Biochem. J. (1977) **165**, 89–96
Printed in Great Britain

Purification and Some Properties of the Protein Component of Tissue Thromboplastin from Human Brain

By EIRIK BJØRKLID and EVA STORM
Institute of Medical Biology, University of Tromsø, Tromsø, Norway

(*Received* 15 *November* 1976)

The protein component of tissue thromboplastin (Factor III) from human brain was purified by extraction of a microsomal fraction with sodium deoxycholate, gel filtration of the extract on Sephadex G-100 and preparative polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The product, apoprotein III, was homogeneous by analytical polyacrylamide-gel electrophoresis, and it induced monospecific antibodies in rabbits and goat as shown by immunodiffusion and immunoelectrophoresis. Amino acid- and carbohydrate-analysis data for apoprotein III are presented. The carbohydrate moiety of the protein consists of fucose, mannose, galactose, *N*-acetylglucosamine and *N*-acetylneuraminate, amounting to a total content of 6.3g/100g. The apoprotein alone had no procoagulant activity. When Factor III was reconstituted by combining the pure apoprotein with a purified lipid fraction from the deoxycholate extract of crude Factor III, a high and optimal procoagulant activity was obtained at a phospholipid/protein ratio of 1.1g/g. Phosphatidylethanolamine alone had a weak but significant ability to restore activity, whereas phosphatidylcholine and phosphatidylserine separately had almost none. Two-component mixtures were on average more effective, and three-component mixtures far more effective, than the single phospholipids. The inclusion of a small amount of phosphatidylserine was very important for high activity.

Tissue thromboplastin (Factor III) can be isolated from different tissues as a protein–phospholipid complex that rapidly initiates the extrinsic pathway of blood coagulation by activating Factor VII. Both components of the complex are required for coagulation activity, as was recognized in some early extraction studies with sodium deoxycholate (Chargaff *et al.*, 1942; Hvatum & Prydz, 1966) and pyridine (Deutsch *et al.*, 1964). With the aid of the detergents sodium deoxycholate and sodium dodecyl sulphate, complete purification of the protein component of Factor III (apoprotein III) from human brain has been obtained. Apoprotein III exhibits procoagulant activity only when incorporated into reconstituted intact membranes (Bjørklid *et al.*, 1973*b*). The protein purified from bovine tissues by Nemerson & Pitlick (1970) or Liu & McCoy (1975*a*) also contained variable amounts of peptidase activity (Pitlick *et al.*, 1971; Nemerson & Pitlick, 1972), which is unrelated to the coagulation activity (Deutsch & Irsigler, 1964; Bjørklid *et al.*, 1973*a*; Nemerson & Esnouf, 1973; Bayer & Deutsch, 1975). Factor III apoproteins from dog tissues were purified to homogeneity by Gonmori & Takeda (1975), who used analytical sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as a test for purity.

By using a slight modification of our previously described procedure for purification of Factor-III apoprotein (Bjørklid *et al.*, 1973*a*) and a more extensive assessment of purity, we present here amino acid- and carbohydrate-analysis data for the product, and a study of the ability of different phospholipids to restore activity to the protein.

Experimental

Purification of Factor III from human brain

Crude Factor III was isolated and split into a protein and a lipid component by extraction of a microsomal fraction with 0.25% sodium deoxycholate in 0.05M-Tris/HCl/0.9% NaCl, pH7.8, and gel filtration on Sephadex G-100 by the method of Hvatum & Prydz (1969) as modified by Bjørklid *et al.* (1973*a*). A column (2.5cm×30cm) of Sephadex G-75 (superfine grade; Pharmacia Fine Chemicals, Uppsala, Sweden) was used for rechromatography of the lipid fraction in 0.05M-$(NH_4)_2CO_3$, pH8.6, and fractions (2ml) were collected. The various phospholipids were further purified as described below. The protein fraction was further purified in preparative sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The discontinuous system described by Laemmli (1970) was used. The separating gels always contained 10g of acrylamide/100ml and 0.27g of *NN'*-methylenebisacrylamide/100ml. Preparative electrophoresis was performed in a Buchler Poly-Prep

200 apparatus with a 75ml separating gel, by using a constant current of 50mA, elution rate 1ml/min, and 4ml fractions were collected. As a routine, the appropriate fractions were pooled and submitted to re-electrophoresis under the same conditions to obtain homogeneous preparations.

For analytical gels the same system (Laemmli, 1970) was used. The gels were stained overnight in 0.05% Coomassie Brilliant Blue R 250 in 20% (w/v) sulphosalicylic acid at room temperature (24°C), then destained and restained as described previously (Bjørklid *et al.*, 1973*a*).

