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**Research Article**

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# The Phospholipid Requirement of Tissue Factor in Blood Coagulation

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**A B S T R A C T** Using a coagulation assay specific for tissue factor, we found that removal of 95% of the tissue factor-phospholipid resulted in a loss of 98% of its biological activity. The activity could be restored, with yields in excess of 100% by combining the extracted tissue factor with either mixed brain phospholipids or highly purified phospholipids. Phosphatidylethanolamine was the most active, followed by phosphatidylcholine. Phosphatidylserine, phosphatidylinositol, and sphingomyelin had little or no activity. In addition, a requirement for unsaturation and the presence of two fatty acids was demonstrated. The activity of phosphatidylcholine was also dependent on the presence of the base. Furthermore, it was shown that activity was not a function of binding of phospholipids to tissue factor, as both active and inactive lipids were equally bound.

## INTRODUCTION

It has been previously demonstrated that tissue factor activates the "extrinsic" blood coagulation mechanism by forming a complex with a plasma protein, factor VII, which then enzymatically activates factor X (1, 2). The material in tissues that interacts with factor VII has not been clearly identified, although it is known to contain both

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protein and lipid (3, 4). In an earlier study we reported that a lipid-poor protein could be extracted from brain, which was capable of activating this pathway of blood coagulation (5). The activity of this material, however, was low and its stability was poor.

The requirement for a lipid-like material for full activity of tissue factor was demonstrated as early as 1946 by Studer (6). He extracted a crude tissue factor preparation with ether-alcohol, and demonstrated that full activity was not obtained until the ether-alcohol soluble material was added to the insoluble residue. Subsequently, Kuhn and Klesse (7) confirmed this observation and suggested that only certain phosphatides could substitute for the naturally occurring lipids. Recently, Deutsch, Irsigler, and Lomoschitz (8) extracted brain tissue factor with pyridine and showed a requirement for the pyridine-soluble components, although the active material was not specifically identified.

The assay system used by the above investigators was based on the ability of the various preparations to accelerate the clotting time of whole plasma. This technique lacks specificity as it reflects the summation of a series of reactions. In the present investigation an assay specific for tissue factor was employed. It was thus possible to demonstrate a specific requirement for lipid in the tissue factor pathway leading to the activation of factor X. Furthermore, the lipid requirement was fully satisfied by phospholipid, and biological specificity was associated with both the polar and non-polar regions of the phospholipid molecule.

## METHODS

Phospholipids<sup>1</sup> were obtained from the following sources: phosphatidic acid (PA) egg, General Biochemical, Chagrin Falls, Ohio; phosphatidylethanolamine (PE) bovine, reduced PE (bovine), lyso PE (bovine), phosphatidylcholine (PC) egg, reduced PC (egg), lyso PC (egg), PI (plant), Supelco, Bellefonte, Pa.; PC (bovine), Applied Science Laboratories, State College, Pa.; sphingomyelin (bovine), Nutritional Biochemicals, Cleveland, Ohio.

These preparations migrated as a single spot or contained a trace contaminant when examined by thin-layer chromatography in two solvent systems. They were used as supplied with the exception of phosphatidic acid, which was converted from the calcium salt to the free acid by the method of Abramson, Katzman, Wilson, and Gregor (9).

All chemicals were of reagent grade. Solvents, with the exception of acetone used for dehydration of brain tissue, were redistilled shortly before use.

*Preparation of tissue factor.* Bovine brains were obtained immediately after slaughter and an acetone powder was prepared as previously described (2). The tissue was extracted by homogenizing the dried powder (10 g) in a Waring Blender for 1 min with 300 ml of 0.15 M sodium citrate (instead of EDTA). The material was then centrifuged at 350 g for 5 min (4°C) and the sediment discarded. The particles in the supernatant were collected by centrifugation (37,000 g, 60 min) washed three times with sodium citrate and three times with distilled water. Finally, the tissue was washed twice with acetone and dried in a rotary evaporator at reduced pressure (25°C). This preparation will be referred to as "native tissue factor."

*Lipid extraction.* Native tissue factor was extracted with butanol (30 min at room temperature; 60 ml per gram of tissue). In order to remove 95% of the lipid phosphorous, it was necessary to repeat the procedure seven times. Finally the material was dried in a rotary evaporator.

The phospholipid content of each preparation was calculated from nitrogen and phosphorus analyses. Tissue factor that had been extracted three times with chloroform:methanol contained 0.016  $\mu$ g P/ $\mu$ g N. This was taken to be nonlipid P and was subtracted from all values. A factor of 25.0 was used to calculate the weight of mixed phospholipids from the phosphorus content; for sphingomyelin and phosphatidylinositol, the factors used were 24.8 and 31.4 (10).

