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Influence of Surface Charge of Phospholipids on their Clot-Promoting Activity.* (27809)

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The identity of the phospholipid participating in blood coagulation has long been the subject of controversy. It has been maintained that phosphatidyl ethanolamine (PE) is the active phospholipid(1,2), while it has also been indicated that phosphatidyl serine (PS) is the active constituent and that its activity could be potentiated by phosphatidyl choline (PC) or other lipids(3,4). Other specific factors have been cited as essential components for activity. For example, Rouser, *et al.*(5) emphasized the importance of unsaturation of the fatty acids and the presence of a free amino group in the phospholipid. Rapport(6) and Theriault, *et al.* (7) reported that potentiation of the activity of PS by PC is greatly increased if the lipids are first mixed in organic solvents and then emulsified. Wallach, *et al.*(2) showed that the pH of the emulsifying buffer was an important factor for optimal activity of the phospholipid. These investigators observed a gradual rise in activity of PE emulsions between pH 5 to 8, then a rapid increase between pH 8 to 10. This change in activity was attributed to the formation of smaller particles due to electrostatic interactions. Recently, Bangham(8) demonstrated that mixed emulsions containing varying amounts of PE plus PC and dicetyl phosphoric acid plus PC showed increasing activity with increasing surface charge.

Certain of these observations showing an increase of activity by mixing 2 phospholipids, or by changing the pH, were confirmed in this laboratory. It was then felt necessary to investigate further the possible influence of physical factors on formation of the prothrombin activator. The experiments reported here include a study of the "thromboplastic" activity of a series of mixed phospholipid emulsions derived from beef brain and egg yolk, and the influence of the surface charge of the lipid particles on this activity.

Materials and methods. Lipid fractions. PS and PE were isolated from beef brain as follows: Brains received within half hour after slaughter were placed immediately in dry ice. Within one hour, the brains were extracted with C:M (chloroform:methanol) 2:1 (v/v) and total lipid extract obtained as described by Rouser *et al.*(9). Most of the steps were performed under a stream of nitrogen. The final extract was dissolved in chloroform and chromatographed immediately on a silicic acid column (2g silicic acid for each mg phosphorus). The neutral lipids were eluted with C:M 9:1, and subsequent use of

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C:M 6:1 eluted PE, PS and cerebroside. The latter fraction was rechromatographed on DEAE cellulose as described by Rouser *et al.* (9). The cerebroside was eluted with C:M 7:1, the PE with C:M 7:3 and, finally, the PS with glacial acetic acid. The bulk of the PE fraction was free from other contaminating lipids as shown by its behavior on thin layer plates. It chromatographed as single spot with an Rf value similar to a standard PE derived from erythrocytes and showed no secondary spots, even when heavily spotted, in a solvent system of chloroform/methanol/water, 95/35/4 or in ethanol/chloroform/water, 5/2/2. A trace amount of PS could easily be detected in such a system. This preparation had a nitrogen to phosphorus ratio of 1.05. The PS fraction was freed of glacial acetic acid by evaporation from the frozen state (lyophilization) and dissolved in C:M 9:1. It migrated on thin layer plates as a single spot in a system of ethanol/chloroform/water, 5/2/2, which showed no traces of PE or other lipids, and it had a nitrogen to phosphorus ratio of 1.00 and a fatty acid ester (determined by infra-red with a triolein standard) to phosphorus ratio of 1.99. Occasionally, this fraction was contaminated by another nitrogen-containing compound(s) as evidenced by N/P ratios above 1.00. In such a case the impurity was removed by rechromatography on silicic acid. C:M 6:1 eluted the impurity prior to the desired PS fraction.

Lecithin (PC) was isolated from egg yolk by silicic acid chromatography as described previously(10) and was shown by infra-red spectroscopy, thin layer chromatography, nitrogen to phosphorus ratio of 1.02 and fatty acid ester to phosphorus ratio of 1.98, to be free of other contaminating lipids.

Phosphatidic acid (PA) was produced enzymatically by the action of phospholipase C from carrot chloroplast preparations on lecithin(11). The reaction mixture was extracted and chromatographed on silicic acid. PA was eluted with increasing amounts of methanol in chloroform from 3 to 10%. The purified PA was free of nitrogen, had a fatty acid to phosphorus ratio of 1.85 and was shown by thin layer chromatography to be free of PC, PE, and PS.