Preparation of phospholipids

After rechromatography the phospholipids were extracted and separated by t.l.c. as described by Otnæss & Holm (1976). The isolated phospholipids were scraped off, eluted with chloroform/methanol (2:1, v/v) and evaporated to dryness in a stream of N_2. The commercial phospholipids used (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) were synthetic pure phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), and chromatographically pure phosphatidylethanolamine (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine) from *Escherichia coli* and phosphatidylserine (1,2-diacyl-*sn*-glycero-3-phosphoserine) and sphingomyelin from bovine brain. They were checked for purity by t.l.c. The isolated phospholipids and the commercial ones were dispersed in 0.25% sodium deoxycholate in 0.05M-veronal/HCl/0.9% NaCl, pH7.35 (Hjort, 1957), by sonication in an MSE 150W ultrasonic disintegrator at an amplitude of 12μm for 3min while the samples were kept in an ice-bath.

Reconstitution of Factor III and its coagulation activity

Apoprotein III was recombined with the various phospholipid fractions by mixing the appropriate volumes and removing the detergents by dialysis against veronal-buffered saline at 4°C (Hvatum & Prydz, 1969). Three changes, each of 2 litres, were used to dialyse 10–20 samples of 0.6ml each. Factor-III procoagulant activity was tested by measuring the coagulation time in a system consisting of 0.1ml of citrated platelet-poor human plasma, 0.1ml of the solution to be tested and 0.1ml of 30mM-$CaCl_2$. The plasma and test solution were mixed, incubated for 3min at 37°C and 0.1ml of prewarmed $CaCl_2$ was added (Hvatum & Prydz, 1966). Reference curves were obtained by testing dilutions of a standard crude Factor-III preparation prepared from human brain as described by Hjort (1957). The activity of this undiluted crude Factor III, corresponding to a clotting time of 13s in the one-stage assay, was taken as 100%. The same standard curve was used for obtaining the activities of phospholipids alone.

Antibody production

Monospecific antisera were raised in rabbits and a goat by multiple subcutaneous injections of completely purified apoprotein III (0.2–0.5mg) in 0.9% NaCl and mixed with Freund's complete adjuvant (Behringwerke A.G., Marburg/Lahn, Germany). After 3 weeks (rabbit) or 5 weeks (goat) the animals were given, at weekly intervals, a booster of apoprotein III in 0.9% NaCl alone or mixed with Freund's incomplete adjuvant (Behringwerke A.G.). Blood was drawn from these and from the control animals and sera were prepared. Immunoglobulin G fractions migrating as a single band in analytical polyacrylamide-gel electrophoresis at pH9.1 (Davis, 1964) were isolated by precipitation with 50%-satd. $(NH_4)_2SO_4$ followed by DEAE-cellulose chromatography as described by Fahey (1967).

A polyvalent antiserum was prepared in a rabbit in a similar way, by using crude Factor III as antigen. The immunoglobulins were isolated by precipitation with 50%-satd. $(NH_4)_2SO_4$.

Immunodiffusion and immunoelectrophoresis

Immunodiffusion was carried out at 4°C in 2mm-thick gels containing 1% agarose (Indubiose A 37; L'Industrie Biologique Française S.A., 92-Gennevilliers, France), 1% Triton X-100 (Bjerrum & Lundahl, 1974; Bjerrum *et al.*, 1975) and a veronal/HCl buffer, pH8.6 (Clarke, 1971). Wells of 4.5mm diameter were cut. The gels used for 'rocket' immunoelectrophoresis (Laurell, 1965) in addition contained isolated antibodies. Ionic detergents were partially removed (at least 90%) and 1% Triton X-100 was added to the apoprotein III-containing samples before immunoprecipitation studies.

Amino acid analysis

Factor-III apoprotein purified by repeated sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was washed free of buffer and dried in a vacuum oven. Then it was either treated with performic acid (Hirs, 1956), dried again and hydrolysed for 24, 48 and 72h in 6M-HCl at 110°C in an oil bath, or hydrolysed directly in 6M-HCl containing 2% (v/v) thioglycollic acid under N_2 (Matsubara & Sasaki, 1969) for the same time intervals. HCl was evaporated off, the hydrolysates were washed once in water and again dried by evaporation. The hydrolysates were then analysed in a JEOL JLC 6 AH amino acid analyser. Tryptophan was also determined spectrophotometrically (Edelhoch, 1967).