*Mixed brain phospholipids.* These were prepared in two ways: *Method 1.* Native tissue factor (10 g) was extracted with 60 ml butanol for 60 min at room temperature. The insoluble material was removed by centrifugation and subsequent filtration through acid-washed filter paper. This preparation contained cerebrosides and neutral lipids in addition to phospholipids. *Method 2.*

<sup>1</sup> Some of the phospholipids used initially were a gift of Dr. Aaron Marcus and were subsequently obtained from the indicated sources.

Native tissue factor was extracted three times with 100 volumes of chloroform:methanol (2:1; v/v) for 30 min at room temperature. The solvent was evaporated under a stream of prepurified nitrogen, and the residue taken up in a minimum volume of chloroform. The material was then separated into neutral lipids and phospholipids plus cerebrosides by silicic acid column chromatography. The eluted phospholipid fraction was used for recombination experiments. Lipids were kept in an atmosphere of nitrogen throughout preparation and storage.

*Recombination of extracted tissue factor and lipids.* This was performed by suspending 25 mg of the tissue in 10 ml of butanol. The lipids (maximum of 7 mg) were dissolved in either butanol, benzene, methanol, or chloroform:methanol (2:1) and added (in a maximum volume of 1.0 ml) to the tissue in butanol. They were gently mixed for 10 min after which the solvents were removed at reduced pressure (30°C). Preliminary experiments revealed that the type of solvent in which the lipids were dissolved had no effect on the activity obtained.

The amount of lipid added to 25 mg of extracted tissue factor was limited to 7 mg. When this was exceeded, recombination did not take place and a precipitate formed during the evaporation procedure. To increase the ratio of lipid to tissue factor beyond this limit, the recombination was performed in successive steps.

*Tissue factor assay.* The assay used was a modification of a procedure previously described (5). Measurements of the initial rate of activation of factor X were made in a system saturated with factors VII and X and in which tissue factor was rate limiting.

Factors VII and X were obtained from bovine serum prepared by defibrination of freshly collected blood that was allowed to stand at room temperature for 4 hr and at 4°C overnight to promote complete coagulation. The erythrocytes were removed by centrifugation. Barium citrate was precipitated in situ by the addition of sodium citrate and barium chloride according to the method of Lewis and Ware (11). The precipitate was washed as previously described (2), and the adsorbed clotting factors were eluted with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (12). The precipitate that formed at this step (BaSO<sub>4</sub> and some protein) was removed by centrifugation and discarded. The soluble proteins were precipitated with additional (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (20 g/liter) and were collected by centrifugation, dissolved in a minimum amount of water, and dialyzed for 36 hr against numerous changes of phosphate buffer (pH 7.0; I 0.05). The material was frozen in aliquots and diluted 1:5 with imidazole buffer (imidazole, 0.05 M; NaCl, 0.1 M; adjusted to pH 7.35 with HCl) immediately before use. This product, which was about 100-fold purified with respect to factor X, contained factors VII and X at concentrations of about 20-fold greater than bovine serum and was used without further purification.

Tissue factor was prepared for assay by homogenization with saline in a Teflon and glass homogenizer at concentrations of 0.5–0.75 mg/ml. It was then subjected to sonic oscillations for 15 sec in ice with a Blackstone BP-2 probe at maximum output (200 w). Saponication, which dispersed the particles, was necessary for the attainment of reproducible results in the assay.

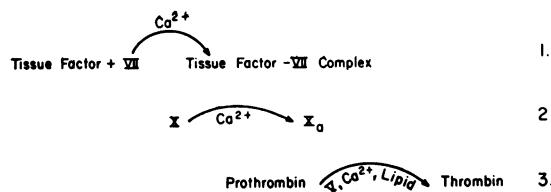


FIGURE 1 A tentative scheme of the reactions of the tissue factor pathway leading to the production of thrombin. In reaction 3, there is probably a direct proteolytic activation of prothrombin by activated factor X (19, 20), and the reaction is accelerated by the indicated cofactors. The possibility of a collateral pathway in which factor V is activated, which in turn converts prothrombin to thrombin, has not been excluded.

