Reactions of activated factor X–phosphatide mixtures in vitro and in vivo

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ABSTRACT The composition, surface properties, and capacity of lipid particles to bind activated factor X can be correlated both with the influence of various phosphatide combinations on activated factor X activity in vitro and on the intensity and duration of the thrombogenic stimulus as measured by a standard bioassay for thrombus formation. The measurable activity of activated factor X in vitro increased as a linear function of lipid concentration from 0 to 40 μmoles/liter. The effectiveness of the lipids examined was in the following decreasing order: phosphatidyl serine–phosphatidylcholine mixture, "cephalin", and phosphatidyl serine alone. An increase in the duration of hypercoagulability with increasing lipid concentration was also observed, but, with regard to the three lipid fractions tested, the in vivo system appeared to be more discriminatory than in vitro assays. Lipid mixtures containing phosphatidyl serine with either phosphatidylcholine, phosphatidyl ethanolamine, or cetyltrimethylammonium bromide markedly enhanced the in vitro activity of activated factor X. Phosphatidic acid–phosphatidylcholine mixtures had a similar but smaller effect, and phosphatidyl ethanolamine–phosphatidylcholine mixtures were inert. The duration of the hypercoagulability was similarly related to the composition of the phosphatide infused. In mixtures containing phosphatidyl serine, the surface charge density of the lipid particles and the binding of the activated factor X activity to lipid showed some correlations with the in vitro activated factor X assay and with the intensity and duration of the thrombogenic stimulus. These data suggest that the catalytic effect of phosphatides on prothrombin activation and their role in the retardation of in vivo compensatory mechanisms directed against circulating activated factor X, are dependent on the affinity of activated factor X for the lipid surface.

SUPPLEMENTARY KEY WORDS coagulant · thrombogenesis · hypercoagulability · phosphatidyl serine · phosphatidyl ethanolamine · phosphatidylcholine · phosphatidic acid · "cephalin" · surface characteristic · lipid-protein binding

A STANDARDIZED TECHNIQUE for the study of hypercoagulability and thrombosis in the large veins of experimental animals has been developed (1). The thrombi which are formed are histologically similar to human thrombi, and the technique, when applied to human subjects, will produce thrombi in the large veins (2). The method has several important features. First, thrombosis is dependent upon the injection into the circulation of an activated species of a clotting factor, thus being in accord with one generally accepted definition of hypercoagulability (3). Second, the application of stasis as an integral part of the technique, permits the recognition of thrombi with doses of activated clotting factors that in freely flowing blood are not thrombogenic. Third, with minor modifications in technique, different plasma and serum fractions, including highly purified individual clotting factors and accessory moieties, can be evaluated for their effect on thrombogenesis. Finally, it is possible to compare the role of the various test fractions in vitro with their function in intravascular coagulation.

We have recently been interested in the role of factor X (Stuart–Prower factor) in thrombosis because both the nonactivated and activated forms can be obtained relatively pure, because activation of factor X represents a focal point in the intrinsic and extrinsic clotting systems, and because the activated species is directly

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; PA, phosphatidic acid; PS, phosphatidyl serine; σ, surface charge density; ψ_G, surface potentials; CTAB, cetyltrimethylammonium bromide.

contributory to thrombin generation in vitro (4, 5). It has been demonstrated recently that, whereas infusions of 630 units of purified nonactivated factor X fail to induce stasis thrombi in rabbits, 5 units of purified activated factor X produce massive thrombi in the jugular veins of rabbits (6). The hypercoagulability induced by infusions of 5 units of activated factor X is transient, persisting for less than 10 sec. When, however, aqueous dispersions of brain lipids are added to the infusate, the intensity and the duration of the hypercoagulable state are markedly enhanced (6). An ultrasonic-disrupted platelet fraction (6) has the same effect as "cephalin" in prolonging the duration of hypercoagulability induced by activated factor X. Several studies in vitro have shown that the participation of phosphatides in blood clotting is dependent at least in part on the physical structure of these substances in an aqueous environment and particularly on their electrokinetic properties (7–9).

