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Research Article

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The proteins were characterized by gel filtration on Sephadex G-200; the activity eluted as a single peak with an apparent mol wt of 425,000. The brain and lung activities were recovered from chromatography on triethyl-aminoethyl-cellulose as single peaks, although the salt concentration required for elution was different for each protein. This suggests that within each organ there is one tissue factor-protein, but there may be molecular differences between brain and lung tissue factor. Alternatively, the chromatographic differences may simply reflect charge differences imparted to the proteins by residual lipids.

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ABSTRACT Soluble proteins were prepared from bovine lung and brain which, when combined with phospholipids, had the biological characteristics of tissue factor. The proteins were solubilized from delipidated (heptane: butanol–extracted) acetone powders with deoxycholate, and precipitated by $(NH_4)_2SO_4$ (30–60% saturation). These soluble proteins, which contained less than 1% phospholipid, were essentially inert in an assay for tissue factor. When they were recombined with phospholipids, however, activity increased by a factor of 500–1000. Phosphatidylethanolamine was the most active specific phospholipid, followed by phosphatidylcholine. Phosphatidylserine was inert. Mixed phospholipids were from two to four times more active than phosphatidylethanolamine. Maximum activity was obtained at phospholipid to protein ratios of 1.5:1 (wt/wt), and when relipidation was performed in deoxycholate followed by dialysis against 0.05 M imidazole–0.375 M NaCl, pH 7.2.

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INTRODUCTION

When aqueous extracts derived from various tissues are added to plasma, the rate of coagulation after recalcification is markedly enhanced. It is now known that this is due to a specific interaction between the tissue extracts (tissue factor) and a plasma protein, factor VII (1, 2). Although the structure of tissue factor is not known, it has been clear for many years that it contains both a protein and lipid moiety (3). In a previous study on the lipid component of tissue factor, it was demonstrated that certain phospholipids were necessary for full biological activity to be achieved (4).

Only some tissues contain significant amounts of tissue factor which strongly suggests that there are specific proteins within the active tissues that account for their coagulant activity. On the other hand, it has been stated that tissue factor is all, or almost all, lipid, (5, 6). Previously, we demonstrated that a lipid-poor protein obtained from brain tissue activated the extrinsic coagulant mechanism, i.e., it functioned as tissue factor (7). This reaction, however, occurred at a very slow rate, and it was therefore uncertain whether this protein represented a specific tissue factor protein, or a nonspecific protease.

In this report, we describe the preparation of soluble proteins from brain and lung that have the biological characteristics of tissue factor. Further, it is shown that the soluble proteins, like the particulate preparations previously reported, have a striking requirement for

phospholipids. After recombination with lipids, these soluble preparations clotted recalcified plasma in less than 12 sec.

The optimal conditions for the restitution of activity by phospholipids were determined for brain and lung proteins and were found to be essentially identical, as were their requirements for specific phospholipids. Preliminary characterization of the proteins was accomplished by gel filtration and ion exchange chromatography. The proteins had similar molecular weights (as determined by gel filtration), but behaved slightly differently on ion exchange chromatography. The chromatographic data suggest that within each organ there is but one active protein (or a family of closely related proteins), and that there may be molecular differences between the tissue factor proteins of lung and brain.

METHODS

Purified phospholipids were obtained from Supelco, Inc., Bellefonte, Pa. These lipids were essentially pure when examined by two-dimensional thin-layer chromatography on silica gel (4), and were used as supplied. Sodium deoxycholate was a product of Mann Biochemicals. Deoxycholic acid-^{14}C was purchased from Tracerlab, Waltham, Mass. This was dissolved in dilute NaOH, titrated to pH 7.4 with HCl, and used without further purification. TEAE *O*-(triethylaminoethyl) cellulose (nominal capacity of 0.44 meq/g) was obtained from Biorad Laboratories, Richmond, Calif., and Sephadex G-200 and Dextran blue 2000 from Pharmacia, Piscataway, N. J.

All other chemicals were of reagent grade and purchased from standard sources. Solvents were distilled shortly before use, with the exception of acetone used to dehydrate the tissues.

Preparation of tissue factor

(*1*) *Brain.* An acetone powder was prepared as previously described (4). The powder was then extracted three times with hepatane: butanol (2:1, 20 ml/g) for 60 min at room temperature. This solvent pair was substituted for the butanol previously used as it proved a more effective solvent for phospholipids: three extractions removed more than 95% of the lipid (chloroform: methanol–extractable) phosphorus. After each wash, the residue was collected by filtration through a double layer of No. 42 Whatman filter paper (which was prewashed with the solvents). The solvents were refiltered until clear (if this was not done, the final yield of activity was reduced). The tissue was dried under a stream of air and stored at −20° until used. Tissue factor protein was solubilized by homogenizing the delipidated powder (in a Thomas No. B29346 glass and Teflon homogenizer at room temperature) with sodium deoxycholate (0.25%, 40 ml/g). The suspension was stirred for 30 min and the insoluble residue removed by centrifugation (37,000 *g*, 30 min, 4°C). The protein concentration of the supernatant was diluted to 3.8 mg/ml with deoxycholate (it usually contained between 4 and 5 mg/ml).