The assay was performed in two stages. All reagents were prewarmed to 37°C at which temperature the procedure was performed. *Stage 1.* The serum fraction containing factors VII and X (0.2 ml) and tissue factor (0.2 ml) was incubated for 5 min. CaCl<sub>2</sub> (0.1 ml, 0.025 M) was added and the incubation continued for 1 min at which time the reaction was stopped by adding an aliquot (0.1 ml) to 0.4 ml of sodium citrate, 0.015 M. *Stage 2.* 0.1 ml of the above was added to 0.1 ml of bovine plasma to which optimum amounts of mixed lipids (13) had been added. 15 sec later, 0.1 ml of CaCl<sub>2</sub> (0.025 M) was added and the clot formation timed. The clotting time was related to the tissue factor concentration and yielded a straight line when plotted on logarithmic coordinates (Fig. 2). In lieu of the factor X-deficient plasma used previously, whole bovine plasma was used in the assay because the former was not available at the time of this study. Similar results were obtained in preliminary experiments using factor X-deficient plasma and whole plasma.

*Characterization of lipids in the brain fractions.* The tissue was extracted three times with about 100 volumes of chloroform:methanol as was done for the preparation of mixed brain phospholipids. An aliquot containing 12–20 µg P was applied to silica gel plates (Camag DO, 400 µ, 20 × 20 cm) and chromatographed in two dimensions. The solvents used were chloroform:methanol:acetic acid:water (25:15:4:2) (14) in the first dimension, and chloroform:acetone:methanol:acetic acid:water (5:2:1:1:0.5) (10) in the second. The chamber was tightly sealed and enclosed in a plastic bag to prevent unequal evaporation of solvent. The lipids were identified by (a) *R*<sub>f</sub>, (b) authentic standards, (c) spraying with ninhydrin (0.2% in acetone, diluted 1:2 with water before use), (d) spraying with Rhodamine 6 G (0.005% in water), (e) spraying with the specific phospholipid stain of Dittmer and Lester (15).

Phospholipids were quantified by spraying the plates with 50% sulfuric acid and charring on a hot plate. The spots were eluted and digested as suggested by Rouser, Siakotos, and Fleischer (10). Recovery of lipid phosphorus averaged 85–90%.

*Chemical methods.* Nitrogen and phosphorus were de-

termined from single aliquots by digesting the samples in perchloric acid at low heat for 2 hr (16). Phosphorus was measured by the method of Fiske and Subbarow (17); and nitrogen, by adding a 0.4 ml aliquot of the digested material directly to 5 ml of Nessler reagent (18).

## RESULTS

*Assay of tissue factor.* A tentative scheme of the tissue factor pathway leading to the production of thrombin is shown in Fig. 1. The assay is based upon coupling reactions 1 and 2 in the first stage, and upon measuring the amount of activated factor X in the second stage. As shown in Fig. 2 the clotting time of the second stage is a function of the tissue factor content.

Since it is known that lipid is required for the optimum conversion of prothrombin to thrombin by activated factor X, factor V, and calcium (21), mixed lipids (13)<sup>2</sup> were added to plasma in an amount (14.7 µg P/ml) found to yield the shortest clotting times; i.e., reaction 3 was not limited by the amount of lipid present in the tissue factor preparation. The maximum amount of lipid phosphorus carried into the second stage of the assay was 1.5 µg/ml, and it was found that varying the final concentration of lipids in the assay by this amount had no effect on the clotting times.

The rate of activation of factor X was linear for several minutes at all concentrations of tissue factor used. In the assay, tissue factor was allowed

<sup>2</sup> This preparation is frequently referred to as "cephalin" but is here called "mixed lipids" to avoid confusion with the trivial nomenclature for some phospholipids.

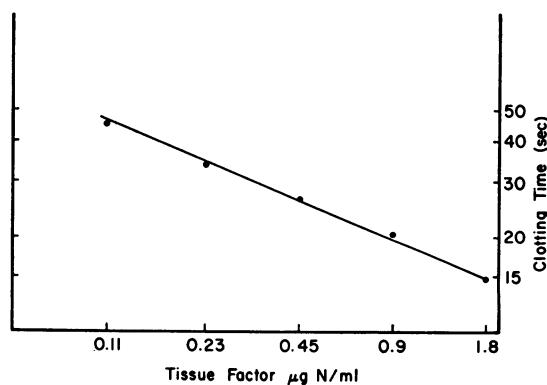


FIGURE 2 Results obtained in the two-stage assay for tissue factor. The indicated amounts refer to the tissue factor concentration (per milliliter) in the first stage of the assay.

to react with factors VII and X for 1 min, thus measuring the initial rate of activation of factor X.

A standard curve was constructed daily using a single preparation of tissue factor which was kept in aliquots at  $-20^{\circ}\text{C}$ . A tissue factor preparation that had the same activity as this material was arbitrarily called 100 U. All data are expressed on the basis of tissue factor protein, which was calculated from the nitrogen content. Each point was determined at least in triplicate at two concentrations of tissue factor. When expressed as a function of tissue factor protein, comparable results were obtained for both concentrations indicating that the lipids present in the tissue factor, which were carried into the second state of the assay, neither enhanced nor suppressed the rate of reaction 3.