The purpose of the present report is to demonstrate that the composition, surface properties, and capacity of lipid particles to bind activated factor X can be correlated both with the influence of the phospholipids on activated factor X activity in vitro and with the intensity and duration of the thrombogenic stimulus. In consequence, it has been possible to suggest a mechanism whereby lipids may facilitate the participation of activated factor X in the generation of experimental stasis thrombi.

METHODS

Activated Factor X

Bovine-activated factor X was isolated from Parke–Davis thrombin by a method previously described (10). In experiments relating to Figs. 1 and 3, the purified material was stabilized in a solution of crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). It was assayed by a modification (1), of the method of Bachmann, Duckert, and Koller (11). The specific activity of the activated factor X was 2000 units[1]/mg of protein. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (12).

Phosphatides

PC and PE were isolated from hen's eggs by solvent extraction and chromatography on columns of silicic acid (13). PA was prepared from egg lecithin by treatment with cabbage-leaf phospholipase (14). PS was isolated from bovine brains (15). The fatty acid composition of each fraction was determined by gas–liquid chromatography of the derived methyl esters on columns of 20% ethylene glycol succinate on 60–70 mesh AS Anakrom support in a Barber-Colman series 5000 gas chromatograph utilizing a hydrogen flame detector. No oxidation products were detected in those fractions. Cetyltrimethylammonium bromide was obtained from Sigma Chemical Co. Individual and mixed phosphatide dispersions were prepared by shaking dried lipid films with 0.145 M NaCl or with Veronal-acetate buffer, pH 7.35 (Michaelis buffer). Dried lipid films were prepared by removing most of the solvent ($CHCl_3$) under a stream of nitrogen. The residue was then desiccated *in vacuo* at room temperature overnight to remove the last traces of organic solvent. The dispersions appeared colorless, very faintly opalescent, and no clumping was observed as the concentrations of lipid examined were in the range 0.0016–0.128 μmoles/ml.

Lipid Extracts

Dispersions of human brain lipids ("cephalin") were prepared by the method of Bell and Alton (16) and contained 2 μg of lipid phosphorus per ml of 0.145 M NaCl.

Experiments In Vivo

The bioassay for the thrombogenicity of the infused fraction was modified from the standard technique for the production of stasis thrombi (1), in that the volume of the infusate, in 0.14 M NaCl, was 1 ml, the marginal ear vein infusion was completed in 2 sec, and the jugular vein segment was ligated (unless otherwise specified) within several sec after the start of the infusion (6). Healthy, fasted male albino New Zealand rabbits, each weighing approximately 1500 g and free of diarrhea, were used in the bioassay. Fasting refers to withdrawal of food, but not of water, for 16 hr preceding the experiment.

Experiments In Vitro

The clot-promoting effects of various lipid fractions were assayed in vitro by a modification of the factor X assay in which a standard solution of activated factor X was used, and the test fraction was substituted for cephalin. The results were expressed in terms of measurable units of factor X activity.

Electrostatic and Electrokinetic Measurements

Surface charge densities (σ) and surface potentials (ψ_G) of the phosphatide mixtures were computed utilizing information on the class composition and fatty acid composition as previously described (17). Partial dis-

[1] 1 unit of activated factor X is defined as the activity that would be produced from 1 ml of normal human plasma, when the precursor is fully activated by Russell's viper venom.

sociation of amino groups in PE and PS was taken into account. Electrophoretic mobilities were obtained by microelectrophoresis (18) and were used to calculate zeta potentials from the following simplified equation

$$\zeta = (4\pi\eta_o/D_o)\cdot\mu$$

where u is the electrophoretic mobility in units of cm^2 sec^{-1} $volt^{-1}$, η_o is the bulk viscosity in poises, and D_o is the bulk dielectric constant. The correction of Davies and Rideal (19) for highly charged surfaces was carried out using the equation

$$\zeta_c = \frac{\eta_o}{D_o}\int_o^{\zeta_o}\frac{D_i}{\eta_i}\,.d\zeta$$

where subscript o refers to bulk values and subscript i refers to values in the interface, and ζ_c refers to corrected zeta potential.

For the complex mixture of brain lipids, the calculations were based on the composition determined by thin-layer chromatography and column chromatography on silicic acid, infrared spectroscopy, and comparison with data in the literature.