The proteins were precipitated from the deoxycholate solution by the addition of solid ammonium sulfate at room temperature. The solution was brought to 30% saturation and the precipitate that formed was removed by centrifugation (37,000 *g*, 15 min, 4°C). The supernatant was adjusted to 60% saturation by the further addition of solid ammonium sulfate. This precipitate was collected as above. The proteins that precipitated at 30% saturation were essentially insoluble in water. When they were to be assayed for biological activity, they were suspended in a small quantity of water and dialyzed against large volumes of imidazole buffer (imidazole, 0.05 M, adjusted to pH 7.35 with HCl); the proteins that precipitated between 30 and 60% saturation readily dissolved in a small volume of water, and were dialyzed as above for 18 to 24 hr before further studies. During dialysis a small amount of insoluble material formed and was removed by centrifugation (37,000 *g*, 30 min). The supernatant, which was moderately turbid, was completely clarified by subsequent ultracentrifugation (Spinco rotor, No. 40, 40,000 rpm, 2 hr). The supernatant obtained from this step was used for the studies below.

(*2*) *Lung.* Bovine lungs obtained immediately after slaughter were minced and homogenized with an equal volume of saline in a Waring Blendor for 15 sec. The large particles were removed by squeezing the homogenate through a double layer of cheese cloth. The remainder was filtered through a double layer of No. 1 Whatman filter paper until clear. The precipitate was extracted with large volumes of acetone and finally dried under a stream of air. All other steps were the same as used for brain except: (*1*) the protein concentration of the deoxycholate extract was adjusted to 3.0 mg/ml, and (*2*) after dialysis, the proteins obtained between 30 and 60% saturation were readily clarified by centrifugation at 37,000 *g* and were usually not ultracentrifuged.

Recombination of lipids and soluble tissue factor

This was performed by mixing 1 volume of aqueous protein solution with 1 volume of lipid dissolved in deoxycholate (0.25%) and 2 volumes of deoxycholate (0.25%). When mixed phospholipids (4) were used, a concentrated stock solution (greater than 5 mg/ml) was freshly prepared, and appropriate dilutions were added to the proteins. When pure phospholipids were used, dilute solutions were made (about 1 mg/ml). Preliminary experiments showed that variable amounts of the lipids were lost from these dilute solutions; consequently, aliquots were analyzed for P, and the lipid content calculated from these determinations.

The deoxycholate was removed from the mixture of protein and lipid by dialysis against large volumes of saline or other solutions (as indicated). Dialysis was continued for 18–48 hr. No differences in activity were observed over this period.

Assay of tissue factor

This was done as previously described (4) by measuring the initial rate of activation of factor X in a system saturated with factors VII and X and in which tissue factor was rate limiting. The assay was calibrated daily using a frozen particulate brain preparation as a reference standard. All determinations were performed in triplicate at two concentrations. Preliminary experiments revealed that the soluble preparations did not require sonication in order to achieve reproducible results, consequently this procedure was omitted. All preparations were diluted so that they contained between 5 and 100 units/ml, the range over which the assay was most reproducible.

TEAE cellulose chromatography

The cellulose was washed with alkali and acid as described for DEAE by Peterson and Sober (8). 12 grams were packed under pressure in a 1 cm column to a height of 20 cm. Before use, the exchanger was cycled with the salt gradient used and finally washed with 2 M NaCl, pH 6.0. After each run, the column was washed with 2 M NaCl and then with starting buffer until the ionic strength of the effluent was that of the latter. The same column was used throughout these experiments.

Sephadex G-200 chromatography

The gel was allowed to swell for 1 wk in saline at room temperature. It was then packed to a height of about 36 cm in a 2.5 cm column. Care was taken to keep the hydrostatic pressure across the column below 10 cm throughout packing and use. Before use, the column was washed for 48 hr with the eluant. Because the elution was performed with buffers of high salt concentration, sucrose was added to the samples until their density exceeded that of the eluant. The void volume of the column was determined before and after each sample was chromatographed by using Dextran blue 2000. The volume of each fraction was measured when a run was completed.

Chemical methods

Protein was measured by an L.C. biuret technique (9), and nitrogen and phosphorus as previously described (4), except that the phosphorus color was developed with ascorbic acid (10) as the reducing agent. Deoxycholate was determined by the method of Mosbach et al. (11). In some experiments, the deoxycholate concentration was beneath the sensitivity of this technique and was quantified with deoxycholate-^{14}C by adding a small aliquot of the specimen to 10-ml of Bray's solution and counting the radioactivity in a liquid scintillation spectrometer. The samples were corrected for quenching by a channels ratio technique.

RESULTS

Solubilization of the tissue factor proteins

Lung. As extraction of the acetone powder with heptane : butanol removed most of the clotting activity, the fractionation procedure was followed by recombining each fraction with mixed phospholipids (Table I). The deoxycholate extract usually contained 4–6 times the total activity of the acetone powder, and solubilized about 75% of the potential clotting activity. Due in part to this increase in total activity, the deoxycholate extract had a specific activity about 37 times that of the acetone powder.