*The relationship of lipid content to biological activity.* Native tissue factor contained 38–45% phospholipid by weight, and averaged 120 U of activity per milligram of protein. The activity of extracted tissue factor could be restored by combining it with the extracted lipids. In each of several preparations, in which the product contained more than 50% phospholipids by weight, greater than 100% recovery of activity was obtained.

Fig. 3 shows the quantitative relationship of specific activity of tissue factor to its phospholipid content. The depicted experiment was performed with mixed brain phospholipids containing cerebrosides and some neutral lipids (Method 1).

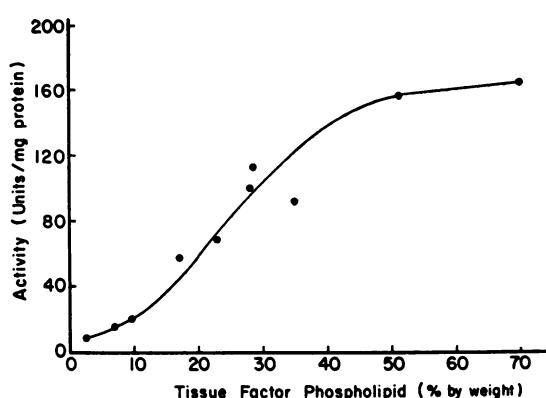


FIGURE 3 The activity of tissue factor as a function of its phospholipid content. Phospholipids were removed from tissue factor by extraction with butanol. Mixed brain phospholipids were then recombined with tissue factor and the activity determined.

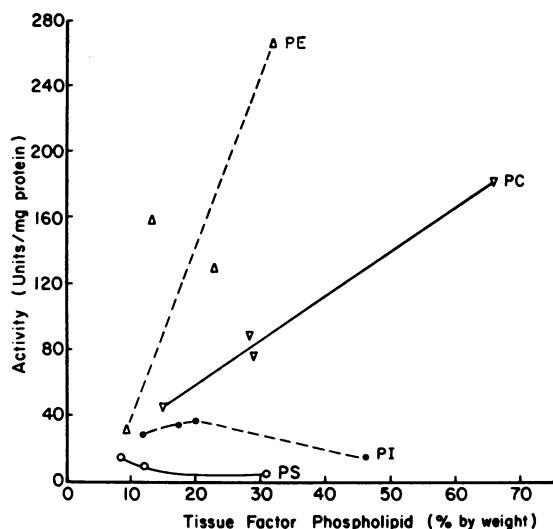


FIGURE 4 The ability of various phospholipids to stimulate tissue factor activity. For abbreviations used, see text.

Similar results (not shown) were obtained with a brain lipid fraction freed of neutral lipids by silicic acid chromatography. When equal amounts of lipid P were added, comparable results were obtained with both preparations, thereby suggesting that only the phospholipids had significant biological activity. Moreover, pure phospholipids could substitute for these fractions (see below). As the mixed phospholipids prepared by Method 1 were more readily obtained, they were used in these experiments.

*Specificity of the phospholipid requirement of tissue factor.* Since tissue factor activity was enhanced in proportion to the phosphorus content of the mixed lipid preparations and was not affected by neutral lipids, purified phospholipids were tested. The ability of individual phospholipids to increase tissue factor activity was highly specific (Fig. 4). Note that PC, which is inert in all other clotting systems (22), had appreciable activity, and that PS, usually active in blood coagulation, was inert.

*Effect of alterations of the PC molecule on activity.* The composition of the nonpolar or fatty acid residues of PC is shown in Table I, and the activity of the compounds is depicted in Fig. 5. The biological activity of bovine and egg PC was virtually identical and their fatty acid composition

TABLE I  
Fatty Acid Composition of Phosphatidylcholine and Some Derivatives\*

Lipid	Fatty acids as per cent of total									
	16:0‡	16:1	17:0	18:0	18:1	18:2	18:3	20:0	20:4	?
PC (egg)	30.3	1.1		16.8	32.2	14.8			4.7	
PC, reduced (egg)	31.4			62.2				6.4		
Lyso PC (egg)	69.3	2.6		23.4	4.7					
PA (egg)	34.0	3.0		20.7	29.8	8.7	1.4		1.4	1.0
PC (bovine)	29.2	2.3	1.1	12.7	44.2	1.8	4.9		2.2	0.16
PC (plant)	18.3			1.0	5.8	14.7	53.3	6.9		

\* Quantification was performed by gas-liquid chromatography. These analyses were kindly performed by Dr. Robert Scheig, Yale Medical School.