Carbohydrate analysis

Purified Factor-III apoprotein was washed free of buffer, and mannitol was added as internal standard. After drying *in vacuo* the preparations were treated for different time intervals with 0.5ml of methanolic

1.5M-HCl at 95°C. Re-*N*-acetylation and silylation was carried out as described by Clamp *et al.* (1971), and the silylated samples were dissolved in carbon tetrachloride and analysed in a 5700 A Hewlett–Packard gas chromatograph; columns [~3.2mm× 1.8m ($\frac{1}{8}$in×6ft)] containing Supelcoport (80–100 mesh) as support and 3% SE-30 (Supelco, Bellefonte, PA, U.S.A.) as stationary phase were used. The oven temperature was increased from 140 to 210°C at 1°C/min; the injection and detection temperatures were 250°C; the electrometer range was 1 and attenuation ×32. Colorimetric analysis of *N*-acetylneuraminic acid was performed by the method of Warren (1959), of hexosamines by that of Johnson (1971) and of fucose as described by Gibbons (1955). Galactose and glucose were measured by using galactose oxidase and glucose oxidase (Behringwerke A.G.) as described by the manufacturers.

Other methods

Proteins were concentrated and/or washed by Amicon Diaflo PM 30 filtration. Protein was measured by a modified (Schachterle & Pollack, 1973) Lowry procedure (Lowry *et al.*, 1951), by the method of Wadell as described by Murphy & Kies (1960) or by a more recent Amido Black/Millipore procedure (Schaffner & Weissmann, 1973). Two of these procedures were used on each preparation. Lipid phosphorus was measured as described by Ames (1966) or by Chen *et al.* (1956).

Results

Purification of apoprotein III

A typical elution diagram obtained by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of gel-filtered Factor-III apoprotein is shown in Fig. 1. The electrophoretic system used here on a preparative scale (Laemmli, 1970) proved superior to the system (Neville, 1971) used earlier (Bjørklid *et al.*, 1973*a*). Occasionally pure protein was obtained after the first preparative electrophoretic run, but as a routine the activity peaks from two electrophoretic runs were pooled and submitted to a second electrophoresis.

Fig. 2 shows a stained analytical sodium dodecyl sulphate/polyacrylamide gel of apoprotein III after completion of the purification procedure. Only a single band is seen. As another criterion of homogeneity, antibodies from goat and rabbit raised against such purified apoprotein-III preparations were allowed to react in immunodiffusion with purified and partly purified (eluate from the gel-filtration step) apoprotein III. The latter contained other proteins in addition to apoprotein III, but only a single, fused line was seen in each case (Plate 1*a*).

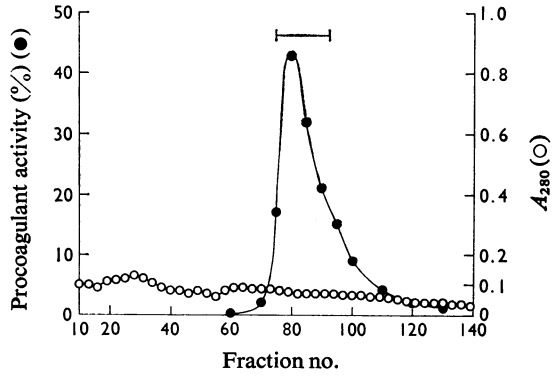


Fig. 1. *Preparative polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of an apoprotein III eluate from a Sephadex G-100 column*
●, Coagulation activity; ○, A_{280}. The pooled fractions are indicated by the horizontal bar. For further details see the Experimental section. The fraction size was 4 ml.

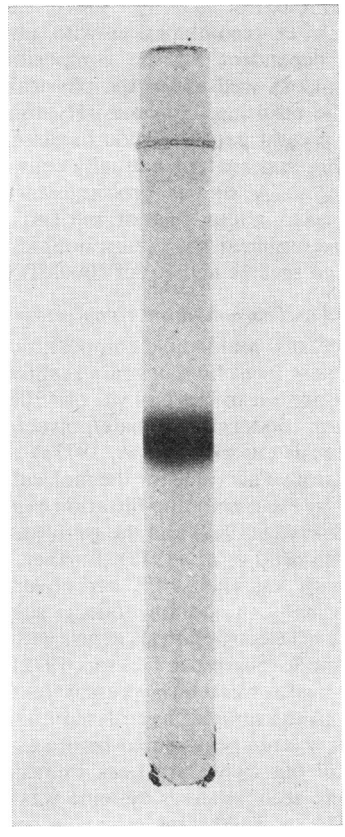


Fig. 2. *Analytical sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified apoprotein III* (25 μg)
For details see the Experimental section.

'Rocket' immunoelectrophoresis of partly purified apoprotein III in gels containing either the rabbit or the goat antibodies (Plate 1*b*) also produced single lines only. These results show that monospecific antibodies towards apoprotein III have been obtained, suggesting that the antigen used to raise these antibodies was without significant antigenic contaminants.

After incubation of crude preparations of Factor III from brain, thyroid and lungs with an equal volume of normal goat immunoglobulin (3.0mg/ml) for 1h at 37°C, all activity remained, whereas activity was completely neutralized when the monospecific goat (10.2mg/ml) or rabbit (3.2mg/ml) antibodies were used under the same conditions (E. Bjørklid, unpublished work). The antibodies raised against crude thromboplastin gave four or five precipitation lines in immunodiffusion against crude apoprotein III, whereas no arcs (except one) were seen against purified apoprotein III. This criterion, however, is not particularly sensitive.