If the deoxycholate was removed by dialysis at this stage, most of the activity precipitated (not shown). If, however, a fraction rich in phospholipids was first precipitated with $(NH_4)_2SO_4$ (30% saturation), then proteins were obtained (precipitating at 60% saturation) that were water-soluble, poor in phospholipid, and that were 58-fold purified. This fraction will be referred to as "soluble proteins." Only a small amount of activity remained in the supernatant from this step.

TABLE I

Preparation of Soluble Tissue Factor Protein from Lung

Preparation	A Protein	B Yield	C Phospho-lipid	D Units (− lipid)	E Units (+ lipid)	F Increase* with lipid (fold)	G Yield‡	H Purification‡
	mg	%	%				%	
Acetone powder	1050	100	28	16,580	—		100	1§
Delipidated powder	1000	95	2	1005	—	—	6	0.06§
Deoxycholate extract	159	15	8	1320	94,500	72	560	37
Residue	841‖	80	1	91	25,600	281	152	2
$(NH_4)_2SO_4$ 30% ppt	114	11	9	1437	8,800	6	52	4.8
60% ppt ¶	19	1.9	0.8	53	17,600	332	105	58
Supernatant	26	2.5	14	0	2,600	—	15	6.3

* Mixed phospholipids.
‡ Yield of activity and purification were based on relipidated preparations.
§ Not relipidated.
‖ Calculated value.
¶ "Soluble proteins."

The degree to which the activity of each fraction was increased by the addition of mixed phospholipids is also shown.[1] In the experiment described in Table I, the deoxycholate residue and the soluble proteins showed about a 300-fold increase, and the deoxycholate extract, 72-fold. As the relipidated specimens were assayed a day later than those without lipid, and the decay of activity at this stage of purification was about 40%/day, the true increases were about 40% greater than those shown. In other experiments the activity of the soluble proteins increased about 1000-fold after the addition of phospholipids.

At each stage of the preparation of the tissue factor protein, the relipidated product was found to yield clotting times proportional to its concentration. Moreover, for these and subsequent experiments, the slope of a plot of the log of the clotting time vs. the log of the tissue factor concentration was linear and paralleled the slope obtained with the standard particulate preparation (4). The parallel slopes strongly suggest that the same biological activity was being measured.

[1] Recombination with lipids was done at the optimal conditions as defined for the soluble proteins (see below). Each fraction was brought to a lipid: protein ratio of about 1.5: 1. The optimal conditions were not determined for the other fractions, but were assumed to be the same as for the soluble proteins. The activity of the other fractions, therefore, may be underestimated.

Brain. The fractionation scheme employed for the brain proteins was similar to that employed for the lung. The results are shown in Table II.

The major difference between brain and lung was that no increase in activity was noted in the deoxycholate extract. In fact, only about 69% of the activity was recovered at this stage. The purification achieved at this step was correspondingly lower: 4.2-fold vs. 37-fold.

The soluble proteins obtained from this experiment increased in activity by a factor of 667 after relipidation. Due to the reasons cited above, this corresponds to a true increase of about 1000-fold.

As deoxycholate has been previously reported to solubilize tissue factor (the "native" lipoprotein) (12, 13), its concentration was determined at several stages of the preparation of soluble brain proteins. After 24 hr of dialysis against large volumes of imidazole buffer, the soluble proteins contained 0.13 mg/ml, and after 48 hr, less than 0.025 mg/ml. After gel filtration (see below), the most active fractions contained less than 0.04 μg/ml (as determined by radioactivity). At each stage the preparations were optically clear, and no activity could be sedimented by ultracentrifugation at 104,000 *g* for 2 hr. It is likely, therefore, that deoxycholate was not required for the solubility of the tissue factor proteins, although interaction of the proteins with trace amounts of the bile salt cannot be ruled out.

TABLE II

Preparation of Soluble Tissue Factor Protein from Brain

Preparation	A Protein	B Yield	C Phospho-lipid	D Units (− lipid)	E Units (+ lipid)	F Increase* with lipid (fold)	G Yield‡	H Purification‡
	mg	%	%				%	
Acetone powder	2160	100	40	247,000	—	—	100	1
Delipidated powder	2000	93	6	8250	—	—	—	0.04§
Deoxycholate extract	351	16	15	2590	169,700	65	69	4.2
Residue	1649‖	76	4.2	75	63,000	840	26	0.33
$(NH_4)_2SO_4$– 30% ppt	76	3.5	22	5806	29,700	5	12	3.5
60% ppt ¶	99	4.6	0.9	75	50,000	667	20	4.9
Supernatant	158	7.3	21	0	650	—	0.3	0.04

* Mixed phospholipids.
‡ Yield of activity and purification were based on relipidated preparations.
§ Not relipidated.
‖ Calculated value.
¶ "Soluble proteins."

