The Isolation of Factor V from Bovine Plasma

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1. A technique for the isolation of factor V from bovine plasma is described.
2. Some of the physicochemical properties of the protein have been measured and the amino acid composition has been determined.

Nolf (1908) was the first to postulate the existence of a clotting factor, which is now identified with factor V; Quick (1943) inferred the existence of a 'labile factor' in plasma, and Owren (1947) described the first patient lacking in this factor, which he called factor V. Subsequently the terms proaccelerin and accelerin (Owren, 1950), and serum accelerator globulin (Ware & Seegers, 1948), were introduced, since it had been observed that bovine factor V was more reactive in serum than in plasma. A similar increase in factor V activity was obtained when partially purified preparations were incubated with thrombin (Hjort, Rapaport & Owren, 1955; Lewis & Ware, 1954). The increase in reactivity of factor V is accompanied by a change in molecular size as judged by gel filtration (Papahadjopoulos, Hougie & Hanahan, 1964) and by a reduction in sedimentation coefficient (Hussain & Newcomb, 1963). However, in most experiments only partially purified preparations of factor V have been used, and, in some experiments, the thrombin preparations may have contained traces of activated factor X (Cole, Koppel & Olwin, 1964).

In the present paper some of the physical and chemical properties of factor V are described.

MATERIALS AND METHODS

Preparation of TEAE-cellulose† eluate. Ox blood for the preparation of plasma was collected in plastic buckets containing 0.1 vol. of 1.34% (w/v) sodium oxalate. About 12 l. of blood was collected from each animal immediately after slaughter, and care was taken to ensure that all the froth was removed from the surface of the blood. The plasma (usually about 4 l.) was obtained after centrifuging the blood at 1000g for 45 min. at 4°, and stirred with BaSO₄ (Judex, Fisons Scientific Apparatus Ltd., Loughborough, Leics.), 100 g./l. of plasma, for 15–20 min. at room temp-

erature. The plasma was freed from BaSO₄ by centrifuging at 1000g for 30 min. at 10°. The plasma was diluted with an equal volume of distilled water and sufficient N-HCl was carefully added to bring the pH to 7.0. The diluted plasma was stirred at room temperature for 15–20 min. with TEAE-cellulose (Serva Entwicklungs Labor, Heidelberg, Germany), 1.5 g. of dry powder/100 ml. of undiluted plasma. The mixture was centrifuged and the TEAE-cellulose was washed twice with a volume of 0.03 M-sodium phosphate buffer, pH 7.0, equal to that of the starting plasma, and then stirred for 15 min. with a volume of 0.4 M-sodium phosphate buffer one-fifth of that of the starting plasma. The eluate obtained after centrifuging was dialysed overnight at 4° against distilled water to lower the buffer concentration to 0.01 M.

Chromatography of TEAE-cellulose eluate. Columns of phosphorylated cellulose (Whatman P.70) were prepared by making a slurry of the cellulose in 0.5 M-sodium phosphate buffer, pH 7.0, and pouring the mixture into a glass column (10 cm. diam.) until the height of the cellulose was about 6 cm. After washing the column with 1 l. of 0.5 M-sodium phosphate buffer the column was equilibrated with 0.01 M-sodium phosphate, pH 7.0, and the height of the packed cellulose adjusted to 6 cm. The dialysed TEAE-cellulose eluate (500 ml.) was centrifuged to remove insoluble protein and poured on to the cellulose column. The fractionation was carried out at room temperature with stepwise increases in the phosphate buffer concentration at pH 7.0; the details of individual experiments are given in the text. The elution of the protein from the column was followed by measuring the extinction of the fractions at 280 mμ in a 1 cm. cell.

Buffers. Sodium phosphate buffers were made by adding NaOH to H₃PO₄ solutions to give the required pH. The molarity refers to the phosphate concentration.

Solutions of tris were adjusted to the required pH with N-HCl, and the molarity of the buffer refers to the final concentration of tris.

