the small amount of phosphatidyl ethanolamine present in the fraction.

The finding of thromboplastic activity in phosphatidyl serine[11] was based on a

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preparation using the FOLCH fractionation technique[25] which does not always remove all of the phosphatidyl ethanolamine from the phosphatidyl serine fraction and recalcified plasma was not used as a clotting test system. Our data are in keeping with the early report of CHARGAFF[1] that phosphatidyl serine is inactive. It is to be noted that SILVER *et al.*[14] report phosphatidyl serine to be strongly inhibitory only when dispersed with the aid of cholate.

The possibility that lecithin can activate phosphatidyl serine[11] has not been studied in this laboratory, although our data indicate that phosphatidyl ethanolamine is not activated by lecithin. The recovery of full activity in the absence of lecithin argues against such activation. Soybean phosphatidyl ethanolamine (free of phosphatidyl serine and plasmalogen) is not activated by lecithin (dipalmitoleyl or soybean lecithin). Direct tests in which lecithin was mixed with phosphatidyl ethanolamine, either as a separate aqueous dispersion or by prior combination in an organic solvent, have shown that in very dilute solutions (in the range of 1 mg %) phosphatidyl ethanolamine may be 2 to 4 times as active with lecithin added. When fresh dispersions are used and every precaution is taken to make exposure to oxygen minimal this effect has not been observed. We have thus concluded that the apparent activation s actually an antioxidant action of lecithin.

Inactivity of ethanolamine plasmalogen

Since it was evident that phosphatidyl ethanolamine was the active phosphatide the question of the structural heterogeneity of this fraction was then investigated. It was necessary to determine whether the aldehyde-containing form was active. DEBUCH[26] has shown that the aldehyde can be split from ethanolamine plasmalogen by hydrolysis in 95% acetic acid at 37° for 15 h. Lysophosphatidyl ethanolamine is produced only from ethanolamine plasmalogen. Table II shows that after such treatment soybean, appendix, and platelet phosphatidyl ethanolamine show little or no decrease in activity. Paper chromatography after acetic acid hydrolysis demonstrated the absence of lysophosphatidyl ethanolamine in soybean, and its presence in appendix and platelet phosphatide preparations. These preparations do not have the high plasmalogen content of brain[26] phosphatidyl ethanolamine. From these studies it

TABLE II

INFLUENCE OF ACETIC ACID HYDROLYSIS ON CLOTTING TIME

Phosphatide mixture	*Concentration* (mg %)*			
	100	*10*	*1*	*Control*
	(sec)**	(sec)	(sec)	(sec)
Soybean untreated***	100	125	155	263
Acid-treated	98	127	158	262
Appendix untreated	79	114	136	250
Acid-treated	78	119	138	250
Platelet untreated	79	88	120	220
Acid-treated	79	89	114	220

* Concentration of phosphatide added to plasma.

** Each value is an average of at least five determinations. A different plasma sample was used for each phosphatide mixture.

*** The untreated samples were dissolved in acetic acid (90%) and incubated at 4°. Treated samples incubated at 37° for 16 hours.

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was apparent that the differences in thromboplastic activity were associated with differences in fatty acid composition. In the following communication it will be shown that thromboplastic activity increases as the degree of unsaturation of the phosphatide fatty acids increases.

Essentiality of the free amino group

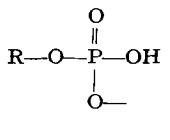
Additional chemical reactions have been performed to determine the essential nature of the various structural features of the phosphatidyl ethanolamine molecule. Removing or blocking the amino group with ninhydrin, acetic anhydride, or succinic anhydride was found to abolish over 99% of the activity of phosphatidyl ethanolamine. These products were found to be approximately 50% as active as the untreated preparations, however, in the venom test system. This is another example of the lack of correspondence of the two test systems. In the recalcified plasma system it seems clear, however, that the amino group is essential for activity.

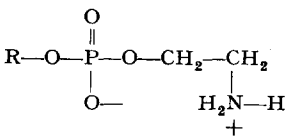
Essentiality of unsaturation of fatty acids

The fact that synthetic phosphatidyl ethanolamine is only slightly active and cannot be fully dispersed in aqueous media whereas natural highly active phosphatidyl ethanolamines are finely dispersed suggests the possibility that the unsaturation of the fatty acids is important because it controls the state of dispersion in water. The possibility that a water-soluble product would have even more activity than the finely dispersed colloidal particles was tested by hydroxylation of the active phosphatide. Hydroxylation with performic acid yielded a product that dissolved in aqueous buffer to give a clear solution without thromboplastic activity. It is also to be recalled that the water-soluble lysophosphatidyl ethanolamine is inactive as are the difficultly dispersed saturated synthetic phosphatidyl ethanolamines. It thus appears most probable that the thromboplastic phosphatide must exist as a finely dispersed colloidal particle.

Summary of phosphatide structure necessary for thromboplastic activity

The two active compounds, phosphatidic acid and phosphatidyl ethanolamine have three properties in common: (1) both phosphatides exist in a finely dispersed colloidal state in aqueous media, each has (2) a negative charge on a phosphate group, and (3) one of the ionic species present at pH 7.4 has a proton-donor group. These relationships are indicated as follows:





The proton-donor group in one case is the acidic hydroxyl and in the other the positively charged quaternary nitrogen. The importance of the proton-donor group is demonstrated convincingly by the inactivity of ninhydrin-treated phosphatidyl ethanolamine in which the proton donor has been replaced by a non-acidic group. The *N*-acetyl derivative shows a similar behavior since the amide nitrogen is no longer

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a proton donor. The inactivity of the *N*-succinyl product can be attributed to the failure of the carboxyl group to function as an effective proton donor in the system.

SUMMARY

1. Highly purified phospholipids and other compounds have been examined for their effect on acceleration of clotting of low platelet recalcified plasma with and without the addition of Russell viper venom.

2. It has been observed that phosphatidic acid and phosphatidyl ethanolamine greatly accelerate the rate of clotting of recalcified low platelet plasma.

3. Rigorous checks of purity including the examination of preparations for water-soluble as well as lipid impurities have shown that the activity observed is not attributable to the presence of impurities in the preparations tested.

4. Phosphatides must contain unsaturated fatty acids to be highly active.

5. The ability of a phosphatide to accelerate the coagulation rate appears to be associated with the formation of a finely dispersed colloidal particle with a negative charge on a phosphate group, and the presence of a proton-donor group.

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Addendum

Since this manuscript was submitted for publication the report of R. KUHN and P. KLESSE (*Naturwissenschaften*, 44 (1957) 352) has come to our attention. The phospholipids that accelerate the clotting time of recalcified plasma in the presence of defatted lung thromboplastic protein were studied by methods similar to those previously used in this laboratory (*Federation Proc.*, 16 (1957) 332). It is evident that the addition of lung thromboplastic protein, like Russell viper venom, abolishes the high degree of structural specificity observed for recalcified plasma. The lung thromboplastic protein appears to be even less specific than venom since lysophosphatidyl ethanolamine will also shorten clotting time in its presence.