The calculation of an exact specific activity for the purified apoprotein III is not feasible at the moment, since its biological activity, which can only be measured after recombination with phospholipid, is highly dependent on the composition of this phospholipid as well as on the physical characteristics of the resulting liposomes (Hvatum & Prydz, 1969; the present paper). Crude Factor-III preparations (3000*g* supernatants) usually contain 5–10mg of protein/ml. A similar procoagulant activity is obtained when about 5μg of purified apoprotein III/ml is recombined with phospholipid, suggesting an increased specific activity of 1000–2000-fold.

Amino acid and carbohydrate composition

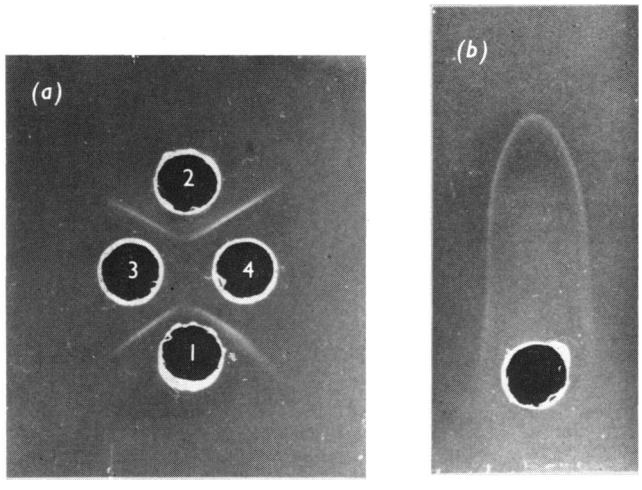
The calculated amino acid composition of Factor-III apoprotein from human brain is shown in Table 1. Approximation to the mol.wt. of 52000 obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Bjørklid *et al.*, 1973*a*) was used in the calculation. This value for the molecular weight is supported by the membrane-filtration experiments of Hvatum & Prydz (1969) and the gel-filtration experiments of Bjørklid *et al.* (1975). Further, sialoglycoproteins with less than 10% carbohydrate behave almost normally in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in gels with 10% (w/v) polyacrylamide (Segrest & Jackson, 1972). A few unidentified peaks were also present in the elution diagrams. In the samples hydrolysed under reducing conditions, a large peak eluted before aspartic acid, and a small but significant peak immediately after proline were seen, whereas cysteine was completely missing. The oxidized samples gave a peak between histidine and lysine. The protein contained 42mol% of hydrophobic amino acids, including all the amino acids more hydrophobic than serine (Nozaki & Tanford, 1971), and the content of arginine and lysine was 9mol%. No attempt was made to determine the contents of asparagine and glutamine.

Table 1. *Amino acid composition of apoprotein III*
Samples containing 300μg of protein were hydrolysed at 110°C for intervals of 24, 48 or 72h, either in 6M-HCl containing 2% thioglycollic acid under N_2 or in 6M-HCl after performic acid oxidation. For the oxidized samples and for serine in the reduced samples the values were obtained by extrapolation to zero time. For the remaining residues in the reduced samples the highest values were used. The values given in the Table are the means of those obtained under reducing and oxidizing conditions, except for half-cystine and aspartic acid (oxidizing conditions) and methionine, tyrosine and tryptophan (reducing conditions).

Amino acid	Content (residues/molecule)		(mol%)
Cys	11.1	(11)	2.6
Asp*	41.7	(42)	9.8
Met	9.3	(9)	2.2
Thr	23.9	(24)	5.6
Ser	24.3	(25)	5.7
Glu*	51.5	(52)	12.2
Pro	24.7	(25)	5.8
Gly	32.5	(33)	7.7
Ala	29.8	(30)	7.0
Val	27.5	(28)	6.5
Ile	21.1	(21)	5.0
Leu	38.1	(38)	9.0
Tyr	16.4	(16)	3.9
Phe	18.6	(19)	4.4
His	8.5	(9)	2.0
Lys	29.1	(29)	6.9
Trp	3.0 (6, 4)†	(6)†	1.5†
Arg	9.3	(9)	2.2
Total		426	

* Including asparagine and glutamine respectively.
† Obtained by spectrophotometry (Edelhoch, 1967).

