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Binding of the Protein Component of Tissue Factor to Phospholipids*

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ABSTRACT: The coagulant activity of tissue factor, a particle-bound lipoprotien, is lipid dependent. Phosphatidylethanolamine and phosphatidylcholine restore activity to delipidated tissue factor protein if lipid and protein are combined in the presence of deoxycholate, then dialyzed. After this recombination procedure, tissue factor activity, protein and phospholipid band isopynically as a complex in a 5–30% sucrose gradient. If deoxycholate is omitted during the recombination, lipid does not bind to tissue factor nor is activity restored. The complex incorporates increasing amounts of phosphatidylethanolamine from 2.5 to at least 7.4 mg of lipid per mg of protein, although the optimal ratio for restoration of activity for these preparations is 5.0 mg of lipid per mg of protein. Phosphatidylcholine, which does not restore activity as effectively as phosphatidylethanolamine, nevertheless, is bound as well. If reduced phosphatidylcholine or lysophosphatidylcholine is substituted for phosphatidylethanolamine, activity is not restored. These phospholipids do interact with the protein, however, since the sedimentation characteristics of each are altered after the recombination procedure. Reduced phosphatidylcholine complexes with apoprotein only at very high lipid to protein ratios. Since a lipoprotein complex containing lysophosphatidylcholine cannot be isolated, it is concluded that these complexes are unstable to centrifugation in a sucrose gradient. Thus, restoration of tissue factor activity requires a specific interaction of lipid and protein which results in a stable lipoprotein complex. Since tissue factor also binds ineffective phospholipids, restoration of activity is considered in terms of phospholipid structure. Although binding to phospholipid is required for restoration of coagulant activity to tissue factor, binding alone does not restore activity.

The role of tissue factor in the initiation of blood coagulation has been studied in detail by Nemerson (1966) and in Williams' laboratory (Williams, 1966; Williams and Norris, 1966). Experiments with purified bovine lung microsomes and crude bovine brain particles have established that tissue factor, in the presence of calcium, forms a complex with factor VII; this complex then enzymatically converts factor X into its activated, enzymatic form.

Tissue factor is a lipoprotein that requires the presence of its lipid component for coagulant activity. The specificity of the lipid requirement has been reported previously from this laboratory (Nemerson, 1968, 1969). Crude tissue factor preparations are 38–45% phospholipid by weight (Nemerson, 1968). The solubilization and partial purification of the protein moiety of tissue factor has been reported earlier (Nemerson, 1969). An accompanying article (Nemerson and Pitlick, 1970), describes in detail the further purification of the apoprotein. Although the apoprotein possesses residual tissue factor activity, recombination of tissue factor with certain phospholipids enhances this activity 500- to 1000-fold.

Crude delipidated brain particles have been demonstrated to bind phospholipid; however, PS[1], which did not restore biological activity, was bound as effectively as PE and PC

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[1] Abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidyl-L-serine; lysoPC, lysophosphatidylcholine; reduced PC, reduced phosphatidylcholine.

which did (Nemerson, 1968). The use of particles, which presumably contained many proteins, led to ambiguous results, as it was unclear to which proteins the lipids bound. The availability of purified soluble apoprotein, however, has now enabled us to explore the role of phospholipid in the restoration of activity. In this study, the binding of purified phospholipids to tissue factor apoprotein has been characterized by determining the behavior of relipidated preparations of the apoprotein during sucrose density gradient ultracentrifugation. The data presented here clearly demonstrate that tissue factor binds not only phospholipids which restore activity, but those which are ineffective as well. Therefore, binding alone is not sufficient for restoration of activity. However, the phospholipids which fail to restore activity are bound very poorly. Complexes formed with lysoPC break up in the centrifugal field, suggesting that these complexes are different from those which contain active lipids.

Materials

Purified bovine PE, bovine PC, egg lysoPC, and reduced PC were obtained from Supelco, Bellefonte, Pa. These lipids were determined to be about 98% pure (Nemerson, 1968).

Mixed brain phospholipids were prepared as previously described (Nemerson, 1968, Method 1).

DEAE-Sephadex, Sepharose 4B and 6B were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

Bovine serum albumin, crystallized and lyophilized, was a product of Sigma Chemical Company, St. Louis, Mo. Sodium deoxycholate was purchased from Mann Research Laboratories, New York, N. Y.

Other reagents were obtained from the usual sources of supply.

Methods

Preparation of the Apoprotein. The apoprotein used in the majority of the experiments reported here was prepared as described in the accompanying paper through the ammonium sulfate fractionation (Nemerson and Pitlick, 1970). The protein was then chromatographed on DEAE-Sephadex and Sepharose 4B. The material in the first peak of the Sepharose 4B Column was pooled and concentrated with an Amicon ultrafilter (XM-100) to a protein concentration of 1 mg/ml. The experiments with PC and PE in the absence of deoxycholate utilized the more refined preparation discussed in the accompanying paper. The material from the second Sepharose 6B column was concentrated to 0.95 mg/ml.

Phospholipid Micellization. Organic solvent was removed from the desired amount of phospholipid with a stream of prepurified nitrogen. The pellet was then suspended in 2–3 ml of 0.25% deaerated deoxycholate and homogenized in a glass homogenizer with a Teflon pestle. The solution was transferred to a 10-ml beaker in a bath of melting ice and sonicated for 5 min in a nitrogen atmosphere with a Blackstone sonicator. The preparations were centrifuged for 30 min at 105,000*g*. The pellet was discarded and the phospholipid concentration of the supernatants, which was used for these experiments, was determined from the phosphorus content of an aliquot digested with perchloric acid according to the method of Chen *et al.* (1956). Reduced PC was sonicated for 30 min and used without centrifugation, since previous experience showed that most of the reduced PC pelleted under the usual conditions.