‡ Designation of fatty acids, No. of carbon atoms: No. of double bonds.

was similar, although egg PC was richer in multiply unsaturated fatty acids.

The experiments depicted in Fig. 5 illustrate the marked dependence of biological activity on the fatty acids present in the phospholipids. Although the differences in saturation between egg and plant PC (47% vs. 25%) were not reflected in biological activity, catalytic reduction of the egg PC resulted in marked loss of activity. Enzymatic hydrolysis of the  $\beta$ -fatty acid produced a phospholipid (lyso PC) virtually devoid of activity. It should be noted that lyso PC contains 93% saturated fatty acids (Table I) and that some reduction of activity may be ascribed to this high degree of saturation, although the dependence of activity on the presence of two fatty acids is suggested by the lower activity of the lyso compound as compared to the fully reduced PC. Enzymatic removal

of the choline moiety, yielding phosphatidic acid, also virtually abolished activity.

Sphingomyelin, which also contains choline as the polar component, had little activity. Although the fatty acid composition was not determined, it is known that the major fatty acids of bovine brain sphingomyelin are 18:0 (40.1%), 24:0 (10.7%), and 24:1 (24.6%) (23). The compound contains about 33% unsaturated fatty acids.

The dependence of activity on the presence of unsaturated fatty acids and on the diacyl form of the lipid was confirmed using PE, lyso PE, and reduced PE. The data (not shown) were comparable to those obtained with the PC derivatives.

*Binding of phospholipids to extracted tissue factor.* In order to determine whether the ability of the lipids to restore biological activity was a function of binding to the tissue factor, the binding of three phospholipids was examined. To do so, a modification of a plasma phospholipid method (24, 25) in which the protein-bound lipids are precipitated with perchloric acid was used. PE, PC, and PS were studied as they had exhibited high, intermediate, and no activity, respectively.

The data in Table II show that PS, with no biological activity, bound at least as well as PE and PC. The differences in activity of these lipids were not due to a lack of binding, but were more likely due to structural differences. No added lipid phosphorus was found in the precipitate when lipids were mixed with tissue factor in the presence of perchloric acid.

*The phospholipids of native and extracted tissue factor.* The composition of these preparations was

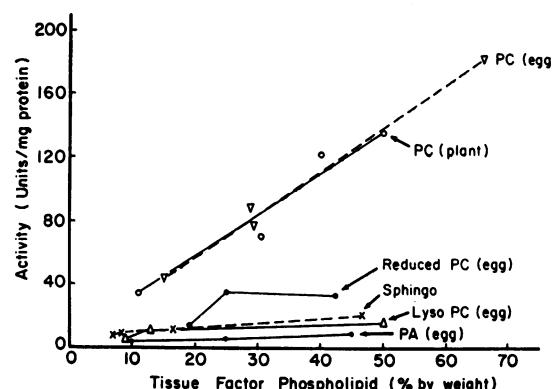


FIGURE 5 The ability of choline-containing phospholipids and some derivatives to stimulate tissue factor. "Sphingo" refers to sphingomyelin. For other abbreviations used, see text.

TABLE II  
*Binding of Lipids to Extracted Tissue Factor\**

Tissue factor preparation	Lipid added	Phospholipid	Expt. 1	Expt. 2
		% by weight	% in ppl	
Extracted	Phosphatidylserine	31	89.5	87.7
Extracted	Phosphatidylethanolamine	32	83.7	82.2
Extracted	Phosphatidylcholine	31	74.6	82.1
Extracted	Mixed lipids + PCA	23	0	0
Native	None	38	85.1	80.0

\* Recombined tissue factor preparations (2-5 mg) were homogenized in 8% perchloric acid (PCA) and centrifuged at 16,300 g for 1 hr. The supernatant solutions were discarded and the precipitates resuspended in PCA. Following an additional centrifugation, the precipitates were analyzed for lipid phosphorus. The per cent of lipid phosphorus recovered in the precipitate is indicated.

determined by extracting them with chloroform: methanol and analyzing the lipids by two dimensional thin-layer chromatography. The data presented in Table III represent the mean of three experiments. Quantitative analysis of the native preparations was similar to that reported for phospholipids of bovine brain tissue (26). It was of interest that the composition of extracted tissue factor differed from that of the native preparation. A similar phenomenon has been reported for other tissues (27).