The carbohydrate content of Factor-III apoprotein obtained by g.l.c. is shown in Table 2. Two terminal sugars were present, fucose and *N*-acetylneuraminate. The amount of the latter was somewhat variable in different apoprotein-III preparations. *N*-Acetylneuraminate (1–2mol/mol of protein) was detected by colorimetry, and the presence of fucose, hexosamine and galactose was also confirmed. In addition, a variable amount of glucose (0.75–8.1 mol/mol of protein) was present in the preparations. This was considered to be a contaminant in the final preparations. The total carbohydrate content of the molecule calculated from the g.l.c. analysis and the protein content was 6.3g/100g.



EXPLANATION OF PLATE 1

Double immunodiffusion (*a*) *and 'rocket' immunoelectrophoresis* (*b*) *with rabbit and goat antibodies against apoprotein III from human brain*

(*a*) The wells contained 25 μl solutions of 2.5 mg of goat immunoglobulin (1), 0.8 mg of rabbit immunoglobulin (2), 25 μg of purified apoprotein III (3) and 100 μg of protein of a gel-filtered detergent extract from crude Factor III (4). (*b*) The gel contained 2.6 mg of goat immunoglobulin/ml; 100 μg of protein of a gel-filtered detergent extract from crude Factor III was submitted to electrophoresis.

Table 2. *Carbohydrate content of apoprotein III*
The results are means±S.D. for three purified preparations isolated from two pooled brains. Each sample contained 90μg of protein. Further details are given in the Experimental section.

	Content (mol of carbohydrate/mol of protein)
Fucose	2.4±1.1
Mannose	6.0±0.7
Galactose	7.4±0.5
N-Acetylglucosamine	2.6±1.0
N-Acetylneuraminate	0.7±0.8

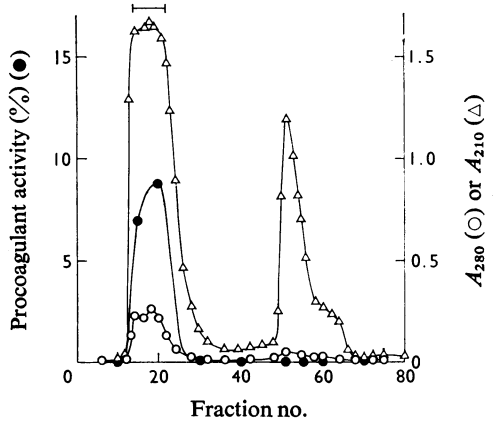


Fig. 3. *Rechromatography on Sephadex G-75 of a lipid fraction eluted from Sephadex G-100*
The fraction rechromatographed was a pool (10ml) of those lipid-containing fractions of the Sephadex G-100 eluate which were active in restoring procoagulant activity when recombined with apoprotein III. ●, Coagulation activity; ○, A_{280}; △, A_{210}. The horizontal bar indicates the pooled fractions. For further details see the Experimental section. The fraction size was 2 ml.

The calculated total number of amino acid residues in an apoprotein-III molecule is 426 (Table 1). A mol.wt. of 50000 can be calculated from the amino acid composition and carbohydrate content.

Procoagulant activity of reconstituted Factor III

To study the activity of Factor III reconstituted from purified apoprotein III and purified phospholipids, the lipid fraction obtained after gel filtration on Sephadex G-100 of the sodium deoxycholate extract was rechromatographed on Sephadex G-75 (Fig. 2). Some u.v.-absorbing (mainly at 210nm) material unable to restore procoagulant activity was thereby removed. The active peak was analysed by t.l.c. (Table 3). As expected, phospholipids, especially phosphatidylcholine and phosphatidylethanolamine, were the most abundant constituents. Cholesterol esters or triacylglycerols were not detected. The relationship between the phospholipid/protein ratio and the coagulation activity obtained when reconstituting Factor III from this whole phospholipid mixture and apoprotein III is shown in Fig. 3. During the dialysis, after recombining apoprotein III and phospholipid, some loss of phospholipids occurred. This loss increased with decreasing amounts of protein and varied from 0 to 40% in the present experiments. It was therefore necessary to determine the optimal phospholipid/protein ratio in the

Table 3. *Composition of the lipid part of Factor III*
The mixture was obtained by rechromatography on Sephadex G-75 of a pool of those lipid-containing fractions of the Sephadex G-100 eluate which were active in restoring procoagulant activity when recombined with apoprotein III. The ranges given were calculated from t.l.c. analysis of three preparations. For further details see the Experimental section.