Relipidation of the Apoprotein. Aliquots (0.2–1.0 ml) of apoprotein were relipidated by mixing them with the appropriate lipids dissolved in deoxycholate according to the method previously described (Nemerson, 1969). The relipidated samples were dialyzed in 500–2000 ml of 0.05 M imidazole·HCl–0.375 M NaCl, pH 7.2 at 4° for 18–24 hr. The deoxycholate (93%) is removed by this dialysis (Nemerson, 1969).

Preparations of the Sucrose Gradient. The sucrose solutions were made with solid sucrose and concentrated buffer solutions for a final composition (at 21°) of 5% (w/v) and 30% (w/v) sucrose in 0.05 M imidazole·HCl–0.375 M NaCl–0.001 M EDTA. At 4°, the pH was 7.2. Linear gradients (4.6 ml) were formed at ambient temperature but with cold solutions and were placed immediately into iced centrifuged buckets. The samples (0.2–0.3 ml) were layered on manually.

Centrifugation Conditions. The gradients were centrifuged for 18–63 hr in a Spinco SW-39 rotor at 1°. Rotor speed was determined from odometer readings over a 3-min period or from the average of the total number of revolutions from the moment of acceleration until deceleration (without braking) was begun.

Collection of Fractions and Analytical Procedures. Gradient fractions (15 drops, $\approx$0.2 ml) were collected from the bottom of the tube using a Büchler piercing unit with light air pressure. The unit was rinsed with distilled water and dried before each tube was pierced. When PE, PC, and reduced PC were centrifuged alone, the lipid was flocculent and stuck to the walls of the centrifuge tube as fractions were collected. Therefore, 0.2-ml aliquots were aspirated from the top of the tube with a 50-μl Eppendorf pipet until the lipids were removed. The tube was then pierced and fractions were collected as usual.

Linearity of each gradient was determined from the refractive index on a temperature compensated hand refractometer (American Optical). Densities of individual fractions were determined from the known refractive indices of the 5 and 30% sucrose solutions and their calculated densities at 1°,[2] the temperature of the rotor during centrifugation.

Protein was determined by method of Lowry *et al.* (1951) using a complete fraction for each determination. Buffered 30% sucrose was used as a blank and bovine serum albumin in water (0.100 mg/ml based on the A_{280}) was used as the standard with a water blank. No correction was made for the effect of sucrose on the color of the reaction. Preliminary experiments with PE showed that the correction of the Lowry color due to PE (based on phosphorus content) was possible (1 mg of PE is equivalent to 0.035 mg of bovine serum albumin) but turbidity prevented such corrections for PC, lysoPC, and reduced PC. Therefore, corrections were not made for any phospholipid, nor could specific activity be determined accurately for the fractions from the gradients.

[2] The weight of water in the buffered sucrose solution was calculated from the known composition at 21°, the temperature at which the solutions were made, and the known densities of sodium chloride (2.165 g/ml), sucrose (1.588 g/ml), and water (0.99802 g/ml) at 21°. The densities of these solutions were then corrected to 1° where the density of water is 0.99993 g/ml. The density of the 5% buffered sucrose solution is 1.0303 g/ml at 1°; the density of the 30% buffered sucrose solution is 1.1230 g/ml under the same conditions.

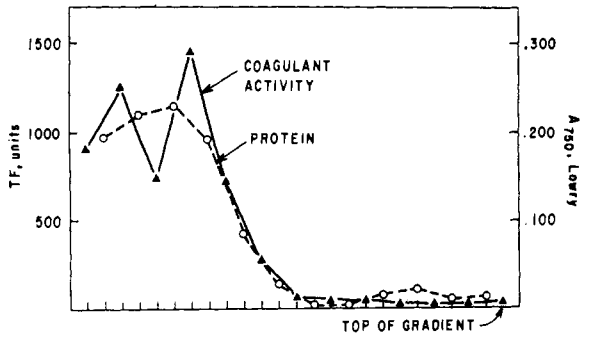

FIGURE 1: Centrifugation of apoprotein (18 hr at 36,400 rpm). A 0.3-ml aliquot (1.0 mg/ml) was layered on the gradient. Activity was located after relipidation with mixed brain lipids at 5 mg per mg of protein.

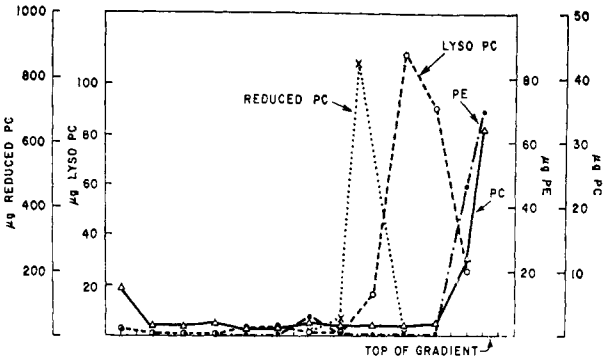

FIGURE 2: Centrifugation (18 hr at 36,500 rpm) of phospholipids after sonication in 0.25% deoxycholate followed by dialysis into buffer (see text). Concentration before dialysis: PE (Lot 43 J), 2.43 mg/ml; PC (Lot 40 D), 1.58 mg/ml; lysoPC (Lot 6003), 1.81 mg/ml; reduced PC (Lot 6016), 3.55 mg/ml. Reduced PC (0.2 ml, sonicated 30 min—see text) layered on gradient with 0.1 ml of buffer; 0.3 mg of other lipids on gradient. PE, PC, and reduced PC were aspirated from the top of the tube in 0.2-ml aliquots before fractions were collected from the bottom.

To locate phospholipid, individual fractions were diluted with 1 ml of H_2O then extracted three times with 3 ml of $CHCl_3$–MeOH (2:1, v/v). The extracts were pooled and evaporated in a hot water bath, then digested with 0.375 ml of 60% perchloric acid. Phosphorus was then determined by the method of Chen *et al.* (1956).