Extracted tissue factor contained 2% phospholipid by weight that could not be removed by several additional butanol extractions. In an attempt to extract this residual lipid, a solvent system recently suggested by Fleischer, Fleischer, and Stoeckenius (27) for completely extracting acidic lipids from mitochondria was employed. This sol-

TABLE III  
*The Phospholipids of Native and Extracted Tissue Factor\**

Lipid	Preparation	
	Native	Extracted
	%	%
Sphingomyelin	21.1	4.2
PC	22.6	5.6
PI	6.1	38.2
PS	14.9	45.9
PE	35.4	5.6

\* Data are expressed as per cent of the total phospholipids. In addition to the compounds listed, trace amounts of PA and lyso PC were detected.

TABLE IV  
*The Effect of Removal of Residual Lipid on the Restoration of Tissue Factor Activity*

Preparation	Phospholipid	Activity
	%	U
1. extracted T.F.*	2.5	6.9
2. 1 + mixed phospholipids†	58.0	145.0
3. reextracted T.F.	0.8	2.2
4. 3 + mixed phospholipids	56.0	29.5

\* "Extracted tissue factor" refers to butanol-extracted material, and "reextracted tissue factor" to extracted tissue factor subsequently treated with acetone:water:ammonia (27).

† Components of preparations 2 and 4 were recombined in butanol.

vent system removed 66% of the residual lipid phosphorus and resulted in a preparation that retained slight activity. The ability to restore maximum activity with mixed brain phospholipids was, however, reduced (Table IV). The specific activity of the reextracted tissue factor was lower than the butanol-extracted brain, but at these levels, the assay is relatively insensitive. Therefore, the question of an absolute requirement for phospholipid cannot be unequivocally resolved on the basis of these data.

## DISCUSSION

The original observation of Studer (6) that a lipid-like material was required for the full activity of tissue factor has been confirmed. Furthermore, investigation has shown that this requirement was fulfilled by phospholipids, and that the requirement was highly specific. Kuhn and Klesse (7) also drew similar conclusions, but their assay was probably measuring the "intrinsic" coagulation system, rather than the tissue factor pathway. These investigators used the clotting time of whole plasma as an assay and obtained times in excess of 50 sec, suggesting that the material tested functioned as phospholipid and not as tissue factor. It is significant that they found PE to be active and PC to be inert. In the present study both PE and PC had significant activity.

Although it has been previously claimed that active preparations could be obtained only in the presence of organic solvents (8), it was found that sonication of the lipid emulsions to form

micelles (28) led to some restoration of activity, averaging about 10% of the maximum obtained when the lipids were dissolved in butanol. Homogenization of the lipids resulted in restoration of about 5% of the maximum activity. In addition, it was found that effective restoration of activity could be accomplished by removing the butanol by prolonged dialysis (48 hr) against water. Evaporation was adopted because of convenience.

Tissue factor, in common with several mammalian and bacterial enzymes (29, 30), exhibits a requirement for phospholipids. Although an appropriate polar group is essential for activity, the nonpolar portion of the molecule also contributes to the activity: catalytic hydrogenation of egg PC significantly reduced activity as did enzymatic removal of the  $\beta$ -fatty acid. In these experiments, the lipids used as starting materials had only minor differences in fatty acid composition and contained the same polar group: choline. Thus, the molecules were essentially identical except for the alterations being studied.

When the binding of phospholipids to tissue factor was studied, it was found that the three lipids tested, PE, PC, and PS, bound to a similar extent irrespective of their biological activity. PS, which is biologically inert, bound to extracted tissue factor somewhat more than did PE which was the most active lipid studied. Thus, PS *per se* was inactive in the tissue factor pathway, and its lack of biological activity was not due to a lack of binding.

The degree of activity of PE was striking, since it was about twice the specific activity of either pure PC or mixed brain phospholipids. If one considers that brain PE is mostly in the plasmalogen form (31) and that plasmalogens may be inert in blood coagulation (32), this finding is even more striking. No attempt was made in these studies to determine the activity of plasmalogens in the tissue factor pathway.

Many authors have stressed the role of charge in determining the biological specificity of phospholipids in coagulation (33-35) and in other enzyme systems (36, 37). It has been held that any micelle would be active in accelerating blood coagulation, provided that its surface charge were appropriate (35). Indeed, it has been shown that micelles formed of mixtures of PS/PC, PE/PS,