Lipid–Protein Binding Experiments

Gel filtration on columns of Sephadex G-200 was used. It had previously been shown that in a mixture of activated factor X and phospholipid, in the absence of calcium ions, the lipid fraction appeared in the void volume (20). If calcium chloride were present in the eluting buffer, the activated factor X and phospholipid formed a macromolecular complex which then emerged in the void volume.

A lucite column, 0.95 × 30 cm, with a total volume of 21.3 ml, was filled with Sephadex G-200 (Pharmacia Fine Chemicals Inc. New Market, N. J.) previously equilibrated with Veronal-acetate buffer, pH 7.35, (Michaelis buffer) containing 0.025 M $CaCl_2$. Samples were applied in a volume of 0.5 ml and were eluted with the same buffer. Fractions of 0.5 ml size were collected into weighed tubes. Fraction sizes were determined by weighing. The void volume (Vo) was determined by the application of 0.5 ml of a solution containing 0.3% w/v Dextran Blue 2000 and was found to be 7.1 ml. The total available volume was determined with sucrose and was found to be 19.0 ml.

Uncombined activated factor X was completely separated from the lipid or lipid–protein complex under these conditions, and hence the proportion of the total protein present in the bound form could be estimated.

Since the total amount of lipid was constant in each experiment, the ratio of protein present in the void volume to total protein could be regarded as a measure of the binding of the protein to the particular lipid or lipid mixture under test.

RESULTS

The Effect of Cephalin and a Binary Mixture of Purified Phosphatides on the Intensity of the Thrombogenicity of Activated Factor X

Infusions of 1.25 units of activated factor X in 1 ml of 1% bovine serum albumin in 0.145 M NaCl without added lipid are not thrombogenic in rabbits. If either cephalin or a binary mixture of PS–PC is added to this subliminal level of activated factor X, the infusates are highly thrombogenic. In Table 1 the effectiveness of these two types of lipid fractions in enhancing the thrombogenicity of activated factor X is compared. It can be seen that an equimolar mixture of PS–PC is effective in amounts as low as 4×10^{-4} μmoles (0.0125 μg of lipid P), whereas with cephalin 24×10^{-4} μmoles (0.075 μg of lipid P) are required. On this basis a binary mixture of PS and PC is approximately 6 times more potent than the complex mixture of brain lipids in cephalin.

Similarly, when the concentration of activated factor X is reduced by making serial dilutions of the working standard, the threshold for thrombus formation induced by activated factor X is lower with PS–PC than with cephalin, this time by a factor of 2 (Table 2). No thrombus was formed when activated factor X (1.25 units/ml) was infused without lipid. The lipid phosphorus concentrations in both the cephalin and the PS–PC mixture are each 2 μg/ml.

Phosphatide Concentration and In Vitro Activity of Activated Factor X

Various phosphatides and phosphatide mixtures were tested for potency by substituting them for cephalin in

TABLE 1 STASIS THROMBI INDUCED IN RABBITS BY 1.25 UNITS OF ACTIVATED FACTOR X INFUSED WITH VARIOUS CONCENTRATIONS OF PHOSPHOLIPIDS

Concentration of Lipid Infusate		Jugular Vein Thrombosis
Cephalin	PS–PC* (1:1)	
μg of P per ml	*μg of P per ml*	
0.50		(+)†
0.250		(+)
	0.25	(+)
0.125		(+)
0.100		(+)
0.075		(+)
0.050		(0)‡
	0.05	(+)
	0.25	(+)
	0.0125	(+)
	0.0100	(0)
	0.0062	(0)

* Dilutions made in 0.145 M NaCl.
† Thrombus formed.
‡ No thrombus formed.