Phospholipid. The emulsions of Inosithin (Associated Concentrates Inc., Woodside, N.Y., U.S.A.) were prepared by dissolving 500 mg. of Inosithin in 10 ml. of diethyl ether; to this was added 10 ml. of 0.01 M-tris-HCl buffer, pH 8.4. The ether was evaporated with a stream of O₂-free N₂. The emulsion was stored at 4° and diluted for use in 0.02 M-tris-HCl buffer, pH 7.5, containing 0.14 M-NaCl.

Citrated bovine plasma. Ox blood was collected in

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† Abbreviations: TEAE-cellulose, triethylaminoethyl-cellulose; Xa, activated factor X.

0.1 vol. of 0.1M-trisodium citrate in a plastic container and then the blood was centrifuged to remove the formed elements.

Factor V-deficient human plasma. Human blood was collected into 0.1 vol. of 0.1M-sodium oxalate, and the plasma obtained from this was incubated for 36 hr. at 37° and then stored at -20°. The factor V activity was in this way reduced to 0.1% of that of normal plasma. Though the amount of prothrombin in the plasma remained unchanged, that of factor X was decreased to between 27 and 35% of normal plasma, which, however, was sufficient for the assay.

Saline extract of brain. This was prepared as described by Biggs & Macfarlane (1962).

Russell's-viper venom. Dried venom was generously provided by the Wellcome Physiological Research Laboratories, Beckenham, Kent.

Assay of factor V. To 0.1 ml. of factor V-deficient human plasma at 37° was added 0.1 ml. of the sample to be assayed, diluted in 0.02M-tris-HCl buffer, pH 7.5, followed by 0.1 ml. of a saline extract of brain, and finally 0.1 ml. of 0.025M-CaCl₂, and the clotting time was measured. A standard curve was obtained by recording the clotting times of fresh dilutions of citrated bovine plasma between 1:10, which was 28 sec., and 1:100, which was 46 sec. The clotting time of the factor V-deficient plasma in the absence of added factor V was 100-110 sec. A plot of the clotting time against dilution gave a straight line on log-log paper. By definition 0.1 ml. of citrated bovine plasma was said to contain 100 units of factor V activity. In some instances where specified the assay was modified and the saline extract of brain was replaced by a mixture of Inosithin and Xa or Russell's-viper venom.

Physical properties of factor V. The measurements except where stated otherwise were made on solutions of the protein in 0.1M-sodium phosphate buffer, pH 7.0. The electrophoretic mobility was measured in a moving-boundary apparatus (Strubin and Co., Basle, Switzerland). Sedimentation-velocity experiments were carried out on a series of dilutions of factor V and the results are expressed at infinite dilution. The molecular weight was calculated by the procedure of Trautman & Crampton (1959). The measurements were made at a rotor speed of 20410 rev./min. and the photographs were taken over a period of 1 hr. The first photograph was taken 20 min. after the rotor had attained the correct speed. Viscosity measurements were made in an Ostwald viscometer at 25°. Measurement of the densities of the buffer and protein solutions were made at 20°, in a linear density gradient, made from mixtures of bromobenzene and commercial paraffin that had been previously equilibrated with 0.2M-KBr. The gradients were calibrated with solutions of KCl. The value of the partial specific volume $\bar{v}$ was obtained from the density measurement.

Optical-rotatory-dispersion curves were plotted with a Polarimatic 62 (Bellingham and Stanley Ltd., London, N. 19) on solutions of factor V in various buffers at 18°.

Protein concentration. This was determined in a differential refractometer (Brice-Phoenix, Phoenix Precision Instrument Co., Philadelphia 40, Pa., U.S.A.).

Immuno-electrophoresis. Electrophoresis of the purified factor V preparations was carried out in 1% agar dissolved in 0.025M-sodium phosphate buffer, pH 7.0, on microscope slides (Grabar & Williams, 1955). The anti-(factor V)

antiserum was obtained from rabbits that had been injected with the TEAE-cellulose eluate by the technique of Gitlin & Janeway (1952).