	Content (g/100g of lipid)
Cholesterol+free fatty acids	12–23
Phosphatidylcholine	34–43
Phosphatidylethanolamine	13–22
Lysophosphatidylcholine	5–16
Sphingomyelin	7–9
Phosphatidylserine	6–7
Phosphatidylinositol	Traces

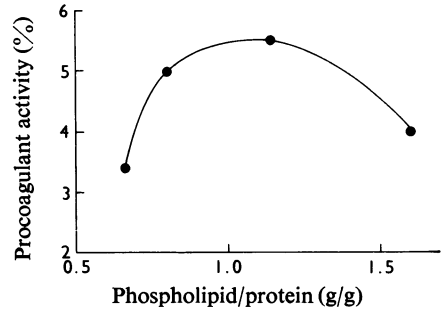


Fig. 4. *Activity of Factor III reconstituted from purified apoprotein III (variable amount) and a phospholipid fraction obtained from a Sephadex G-75 eluate (fixed amount of 10μg of phospholipid before dialysis)*
Each point represents results from five individually dialysed samples of 0.6ml each, which were then pooled and assayed for coagulation activity and phospholipids. The latter values were used in calculating the phospholipid/protein ratios. For further details see the text.

Table 4. *Procoagulant activity of reconstituted Factor III*

Factor III was reconstituted from purified apoprotein III (9 μg) and different pure phospholipids (0.1 mg of the commercial phospholipids, and 0.14 mg, 0.07 mg and 0.04 mg of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine from human brain respectively). The final volume was 0.6 ml. For further details see the Experimental section.

Phospholipid	Source	Procoagulant activity (%) Phospholipid + apoprotein III	Phospholipid alone
Phosphatidylcholine	Human brain	0	0
	Synthetic	0	0
Phosphatidylethanolamine	Human brain	1.0	0
	Escherichia coli	3.4	0
Phosphatidylserine	Human brain	0	0
	Bovine brain	0.7	0
Sphingomyelin	Bovine brain	0	0

Table 5. *Procoagulant activity of reconstituted Factor III*

Factor III was reconstituted from purified apoprotein III (9 μg in 0.3 ml) and mixtures of pure commercial phospholipids (a total amount of 0.1 mg in 0.3 ml before dialysis). For further details see the Experimental section.

Phospholipids	Procoagulant activity (%) Phospholipids + apoprotein III	Phospholipid alone
Phosphatidylcholine (PC)	0	0
Phosphatidylethanolamine (PE)	3.0	0
Phosphatidylserine (PS)	0.6	0
PC/PE (1 :2)	0.6	0
PC/PS (1 :2)	2.4	0.10
PE/PS (1 :1)	7.0	0.15
PC/PE/PS (1 :1 :1)	26	0.58
PC/PE/PS (1 :1 :0.22)	60	1.1

recombined preparations after the dialysis step, and a value of 1.1 was found, corresponding to a ratio of about 80 phospholipid molecules per apoprotein molecule.

The ability of single phospholipids to restore procoagulant activity to Factor-III apoprotein is illustrated in Table 4. The phospholipid loss during dialysis ranged from 0% (phosphatidylcholine alone) to about 40% (phosphatidylethanolamine alone), and was 6–30% for the mixtures. In view of the loss, the phospholipid/apoprotein III ratio used was about eight times higher than the optimum found for the mixture. This value is close to the phospholipid/protein ratio in crude Factor III in which several proteins are present. Sufficiently high procoagulant activities were obtained without any need to concentrate the apoprotein-III preparation. Recombination of apoprotein III and the phospholipid mixture in the same concentrations and ratio as used here with single phospholipids will yield high procoagulant activity (40–50%). Table 4 shows that phosphatidylethanolamine, both from human brain and from *Escherichia coli*, had a significant potency for restoring coagulation activity. Phosphatidylserine from bovine brain had a weak effect, whereas that purified from human brain was quite ineffective. The recombined preparations obtained with phosphatidylcholine and sphingomyelin were devoid of activity. When apoprotein III was omitted, none of the phospholipids gave any activity in the thromboplastin assay.

Combinations of two phospholipids and apoprotein gave more active thromboplastins than was the case with the single ones (Table 5). Thus phosphatidylethanolamine + phosphatidylserine was more effective than either component alone, and phosphatidylcholine + phosphatidylserine gave significant activity, although either component alone was almost ineffective. However, although phosphatidylethanolamine alone gave an active product when combined with apoprotein III, when mixed with phosphatidylcholine it gave almost no activity. The results show that phosphatidylserine is markedly more effective in the two-component mixtures than any of the other

phospholipids. The three-component mixtures were far more active than the two-component ones. A relatively small amount of phosphatidylserine (about 10% of total phospholipid) gave the most active product. This most active mixture had a composition which to some extent resembles that of the endogenous Factor-III phospholipid mixture (Tables 3 and 5), and the exogenous phospholipids gave the reconstituted Factor III an activity comparable with that of native Factor III. The relative cephalin-like activities of the phospholipid mixtures paralleled their relative activities in restoring Factor-III activity (Table 5).