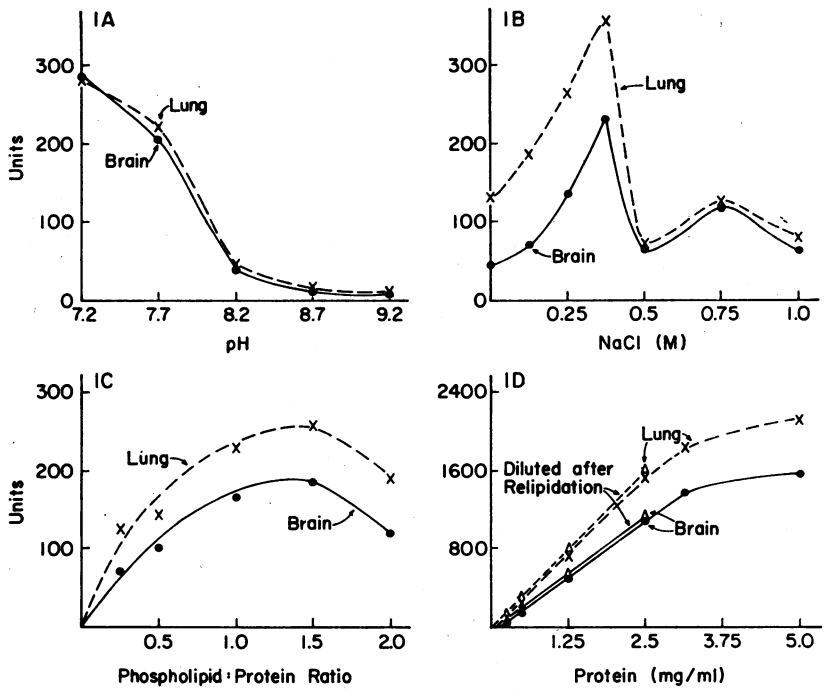

FIGURE 1 Conditions for optimal relipidation. The results in A, B, and C are expressed as units/mg protein. In each instance, the protein concentration was 1 mg/ml before relipidation and the preparations were adjusted to standard conditions (see Results) before assay. In A, the buffer was 0.05 M imidazole–0.375 M NaCl adjusted to the particular pH at 4°C. In B, the buffer was 0.05 M imidazole, pH 7.2 at 4°C with increasing salt concentration. In both A and B, the phospholipid-to-protein ratio was 1.5 : 1. For C, the buffer was 0.05 M imidazole–0.375 M NaCl, pH 7.2, at 4°C. In D, the protein concentration was varied, but the ratio of lipid-to-protein was constant (1.5 : 1). The buffer was the same as in C. The results are expressed as units/ml. The activities of samples that were relipidated at high protein concentrations and then diluted were the same as samples that had been relipidated under dilute conditions (i.e., the specific activity of the relipidated sample was independent of the starting protein concentration).

Activity of the relipidated soluble proteins in a recalcified plasma clotting system

To relate the units described in this paper to the widely used assay of tissue factor (acceleration of the recalcified plasma clotting time), soluble proteins of brain and lung that were relipidated with mixed phospholipids were used as reagents in a "one-stage prothrombin time." A lung preparation containing 0.77 mg protein/ml and 1250 units/ml accelerated the clotting time of recalcified plasma from 75 to 11.4 sec (equal volumes of tissue factor, plasma, and 0.025 M $CaCl_2$). Serial twofold dilutions of the lung preparation clotted the plasma in 12.8 and 14.2 sec. A brain preparation containing 0.68 mg protein/ml and 1030 units, coagulated the plasma in 11.7 sec. Twofold dilutions of this material clotted the plasma in 13.0 and 14.8 sec.

Conditions for relipidation [2]

The effects of pH, salt, and the lipid-to-protein ratio on the recovery of activity were determined. The results are depicted in Fig. 1 (A, B, and C). The data are expressed as units/mg protein (added to the relipidation system). These experiments were performed with different preparations, which accounts for the varying specific activities shown.

After the optimal conditions were determined in preliminary experiments, the studies shown were performed at these conditions, except for the variable being studied. In each instance, all the components (protein, lipid, and deoxycholate) were adjusted to the appropriate pH and

[2] As the physical binding of phospholipids to the soluble proteins was not investigated, "relipidation" and "recombination" are used in a functional sense.

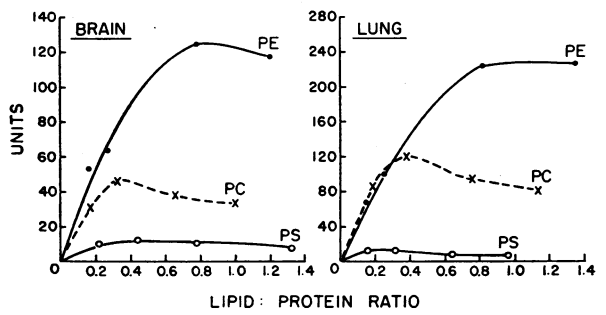

FIGURE 2 The requirement of brain and lung proteins for specific phospholipids. The conditions for relipidation were the same as Fig. 1 C. Abbreviations used: phosphatidylethanolamine, PE; phosphatidylcholine, PC; and phosphatidylserine, PS.

salt concentrations and were dialyzed overnight against a buffer of the same composition. Before assay, all preparations were adjusted to the same ionic strength and pH, 0.15 and 7.35. The data presented, therefore, reflect only the effects on relipidation and not on the assay itself.