PE/PC, and PA/PC were all equally active, provided the net charge on the micelles was optimally negative. In the present study, it was found that only PE and PC exhibited significant activity. The extracted tissue factor used in these experiments contained about 2% by weight of phospholipids, of which PS and PI comprised 84%. It could be argued that PE and PC were active in this system, because in combination with the residual acidic lipids PE and PC had the appropriate charge. This is considered unlikely for two reasons. First, in the experiments previously described, the ratio of phospholipids was optimal over a very narrow range, and activity fell off markedly when one component was increased significantly. In the present experiments, a linear response of activity was noted when PC was present in a molar excess from about 5 to 35 with respect to the residual lipids. A narrower range of concentrations of PE was tested, but the response was linear with molar excesses from 4 to 15. With such a wide range in the ratios of added to residual lipid, the resultant charge would be expected to vary widely. This would be inconsistent with the linear response noted in tissue factor activity if charge were a major determinant of activity. In addition, at the pH (7.35) at which the present experiments were performed, PE is intermediate in charge between PS and PC, with PS being strongly negatively charged, PE weakly so, and PC zwitterionic. The observed results, PE > PC > PS, are in an order not conforming to their respective charges. Two explanations for these differences should be considered: (1) a different clotting system was employed, the tissue factor system, and that the biological requirements of this system differ from "intrinsic" coagulation; (2) the lipids used were in a different physical state from those used in the cited experiments. The tissue factor lipids were in the form of recombined lipoproteins, whereas those used in "intrinsic" systems were in the form of micelles. No experiments have been performed with respect to the relationship of the physical state of lipids to their specificity in clotting systems, but Marcus and associates (38) have shown that lipoprotein-lipid in the form of platelet membranes is about 20-fold more active than the same lipids in emulsions. They did not determine, however, whether lipids that were inactive as micelles

might be active as lipoproteins; that is, whether alterations in the physical state of the lipids altered their biological activity. Although this phenomenon has not been demonstrated for clotting systems, Thomas (39) has shown that the substrate specificity of a bacterial cyclopropane synthetase varied, depending on whether the phospholipid acceptor was in a micellar form or whether it was dried on a filter paper disc: in micelles, phosphatidylethanolamine > phosphatidylglycerol, whereas on the discs the order was reversed. Perhaps a similar mechanism is operative in blood coagulation.

Finally, it should be emphasized that the extracted tissue factor used in these experiments contained some residual phospholipids. It is not clear whether residual lipids are an absolute requirement for the activation of tissue factor with added lipids, but attempts to remove all the residual acidic lipids have resulted in preparations with reduced activity. It is possible that residual lipid is not required and that the extraction technique resulted in denaturation of the tissue factor. Alternatively, it may be that the recombination with added lipids occurs via a lipid-lipoprotein interaction, which thereby requires some residual lipids. These questions cannot be resolved at the present time.

#### ACKNOWLEDGMENTS

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#### REFERENCES

1. Williams, W. J., and D. G. Norris. 1966. Purification of a bovine plasma protein (factor VII) which is required for the activity of lung microsomes in blood coagulation. *J. Biol. Chem.* **241**: 1847.
2. Nemerson, Y. 1966. The reaction between bovine brain tissue factor and factors VII and X. *Biochemistry*. **5**: 601.
3. Howell, W. H. 1912. The nature and action of the thromboplastic (zymoplastic) substance of the tissues. *Am. J. Physiol.* **31**: 1.
4. Chargaff, E., A. Bendich, and S. S. Cohen. 1944. The thromboplastin protein: structure, properties, disintegration. *J. Biol. Chem.* **156**: 161.
5. Nemerson, Y., and T. H. Spaet. 1964. The activation of factor X by extracts of rabbit brain. *Blood*. **23**: 657.
6. Studer, A. 1946. Contribution à l'étude de la thrombokinase. In Jubilee Volume dedicated to Emile Christophe Barell. The Roche Companies, Basel. 229.
7. Kuhn, R., and P. C. Klesse. 1957. Zur chemischen konstitution des Lipoids der Thrombokinase. *Naturwissenschaften*. **44**: 352.
8. Deutsch, E., K. Irsigler, and H. Lomoschitz. 1964. Studen über Gewebethromboplastin. I. Reinigung, chemische Charakterisierung und Trennung in einen Eiweis- und Lipoidanteil. *Thromb. Diath. Haemorrhag.* **12**: 12.
9. Abramson, M. B., R. Katzman, C. E. Wilson, and H. P. Gregor. 1964. Ionic properties of aqueous dispersions of phosphatidic acid. *J. Biol. Chem.* **239**: 4066.
10. Rouser, G., A. N. Siakotos, and S. Fleischer. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids*. **1**: 85.
11. Lewis, M. L., and A. G. Ware. 1953. A simple procedure for separation of prothrombin and accelerator globulin from citrated human plasma. *Proc. Soc. Exptl. Biol. Med.* **84**: 636.
12. Aronson, D. L., and D. Ménaché. 1966. Chromatographic analysis of the activation of human prothrombin with human thrombokinase. *Biochemistry*. **5**: 2635.
13. Bell, W. N., and H. G. Alton. 1954. A brain extract as a substitute for platelet suspensions in the thromboplastin generation test. *Nature*. **174**: 880.
14. Skipsky, V. P., R. F. Peterson, and M. Barclay. 1964. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* **90**: 374.
15. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin layer chromatograms. *J. Lipid Res.* **5**: 126.
16. McIlwain, H., and R. Rodnight. 1962. Quoted in Ansell, G. B., and J. N. Hawthorne. *Phospholipids*. Elsevier Publishing Co., Amsterdam. 57.
17. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**: 375.
18. Vanselow, A. P. 1940. Preparation of Nessler's reagent. *Ind. Eng. Chem. (anal. ed.)* **12**: 516.
19. Milstone, J. H. 1964. Thrombokinase as prime activator of prothrombin: historical perspectives and present status. *Federation Proc.* **23**: 742.
20. Jobin, F., and M. P. Esnouf. 1967. Studies on the formation of the prothrombin converting complex. *Biochem. J.* **102**: 666.
21. Milstone, J. H. 1951. Fractionation of plasma globulin for prothrombin, thrombokinase and accessory thromboplastin. *J. Gen. Physiol.* **35**: 67.
22. Marcus, A. J. 1966. The role of lipids in blood coagulation. *Advan. Lipid Res.* **4**: 1.
23. O'Brien, J. S., and G. Rouser. 1964. The fatty acid composition of brain sphingolipids: sphingomyelin, ceramide, cerebroside, and cerebroside sulfate. *J. Lipid Res.* **5**: 339.