Discussion

Attempts at purifying the protein component of tissue thromboplastin have been hampered by its presence in a subcellular particulate fraction. It has a marked tendency to aggregate at all stages of the purification procedure (Bjørklid *et al.*, 1973*a*). By omitting any treatment of the tissue with organic solvents, and by detaching the protein from the subcellular particles with detergents which then are present throughout the purification procedure, a homogeneous and apparently pure protein free of aggregates has been prepared. The purity has been checked with different methods by using detergents in the systems. The use of a microsomal fraction as starting material in the purification does not imply that apoprotein III is located in the endoplasmic reticulum, since the fraction contains different subcellular membranes and particles.

There are only minor differences in the amino acid mol% composition of the apoprotein III reported here and that reported for the apoprotein III of dog brain (Gonmori & Takeda, 1975) and bovine brain (Liu & McCoy, 1975*a*). The bovine preparation had a higher content of alanine and a low content of half-cysteine and tryptophan, and the dog preparation a higher content of arginine and a lower content of glycine compared with the other preparations. Although the dog preparation had an overall similarity in amino acid composition, its molecular weight differed markedly from that of the others, i.e. 80000 (Gonmori & Takeda, 1975) as against 52000 (Bjørklid *et al.*, 1973*a*) and 56000 (Liu & McCoy, 1975*a*). The content of hydrophobic amino acids (42%) in apoprotein III from human brain does not differ much from the average content in water-soluble proteins. Since the protein has a marked tendency to aggregate in detergent-free solution, these amino acids are probably clustered on the molecule to some extent. A cluster of hydrophobic amino acids on its surface might be sufficient to keep the protein dissolved in cellular membranes.

Factor-III apoprotein from human brain has a carbohydrate content of about 6%. The presence of mannose accords with the observation that Factor III binds concanavalin A (Pitlick, 1975). Preliminary isoelectric-focusing experiments (E. Bjørklid, unpublished work) indicate that the protein is microheterogeneous and somewhat acidic, and this may be partially explained by the presence of *N*-acetylneuraminate. The carbohydrates detected in apoprotein III are often found in membrane proteins, and hence their presence is consistent with the supposition that the protein is localized on cellular membranes. The carbohydrate moiety is most probably linked to the protein via asparagine. An ordinary sequence such as Asn-GlcNAc-(Gal-Man-)$_2$ agrees fairly well with our data.

Factor III reconstituted from pure apoprotein III and single phospholipids had little or no procoagulant activity. In previous experiments (Nemerson, 1969; Pitlick & Nemerson, 1970; Liu & McCoy, 1975*b*) the ability of phosphatidylethanolamine and to a lesser extent of phosphatidylcholine to restore coagulation activity was observed, whereas phosphatidylserine and phosphatidylinositol were ineffective in this respect. With our preparations too, phosphatidylethanolamine gave the best results, but phosphatidylserine was more effective than phosphatidylcholine, the latter being unable to restore any activity. Binary phospholipid systems based on these latter three phospholipids were on the average more effective than the single phospholipids, and ternary systems very much more effective. In contrast with this, Pitlick (1975) found that phosphatidylethanolamine had a better activity-restoring capacity than a mixed brain-lipid preparation or a mixture of phosphatidylserine and phosphatidylcholine. This discrepancy may largely reside in differences in the exact composition of the lipid mixtures used. Whether the lipid composition influences the optimal phospholipid/protein ratio is not known, but such an influence might explain the higher optimal ratio found with the rechromatographed lipid mixture compared with the mixture used previously (Bjørklid *et al.*, 1973*a*).

The mixing of phospholipids may cause the higher procoagulant activity by allowing a more effective distribution on the membrane surface of the different groups which participate in the binding and activation of the substrate(s) and modulate the binding and conformation of apoprotein III. Phosphatidylcholine and phosphatidylethanolamine have previously been shown to bind a water-soluble bovine apoprotein-III preparation, whereas lysophosphatidylcholine and reduced phosphatidylcholine interacted only weakly with the protein and did not restore procoagulant activity (Pitlick & Nemerson, 1970). Our results indicate that phosphatidylserine plays a special role, since a limited amount of this phospholipid, when introduced into a phosphatidylcholine+phosphatidylethanolamine mixture, increases the

activity 100-fold. Phosphatidylserine differs from phosphatidylethanolamine in having a carboxyl group that extends into the aqueous phase surrounding the membranes. Phosphatidylserine is also a necessary component for phospholipid membranes to be highly active in accelerating coagulation in the interaction of Factors II and X_a. This is due to its negative charge at physiological pH and its corresponding ability to bind Factors II and X_a through Ca^{2+} bridges (Bull *et al.*, 1972). Our experiments also indicate a high procoagulant effect of phosphatidylserine in the intrinsic system, i.e. when apoprotein III is not present in the membranes. Consistent with our observations on phosphatidylserine alone, this phospholipid or phosphatidic acid alone produces a prothrombinase complex with very low procoagulant activity (Bull *et al.*, 1972; Subbaiah *et al.*, 1976). Phosphatidic acid, which is also negatively charged at physiological pH, does not promote coagulation to the same extent as phosphatidylserine (Bull *et al.*, 1972). In addition to the charge effect, phosphatidylserine may therefore have some more specific effect on the protein–phospholipid interaction or on the subsequent enzymic proteolysis.

