Complexes of Prothrombin with Calcium Ions and Phospholipids*

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SUMMARY

The ability of phospholipids to bind prothrombin in the presence of Ca2+ ions has been studied as a function of lipid composition. Two distinct but complementary methods have been devised to evaluate binding. The first method involves gel filtration on Sephadex G-200 at pH 9.0 to separate the lipid-bound and free prothrombin; the second involves precipitation of the complexes at pH 6.5. Phosphatidylcholine (PC) or a mixture of this lipid with phosphatidylethanolamine (PE) bound very little prothrombin, whereas mixtures of phosphatidylserine (PS) with PC were very effective in binding and the complexes that formed were highly active in the one-stage prothrombin assay. PS alone, phosphatidic acid (PA) alone, PA-PS mixtures, and PA-PC mixtures containing high proportions of PA, bound prothrombin very effectively, but the complexes formed were much less active than when PS-PC was used. Bound protein could be much more readily removed from PS-PC than from PS-PA by treatment of the complexes with EDTA. It was suggested that an optimal surface condition is required to bind reversibly prothrombin prior to conversion to thrombin. Anticoagulant effects ascribed to acidic phospholipids may be due to irreversible binding of the clotting factors to lipid surfaces.

EXPERIMENTAL PROCEDURE

Materials

(NH4)2SO4 (special enzyme grade, Lot V 4035) was obtained from Mann Research Laboratories and BaSO4 from Merck. EDTA (Lot 761551) and sodium dodecyl sulfate (Lot 790430) were obtained from Fisher Scientific; 2-mercaptoethanol was obtained from Eastman. Diisopropyl fluorophosphate was obtained from Aldrich Chemical Co. and was prepared as a 1 M solution in isopropanol. Sephadex G-150 (Lot 860), Sephadex G-200 (Lot 8706), and Sepharose 4B (Lot 8762) were obtained from Pharmacia. DEAE-cellulose (Whatman DE-32) was obtained from Whatman Research Laboratories, Ltd. Cetyltrimethylammonium bromide was obtained from Sigma Chemical Co. Prothrombin-deficient substrate plasma was prepared according to Koller et al. (6) or was obtained as Factor II- and VII-deficient plasma from Sigma Chemical Co. Standard normal plasma was obtained from ADME (Miami, Fla.). Russell Viper Venom (Burrough's Wellcome Stypven) was obtained from Warner-Chilcott (Scarborough, Ont.). Rabbit brain acetone powder for thromboplastin (type 2, fine ground, without sodium citrate, desiccator dried) was obtained from Pel-Freez Biologicals, Inc. Normal plasma was obtained from DADE (Miami, Fla.). Rabbit brain acetone powder for thromboplastin (type 2, fine ground, without sodium citrate, desiccator dried) was obtained from Pel-Freez Biologicals, Inc. and was suspended in 0.14 M NaCl at a concentration of 3 g per 100 ml and stored in portions at -20°C. Brain lipid extract (cephalin) was prepared according to Bell and Alton (7) as a stock solution containing 120 μg of phosphorus per ml and was made to a final concentration of 2 μg of phosphorus per ml in Michaelis buffer, pH 7.35.

Phosphatidylcholine and phosphatidylethanolamine were prepared from hen eggs by published methods (8, 9). Phosphatidylserine was prepared from beef brains by modification (10) of the method of Kowser et al. (11). Phosphatidic acid was prepared by the method of Kowser (12) and converted to the sodium form according to Ansell and Hawthorne (13). On standing in chloroform, however, it reverted to the acid form as judged by thin layer chromatography on Silica Gel G or H.
liters with water. Scintillation fluid was prepared according to Bray (14).

Buffers

Veronal-acetate buffer (pH 6.3 to 9.0) was prepared by dissolving sodium acetate trihydrate (19.528 g), sodium diethylbarbiturate (29.528 g), and 34 g of sodium chloride in 4 liters of water. The pH of the solution was adjusted as required with either 1 N HCl or 1 N NaOH and the volume made up to 5-4 liters with water.