TABLE 2 STASIS THROMBI INDUCED IN RABBITS BY VARIOUS CONCENTRATIONS OF ACTIVATED FACTOR X INFUSED WITH PHOSPHOLIPIDS

Activated Factor X Infused†		Jugular Vein Thrombosis
Cephalin*	PS–PC*	
units/ml		
0.625	—	(+)‡
0.313	—	(+)
—	0.313	(+)
0.156	—	(+)
0.078	—	(0)§
	0.078	(+)
0.039	—	(0)
	0.039	(0)

* 2 μg of lipid P.
† Dilution made in 1% bovine serum albumin in 0.145 M NaCl.
‡ Thrombus formed.
§ No thrombus formed.

the modified factor X assay. Control tests were performed in which activated factor X and lipid were separately omitted. In neither control experiment was activity observed. When cephalin was used as the lipid component, values in the range 8–9 units/ml of activated factor X were obtained. We first investigated the effects of varying concentrations of lipids on measurable factor X activity in vitro. The range from 0 to 0.14 μmole/ml was examined. The lipid fractions were PS alone, PS–PC (1:1), and cephalin. In each case the activity increased as a linear function of concentration from 0 to 0.05 μmole/ml and then reached a plateau above this limit (Fig. 1). Over the concentration range examined, the decreasing order of effectiveness was PS–PC (1:1), cephalin, and, finally, PS alone. Although the differences were very small under these conditions, the order of effectiveness was maintained at all concentrations examined.

Phosphatide Concentration and the Duration of Hypercoagulability Induced by Activated Factor X

The duration of hypercoagulability induced by mixtures containing a constant level of activated factor X plus varying amounts of phosphatides is shown in Fig. 2. Over the concentration range examined (0–0.064 μmole/ml), the duration of hypercoagulability increased with increasing concentration of phosphatide. The order of effectiveness of the three preparations tested (PS–PC, cephalin, and PS alone) was maintained throughout this concentration range. PC by itself was inactive.

Dependence of Activity In Vitro on Lipid Composition and Surface Potential

Mixtures containing PS with PC, PE, or cetyltrimethylammonium bromide (CTAB) are extremely active,

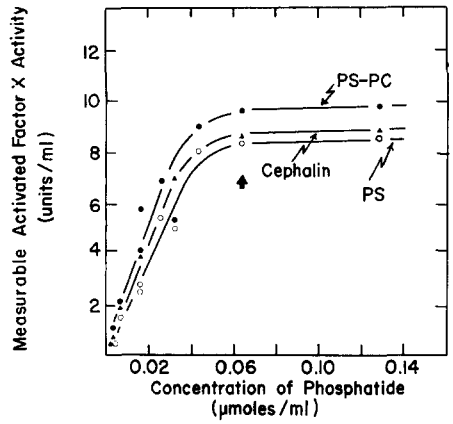

FIG. 1. Dependence of activated factor X activity in vitro on the concentration of phosphatide present. ●, PS–PC mixtures; ○, PS; ▲, cephalin. Position of arrow indicates concentration of the lipid mixture used in routine tests.

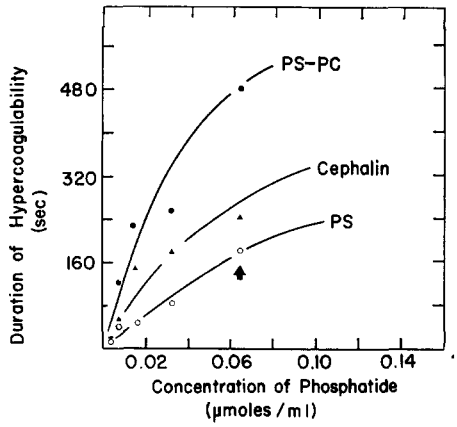

FIG. 2. Duration of hypercoagulability induced in rabbits by activated factor X as a function of the concentration of phosphatide present in the infusate. ●, PS–PC mixtures: ○, PS; ▲, cephalin. Position of arrow indicates concentration of the lipid mixture used in routine tests.

whereas mixtures such as PE–PC are inactive. Mixtures of PA and PC exhibited behavior qualitatively similar to that of PS and PC, but the activity displayed was at a greatly reduced level. Fig. 3 shows a series of curves in which the activities of various phosphatide mixtures are plotted against composition and surface potential. The computed surface potential and the corrected zeta potential were coincident in all cases examined and are, therefore, not shown separately. It can be seen that the activity of mixtures of acidic and zwitterionic lipids increases with increasing surface potential to a maximum at about −75 mv, and subsequently decreases. This optimum corresponds to a surface charge density of about 30,000 esu cm^{-2}. A small secondary optimum was reproducibly observed at about −130 mv (75,000 esu cm^{-2}).