Nitrogen estimations. These were carried out by the standard micro-Kjeldahl method.

Amino acid analysis. This was done on a Technicon amino acid analyser (Technicon Instruments Ltd., Chertsey, Surrey) with the elution procedure of Thomson & Miles (1964). The analyses were carried out on samples of factor V that had been hydrolysed with 6N-HCl for 22, 48, 68 and 96 hr. at 108°. Tryptophan was determined on separate samples by the colorimetric method of Spies & Chambers (1948). Cystine and cysteine were estimated as cysteic acid, on samples of factor V which were oxidized with performic acid (Hirs, 1956) before hydrolysis and amino acid analysis.

RESULTS

Assay of factor V. In the factor V assay the dilution curves given by the partially purified or purified preparations of factor V did not always give slopes parallel to that obtained when dilutions of bovine plasma were used. Further, when the partially purified or purified preparations were assayed by using mixtures of Xa, or Russell's-viper venom and Inosithin, instead of saline extract of brain, and estimated relative to citrated plasma by the various modifications of the factor V assay, the values obtained varied markedly from one type of assay to the other. For these reasons the determination of the factor V activity in the purified fractions relative to plasma was difficult to make. This discrepancy was taken as an indication of the presence of interfering substances present in the crude preparations. However, assays of different samples of factor V at the same stage of purification by each of the different types of assay gave consistent results.

Chromatography of the TEAE-cellulose eluates on phosphorylated cellulose. The fractionation of the TEAE-cellulose eluate derived from 1 l. of plasma, on a column of phosphorylated cellulose (6 cm. x 5.5 cm.), is shown in Fig. 1. Six fractions were obtained and 50% of the factor V activity added to the column was recovered in fraction 6. The distribution of factor V activity and the specific activity of each of the fractions is given in Table 1. The TEAE-cellulose eluate had a faint blue colour. This colour, which was eluted as a single band with fraction 3, was due to caeruloplasmin. In many experiments fibrin-like material was eluted towards the end of fraction 3, and persisted through to the later fractions; because of this the dead space under the column was washed with 0.15M-sodium phosphate buffer, pH 7.0, to remove any remaining strands before the elution of factor V.

For the routine separation of factor V larger columns (diam. 10 cm.) were used, the height of the packed cellulose remaining at 6 cm. The optimum

Fig. 1. Fractionation of a TEAE-cellulose eluate on a column (6cm. diam $\times$ 5.5cm. high) of phosphorylated cellulose. The chromatogram was developed at room temperature by stepwise elution with phosphate buffers, at the molarities indicated at the top of the Figure, at pH 7.0. The column was eluted at a flow rate of 540 ml./hr. and the volume of each fraction was 45 ml.

Table 1. *Distribution of factor V in the fractions obtained from the phosphorylated cellulose column*

For details of columns and fractions see Fig. 1.

Fraction no.	Factor V recovered (%)	Protein (%)	Sp. activity (units/ μ g.)	Purification*
1	0	67	—	—
2	0	1.8	—	—
3	1	7.4	—	—
4	1.5	1.4	2.0	164
5	5.0	1.8	9.8	754
6	50	2.7	53.2	4000
7	2.0	1.6	2.2	170

* Relative to pooled citrated plasma.

load of TEAE-cellulose eluate for the larger columns was 500ml. The columns were eluted with the same solvents as those used for the smaller columns, and the flow rate was increased to 1.5 l./hr. The results obtained from six experiments on the larger columns are summarized in Table 2. The recovery of biological activity of the factor V exceeded that put on the column in the TEAE-cellulose eluate. This may be due to the removal of a substance which interferes with the assay of factor V activity, or because the protein becomes more reactive on chromatography.