Phosphate buffers were prepared from Na₂HPO₄ and Na₂H₂PO₄, at concentrations ranging from 0.05 M to 0.175 M. The pH of the buffers ranged from 7.2 to 7.4, and adjustments were made when necessary with 1 N HCl or 1 N NaOH. Tris-HCl buffer, pH 7.35, was prepared as a 0.145 M solution of Tris (Sigma) in water adjusted to pH 7.35 with 0.145 M HCl.

Phosphorus Determinations

Phospholipid phosphorus was determined according to the method of King (15). Total phospholipid was determined using a conversion factor of 25 for PS, PC, phosphatidylethanolamine, and PE, and 21 for PA.

Protein Determinations

Protein concentrations were determined either by absorbance of solutions at 280 nm or by the method of Lowry et al. (16).

Coagulation Assays

The column fractions were adjusted to pH 7.35 and 0.025 M CaCl₂ and were then assayed for prothrombin by one-stage assays. Either the one-stage tissue thromboplastin assay of Koller et al. (9) was used in conjunction with the prothrombin-deficient substrate plasma (6) or the one-stage RVV-cephalin assay according to Hjort et al. (17) was used in conjunction with Factor II- and VII-deficient plasma (Sigma). This latter system was also used for the assay of the coagulant activity of the different phospholipid mixtures, the latter being substituted for cephalin (7). Ten-fold dilution of a standard normal plasma (DADE) was used as a source of prothrombin in the latter system. The assays were calibrated by measuring the clotting times obtained with various dilutions of the standard plasma and plotting these against units of activity on log-log scales.

Prothrombin

Prothrombin was prepared by a modification of the method of Cox and Hanahan (18) in which the citrate elution from DEAE-cellulose was replaced by phosphate elution according to Seegers and Landaburu (19). In the gel filtration step, Veronal-acetate buffer, pH 6.2, containing 10⁻⁴ M disopropyl fluorophosphate and 10⁻⁴ M EDTA was used for elution. Both of these chromatographic procedures were carried out at 4°C. The product, specific activity 1 to 2 units per mg, appeared as a single homogeneous species when examined over a concentration range from 1 to 15 mg per ml by sedimentation velocity in the ultracentrifuge, with s₂₀,w = 4.98. The molecular weight of the prothrombin obtained was measured using both conventional (low speed) and meniscus depletion methods (20). Results from the high speed equilibrium runs indicated Mw values of 75,500 ± 1,000 for sample concentrations of 0.5 to 1.5 mg per ml in 0.15 M KCl, 0.001 M Tris, pH 7.4. Low speed equilibrium data using the same solvent system showed a range of Mw's varying with concentration across the cell. Low concentration regions of the cell showed Mw's from 55,000 to 65,000, whereas high concentration extremes indicated Mw's of 70,000 to 85,000. Intermediate concentration Mw's were fairly constant. Extrapolation of this flat region of the curve to zero concentration gave a Mw of 67,500 ± 1,500. For all calculations ß was taken as 0.70 (18, 21). A single band was observed with this preparation on disc electrophoresis at pH 8.6 (22). After treatment with 1% 2-mercaptoethanol and 2%, sodium dodecyl sulfate, the protein was subjected to electrophoresis on 5% acrylamide gel in 0.1 M phosphate buffer, pH 7.2, with 0.1% sodium dodecyl sulfate (23). The molecular weight of the resulting single component was estimated to be 84,000 ± 3,000 using aldolase, bovine serum albumin, cytochrome c, chymotrypsinogen, and ovalbumin as standards (24).

The amino acid composition determined from 24-hour, 48-hour, and 72-hour hydrolysates was very similar to that reported by Cox and Hanahan (18). Factor X was not detectable by the standard procedures of Baehmann et al. (25). No fibrinogen clotting activity was observed in 18 hours when 14 units of prothrombin were incubated with 0.2 ml of fibrinogen (2 mg per ml) at pH 7.35 and 37°C.

Preparation of Lipid Dispersions for Gel Filtration and Precipitation Experiments

All phospholipids used in gel filtration and precipitation experiments were prepared at concentrations of 5 mg per ml in distilled, deionized water. pH adjustments were made with microliter amounts of 1 N KOH or 1 N HCl. Uniformly labeled [¹⁴C]PC was added to each sample for use as a tracer to measure phospholipid concentration. The final concentration of added [¹⁴C]PC was never greater than 0.5 μg per ml. Final specific activities were of the order of 30 cpm per μg of phospholipid.