The effect of pH was studied between 7.2 and 9.2, because preliminary experiments showed a marked loss of activity when the proteins were exposed to buffers beyond this range. Exposure of the proteins to the indicated salt concentrations had no effect on the recoverable activity when relipidation was performed at standard conditions. In addition to the imidazole buffers used, Tris-chloride and phosphate buffers of the same ionic strength were tested; phosphate was inhibitory and Tris gave results equal to imidazole. The latter was used because of its higher buffering capacity at pH 7.2.

The recovery of activity at increasing protein concentrations (at a lipid to protein ratio of 1.5:1) was determined (Fig. 1 D). It is apparent that recovery was linear up to protein concentrations of 2.5 mg/ml (lung) and 3.0 mg/ml (brain). Above these values, the recovery decreased. The preparations relipidated at protein concentrations of 2.5 mg/ml were diluted after relipidation. As shown in the figure, the activity was virtually identical in the preparations relipidated and then diluted, and in those diluted and then relipidated. This experiment also shows that the small amount of residual deoxycholate present in the assay had no effect on the clotting times as an eightfold dilution of the preparation diluted after relipidation yielded activity proportional to its protein content.

Requirement of the brain and lung proteins for specific phospholipids

The specificity of the phospholipid requirement of the tissue factor proteins was determined by relipidating them with purified lipids (Fig. 2). Of note is that both proteins had highly specific requirements that were essentially identical and are similar to those previously reported for brain particles (4). In contrast to the particles, however, purified lipids were not as effective as mixed brain lipids in restoring activity. Lung proteins relipiated with mixed lipids had 944 units/mg, and brain, 200. These values were about 4.1 and 1.6 times greater than those achieved with phosphatidyl ethanolamine, the most active phospholipid studied.

Chromatographic characterization of the soluble proteins

The soluble proteins were analyzed by gel filtration on Sephadex G-200 (Fig. 3). The depicted elution patterns are representative of duplicate experiments. The re-

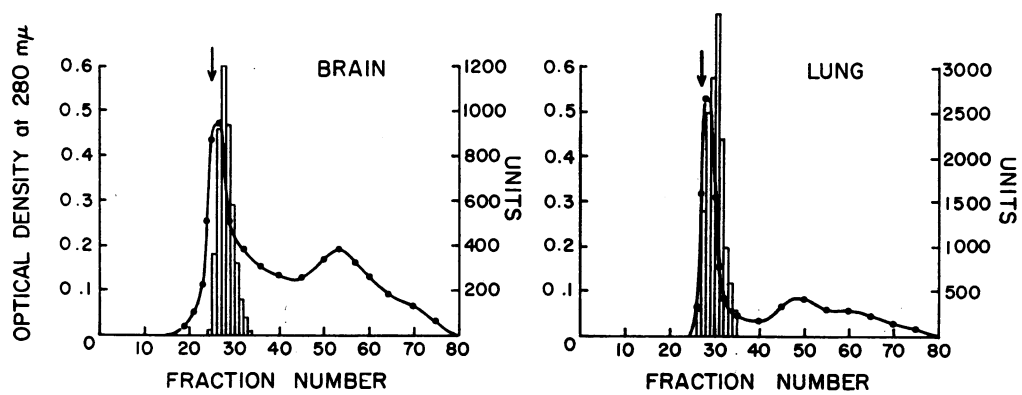

FIGURE 3 Sephadex G-200 chromatography of brain and lung proteins. The chromatography was performed at 4°C on a column 36 × 2.5 cm. The eluant was 0.375 M NaCl in 0.05 M imidazole, pH 7.2. A sample of 2-ml (containing 12.2 mg protein, brain; and 18.9 mg protein, lung) was applied to the column. Fractions of 1.9 ml were collected. The flow rate was about 6 ml/hr. Protein is indicated by the solid lines, and activity (units/ml) by the bars. The arrows denote the void volume.

covery of activity of the lung proteins was 240%, and of the brain, 65%. The lung protein was purified 14-fold by gel filtration, but if the increase in activity is taken into account, the purification was sixfold. The brain protein was purified about 2.5-fold by this technique.

The void volume of the column used for the brain was 48.5 ml, and the elution volume of the fractions containing the most activity was 56 ml. The void volume of the column used for the lung was 53 ml, and the elution volume, 61 ml. The ratio of the elution volumes to the void volumes was 1.15, corresponding to a mol wt of about 425,000 (Fig. 4) (14). The extrapolated exclusion limit for these columns (for globular proteins) was about 680,000.

The proteins were also characterized by TEAE chromatography (Fig. 5). The proteins from each organ were chromatographed alternately on the same column three times each. In two experiments, selected fractions were relipidated. In the other, 10-fraction pools were relipidated.