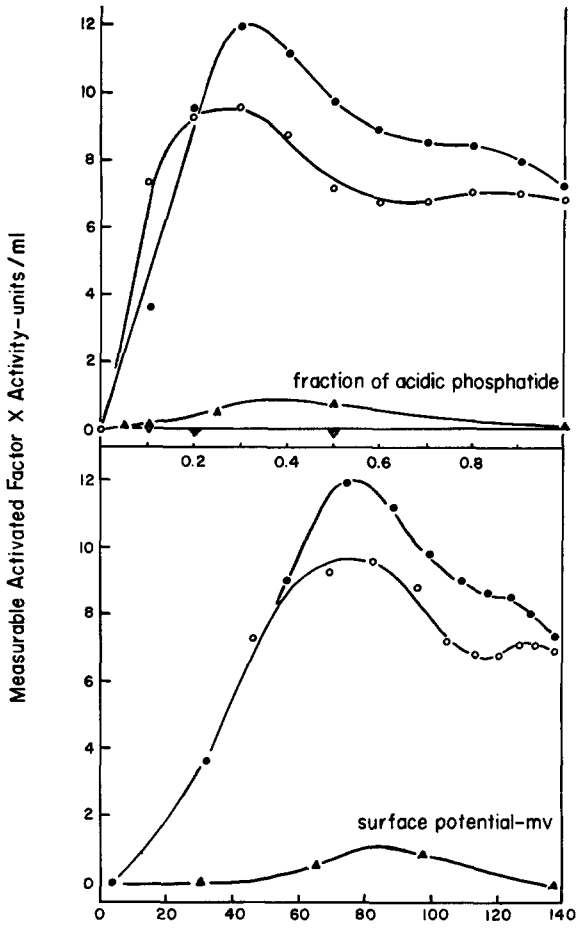

FIG. 3. Factor X activity in vitro as a function of composition, and surface potential (ψ_G) when assayed by the modified factor X assay. Per cent composition on the abcissa refers to the percentage of acidic lipids in the mixture, on a molar basis. The lipid concentration in each dispersion tested was 0.064 μmole/ml. ●, PS–PC mixture; ○, PS–PE mixture; ▲, PA–PC mixture; ▽, PE–PC mixture.

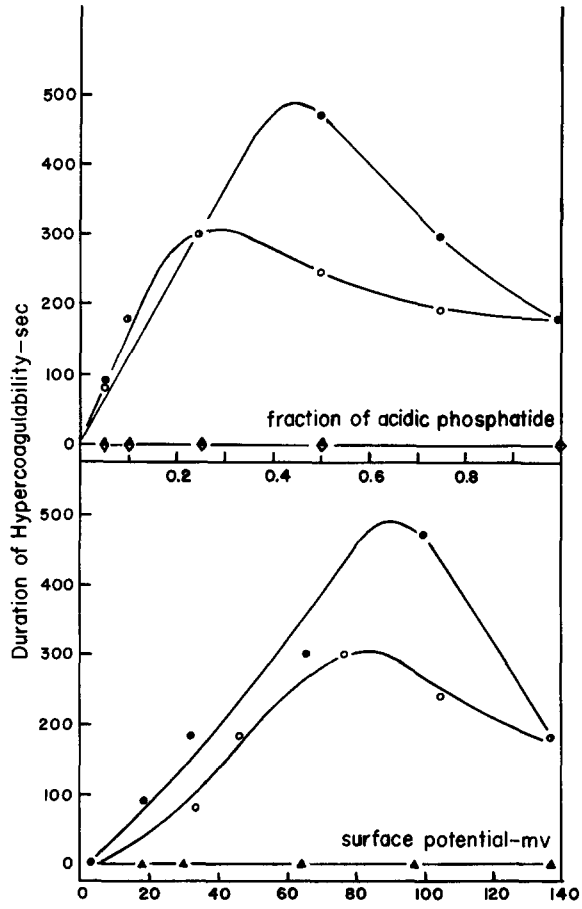

FIG. 4. Duration of hypercoagulability induced in rabbits as a function of composition, and surface potential (ψ_G) of the phosphatides. Per cent composition on the abscissa refers to the percentage of acidic lipids in the mixture, on a molar basis. The lipid concentration in each dispersion tested was 0.064 μmoles/ml. ●, PS–PC mixture; ○, PS–PE mixture; ▲, PA–PC mixture; ▽, PE–PC mixture.