Solvent was removed from measured volumes of stock phospholipid solutions in either chloroform or diethyl ether by streaming nitrogen over the samples. Further drying was carried out by placing samples in a vacuum oven at room temperature overnight. The phospholipids were then dispersed in water by hand shaking. After adjusting the pH, the dispersions were sonicated using a Raytheon DF 101 10 kHz sonic oscillator at maximum power setting. Total sonication time was from 15 to 45 min as required for samples to become transparent. The phospholipids were then dispersed in water by hand shaking. After adjusting the pH, the dispersions were sonicated using a Raytheon DF 101 10 kHz sonic oscillator at maximum power setting. Total sonication time was from 15 to 45 min as required for samples to become transparent. Sonication was interrupted every 5 to 10 min to check and adjust pH. A circulating coolant kept sample temperatures below 10°C during sonication.

Preparation of Lipid Dispersions for Microelectrophoresis and Clotting Assays

For electrophoretic measurements, dried lipid films were dispersed at a concentration of 227 μg per ml in 3.8 ml of 0.145 M NaCl by vigorous manual agitation. The dispersions were al-
lowed to equilibrate overnight at room temperature and then 0.2 ml of 0.145 M Tris-HCl buffer, pH 7.35, was added.

Dispensers for clotting assays were prepared in a similar manner but at a concentration of 45.4 μg per ml in Michaelis buffer, pH 7.35.

**Microelectrophoresis**

Measurements of electrophoretic mobility were made as described in a previous publication (26).

**Evaluation of Lipid-Protein Interactions**

**Method 1. Analytical Gel Filtration on Sephadex G-200 at pH 9.0**—Gel filtration experiments were carried out using Pharmacia K 3/30 columns (0.9-cm diameter x 30-cm height) packed with Sephadex G-200. Samples were eluted at 4° with Veronal-acetate buffer, pH 9.0, containing CaCl₂. CaCl₂ concentration was 0.025 M for PC and PS-PC mixtures and 0.010 M for the remaining phospholipid mixtures. Elution volume was monitored by weighing eluate fractions. Fraction size ranged from 0.4 to 0.5 ml. Flow rates were 1.5 to 2.2 ml per hour.

When PC or PS-PC mixtures were to be used, the column samples were prepared by mixing in order 0.15 ml of phospholipid suspension (5 mg per ml), 0.15 ml of prothrombin solution (3.8 to 7.3 A₂₈₀ units per ml), and 0.15 ml of 0.075 M CaCl₂. When other phospholipids were used, the column samples were prepared by mixing in order 0.11 ml of phospholipid suspension (5 mg per ml), 0.11 ml of prothrombin solutions (7.3 A₂₈₀ units per ml), and 0.22 ml of 0.020 M CaCl₂. The pH of each component solution had been adjusted to 9.0 prior to mixing. CaCl₂ solutions were added slowly from a 500-μl syringe with constant swirling. Samples were incubated at 4° for 20 min before applying to the column. Applied sample size was 0.40 ml.

Fractions were analyzed directly for prothrombin activity, ¹⁴C radioactivity, and, in some cases, protein (16). Usually, a separate column was run with larger fractions (1 to 1.5 ml) to facilitate protein determinations. No attempt was made to disrupt any complexes formed before carrying out the assays.

Counting procedure involved counting 0.10-ml samples of each fraction in 10 ml of Bray's solution for 4 min with a Nuclear-Chicago MK 1 scintillation counter. Counts were converted to total phospholipid using calculated specific activities. Recovery of ¹⁴C ranged from 70 to 90%. Quenching and self-absorption effects were ignored.

**Method 2. Analysis of Precipitates Formed at pH 6.5**—Solutions were prepared by mixing, in order, 0.20 ml each of sonicated phospholipid, pH 6.5 (5 mg per ml), prothrombin solution, pH 6.5 (7.3 A₂₈₀ units per ml), and 0.030 M CaCl₂, in Veronal-acetate buffer, pH 6.5. The CaCl₂ solution was added carefully through a syringe with constant mixing. Control solutions were prepared in the same manner except that the prothrombin solution was replaced by an equivalent volume of Veronal-acetate buffer, pH 6.5.