Protein fractions. Factor VIII was a bovine, anti-hemophilic globulin (AHG) preparation (Maw's Laboratories). Factor V was a 33-45% ammonium sulfate precipitate of $Al(OH)_3$ treated bovine plasma. Factors IX and X were obtained from bovine serum after adsorption on $BaSO_4$ and elution with 5% trisodium citrate. All 3 fractions were maintained in a lyophilized state at –20°C.

Thromboplastic activity tests. Each of the 3 protein fractions was dissolved in 0.14M sodium chloride in optimal concentration before each series of tests. One volume of each of the protein solutions was mixed with one volume of Tris/HCl buffer, 0.14M, pH 7.4, and one volume 0.025M $CaCl_2$ and the whole incubated for 10 minutes at 37°. The above mixture (A) contained "product 1" activity, as described by Bergsagel and Hougie(12) and Spaet *et al.*(13) and could convert prothrombin to thrombin in the presence of "cephalin" or platelets. Two tenths ml of A was mixed with 0.1 ml of lipid emulsion and this mixture (B) was tested after 3 and 5 minutes for its ability to activate prothrombin. This assay was accomplished by transferring 0.1 ml B to a tube containing 0.1 ml of citrated, platelet-poor, normal human plasma and 0.1 ml .025M $CaCl_2$. Clotting times ranged from 6.5 seconds for the most active emulsions to 80 seconds for the 0.14M sodium chloride control. The activity of the different emulsions was checked at concentrations ranging from 900 μg to 1 μg of lipid per ml of test mixture. The activity was plotted as minimum concentration of lipid necessary for a 10-second clotting time.

Lipid emulsification. An aliquot of lipid or mixture of lipids of approximately 0.5 mg total phosphorus was taken to dryness in a 500-ml round-bottom flask. The pressure, after every evacuation in this and in the following steps, was restored with nitrogen. The lipid was dissolved in 10 ml of diethyl ether, a few glass beads added, plus 20 ml methanol and 20 to 30 ml of distilled water. (If the emulsions were to be prepared at pH 7.4, a sufficient amount of buffer was added for a final concentration of 0.03M.) The mixture

was then placed on a rotary evaporator, where temperature was kept below 30°C. When the volume was approximately 10 ml, an additional 10 ml of water were added and evaporation continued to a volume of about 3 to 4 ml. The emulsion was then removed by pipetting and brought to a final volume of 5 ml. These samples were stored at 5° and were quite stable.

Mixtures of PS in PE, PS in PC, PA in PC, and PE in PC in varying relative concentrations, as well as the individual lipids alone, were emulsified in water (pH 5.0) and in Tris buffer at 7.4. The physical appearance of these emulsions varied according to the individual lipid present, the pH and ionic strength of the medium. In general, the higher the pH and lower the ionic strength, the clearer the resulting emulsion. In many instances, the mixtures were nearly water-clear. It should be noted that when a number of these emulsions were run on the analytical electrophoresis apparatus, a single band of electrophoretically homogenous material was obtained.

Electrophoretic mobility. All emulsions under test were diluted one to ten with a sodium chloride-Tris buffer to a final molarity of 0.03M Tris buffer and 0.115M sodium chloride and a final pH 7.4 immediately before the experiment. The apparatus employed was similar to that described by Bangham *et al.*(14). Rate of movement of the microscopically visible particles was measured at 36.0° ± 0.1°C under an applied current. Usually, the speed of more than 10 individual particles was averaged and the electrophoretic mobility calculated. The results were expressed as cm^2 sec^{-1} $volt^{-1}$.

Results. There appears to be a definite correlation between the electrophoretic mobility of the various phospholipid mixtures and their "thromboplastic" activity (Fig. 1). All 3 series of different mixtures of phospholipids developed maximal activity when they possessed nearly the same charge as expressed by their electrophoretic mobility.