Fractions 30–40 contained the most activity in the lung preparation, and 40–50 in the brain. It can be seen in Fig. 5 that the peak activity of the lung proteins

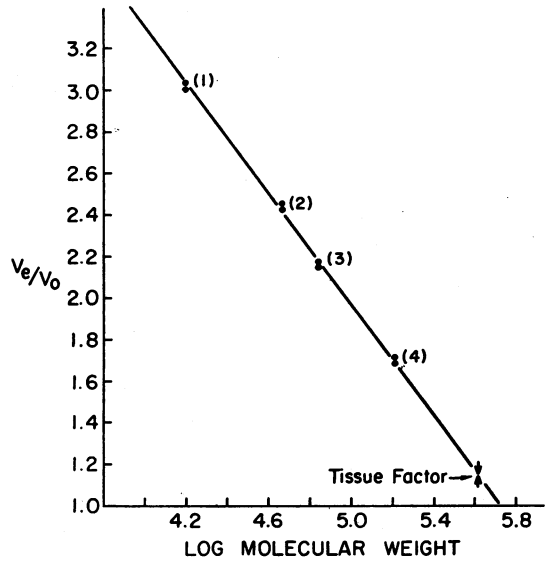

FIGURE 4 Calibration of the Sephadex columns for molecular weight determination. The markers were: (*1*) myoglobin, (*2*) ovalbumin, (*3*) human serum albumin, and (*4*) human gamma globulin. Conditions were the same as Fig. 3. Tissue factor from both organs eluted with a Ve/Vo of 1.15.

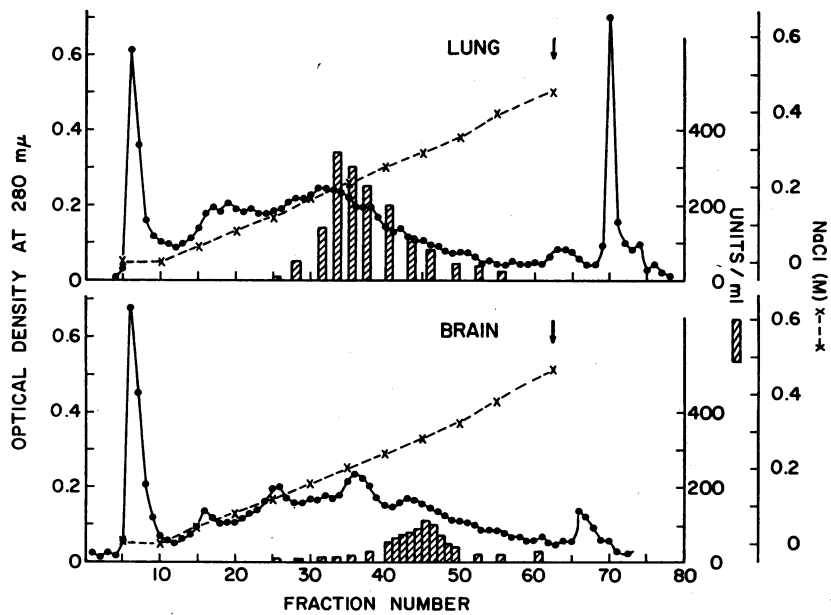

FIGURE 5 Chromatography of brain and lung proteins on TEAE cellulose. The columns were eluted at 4°C with linear gradients of NaCl in 0.05 M imidazole, pH 7.2. Samples of 9.0-ml containing 60-mg protein were applied to a column of 1 × 20 cm containing 12 g of the cellulose. The flow rate was 12 ml/hr and 6-ml fractions were collected. The solid lines denote protein; the bars, activity (units/ml); and the dashed line, the NaCl concentration of the effluent (calculated from the refractive index). The arrow indicates the point at which 2 M NaCl was added. The same column was used for all experiments. Before relipidation all samples were adjusted to 0.375 M NaCl.

eluted at a NaCl concentration of 0.2 M, and the brain at about 0.28 M.

The recovery of activity from both preparations was between 15 and 22%, and the purification about 1.8-fold.

DISCUSSION

The results of these experiments show that tissue factor is a lipoprotein containing a specific protein component. This conclusion is supported by the observation that when the soluble, delipidated brain and lung proteins were chromatographed, only one peak yielded significant activity after relipidation (Figs. 3 and 5). The data also show the tissue factor proteins of brain and lung to be similar, but, perhaps, not identical (Fig. 5).

The conditions for optimal recovery of activity after relipidation were strikingly similar for both preparations, as were their requirements for specific phospholipids. Activity was markedly affected by the amount of lipid added, the pH, and the salt concentration (Fig. 1). Maximum activity was obtained with lipid-to-protein ratios of about 1.5:1, which was somewhat higher than that found with brain particles (4). The effect of pH was striking, maximal activity being obtained at 7.2, and very little at 8.2. Whether this was due to conformational changes in the protein, to ionization of a group on the protein (with a pK of about 7.5), or to ionization of a group on a lipid (perhaps PE), cannot be ascertained from the experiments performed. The salt curve was found to be biphasic, which suggests that at least two functions were affected by the salt. The fact that recovery of activity was not linear at protein concentrations above 2.5–3.0 mg/ml may reflect a tendency of the proteins to aggregate in concentrated solutions, or a change in the physical (micellar) state of the lipids. Although these data cannot be explained in detail, they defined the conditions for maximal recovery of activity and all subsequent experiments were performed at these conditions.