Samples were allowed to remain at 4° for approximately 24 hours and were then centrifuged at full speed in a clinical centrifuge (International model CL). The supernatants were removed and the precipitates washed twice with 0.2 ml of 0.010 M CaCl₂-Veronal-acetate buffer, pH 6.5. Precipitates were then suspended in 1.0 or 2.0 ml (depending on the amount of precipitate) of 0.010 M CaCl₂ in Veronal-acetate buffer, pH 9.0. Supernatants and precipitate suspensions were then checked for protein (16), prothrombin activity, and ¹⁴C radioactivity. Scintillation counting procedure involved putting 0.10-ml samples in 10 ml of Bray's solution and counting for 20 min.

**RESULTS**

**Method 1. Gel Filtration on Sephadex G-200 at pH 9.0**

**Gel Filtration of Prothrombin with PC, PS, and PS**—When sonicated dispersions of phospholipids containing [¹⁴C]lecithin were filtered through standardized columns of Sephadex G-200 at pH 9.0 in the presence of 0.025 M CaCl₂, the radioactivity was recovered quantitatively as a single peak in the void volume (Kᵥ = 0). When prothrombin samples were subjected to gel filtration under those conditions, both the clotting activity and the protein material were partially retained in the gel (Kᵥ = 0.42). Previously incubated mixtures of prothrombin, 0.025 M CaCl₂ and phospholipids were then examined likewise. In every case the radioactivity was found in the void volume component whereas the prothrombin activity was distributed between this component and the component having Kᵥ = 0.42. In the experiments illustrated in Fig. 1, the phospholipid composition was varied systematically from 100% PC to 100% PS to determine the influence of this parameter on the distribution of protein and on the distribution of prothrombin activity. It can be seen (Fig. 1A) that when the lipid was entirely PC the amounts of protein and of prothrombin activity found in the void volume were negligible. The elution profiles were essentially identical with those of prothrombin chromatographed in the absence of lipid.

It was found that increasing the proportion of PS from 0 to 30% of the total phospholipid progressively increased the amount of protein and prothrombin activity eluted in the void volume fractions and concurrently decreased the relative proportion in the retained fractions (Fig. 1, A to D). Above 30% PS the amount of activity recovered in the void volume decreased again without reappearing in the retained fractions (Fig. 1, G and F). Most of the protein material was present in the void volume under these conditions. The use of 0.010 M CaCl₂ instead of 0.025 M CaCl₂ did not significantly affect the extent of binding of prothrombin to PS-PC (50:50). When previously incubated samples of prothrombin, PS-PC (30:70), and Ca²⁺ were treated with 0.030 M EDTA, most of the protein appeared in the retained fractions (Fig. 2A).

**Gel Filtration of Prothrombin with PA-PC**—In a similar manner gel filtration of a previously incubated mixture containing prothrombin, 0.010 M CaCl₂ and equal weights of PA and PC was carried out. The results are presented in Fig. 3A. The amount of protein transferred to the void volume was comparable with the corresponding PS-PC mixture but, in contrast, PA-PC was quite ineffective in transferring prothrombin activity to the void volume fractions.

**Gel Filtration of Prothrombin with PE-PC**—One lipid mixture, containing equal weights of PE and PC, was tested (Fig. 3B). A rather small proportion of the total protein and of the total prothrombin activity was recovered in the void volume.

**Gel Filtration of Prothrombin with PA, PS-PA, and PS**—All mixtures of PA and PS, as well as the single lipids (Fig. 4), gave comparable results. In every case, most of the protein and lipid appeared in the void volume fractions but the activity of these fractions was always quite low in comparison with PS-PC mixtures. When previously incubated samples...
Gel Filtration on Sepharose 4B—It was expected that the retention characteristics of this gel might provide a better resolution of the two components separated on Sephadex G-200. However, in the presence of 0.01 M CaCl₂ it was found that mixtures containing acidic phospholipids were not eluted from the columns. Subsequently, the gels were carefully extruded, separated into layers, and the radioactivity was measured in each layer. Most of the ¹⁴C label was found to have accumulated at the top of the column where the sample first contacted the gel. This effect was not found when PC alone was used, when PS-PC-prothrombin mixtures were applied or when CaCl₂ was completely omitted. Microelectrophoretic observation of the Sepharose particles in the buffer used for chromatography revealed movement of the particles towards the anode.