In graph A, curve I, which represents the results obtained with emulsions originally prepared in water (pH 5.0), the maximum activity developed at approximately 40% PS in

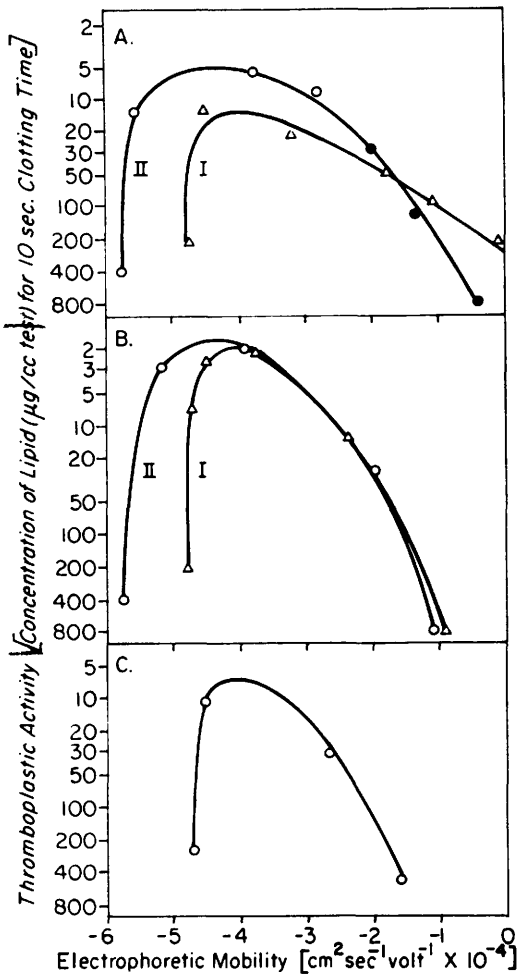

FIG. 1. Correlation of electrophoretic mobility of lipid emulsions with thromboplastic activity. The individual points, as shown from left to right on above curves represent results obtained with the following lipid mixtures (as indicated by w/w ratios based on phosphorus values) and prepared at indicated pH values. Determinations of mobility and activity were performed at pH 7.4 and ionic strength .14 for all emulsions. *Graph A:* Curve I, △ PS/PE (pH 5.0) 100/0, 40/60, 6/94, 3/97, 1/99, 0/100; Curve II, ○ PS/PE (pH 7.4) 100/0, 50/50, 10/90, 5/95, ● PE/PC (pH 7.4) 90/10, 80/20, 50/50. *Graph B:* Curve I, △ PS/PC (pH 5.0) 100/0, 70/30, 50/50, 20/80, 10/90, 1/99; Curve II, ○ PS/PC (pH 7.4) 100/0, 50/50, 20/80, 10/90, 5/95. *Graph C:* ○ PA/PC (pH 7.4) 100/0, 50/50, 20/80, 10/90.

PE at an approximate mobility value of 4.5 × 10^{-4} cm^2 sec^{-1} $volt^{-1}$. Both PS and PE alone showed very little activity when the emulsions were prepared in water (pH 5.0). On the other hand, when emulsions were prepared at pH 7.4, as illustrated in curve II

(graph A), maximum activity developed at about 10% PS in PE, but at the same mobility value as in curve I. The PE emulsions prepared at pH 7.4 exhibited a much more negative mobility and concomitantly a higher activity. To obtain the data for the less negative mobilities shown in curve II (graph A), it was necessary to use emulsions of PE mixed with PC. The activity dropped progressively to zero when mobility decreased to less negative values.

In graph B the mobilities of a series of emulsions of PS in PC are plotted *versus* their activities. The maximum thromboplastic activity developed for mixtures having nearly the same mobility value as indicated in graph A, and at relative concentrations of 40% PS in PC.

In graph C, which represents results obtained with emulsions of PA in PC, it is clear that the emulsions which possess the optimal negative mobility found in previous experiments, again exhibit maximal activity.