The kind help of R. Flengsrud, T. Holm, S. Johnsen and A. B. Otnaess is gratefully acknowledged.

References

Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118

Bayer, P. M. & Deutsch, E. (1975) *Acta Haematol.* **54**, 165–171

Bjerrum, O. J. & Lundahl, P. (1974) *Biochim. Biophys. Acta* **342**, 69–80

Bjerrum, O. J., Bhakdi, S., Bøg-Hansen, T. C., Knüfermann, H. & Wallach, D. F. H. (1975) *Biochim. Biophys. Acta* **406**, 489–504

Bjørklid, E., Storm, E. & Prydz, H. (1973*a*) *Biochem. Biophys. Res. Commun.* **55**, 969–976

Bjørklid, E., Otnæss, A. B., Storm, E., Prydz, H., Johansen, B. V. & Frøholm, L. O. (1973*b*) *Thromb. Diath. Haemorrh.* **30**, 509–518

Bjørklid, E., Storm, E., Østerud, B. & Prydz, H. (1975) *Scand. J. Haematol.* **14**, 65–70

Bull, R. K., Jevons, S. & Barton, P. G. (1972) *J. Biol. Chem.* **247**, 2747–2754

Chargaff, E., Moore, D. H. & Bendich, A. (1942) *J. Biol. Chem.* **145**, 593–603

Chen, P. S., Jr., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758

Clarke, H. G. M. (1971) *Methods Immunol. Immunochem.* **3**, 287

Clamp, F. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–344

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427

Deutsch, E. & Irsigler, K. (1964) *New Istanbul Contrib. Clin. Sci.* **7**, 89–105

Deutsch, E., Irsigler, K. & Lomoschitz, H. (1964) *Thromb. Diath. Haemorrh.* **12**, 12–34

Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954

Fahey, J. L. (1967) *Methods Immunol. Immunochem.* **1**, 321–329

Gibbons, M. N. (1955) *Analyst (London)* **80**, 268–276

Gonmori, H. & Takeda, Y. (1975) *Am. J. Physiol.* **229**, 618–626

Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621

Hjort, P. F. (1957) *Scand. J. Clin. Lab. Invest. Suppl.* **9**, 27

Hvatum, M. & Prydz, H. (1966) *Biochim. Biophys. Acta* **130**, 92–101

Hvatum, M. & Prydz, H. (1969) *Thromb. Diath. Haemorrh.* **21**, 217–222

Johnson, A. R. (1971) *Anal. Biochem.* **44**, 628–635

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Laurell, C. B. (1965) *Anal. Biochem.* **10**, 358–361

Liu, D. T. H. & McCoy, L. E. (1975*a*) *Thromb. Res.* **7**, 199–211

Liu, D. T. H. & McCoy, L. E. (1975*b*) *Thromb. Res.* **7**, 213–221

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Matsubara, H. & Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun.* **35**, 175–181

Murphy, J. B. & Kies, M. W. (1960) *Biochem. Biophys. Acta* **45**, 382–384

Nemerson, Y. (1969) *J. Clin. Invest.* **48**, 322–331

Nemerson, Y. & Esnouf, M. P. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 310–314

Nemerson, Y. & Pitlick, F. A. (1970) *Biochemistry* **9**, 5100–5105

Nemerson, Y. & Pitlick, F. A. (1972) *Prog. Hemostasis Thromb.* **1**, 1–38

Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246**, 6328–6334

Nozaki, Y. & Tanford, C. (1971) *J. Biol. Chem.* **246**, 2211–2217

Otnæss, A. B. & Holm, T. (1976) *J. Clin. Invest.* **57**, 1419–1425

Pitlick, F. A. (1975) *J. Clin. Invest.* **55**, 175–179

Pitlick, F. A. & Nemerson, Y. (1970) *Biochemistry* **9**, 5105–5113

Pitlick, F. A., Nemerson, Y., Gottlieb, A. J., Gordon, R. G. & Williams, W. J. (1971) *Biochemistry* **10**, 2650–2657

Schacterle, G. R. & Pollack, R. L. (1973) *Anal. Biochem.* **51**, 654–655

Schaffner, W. & Weissmann, C. (1973) *Anal. Biochem.* **56**, 502–514

Segrest, J. P. & Jackson, R. L. (1972) *Methods Enzymol.* **28**, 54–63

Subbaiah, P. V., Bajwa, S. S., Smith, C. M. & Hanahan, D. J. (1976) *Biochim. Biophys. Acta* **444**, 131–146

Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975