The biological activity of tissue factor was determined in the two stage assay previously described (Nemerson, 1968). Preliminary experiments showed that 10% (w/v) sucrose (the maximum concentration in the assay) had no effect upon the assay. If the material which was applied to the gradient was biologically active (apoprotein relipidated with PE or PC), the fractions were assayed directly. In cases where the starting material was not active (apoprotein or apoprotein relipidated with ineffective lipids), 0.2 ml of mixed brain lipids in 0.25 or 0.5% deoxycholate were added to the individual fractions for a final ratio of 5 mg of lipid per mg of protein based upon protein determinations of the alternate fractions. The samples were dialyzed as above before assay.

Results

Sedimentation of the Apoprotein. After 18-hr centrifugation at 36,000 rpm the bulk of the protein was found in the lower third of the gradient (Figure 1). Tissue factor activity was located by relipidating the gradient fractions with mixed brain lipids in deoxycholate, followed by dialysis. When each fraction was assayed, two peaks of tissue factor activity were found. However, if only alternate fractions were assayed, activity usually appeared as one broad peak.

Centrifugation of Phospholipids. PC, lysoPC, reduced PC, and PE were prepared in deoxycholate, as for relipidation, then dialyzed against buffer and centrifuged individually for 18 hr. While some phospholipid was found deep in the gradient, the major portion remained in the less dense area of the gradient (Figure 2). PC and PE were both located at the top (density of less than 1.0303 g/ml). LysoPC was found at a density of 1.044 g/ml and reduced PC at a density of 1.057 g/ml. Similar results were obtained when samples were prepared in the absence of deoxycholate.

Effect of Deoxycholate on the Centrifugal Behavior of Lipid and Protein. Although tissue factor protein does not require deoxycholate to remain soluble, and apparently binds very little deoxycholate (Nemerson, 1969), it was not known how residual deoxycholate in the protein and lipid preparations would affect their sedimentation characteristics. Therefore, apoprotein or PE was dissolved in 0.25% deoxycholate, the concentration used in the relipidation procedure, and the sedimentation behavior of each was studied.

Apoprotein was centrifuged for 18 hr after it was layered on the gradient. The results were virtually the same as with the apoprotein in buffer. Therefore, deoxycholate was neither binding to the protein in sufficient amount to alter its density, nor was it disaggregating it into smaller units. PE, homogenized and sonicated in deoxycholate, was layered on the gradient and centrifuged for 18 hr. It was found slightly into the gradient at a density of 1.048 g/ml. PE which had been prepared in deoxycholate and dialyzed, banded at the meniscus (Figure 2). Thus, it appears that deoxycholate in high concentration binds to PE, slightly increasing its density, although it still comes to equilibrium far away from the region where apoprotein is found after this centrifugation time. Significantly, PE prepared in the same manner, but dialyzed prior to centrifugation, has the same apparent density as PE which has never been in deoxycholate. Thus, the deoxycholate used to promote the recombination of lipid and protein does not alter the sedimentation characteristics of the apoprotein, nor does it increase the density of PE which was dialyzed before centrifugation.

Centrifugal Behavior of Biologically Active Relipidated Protein. Maximal activity for the protein used in the experiments presented here was obtained when phospholipids (mixed brain lipids or PE) were added in the presence of 0.25% deoxycholate at a ratio of 5 mg of lipid per mg of protein. At this relipidation ratio, however, the protein, if fully bound to lipid, would not enter far enough into the gradient to separate it from the position of free PE. In order to demonstrate clearly that tissue factor activity is bound to phospholipid, a low relipidation ratio (2.5 mg per mg of protein) which partially restores activity was chosen so that the expected complex would be located intermediate to the position of apoprotein and phospholipid. The relipidation ratio was increased to optimal and greater than optimal amounts in order to deter-

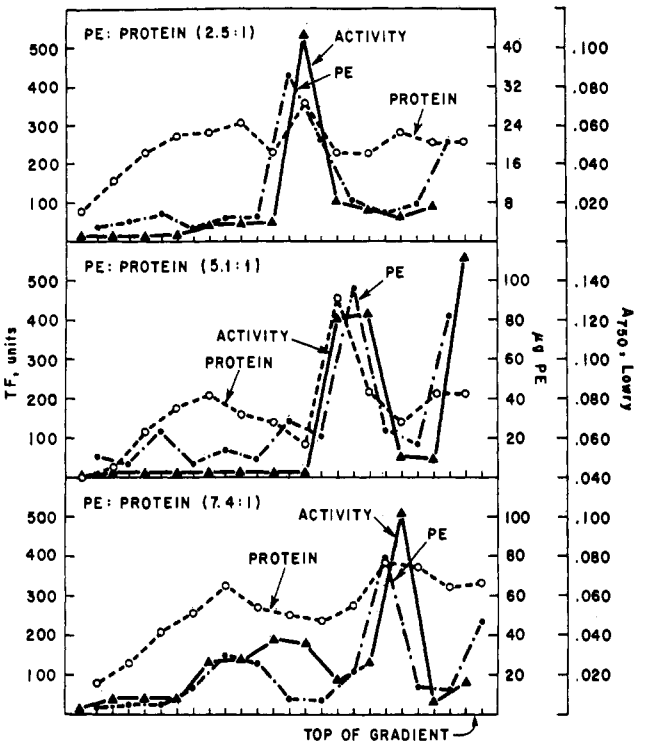

FIGURE 3: Centrifugation (18 hr at 36,700 rpm) of apoprotein relipidated with increasing amounts of PE (Lot 29 J). Lipid to protein ratio, final protein concentration: 2.5 mg of PE/mg, 0.25 mg of protein/ml; 5.1 mg of PE/mg, 0.316 mg of protein/ml; 7.4 mg of PE/mg, 0.238 mg of protein/ml. Each (0.3 ml) was layered on the gradient. Activity was located by assaying fractions directly.