Discussion. Evidence is presented that thromboplastic activity of phospholipid emulsions is correlated with the electrophoretic mobility exhibited by the phospholipid particles present. The electrophoretic mobility values are an expression of the zeta potential and are related to the surface charge of the particles. In all cases tested, there is an optimal negative surface charge at which the lipid particles exhibit their maximal "thromboplastic" activity. When the particles carry a more negative or a less negative charge than the optimal, a diminished activity is noted. Specifically, PS alone, when emulsified at pH 7.4, carried a charge more negative than the optimal and exhibited very little activity. The same lipid emulsified in water (pH 5) had a charge nearer to the optimal and possessed a higher activity. When PS was mixed with increasing amounts of PC, which is neutral at the two pH values, and emulsified, the resulting emulsions had progressively lower surface charge, while their "thromboplastic" activity reached a maximum and subsequently diminished at even lower surface charge. On the other hand, the surface charge of emulsions of PE alone was less negative than the optimal and again depended on the circumstances under which they were originally prepared. There was a difference in charge between emulsions made in water at pH 5 and in buffer at pH 7.4 with the more negative particles possessing a higher "thromboplastic" activity.‡ Even at pH 7.4, the charge was less negative than the optimal and the addition of lecithin which lowered the charge also diminished the activity. Only when the negative charge of PE was increased by addition of PS could one observe the maximal activity. The same phenomenon could be observed when PE or PS was replaced by phosphatidic acid which is not believed to take part in the physiological process of activation of prothrombin.

The electrophoretic mobility values given here differ somewhat from the values given by Bangham(8) and also from the values obtained in this laboratory by the free boundary method. The discrepancies could be due to differences of pH, temperature, or focusing during electrophoresis. We have already shown that presence or absence of buffer during emulsification can change the mobility as well as the activity of certain lipids. Nevertheless, the conclusions drawn from the relative values obtained with a number of emulsions under the same conditions can still be valid.

Our results suggest that there is no correlation between thromboplastic activity as measured by our test system and any particular phospholipid. It appears that the ability of a lipid emulsion to participate in activation of prothrombin depends largely on physicochemical properties, *e.g.*, surface charge (zeta potential), of the micellar structures as a whole. It must be borne in mind that other aspects of surface chemistry and structure of the lipids cannot be excluded. It should also be emphasized that the participation of phospholipids in the physiological process of coagulation could be quite different from the picture given here. Nevertheless, one can

‡ The actual experimental point for PE alone, emulsified at pH 7.4 is not represented in Fig. 1. It was not possible to obtain reproducible emulsions of this lipid alone at 0.03 ionic strength. It is believed, however, that its value should be close to that obtained for a 90% PE - 10% PC mixture.

gain important information in an artificial, *in vitro* system, which can later be applied to the physiological process.

Summary. Electrophoretic mobilities and thromboplastic activities of emulsions of mixtures of phospholipids derived from beef brain and egg yolk were determined. The studies included mixtures of varying amounts of phosphatidyl serine in lecithin, phosphatidyl serine in phosphatidyl ethanolamine, and phosphatidic acid in lecithin. It was concluded that the manifestation of "thromboplastic activity" by a particular phospholipid emulsion will depend largely upon the surface charge of the particles. The possibility of other factors involved was not excluded.

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Studies on Fluorescent Antibody Staining II. Inhibition by Sub-Optimally Conjugated Antibody Globulins.* (27810)

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The principle followed until recently for preparing fluorescent antibody reagents has been to attach to antibody globulins as many fluorescent groupings as possible without precipitating the antibody. Then, to reduce the large amount of resulting non-specific fluorescence, it has been customary to subject the fluorescent antibody to multiple absorptions with tissue powders, charcoal, or resins.

Recent reports(1-3) have shown that the attachment of fluorescein isothiocyanate (FITC) to protein is not a uniform process, and that within each fluorescent antibody preparation there may be a marked variation in the number of fluorescent groupings per protein molecule. Furthermore, it has been shown that the specific and the non-specific staining properties of fluorescent antibodies are related to the number of fluorescent groupings conjugated with antibody. Thus, homogeneous fractions of fluorescent antibodies with fluorescein:protein (F:P) ratios of 2 to 3.5×10^{-3} have satisfactorily bright specific staining and negligible non-specific staining (*cf* 1).‖ Fractions with higher F:P ratios stain both non-specifically and specifically. These findings have led to the realization that it is not desirable to couple more than a certain chosen number of fluorescent groupings.

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‖ The F:P ratio is a relative measure of the weight of fluorescein and protein in a fluorescent protein conjugate (Table I).