24. Zilversmit, D. B. 1958. Phosphatides in plasma. In *Standard Methods of Clinical Chemistry*. D. Seligson, editor. Academic Press Inc., New York. 2: 132.
25. Zilversmit, D. B., and A. K. Davis. 1950. Microdetermination of plasma phospholipids by trichloroacetic acid precipitation. *J. Lab. Clin. Med.* 35: 155.
26. Rouser, G., A. J. Bauman, G. Kritchevsky, D. Heller, and J. S. O'Brien. 1961. Quantitative chromatographic fractionation of complex lipid mixtures: brain lipids. *J. Am. Oil Chemists' Soc.* 38: 544.
27. Fleischer, S., B. Fleischer, and W. Stoeckenius. 1967. Fine structure of lipid-depleted mitochondria. *J. Cell Biol.* 32: 193.
28. Fleischer, S., and G. Brierley. 1961. Solubilization of cholesterol in phospholipid micelles in water. *Biochem. Biophys. Res. Commun.* 5: 367.
29. Fleischer, S., G. Brierley, H. Klouwen, and D. B. Slautterback. 1962. Studies of the electron transfer system. XLVII. The role of phospholipids in electron transfer. *J. Biol. Chem.* 237: 3264.
30. Rothfield, L., and M. Pearlman. 1966. The role of the cell envelope phospholipid in the enzymatic synthesis of bacterial lipopolysaccharide. *J. Biol. Chem.* 241: 1386.
31. O'Brien, J. S., D. L. Fillerup, and J. F. Mead. 1964. Quantification and fatty acid and fatty aldehyde composition of ethanolamine, choline, and serine glycerophosphatides in human cerebral grey and white matter. *J. Lipid Res.* 5: 329.
32. Zilversmit, R. D., A. J. Marcus, and H. L. Ullman. 1961. Plasmalogen in human blood platelets. *J. Biol. Chem.* 236: 47.
33. Rapport, M. M. 1956. Activation of phospholipid thromboplastin by lecithin. *Nature.* 178: 591.
34. Bangham, A. D. 1961. A correlation between surface charge and coagulant action of phospholipids. *Nature.* 192: 1197.
35. Papahadjopoulos, D., C. Hougie, and D. J. Hanahan. 1962. Influence of surface charge of phospholipids on their clot-promoting activity. *Proc. Soc. Exptl. Med. Biol.* 111: 412.
36. Bangham, A. D., and R. M. C. Dawson. 1959. The relation between the activity of a lecithinase and the electrophoretic charge of the substrate. *Biochem. J.* 72: 486.
37. Chung, A. E., and J. H. Law. 1964. Cyclopropane fatty acid synthetase: partial purification and properties. *Biochemistry.* 3: 967.
38. Marcus, A. J., D. Zucker-Franklin, L. B. Safier, and H. L. Ullman. 1966. Studies on human platelet granules and membranes. *J. Clin. Invest.* 45: 14.
39. Thomas, P. J. 1966. Stearic requirements for the enzymatic synthesis of cyclopropane fatty acids. Ph.D. dissertation. Harvard University, Cambridge. 67-68.