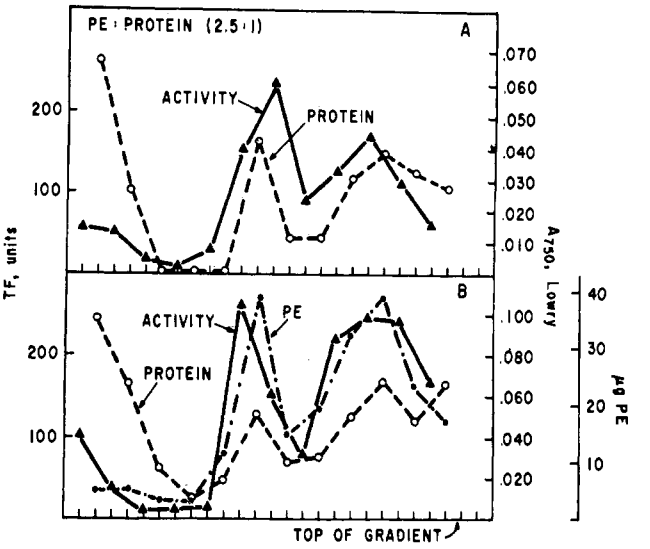

FIGURE 4: Centrifugation (63 hr at 36,400 rpm) of apoprotein relipidated with PE (Lot 29 J). 2.5 mg of PE/mg of protein; final protein concentration, 0.526 mg/ml. (A) 0.3 ml incorporated into the 5% sucrose solution; (B) 0.3 ml layered on gradient. Activity was located by assaying fractions directly.

mine whether a finite lipid to protein ratio is established in the most active complex or whether the fully activated complex incorporates excess lipid.

Apoprotein was relipidated with PE at 2.5, 5.1, and 7.4 mg of PE per mg of protein, layered on the gradient and centrifuged for 18 hr. After centrifugation, bands were visible in the tube at 1.5, 1.2, and 0.7 cm below the meniscus for the respective ratios. The results of chemical analyses and assays for coagulant activity of the fractions collected from this gradient are presented in Figure 3. In contrast to the sedimentation pattern of the apoprotein, protein of the relipidated preparation was found in the center of the gradient. As the lipid to protein ratio was increased, the protein shifted to the top of the gradient. The bulk of the phospholipid moved from the meniscus into a denser portion of the gradient and is coincident with one of the protein peaks. Thus the relipidation procedure created a complex of lipid and protein with a density intermediate to the two components. Activity was determined directly on the fractions and was found to be coincident with the protein and phospholipid peak. The density of the complex formed with 7.4 mg of PE per mg of protein was less than that formed with 5.0 mg of lipid (although the latter was optimal for coagulant activity). Thus the complex incorporates more lipid than is required for complete restoration of biological activity.

The isopycnic point for apoprotein relipidated with PE was determined in two ways. On several occasions, a preparation which had been relipidated with 2.5 mg of PE per mg of protein was centrifuged for several days to determine that the complex had reached equilibrium. As an alternate verification of equilibrium, the sample was distributed throughout the gradient by incorporating it into the 5% sucrose solution as the gradient was poured. Figure 4A compares incorporation of the sample with layering (Figure 4B) after both were centrifuged for 63 hr. Both tubes had visible bands at 2.0 cm below the meniscus. In both instances, protein was found up into the gradient and at the top as well. Phospholipid, measured on the layered sample, was found down in the gradient, coincident with the protein and activity peaks. Activity in the incorporated sample was found at densities of 1.072 g/ml and 1.048 g/ml; the activity peaks of the layered sample were at 1.077 g/ml and 1.043 g/ml. The similarity of the patterns of these two samples demonstrates that equilibrium has been reached; the location of the activity peak at 1.069 g/ml in Figure 3 suggests that 18 hr is sufficient time for the sample to reach its isopycnic point.

The occurrence of two activity peaks as seen in the equilibrium experiment has been noted on several occasions. While a sharp peak with the bulk of the activity is found in the density region of 1.070 g/ml at the relipidation ratio of 2.5 mg of PE per mg of protein, lipid-bound activity has also been recovered either just under the meniscus or between the meniscus and this peak. Since the variable occurrence of a second activity peak has been observed with the same protein preparation and same lot of lipid, an explanation for its cause is not yet forthcoming.

Since PC does not restore activity as effectively as PE (Nemerson, 1969), it was of interest to determine if this was a result of ineffective binding of PC. Therefore, the more purified apoprotein (see Methods) was combined with PC at 2.5 and 5.0 mg of PC per mg of protein and compared with the same protein to which PE was added. At a ratio of 2.5 mg of PE per mg of protein, the visible band in the centrifuge tube was found between 1.3 and 1.7 cm below the meniscus,