Dependence of the Duration of Hypercoagulability on the Lipid Composition and Surface Potential

The duration of hypercoagulability induced in experimental animals can be related to the composition and surface potential of the phosphatides. These results are shown in Fig. 4. Mixtures containing PS are found always to be active, whether mixed with PC, PE, or CTAB. The optimal proportion of PS is 35% of the total lipid, corresponding to a surface potential of −80 mv. No activity can be observed with mixtures of PE and PC or with PA and PC.

Binding Ratios for Activated Factor X–Phosphatide Complexes

The capacities of five phosphatide preparations to bind activated factor X in the presence of 0.025 M $CaCl_2$ are shown in Table 3. When PC is used only a small amount of protein cochromatographs with the emulsified lipid, this amount being steadily increased by admixture with increasing amounts of PS. An equimolar ratio of PS–PC appears optimal, and further increase in the ratio of PS–PC displaces a small proportion of the bound protein.

TABLE 3 BINDING RATIOS FOR ACTIVATED FACTOR X–PHOSPHATIDE COMPLEXES IN THE PRESENCE OF 0.025 M $CaCl_2$

Lipid Composition		Surface Potential of Lipid Particle	Bound Activated Factor X / Free Activated Factor X
PC	PS		
%	%	*mv*	
100	0	−3	0.1
90	10	−32	1.3
75	25	−67	4.9
50	50	−99	5.9
0	100	−137	5.3

* Total lipid P concentration equals 2.0 μg/ml.

DISCUSSION

Numerous investigators have attempted to identify which of the naturally occurring phosphatides present in lipid extracts of tissue and platelets participates in blood clotting. These reports have been adequately reviewed by Marcus (21). In 1961, Bangham reported that the activity of PC could be enhanced by the addition of dicetylphosphoric acid (7). He related this increase of activity to the increase in the negative electrophoretic mobility of the lipid particles. This relationship was confirmed by the use of mixtures of purified acidic and zwitterionic phosphatides (8, 9). It has been shown by numerous investigators that, for the elaboration of prothrombin activator, activated factor X, factor V, phosphatides, and calcium ions are required. The omission of any of these components greatly reduces the prothrombinase activity. The complete activator is believed to be a sedimentable lipid–protein complex (20, 22–25).

Relating these observations to the in vivo situation, we find that, whereas the hypercoagulability induced by the infusion of highly purified activated factor X into fasted rabbits persists only for a few sec, the addition of cephalin or a sonicated platelet fraction to the infusate increases the intensity, as well as the duration of the hypercoagulability, severalfold (6). The finding that the thrombogenicity of activated factor X is increased by cephalin suggests that there is a specific rapid formation of prothrombin activator in vivo when the activated factor X–cephalin fraction is infused; the other factors, factor V and calcium, are already present in circulating blood and are not rate-limiting in the formation of prothrombin activator. In this connection it should be noted that cephalin is not thrombogenic by itself, and neither confers thrombogenicity on nonactivated factor X nor augments the thrombogenicity of infusions of purified thrombin (26).

If the above view of the role of activated factor X and phosphatides in experimental thrombogenesis is correct, then we may expect a very high level of thrombogenic potency when such mixtures are infused into the intact animal. This is emphasized by comparison of our results with those of other investigators who used different substances to induce intravascular coagulation in the stasis model, but without the addition of an activated clotting factor. Thus, with an amount of activated factor X alone that is subliminal, we observe that stasis thrombi form when 24×10^{-4} μmoles of cephalin are infused with the activated factor X. When an equimolar mixture of PS–PC is substituted for the cephalin, only 4×10^{-4} μmoles of phosphatide are required to induce a stasis thrombus (Table 1). In the converse experiment, 6.4×10^{-2} μmoles of an equimolar mixture of PS–PC initiate thrombus formation when infused with 7.8×10^{-2} units of activated factor X (Table 2).