Gel filtration on Sephadex G-200 showed both proteins to have essentially the same molecular size. Assuming a globular shape, their mol wt were found to be about 425,000 (Fig. 4). If, in fact, the proteins are elongated, their molecular weights will be substantially lower than the gel filtration experiments indicate (14).

The response of the soluble tissue factor proteins to purified phospholipids (Fig. 2) was qualitatively similar to the data previously reported (4) for particulate brain tissue factor: PE > PC > PS. This suggests that the solubilized activity was the same as that measured in the particles. The response to mixed phospholipids, however, was different in these preparations: mixed lipids were less active than PE in the particles, but 2–4 times more active than PE in the soluble proteins from both organs. The reason for this discrepancy is not apparent, but may relate to the fact that the experiments with particles were done in organic solvents in which PE formed a clear solution. In contrast, the present experiments used PE suspended in deoxycholate which yielded a turbid suspension. Thus, the lipid was not in a true micellar state. In contrast, the mixed lipids formed a slightly opalescent solution in deoxycholate. Moreover, it should be noted that the relipidations were performed at conditions that were optimal for mixed lipids, but which may not have been so for PE. In any event, the definitive experiments must await purification of the tissue factor proteins, as there undoubtedly were other lipophilic proteins present which could affect the results.

The role of phospholipids in the tissue factor system is obscure, as is their role in other lipid-requiring enzyme systems. Since the original observation by Fleischer, Klouwen, and Brierley (15) that phospholipids were required for mitochondrial electron transport, similar lipid-dependence has been demonstrated for several classes of enzymes, e.g., hydrolytic (glucose-6-phosphatase) (16), synthetic (the transferases required for bacterial cell envelope synthesis) (17), and now for the tissue factor system in blood coagulation. In none of these systems is the function of lipids known in any detail. In most instances, however, binding of lipid to protein was required for the restitution of activity. Binding of lipids to the soluble tissue factor proteins has not been demonstrated, although it was previously shown for the particulate preparations (4). It is likely, therefore, that binding to the proteins has occurred in our preparations, and experiments designed to investigate this phenomenon are now in progress.

Deoxycholate solubilized tissue factor but was not required for maintaining the solubility of the lipid-poor proteins. Deoxycholate was first reported to solubilize tissue factor by Chargaff who found that tissue factor (lung) came out of solution when the deoxycholate was removed (12). This was subsequently verified by Hvatum and Prydz (13) who, in addition, found that in the presence of deoxycholate, tissue factor was retarded by Sephadex G-200, but only when the eluent contained deoxycholate. The difference between these preparations and the ones described here is that our preparations were delipidated before extraction with deoxycholate. Bile salts, however, were not required for the solubilization of delipidated tissue factor–NaCl, 0.15 M and 1.0 M–solubilized 10–20% of the activity, but deoxycholate was used because of its higher yield.

The over-all yield of activity in the soluble $(NH_4)_2SO_4$ fraction of lung protein (obtained between 30 and 60% saturation) was about 100% of the acetone powder (Table I, column G). After relipidation, this material contained 500–700 units of activity/mg protein, representing a purification of 40–60-fold. This, however, cannot be interpreted strictly, as there was a 4–6-fold increase in total activity in the deoxycholate extract of the delipidated powders. The explanation for this is not

clear, but an increase in activity was also observed by Williams during the purification of a particulate lung tissue factor preparation (18). It should be noted that William's preparation was not exposed to bile salts. The observed increases in activity could be accounted for by the presence of an inhibitor in the acetone powder that was not solubilized with the tissue factor. The presence of a soluble inhibitor, however, is indicated by the finding that Sephadex filtration increased the total activity of the soluble protein by 240% (Fig. 3).

The yield of brain tissue factor was lower than lung at each step (Table II, column G), and the purification of the soluble $(NH_4)_2SO_4$ fraction (precipitating between 30–60% saturation) was also lower (4.9-fold vs. 58-fold). No increase in total activity was observed in the deoxycholate extract (the combined yield of the soluble extract and insoluble residue was about 100%), nor did gel filtration increase the total activity. Thus, there was no evidence for the presence of an inhibitor in the brain preparations.

Although the fractions chromatographed on TEAE and Sephadex contained only one peak of activity (Figs. 3 and 5), it is not certain that only one active protein was present in the acetone powders. A single extraction with deoxycholate solubilized 65–80% of the total activity. An additional extraction (data not shown) solubilized about 75% of the residual activity, indicating that most, if not all, of the activity in the delipidated powders was soluble in deoxycholate. The material that precipitated at 30% saturation with $(NH_4)_2SO_4$, however, was not water-soluble. This could be interpreted to imply the presence of another tissue factor protein in each organ. Alternatively, it might simply be that undissociated lipoprotein precipitated at this concentration of $(NH_4)_2SO_4$. This concept is in accord with the high lipid content (Tables I and II, column C) of the 30% precipitates. Attempts to approach this by completely removing the phospholipids from the acetone powders resulted in preparations with much reduced total activity, so that this question cannot be resolved at the present time.