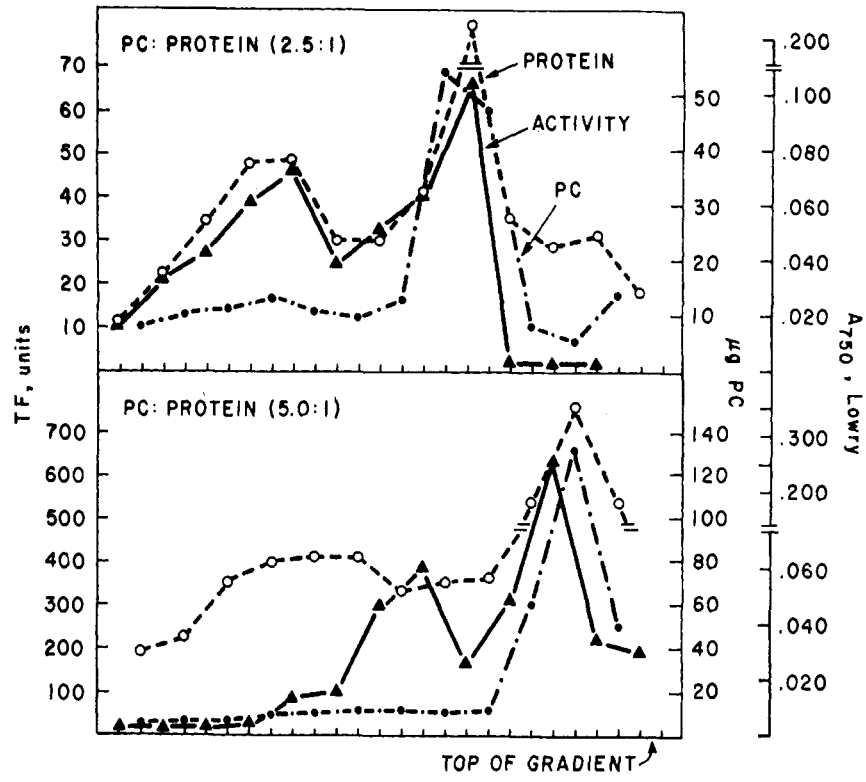

FIGURE 5: Centrifugation (18 hr at 36,500 rpm) of apoprotein relipidated with increasing amounts of PC (Lot 40 D). Lipid to protein ratio, final protein concentration: 2.5 mg of PC/mg, 0.58 mg of protein/ml; 5.0 mg of PC/mg, 0.417 mg of protein/ml; 0.3 ml was layered on the gradient. Activity was located by assaying directly.

while the sample relipidated with the same amount of PC banded more sharply at 1.2–1.4 cm. The sample relipidated with 2.5 mg of PC per mg of protein, had two activity peaks with densities of 1.100 g/ml and 1.063 g/ml (Figure 5), whereas the sample relipidated with the same amount of PE had a single, broad peak with maximal activity at a density of 1.065 g/ml (not shown).

The sample combined with 5.0 mg of PC per mg of protein has about the same specific activity as that formed with 2.5 mg of PE per mg of protein (3350 and 3710 units per mg, respectively). The experiments illustrated in Figures 3 and 5 show that the specific activity of the sample applied to the gradient does not correlate with the density of the complex formed. That is, PC added buoyancy to the protein, but did not correspondingly increase its activity. It is, of course, possible that the peak itself had a higher specific activity than the total preparation applied to the gradient. Turbidity in the samples containing PC prevents an accurate protein determination and so the specific activity of the peak cannot be calculated using these data.

Behavior of Apoprotein Combined with Active Lipid in the Absence of Deoxycholate. PE was prepared as usual in 0.25% deoxycholate, then dialyzed against buffer before addition of the protein. The protein and lipid were dialyzed together as usual, but in the absence of deoxycholate. The sample, which had no more coagulant activity than the apoprotein, was applied to the gradient. More than half the protein was found at the bottom of the gradient (Figure 6) in the region where apoprotein alone was found. Phospholipid was present only in the top fraction. Alternate fractions were relipidated with mixed brain lipids in deoxycholate and the activity was found in the lower third of the gradient. PE in the presence of deoxycholate is the most effective phospholipid in restoring activity, but in the absence of deoxycholate activity is not restored and tissue factor is not bound to the phospholipid.

Centrifugal Behavior of Apoprotein Relipidated with Ineffective Phospholipids. Recombination of the apoprotein with 1.6 mg of lysoPC per mg of protein did not stimulate activity. While this is less phospholipid on a weight basis than was used in the experiments with PE, the molar ratio is the same as PE at 2.5 mg per mg of protein. After 18-hr centrifugation, protein and phospholipid were found throughout the gradient

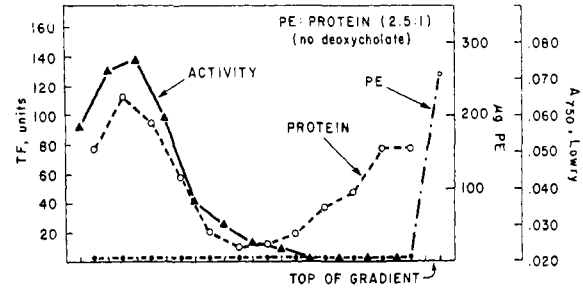

FIGURE 6: Centrifugation (18 hr at 36,900 rpm) of apoprotein combined with PE (Lot 43 J) in the absence of deoxycholate (see text). 2.5 mg of PE/mg of protein; final protein concentration, 0.324 mg/ml. A 0.3-ml aliquot was layered on the gradient. Activity was located after relipidation with mixed brain lipids at 5 mg/mg of protein.