The most convenient way to concentrate factor V was by adsorption on and elution from a small phosphorylated cellulose column (4cm. diam. $\times$ 3cm. high). Fraction 6 in 0.3M-sodium phosphate buffer containing about 30–40mg. of protein was diluted with 2.3 vol. of cold distilled water and applied to the column at a flow rate of 8ml./min. at room temperature. The adsorbed protein was eluted with 0.3M-sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml./min. The protein concentration of the eluate was increased ten times relative to fraction 6. If further concentration was required this was achieved by ultrafiltration. The final product corrected the coagulation defect found in the plasma taken from the human blood with congenital factor V deficiency, and no other clotting factor was detected.

Analysis of this material in the ultracentrifuge showed that these preparations were contaminated with a small amount of substance with S_{20} 32s. This component was readily removed by differential ultracentrifugation.

The purified factor V on immunoelectrophoresis gave a single precipitin band at an antigen concentration as high as 8mg./ml. against the rabbit anti-(factor V) antiserum.

Stability of factor V. In preliminary experiments factor V was eluted from TEAE-cellulose with 0.5M-sodium chloride buffered with 0.15N-hydrochloric acid adjusted to pH 8.0 with tris. It was observed that these eluates gradually lost their

Table 2. *Yield of factor V and degree of purification at the various stages of preparation*

	Yield of factor V (% of starting material)						Factor V† (units/ml.)	Concn. of protein† (mg./ml.)	Purification†
	1	2	3	4	5	6			
Bovine plasma	100						1000	74	1
BaSO ₄ -adsorbed plasma	70						700	74	0.7
TEAE-cellulose	64	52	46	126	40	64	3250	3.3	73
Phosphorylated cellulose column* (fraction 6)	96	78	28	227	66	70	11000	0.14	6.000

* Column used was 10cm. diam. $\times$ 6cm. high; for details see the text.

† Average values from six preparations.

Fig. 2. Reversible inactivation of factor V in TEAE-cellulose eluates by NaCl. The eluate (▲—▲) in 0.5M-NaCl buffered with 0.15N-HCl-tris buffer, pH 8.0, was dialysed (△—△) after 8 days against 0.05N-HCl-tris buffer, pH 8.0. The two arrows indicate the period of dialysis.

activity, which could be restored by dialysing them against 0.05N-hydrochloric acid adjusted to pH 8.0 with tris (Fig. 2). In other experiments the loss in activity was prevented by dialysing freshly prepared eluates against distilled water or 0.02M-sodium phosphate buffer, pH 7.0. In view of the adverse effect of 0.5M-sodium chloride, the cellulose was subsequently eluted with 0.4M-sodium phosphate buffer, pH 7.0, and the eluate was dialysed against distilled water to lower the buffer concentration to 0.01M before chromatography.

When purified factor V was stored in 0.3M-sodium phosphate buffer, pH 7, at 4° there was a gradual loss in biological activity. In some instances the activity of these preparations could be regenerated by adsorption and elution of the protein on a small column of phosphorylated cellulose.

Solutions of pure factor V that had been frozen were completely inactive, whereas control samples kept at 4° or room temperature were fully active. However, if factor V was dissolved in 50% (v/v) glycerol in 0.01M-tris-lactic acid buffer, pH 7.5, and stored at -20° the protein retained its activity indefinitely.

Factor V dissolved in 0.02M-tris-hydrochloric

Table 3. *Effect of concentration on the sedimentation coefficient of purified factor V*

Protein was sedimented in 0.1M-sodium phosphate buffer, pH 7.0.

Concn. of protein (mg./ml.)	S_{20} (s)
10.0	5.8
9.6	6.0
8.0	6.5
4.5	7.3
0.5	8.2

acid buffer, pH 7.5, retained over 80% activity on storage at 4° for 24hr. The addition of 0.16M-calcium chloride or 0.16M-magnesium chloride to the buffer had an insignificant effect on the rate of decay of its biological activity. When 1mM-EDTA at pH 7.5 was added to the buffer in place of the metal ions the protein lost 80% of its activity after 30min. at room temperature; a similar effect was also observed with 10mM-sodium citrate buffer, pH 7.5.