The purifications achieved after gel filtration (*circa* 700-fold for lung and 13-fold for brain) are about the same as reported for particulate preparations (18, 19), although the brain is somewhat lower. It is clear from inspection of the chromatograms (Figs. 3 and 5), and from disc gel electrophoresis (not shown) that the proteins are grossly impure. Techniques for further purification of these proteins are being explored in our laboratory.

Organ specificity of tissue factor is implied by the observed differences between lung and brain tissue factor on TEAE chromatography (Fig. 5). This question has been explored by other investigators: Weiss, Eichelberger, and Crosby (20), using relatively crude particulate preparations, found that the total extractable activity was higher in lung than in brain. In addition, they found that brain tissue factor was more dependent on the factor VII concentration in the plasma than was lung. Williams, who studied the binding of activated factor X to highly purified particulate preparations of brain and lung, found that binding to brain was much tighter than to lung (18, 19). These experiments, whereas unquestionably demonstrating differences between brain and lung particles, do not necessarily indicate organ specificity of the tissue factor protein. It is likely, for example, that even these highly purified preparations contained contaminants which could have modified the activity of tissue factor and led to the observed differences.

The TEAE chromatography described in this paper yielded reproducible results, and it may be said with certainty that the brain and lung preparations had different chromatographic characteristics. Whether this represents true organ specificity of the tissue factor proteins, or is due to subtle differences in the residual lipids cannot be answered on the basis of the available data. The possibility that these differences may have been due to nonprotein components of the preparations must be stressed as it is known that small amounts of fatty acids can alter the electrophoretic mobility of proteins (21). Presumably, then, other charged lipids (e.g., phospholipids) could alter the charge on proteins and, hence, their behavior on ion exchange chromatography.

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REFERENCES

1. Williams, W. J., and D. G. Norris. 1966. Purification of a bovine plasma protein (factor VII) which is required for the activity of lung microsomes in blood coagulation. *J. Biol. Chem.* **241**: 1847.
2. Nemerson, Y. 1966. The reaction between bovine brain tissue factor and factors VII and X. *Biochemistry.* **5**: 601.
3. Howell, W. H. 1912. The nature and action of the thromboplastic (zymoplastic) substance of the tissues. *Amer. J. Physiol.* **31**: 1.
4. Nemerson, Y. 1968. The phospholipid requirement of tissue factor in blood coagulation. *J. Clin. Invest.* **47**: 72.
5. Hecht, E. R., M. H. Cho, and W. H. Seegers. 1958. Thromboplastin: Nomenclature and preparation of protein-free material different from platelet factor III or lipid activator. *Amer. J. Physiol.* **193**: 584.
6. Hecht, E. 1967. Chemical nature of human brain thromboplastin. *Nature.* **214**: 197.
7. Nemerson, Y., and T. H. Spaet. 1964. The activation of factor X by extracts of rabbit brain. *Blood.* **23**: 657.
8. Peterson, E. A., and H. A. Sober. 1956. Chromatography

of proteins. I. Cellulose ion-exchange adsorbents. *J. Amer. Chem. Soc.* **78**: 751.
9. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the I. C. biuret reaction. *J. Biol. Chem.* **177**: 751.
10. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**: 1756.
11. Mosbach, E. H., H. J. Kalinsky, E. Halpern, and F. E. Kendall. 1954. Determination of deoxycholic and cholic acids in bile. *Arch. Biochem. Biophys.* **51**: 402.
12. Chargaff, E. 1948. Studies on the mechanism of the thromboplastic effect. *J. Biol. Chem.* **173**: 253.
13. Hvatum, M., and H. Prydz. 1966. Studies on tissue thromboplastin. I. Solubilization with sodium deoxycholate. *Biochim. Biophys. Acta.* **130**: 92.
14. Whitaker, J. R. 1963. Determination of molecular weights of proteins by gel filtration on sephadex. *Anal. Chem.* **35**: 1950.
15. Fleischer, S., H. Klouwen, and G. Brierley. 1961. Studies of the electron transfer system. XXXVIII. Lipid composition of purified enzyme preparations derived from beef heart mitochondria. *J. Biol. Chem.* **236**: 2936.
16. Duttera, S. M., W. L. Byrne, and M. C. Ganoza. 1968. Studies on the phospholipid requirement of glucose 6-phosphatase. *J. Biol. Chem.* **243**: 2216.
17. Rothfield, L., and M. Pearlman. 1966. The role of the cell envelope phospholipid in the enzymatic synthesis of bacterial lipopolysaccharide. *J. Biol. Chem.* **241**: 1386.
18. Williams, W. J. 1964. The activity of lung microsomes in blood coagulation. *J. Biol. Chem.* **239**: 933.
19. Williams, W. J. 1966. The activity of human placenta microsomes and brain particles in blood coagulation. *J. Biol. Chem.* **241**: 1840.
20. Weiss, H. J., J. W. Eichelberger, Jr., and W. H. Crosby. 1961. Studies on the differences in activity of lung and brain thromboplastin. *J. Clin. Invest.* **40**: 205.
21. Lewis, U. J., E. V. Cheever, and B. K. Seavey. 1968. Influence of fatty acids on the electrophoretic behavior of proteins with special reference to pituitary hormones and thyroglobulin. *J. Biol. Chem.* **243**: 260.