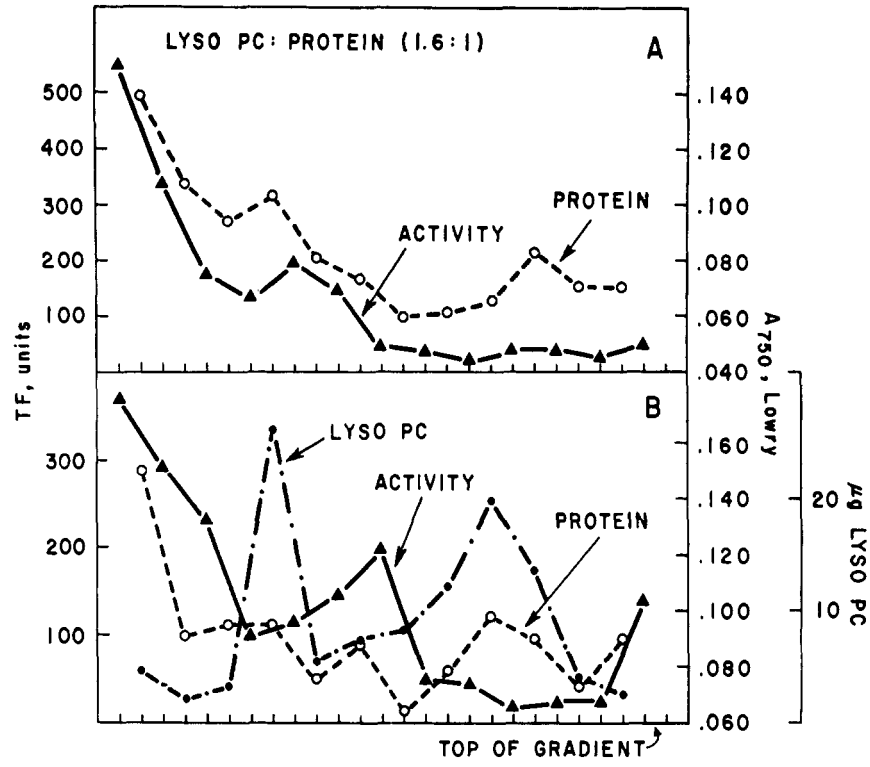

FIGURE 7: Centrifugation (47 hr at 36,400 rpm) of apoprotein relipidated with lysoPC (Lot 6003). 1.6 mg of lysoPC/mg of protein; final protein concentration, 0.5 mg/ml. (A) 0.25 ml incorporated into 5% sucrose solution; (B) 0.25 ml layered on gradient. Activity was located after relipidation with mixed brain lipids at 5 mg/mg of protein.

(not shown). Activity was located by recombining individual fractions with mixed brain lipids in the presence of deoxycholate. Tissue factor activity was also found closer to the center of the gradient than apoprotein under the same conditions. However, the protein, phospholipid and activity peaks were not coincident as they had been in experiments with samples relipidated with PE. Furthermore, increasing the relipidation ratio to 2.5 and 5.0 mg of lysoPC per mg of protein did not appreciably alter the location of the components. The fact that, in all of these instances, the position of the components after relipidation with lysoPC differed from their native position suggested that some sort of complex had been formed.

In order to determine whether the lysoPC was bound to a small fraction of the protein and had reached an isopycnic point in the gradient or whether tissue factor had been bound to the lipid and the complex was breaking up, the protein was relipidated with 1.6 mg of lysoPC per mg and centrifuged for 47 hr. The results of prolonged centrifugation are presented in Figure 7. When a sample was layered on the gradient, protein, activity and phospholipid failed to coincide (Figure 7B) as they had for the PE experiments (Figures 3 and 4). Considerable phospholipid was found deep in the gradient, indicating that it had bound to protein. Activity, determined after relipidation with mixed lipids, was toward the top of the gradient, suggesting that tissue factor itself had been bound to the lysoPC.

A sample relipidated with lysoPC was also incorporated into a gradient and centrifuged for 47 hr. In this case, protein was found distributed throughout the gradient, but the activity peak, again determined after addition of mixed lipids, was located at the bottom of the gradient (Figure 7A). These locations are different from those found when the sample was layered on. This demonstrates that equilibrium had not been reached and that the lipid–protein complex was indeed unstable.

Relipidation of the protein with reduced PC also failed to restore coagulant activity, but at a relipidation ratio of 2.5 mg per mg of protein, a very sharp band was observed 1.4 cm below the meniscus after 18-hr centrifugation. Reduced PC alone banded at 1.2 cm below the meniscus. The activity, located by relipidation of individual fractions, was in a slightly broader peak than apoprotein alone (Figure 8). Phosphorus peaked at a density of 1.063 g/ml while the phosphorus peak of reduced PC alone was found at a density of 1.057 g/ml. When the relipidation ratio was increased to 10.6 mg per mg of protein, about half the activity and most of the protein was found coincident with the phospholipid in a region between 1.059 g/ml and 1.078 g/ml, still more dense than phospholipid alone. Thus, at high lipid to protein ratios, reduced PC formed a stable, but inert, complex with tissue factor protein.

Discussion

When centrifugal conditions are held constant, alteration of the sedimentation characteristics of a given molecule reflects a change in one or more of the parameters of size, shape, and density. Thus, the marked difference in sedimentation of the apoprotein observed after relipidation reflects such a change.

The fact that the relipidated preparations sedimented less

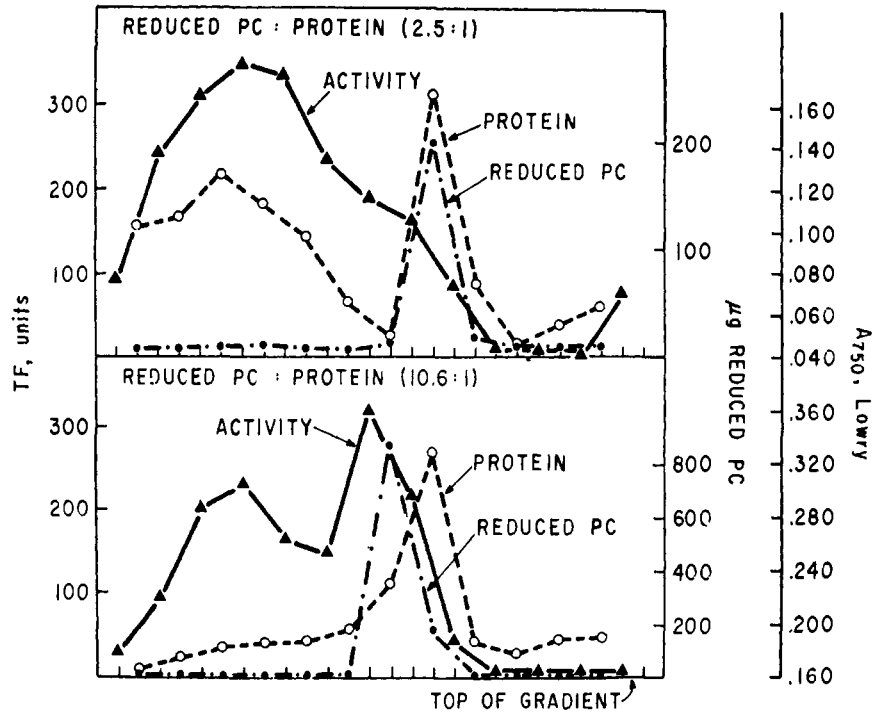

FIGURE 8: Centrifugation (18 hr at 36,500 rpm) of apoprotein relipidated with reduced PC (Lot 6016). Lipid to protein ratio, final protein concentration: 2.5 mg of reduced PC/mg, 0.5 mg of protein/ml; 10.6 mg of reduced PC/mg, 0.25 mg of protein/ml; 0.3 ml was layered on the gradient. Activity was located after relipidation with mixed brain lipids at 5 mg/mg of protein.

far than the apoprotein could be interpreted to mean that the relipidation procedure disaggregated the protein. This argument is not tenable, however, as it was shown that active complexes with PE were at equilibrium since (1) prolonged centrifugation of the samples did not alter their location in the gradient and (2) the activity of samples incorporated in the gradient rose to the same point in the gradient as the activity from the sample which had sedimented from the top. The only explanation for these observations is that the material reached its isopycnic point in the gradient and was, therefore, less dense than the apoprotein. It should be noted, however, that the data do not rule out changes in size and shape of the protein as well as in its buoyancy.