Method 2. Analysis of Precipitates Formed at pH 6.5

It was found that mixing acidic phospholipids with 0.01 M or 0.025 M CaCl₂ at pH 6.5 resulted in the formation of precipitates which could be readily separated by centrifugation. Under specified conditions, some prothrombin could be recovered along with ¹⁴C radioactivity in such precipitates. These observations formed a basis for the analysis of insoluble complexes of prothrombin with different phospholipid mixtures.

Precipitation of Prothrombin and PS-PC—Fig. 5 shows the distribution of ¹⁴C radioactivity, protein material, and prothrombin activity between the supernatant and washed precipitates when PC, mixtures of PS with PC, and PS alone were used. It can be seen (Fig. 5A) that negligible amounts of lipid precipitated when the content of PS in the mixtures was equal to or less than 20% of the total phospholipid. At 40% PS, significant precipitation was observed in the absence of added prothrombin, but when the protein was present this seemed to afford some protection against precipitation. At 60% PS or higher, most of the lipid was precipitated under these conditions in the presence or absence of prothrombin. Fig. 5B shows that about 60% of the total protein present was also precipitated along with the lipids. Fig. 5C shows that the total coagulant activity measurable in the supernatant plus precipitate increased with increasing percentage of PS up to 60% then decreased again. This was also true of activity present in the precipitate alone. Therefore, comparison of Fig. 5, B and C, shows that protein bound with phospholipid in the precipitate is unable to manifest optimal activity when the percentage of PS exceeds 60% of the total phospholipid present.
FIG. 2. Chromatography of EDTA-treated mixtures of prothrombin, CaCl₂, and sonicated phospholipid dispersions on Sephadex G-200 columns. Mixtures of 0.73 A₂₈₀ unit of prothrombin (0.1 ml) CaCl₂ (0.1 ml, 0.075 M), and 0.5 mg of total phospholipid (0.1 ml) were incubated at 4° for 20 min followed by addition of 0.1 ml of 0.120 M EDTA and further incubation for 20 min. The mixtures were then applied to the columns. Phospholipid ratios are expressed as w/w. Columns were equilibrated and samples were eluted with Veronal-acetate buffer, pH 9.0. Both phospholipid dispersions contained [³⁵]PC (~0.05 μg per ml) as a radioactive tracer. Phospholipid concentrations were calculated from specific activities 0—0, prothrombin activity; 0—0, phospholipid concentration; 0—0, protein level expressed as absorbance at 750 nm (according to Reference 16).

FIG. 3. Chromatography of previously incubated mixtures (total volume 0.4 ml) of prothrombin (0.73 A₂₈₀ unit), CaCl₂ (0.010 M), and sonicated phospholipid dispersions (0.5 ml of total phospholipid) on Sephadex G-200 columns. Phospholipid ratios are expressed as w/w. The mixtures were incubated at 4° for 20 min prior to column application. Columns were equilibrated, and samples were eluted with Veronal-acetate buffer, pH 9.0, containing 0.010 M CaCl₂. Both phospholipid dispersions contained [³⁵]PC (~0.05 μg per ml) as a radioactive tracer. Phospholipid concentrations shown were calculated from specific activities. 0—0, prothrombin activity; 0—0, phospholipid concentration; 0—0, protein level expressed as absorbance at 750 nm (according to Reference 16).

Precipitation of Prothrombin with PA-PC—The results are shown in Fig. 6. Again there was negligible phospholipid precipitation when the acidic species constituted less than 20% of the total lipid (Fig. 6A). At levels of 40% PA and 60% PA, the presence of prothrombin appeared to enhance phospholipid precipitation. The protein material itself was found largely in the precipitates at 60% PA and higher and appeared to bind slightly more effectively than to the equivalent PS-PC mixtures. In spite of this, the coagulant activity localized in these precipitates was very low. When PA alone was used no activity was found either in the precipitate or in the supernatants.