In the present study we have attempted to define more precisely the surface characteristics of the phospholipid components. Based upon the class composition and fatty acid composition of each particular phosphatide mixture, we calculated the corresponding surface charge densities and surface potentials. It should be emphasized that these electrostatic parameters provide theoretical values and that their calculation involves a number of assumptions (17). Direct measurement of surface potential in the bulk phase has to be carried out by methods which involve motion of either the particles or the bulk fluid, such as microelectrophoresis or streaming-cell measurements. The potential thus measured is, therefore, an electrokinetic parameter, the zeta potential. We have measured the electrophoretic mobilities of each of the preparations used and have utilized the data to calculate zeta potentials. The correction of Davies and Rideal (19) was used to allow for the enhanced viscosity and reduced dielectric constant in the interface at high surface charge density. With the use of these procedures, the electrostatic and electrokinetic potentials were found to be coincident within experimental error over the whole range of composition studied. These potentials can now be related with a high degree of confidence to the other parameters studied with these systems.

Table 3 shows binding ratios for activated factor X–phosphatide complexes in the presence of 0.025 M calcium ions. There appears to be a definite relationship between the composition and surface potential of a particular lipid surface and the ability of that lipid to bind activated factor X. Similar observations have been made in the case of prothrombin binding to lipid surfaces.[2] Subsequently it was suggested (27) that prothrombin activation involves the following steps: (*a*) binding of prothrombin to the lipid moiety of prothrombin activator; (*b*) conversion of prothrombin to thrombin as a result of interaction with activated factor X at the lipid–water interface, and (*c*) release of thrombin into the bulk aqueous phase.

The evidence presented here is in line with the concept that prothrombin activation is catalyzed by activated factor X (4, 5) in a heterogeneous system which exerts a specific directive influence on the reaction (27), and that this catalytic effect is dependent on the surface structure of the lipid component. Since the binding experiments described here were restricted to mixtures of PS and PC, no conclusions can be drawn regarding specific features of the lipid surface which might determine the binding affinity.

[2] Barton, P. G. Unpublished observations.

The role of phosphatides appears to be closely linked to their surface potential, when the activity is examined in vitro. The optimal composition of lipids vary somewhat depending on the component species present, but the optimal electrostatic and electrokinetic parameters fall within a narrow range of values. These data confirm the results o other investigators using different assay systems (7–9). The failure of PA–PC mixtures to provide an effective substitute for PS–PC is worthy of comment. Daemen, van Arkel, Hart, van der Drift, and van Deenen (28) have previously noted that synthetic phosphatidic acids as well as isolated cardiolipin (diphosphatidyl glycerol) are poor substitutes for phosphatidyl serines, either individually or in binary mixtures with phosphatidyl ethanolamines. The data of Papahadjopoulos, Hougie, and Hanahan (8) also indicate that PA–PC mixtures are less effective than PS–PC mixtures at any given electrophoretic mobility. These findings suggest that some facet of the lipid-water system, other than the charge density, may also be important for participation in this reaction sequence. In recent years much progress has been made in the determination of the phase structures of single and mixed liquid crystals of phosphatides and it is known that, depending on their composition and method of preparation, they can vary considerably in molecular architecture (29, 30) and in gross hydrodynamic properties (31). The procoagulant function of these structures may be dependent, for example, on the ion-binding properties of the surfaces, on the presence or absence of specific chemical groups such as carboxyl groups, or on the size and arrangement of the aqueous compartments separating the lipid regions. In this regard preliminary experiments using a mixture of PS and the cationic detergent CTAB show this colloid to be active in vitro and in vivo. Thus, the action of PS is not dependent on the aggregate containing only phosphatides.

If the coagulation sequence is to be related to the thrombotic process, it becomes necessary to demonstrate that the mechanism operative in vitro is applicable to the elaboration of fibrin intravascularly. It has been shown that the ability of any particular phosphatide dispersion to participate in the formation of prothrombin activator in vitro depends largely on the physicochemical properties of the colloidal particles. These observations have been extended in the present study by using a standard animal model for the formation of activated factor X and a variety of phosphatides. In a series of experiments shown in Fig. 4, it was found that those lipid mixtures exhibiting maximal procoagulant activity in vitro potentiated in the living animal the intensity and duration of the hypercoagulability of infused activated factor X. Comparisons of Fig. 1 with Fig. 2 and of Fig. 3 with Fig. 4 indicate that results of experiments in vivo could be predicted in part from the results of tests in vitro. The dependence on lipid concentration and composition is broadly similar for both types of results.