The change in density of the protein can be attributed only to binding to the less dense lipids; or, conversely, the increase in the density of the phospholipids can be attributed only to binding to the more dense protein. Control experiments in which the protein was studied with only one of the other two components of the relipidation procedure (lipid or deoxycholate) showed that the individual sedimentation characteristics were unchanged. Likewise, when the PE was centrifuged with only one of the relipidation components, no significant change in its location in the gradient was noted. Thus, as a result of combining protein, lipid and deoxycholate, followed by dialysis, an active complex was formed with a resultant density intermediate to that of protein or lipid. Moreover, the density of the complex was dependent upon the lipid to protein ratio.

The results of these experiments show that binding of tissue factor to phospholipid is required for activity. When the protein was simply added to an active lipid in the absence of deoxycholate, activity was not restored, nor did binding occur. It is clear, however, that binding to lipid *per se* is not sufficient to restore activity to tissue factor; reduced PC and lysoPC bound to tissue factor, but did not restore activity. The data do show, however, that lipids which stimulate biological activity readily form more stable complexes with tissue factor than does the inert lysoPC. PE formed complexes which quickly reached equilibrium in the gradients and which remained intact for at least 63 hr. It is noteworthy that the forces operative during centrifugation tend to dissociate the complex by working to sediment the dense protein and float the buoyant lipid.

The complexes formed with lysoPC behaved differently from the active complexes during centrifugation. The phospholipid peak of the lysoPC sample did not coincide with the bulk of the protein or activity, although it was found deep in the gradient, indicating that it had been bound to protein which had increased its density. At 47 hr, the location of activity, protein, and phospholipid were at different positions in the gradient than they had been at 18 hr. It seems unlikely that this discrepancy occurs because the original complex is still sedimenting—the patterns for PE are similar at 18, 45, and 63 hr of centrifugation (Figures 3 and 4). However, if the composition of the initial complex were changing during sedimentation due to the centrifugal and buoyant forces at work, its position in the gradient would shift as its composition changed. As further evidence that a lysoPC–protein complex does not withstand centrifugation and reach an isopycnic point, the location of protein and activity in the gradient is a function of whether the sample sediments from the top or is dispersed throughout the gradient.

Reduced PC formed a stable, but biologically inert complex with the tissue factor protein. It appears, however, as if

the affinity of reduced PC for the protein is less than that of the active lipids. At a ratio of 2.5 mg of reduced PC per mg of protein, little tissue factor was bound to lipid. At 10.6 mg of reduced PC per mg of protein a complex was formed, as evidenced by the coincidence of lipid, protein, and activity in a single peak. Even at this high ratio, however, much of the activity was not incorporated into the complex (Figure 8). It may be that this phenomenon is a result of the inability of reduced PC to form micelles (see below) which would result in markedly less surface area of lipid available to interact with the protein.

As the tissue factor activity is restored with increasing amounts of PC or PE, more phospholipid is bound in the active complexes. At the ratios studied here (2.5 to 7.4 mg per mg of protein), lipid–lipid interactions are most likely occurring as well as lipid–protein interactions. Thus, activity may be due to lipid–protein interactions by which PE and PC, but neither lysoPC nor reduced PC, induce the protein to assume a specific conformation. Alternatively, stronger lipid–lipid interactions may occur with PE and PC, and be required for activity.

A minimal lipid–protein complex without lipid–lipid interactions could not be distinguished isopycnically from apoprotein in these experiments. At 1 mg of lipid per mg of protein, the calculated density of the complex is the same as the 30% sucrose solution. The complex would, therefore, sediment to the same region as the apoprotein. Thus, density gradient centrifugation cannot distinguish between unstable lipid–lipid and lipid–protein interactions. Different techniques must be employed to determine whether the protein is held in a less reactive conformation by the ineffective phospholipids or whether the protein conformation is the same in all instances, but that an external lipid shell, manifested here as an unstable complex, prevents interaction of the protein with its substrate.

The lipid–lipid interactions vary considerably from one phospholipid to another as manifested by different micellar properties. The number of fatty acid chains in the phospholipid and their degree of saturation play an important role in the binding to tissue factor and the restoration of activity. These parameters are also reflected in the micellar properties of the phospholipids. In aqueous solutions, egg and brain PC form large micelles (mol wt 2×10^8) in a coiled configuration (Gammack *et al.*, 1964; Attwood and Saunders, 1965) while egg lysoPC forms a smaller (mol wt 9.5×10^4) globular structure (Perrin and Saunders, 1964; Saunders, 1966); reduced PC may not even form micelles at room temperature (Fleischer *et al.*, 1961). Brain PE micelles are probably similar in size to those of PC (Gammack *et al.*, 1964).