Electrophoretic Mobility and One-stage Prothrombin Activity—From the foregoing it was evident that PA mixed with PC could not effectively substitute for PS mixed with PC in manifesting the coagulant activity of prothrombin. Since PA and PS are both acidic phosphatides, this result appeared to contravene the suggestion that phospholipids merely provide an appropriate negative surface charge for coagulant protein interactions (see "Discussion"). Consequently, we examined the relationship between electrophoretic mobility and one-stage prothrombin
Fig. 5. Distribution of 14C radioactivity (A), protein (B) (determined according to Reference 16), and prothrombin activity (C) in the precipitates and supernatants from mixtures of sonicated PS-PC dispersions (1.0 mg of total phospholipid in each mixture) with prothrombin (1.46 $A_{280}$ units) and CaCl$_2$ (0.010 M) at pH 6.5. Compositions of the phospholipid dispersions are expressed as w/w, and all mixtures contained $[^{14}C]$PC (~0.05 pg) as a radioactive tracer. The preparation of these mixtures is described in the text. Closed portions of the histograms represent precipitates, and open sections denote supernatants. Mixtures prepared in the presence of prothrombin are indicated by +, while those prepared in its absence are designated -. F. II, Factor II, prothrombin.

activity when a series of PS-PA mixtures was used as lipid substitute for cephalin in the one-stage assay with Russell Viper Venom (17). The results are shown in Fig. 7A. It can be seen that the electrophoretic mobility of the phospholipids was essentially invariant as the percentage of PS was progressively increased (Fig. 7A). At the same time, the one-stage assay showed a manifestation of clotting activity which was directly related to the content of PS in the mixtures (Fig. 7B). For comparative purposes the one-stage activities of equivalent amounts of PS alone and PS mixed with PC are shown in Fig. 7. It is evident that PA, when mixed with PS, exerted a slight inhibitory effect on clotting activity, whereas PC exerted a striking promotional effect.

DISCUSSION

Gel filtration at pH 9.0 followed by analysis of one-stage prothrombin activity, protein, and $^{14}$C radioactivity gave results which reflected both the extent of binding of prothrombin to phospholipids as well as the level of activity in the lipid-protein complexes formed. The desired information could be extracted by comparing the amounts of prothrombin activity in the void volume and retained components with the amounts of protein recovered from the same regions of the chromatogram. Precipitation at pH 6.5 was used for making additional comparisons of protein and one-stage prothrombin activity in the
complexes. The disadvantage suffered in the latter technique was that the amounts of protein precipitated were dependent on the extent to which the phospholipids themselves were precipitated. When the data obtained from both methods were compared, however, valid conclusions could be drawn with regard to the extent of binding and the level of the activity of each of the complexes.

It has been known for some years that certain combinations of phospholipids can act as substitutes for platelets during blood coagulation, whereas other combinations are ineffective (7, 27). It seems probable that the acceleratory effect of phospholipids on blood coagulation is dependent on their ability to provide a catalytic surface in the aqueous medium for protein-protein interactions. Conflicting data regarding the requirement for a specific phosphatide species or specific combinations of phosphatides appeared to be uniquely resolved by the correlation of procoagulant activity with the sign and magnitude of the surface charge on the lipid particles (28-30).

This hypothesis was based mainly on the demonstration of a relationship between the activity of various phospholipides in clotting tests and their negative electrophoretic mobility. Some deviations from this generalization were subsequently reported, however (31, 32). In particular, PA previously mixed with PC was quite ineffective compared with PS-PC at any given electrophoretic mobility. Furthermore, several authors have reported that phosphatidylserines exhibit procoagulant activities at low concentrations but anticoagulant activities at higher concentrations, especially when solubilized with sodium deoxycholate or serum albumin (33-37). No satisfactory explanation for this finding has been given hitherto.