More detailed comparison of the two sets of data reveals differences which can provide some clues as to the mechanism of the regulation of blood coagulability in the intact animal. For example, we note that the differences between PS–PC, cephalin, and PS alone, observed in vitro (Fig. 1) are considerably accentuated in vivo (Fig. 2). This is the first indication we have that the intact animal is actually more discriminatory with regard to lipid–protein interactions than are the various assay systems in vitro. We have referred already to the lower activity of PA–PC mixtures as compared with PS–PC mixtures. In vivo mixtures containing PS are maximally active, following an identical relationship between surface potential and biological activity with that observed in vitro. No activity whatever could be detected with mixtures containing PA at any negative surface potential, again an accentuation of the differences observed with these lipids in vitro. Such differences may reflect the existence of mechanisms within the animal body designed to remove or neutralize the circulating coagulant materials. Evidence is available that there exists in plasma an inhibitor of activated factor X (32, 33). It has also been shown that activated factor X disappears from liver perfusates (34, 35). Both the greater intensity and prolongation of hypercoagulability induced by the activated factor X–phosphatide mixture over the activated factor X alone suggest that its neutralization, whether by circulating inhibitors or clearance mechanisms, is different from that of activated factor X alone. Thus, in fasting animals the soluble activated factor X is promptly inactivated, a result not so readily achieved in fed animals when the activated factor X is bound to lipid in the presence of calcium ions. We can therefore suggest a dual role for phosphatides in thrombogenesis. There is a catalytic effect on prothrombin activation as well as a retardation of compensatory mechanisms directed against activated factor X. The results presented here suggest that both of these effects are dependent on the affinity of activated factor X for the lipid surface.

Despite reports in the literature to the contrary (36, 37), we have never observed any marked anticoagulant effect of PS. Several reasons may be advanced for this discrepancy. First, it has been shown that the demonstration of anticoagulant activity is dependent on the type of test used (38). For example, while most clotting tests are inhibited by the addition of PS, those related specifically to activated factor X are accelerated. Secondly, the influence of PS is concentration dependent. Demonstration of the inhibitor effect in vivo requires amounts as large as 30–50 mg/kg of

body weight (36, 38), whereas our studies have been limited to a range of 0–70 μg/kg of body weight. Thirdly, the inhibitory effect of PS has only been striking when the lipid was first treated with bile salts or albumin solutions (39, 40).

Aside from positive correlations between in vitro and in vivo reactions of activated factor X, one can ask what the relevance of these observations is to the possible occurrence of hypercoagulability in man. Activation of factor X in vitro can be initiated by activation of the intrinsic clotting system (41), via the extrinsic system by organ extracts (42), by Russell's viper venom (43), and by trypsin (44). The possibility that activated factor X formed by any of these mechanisms might be thrombogenic would be immediately opposed by compensatory mechanisms which include endothelial fibrinolytic activity (45), circulating inhibitors (32, 33), liver clearance (34, 35), and adequate blood flow (46). One might speculate, however, on the possibility of an equilibrium condition comprising both formation and removal reactions such that in the steady state there would be a fixed level, albeit a very low one, of activated factor X in circulating blood and one certainly not measurable by current techniques. Assuming a platelet count of 350,000/mm³ and a blood volume of 80 ml/kg of body weight (47), it can be calculated from data in the literature (48) that the required amount of lipid would be liberated in the form of platelet factor 3 by destruction of less than 0.5% of the circulating platelets. If we now assume that the level of circulating activated factor X is in the range of 0.001–0.01 units/ml of blood, then it is evident from Table 2, that the blood would remain fluid. If at this point lipid or lipoprotein with the appropriate surface structure was released into the fluid blood through damage to less than 2000 circulating platelets, a hypercoagulable state would then exist in areas of retarded blood flow (Table 2, column 2). This speculation is put forth, although we are aware of the controversy concerning the normal occurrence of continuous intravascular coagulation (46), to suggest how the data obtained in this study can be used in a semiquantitative way to provide a basis for conceptualization of systemic hypercoagulability.

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