Although the micellar size of PE and PC is the same, the fatty acids of PE have a greater degree of unsaturation, and therefore, a greater cross sectional area than those of PC. When synthetic dipalmitoylPC and -PE are compared using the monolayer technique (Phillips and Chapman, 1968), PE tends to form a more compact film than PC; distearoylPE is also more stable to temperature changes than distearoylPC (Chapman and Fluck, 1966). The ethanolamine head group is not only less bulky than the choline group, but it also interacts more strongly than those of PC with neighboring head groups. Thus, the properties of naturally occurring PE must reflect both the expansive nature of the unsaturated fatty acids and the cohesive nature of the polar head groups. Indeed, this seems to be the case. X-Ray diffraction studies of PE and PC in the liquid crystalline state have demonstrated a closer spacing for PC (Papahadjopoulos and Miller, 1967); however, PE is much more permeable than PC to both K^+ and Cl^- (Papahadjopoulos and Watkins, 1967). It should also be noted that PE does not form stable structures, but rather has a tendency to aggregate, particularly if air oxidation is not controlled (Papahadjopoulos and Miller, 1967). Thus, the nuances of the crystalline nature of the fatty acid chains, as well as the interactions of the polar head groups affect the relationship of one phospholipid molecule to another. Both factors probably play a large role in the binding of a particular phospholipid to tissue factor with consequent restoration of activity.

In the activity assay, tissue factor first complexes with factor VII and calcium (Nemerson, 1966; Williams and Norris, 1966); this complex then enzymatically converts factor X into its coagulant form. Experiments with the UDP galactose: lipopolysaccharide-α-3-galactosyl transferase system have demonstrated that an ineffective phospholipid, didecanoylPE, binds to the lipoplysaccharide but, in contrast to the active PE–lipopolysaccharide complex, this complex will not form a ternary complex with the enzyme (Endo and Rothfield, 1969). A similar phenomenon may occur with tissue factor, and experiments to determine the effect of lipid on the tissue factor–factor VII reaction are in progress. Since the assay system for tissue factor activity couples the reactions of factors VII and X, the effect of phospholipid upon tissue factor activity may be reflected in either one of these two steps.

Finally, since the apoprotein seems to exist in at least two forms distinguishable by ultracentrifugation, disc gel electrophoresis and gel filtration (Nemerson and Pitlick, 1970), the possibility exists that the lipoproteins created by these two forms may be different. As noted above, two peaks of activity were frequently observed in the isopycnic centrifugations of the lipoprotein; it is not yet clear, however, whether these two lipoprotein peaks correspond to two forms of apoprotein or are a function of the lipid–protein interactions.

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Nitrocytochrome *c*. I. Structure and Enzymic Properties*

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ABSTRACT: Cytochrome *c* was nitrated with tetranitromethane at pH 8.0.

A modified enzyme nitrated specifically at the tyrosyl residue in position 67 was purified. This nitrocytochrome *c* was found to be unable to restore the respiratory function of cytochrome *c* depleted mitochondria. In the presence of cyanide, which forms a complex with cytochrome *c*, the described nitration did not take place.

Specific chemical modifications of amino acid side chains are often used to establish whether particular amino acid residues in a protein are required for its biological activity. Experimentally, tyrosine is one of the most accessible residues of proteins (Vallee and Riordan, 1969). Horse heart cytochrome *c* contains four tyrosyl residues. Their ionizations were studied by spectropolarimetry (Ulmer, 1966, and references cited therein), and their reactivities by acetylation reactions. The present communication reports the preparation using tetranitromethane, and the enzymic activity of nitrotyrosyl-67-cytochrome *c*. The accompanying paper describes the physicochemical properties of this modified enzyme (Schejter *et al.*, 1970). A preliminary report has been given (Schejter and Sokolovsky, 1969) and similar studies were reported also by Skov *et al.* (1969).

Materials

A crystalline preparation of cytochrome *c* from horse heart was a gift of Dr. E. Margoliash. Commercial horse heart cytochrome *c*, type II, was obtained from the Sigma Chemical Co., and purified on Amberlite CG-50 (Margoliash and Walasek, 1967). Tetranitromethane was obtained from Fluka AG; α-chymotrypsin, three-times crystallized, from Worthington; and Dowex AG 50-X2 (200–400 mesh) was obtained from Bio-Rad. All other chemicals were of the best grade available.

Methods

Concentrations of native cytochrome *c* were determined by the absorbance of the reduced form at 550 mμ using a molar absorptivity of 2.77×10^4 M^{-1} cm^{-1} (Margoliash and Frohwirth, 1959). The concentration of nitrated cytochrome *c* was determined by amino acid analysis.

A Zeiss PMQ spectrophotometer was used for measurements of absorbance at single wavelengths, and Cary 15 or 14 recording spectrophotometers were employed for determination of absorption spectra.

The pH of solutions was measured on a Radiometer Model 26 pH meter equipped with a glass-calomel combination electrode.

Amino acid analysis were performed with a Beckman-Unichrome amino acid analyzer according to the procedure of Spackman *et al.* (1958). Samples were hydrolyzed in constant-boiling HCl in evacuated sealed tubes in the presence of 10 μl of phenol, at 110° for 22 hr. Tryptophan was determined on the unhydrolyzed protein using *N*-bromosuccinimide (Spande and Witkop, 1967), by the reaction with dimethylaminobenzaldehyde (Spies and Chambers, 1949), and after acid hydrolysis in the presence of 2% thioglycolic acid (Matsubara and Sasaki, 1969). Methionine was also determined after alkaline hydrolysis (Neumann, 1967). Quantitative determinations of nitrotyrosine were obtained from amino acid analyses as described by Sokolovsky *et al.* (1966).

Paper chromatography was carried out on Whatman No. 3MM paper using 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v) as the solvent.

Cytochrome c activity was estimated by the method of Jacobs and Sanadi (1960).

Nitration was performed by addition of tetranitromethane in 95% ethanol to a solution of ferricytochrome *c* (2–2.5 mg/ml) in 0.1 M Tris–0.1 M KCl (pH 8.0) at room temperature. The final concentration of ethanol was always less than 4%. The reaction was terminated by passing the mixture through a Bio-Gel P-4 column in 0.04 M ammonium bicarbonate (pH 8.0). In experiments using cyanide, 0.1 M KCN was substituted for 0.1 M KCl.

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