Pure PC is essentially inert in clotting systems (Reference 38 and Fig. 7B) and failed to bind prothrombin in the presence of 0.025 M Ca2+ ions (Fig. 1A). A mixture containing equal amounts of PE and PC showed only a marginal increase in the extent of binding of prothrombin (Fig. 2B). Progressively increasing the percentage of PS mixed with PC up to 30% PS increased both the coagulant activity of the lipid (References 29 to 31 and Fig. 7) and the amount of bound prothrombin (Fig. 1, B to E). When PS-PC mixtures containing more than 50% PS were used or when PS alone was used, approximately the same amount of protein was bound to the lipid as before but the complexes showed decreased activity (Figs. 1, F and G, 5B, and 6B). To account for these results we suggest that a tight binding of prothrombin to lipids is necessary for conversion of prothrombin to thrombin, but that when the protein is more firmly attached either this conversion cannot take place or the thrombin formed cannot be released from the lipid surface and thus remains inactive.

Since PA cannot substitute as a procoagulant for PS in mixtures with PC, we next studied binding of prothrombin to PA and to PA-PC mixtures. It was found that these lipids also bound protein quite effectively (Figs. 3A and 6B), but the complexes were even less active (Figs. 3A, 4A, and 6C) than when PS alone was used. Thus, PA may have even more affinity for prothrombin than has PS at a given charge density. Several explanations may be offered as to why this should be so. (a) The extent of binding may be determined by the presence of specific chemical groups such as phosphate, carboxyl, or amino groups; (b) the size and shape of the lipid aggregates or their molecular architecture may be affected by the phospholipid composition; (c) the approach of a protein macromolecule to the lipid surface may be restricted by the bulkier phosphoserine headgroups of PS in the interface as compared with the more compact phosphate groups of PA.

Since PA and PS both carry one to two net negative charges per molecule at pH 7.35, it was not expected that varying the proportions of these two species in binary mixtures would greatly affect their electrophoretic mobility. This was indeed found to be the case (Fig. 7A). The ability of such mixtures to substitute for Cephalin in the one-stage test (17) was found to be independent of the electrophoretic mobility and was related only to the amount of PS present in each mixture (Fig. 7B). In contrast with PC which potentiated the coagulant activity of PS, PA exerted a small but definite inhibitory effect on PS. This again could be related to the greater ability of PA to bind free prothrombin in the form of an inactive complex. Careful inspection of Fig. 4, A to E, shows that increasing the percentage of PA in PA-PS mixtures removes progressively more of the free prothrombin component into a low activity complex.

The tighter binding of prothrombin to PS-PA as compared with PS-PC was confirmed by treating the complexes with EDTA. Although in both cases this treatment caused an apparent loss of activity of prothrombin, probably due to chelation of Ca2+ in the subsequent clotting assay, a significant difference in the amounts of protein transferred back to the free form was evident. Whereas the complex containing PS-PC (30:70) retained only about 20% of the total protein present (Fig. 2A), the complex containing PS-PA (25:75) retained over 50% after EDTA treatment (Fig. 2B). This may indicate that, in complexes involving PA, either Ca2+ incorporated into the lipid-protein complex is more firmly bound or bonds not involving Ca2+ are formed.

The experiment with Sepharose 4B also shed some light on the type of binding involved in formation of these complexes. The gel is prepared from agar and may contain residual agarpectin with carboxyl and sulfate groups (39-41). This suggestion is supported by the negative electrophoretic mobility of the beads. Presumably, acidic phospholipids can then cross-link to the gel via Ca2+ bridges. The failure of the gel to bind the lipid-protein complexes suggests that a similar type of mixed chelate may be involved in stabilizing these complexes. PC itself binds Ca2+ only weakly (49) and also did not attach to Sepharose 4B or bind prothrombin.

Finally, the demonstration of a tighter binding of prothrombin to PS alone to form a low activity complex may help to explain the apparent anticoagulant activity of this lipid when used at high concentrations (33-37). Presumably, adsorption of most of the prothrombin present in plasma to PS would render the protein unavailable for activation to thrombin. On this basis the data obtained with PA alone predict that a similar or more powerful anticoagulant activity might be elicited by high concentrations of this phospholipid.

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