Physical and chemical properties of factor V. There was considerable dependence of the sedimentation coefficient on the concentration of factor V (Table 3). At a concentration of 10mg./ml. factor V had $S_{20,w}$ 5.8s; at infinite dilution the corrected sedimentation coefficient $S_{20,w}^0$ was 8.68s.

From the measurement of the densities of factor V solutions at various concentrations a value 0.73 was obtained for the partial specific volume.

The molecular weight was determined on three separate preparations of factor V at different concentrations (4.7, 2.87 and 2.07mg./ml.); the values obtained were 294120, 287730 and 291720 respectively.

The electrophoretic mobility of the protein was measured at pH 7.0 rather than pH 8.6 because of the greater stability of the protein at the lower pH. Under these conditions the mobility was 3.3×10^{-5} v/cm.²/sec.

The optical rotatory dispersion curves were plotted at 18° in both 0.5M-sodium chloride buffered with 0.02M-sodium phosphate, pH 7.3, and in 50% (v/v) glycerol buffered with 5mM-sodium phosphate, pH 7.3. The helical content of the protein was calculated from the dispersion curves by the procedure of Moffitt & Yang (1956). In both these solvents linear plots (Fig. 3) were obtained which had b_0 0, and indicated that under these conditions the protein had a disorganized structure. However, when the protein was dialysed for 48hr. against 0.1M-phosphate buffer, b_0 60 was found, which suggests that there was a slight change in the tertiary structure of the protein.

Fig. 3. Moffit & Yang (1956) plots of factor V in different solvents at 18°. ○, Factor V (580 μ g./ml.) dissolved in 50% glycerol + 0.01M-sodium phosphate buffer, pH 7.4. ●, Factor V (340 μ g./ml.) dissolved in 0.5M-NaCl buffered with 0.01M-tris-HCl buffer, pH 7.3. △, Factor V (230 μ g./ml.) dissolved in 0.1M-sodium phosphate buffer, pH 7.4.

The amino acid content of factor V is given in Table 4. The values for those amino acids that are partially destroyed during hydrolysis were obtained by extrapolation to zero time. The value given for the amino acids that are released slowly is the maximum value obtained, there appearing to be complete hydrolysis after 96hr. The value for ammonia is given at zero time and represents the amide nitrogen and ammonia release from sources other than amino acids. Table 4 shows that the recovery of the nitrogen reacting with ninhydrin accounts for 94% of the total nitrogen applied to the analyser column. This is in contrast with the finding that the weight of the amino acid residues and ammonia accounts for only 73% of the weight of material applied to the column. The composition of the remaining 21% has not yet been investigated.

DISCUSSION

The factor V obtained by the procedure described above is a homogeneous protein fraction, as judged by the criteria of a single component on sedimentation and a single band after immunoelectrophoresis. The molecular weight 290 000 found for the preparation is considerably higher than that (180 000) obtained from sedimentation data by Hussain & Newcomb (1963) and the value (98 800) calculated by Aoki, Harmison & Seegers (1963) on the basis of amino acid analysis. On the other hand, gel-filtration studies of plasma proteins (Lewis, 1964) indicate that factor V has a molecular weight in excess of 200 000 and Papahadjopoulos *et al.* (1964), using purified preparations of factor V, arrived at the same conclusion.

Factor V has always been recognized as an unstable protein; although no specific attempt has

Table 4. Amino acid composition of bovine factor V

Amino acid	Analysis	
	(μ mole found)	(moles/ 10^5 g. of protein)
Asp	0.282	60.8
Thr	0.192	41.4
Ser	0.252	54.3
Glu	0.350	75.4
Pro	0.215	46.3
Gly	0.221	47.6
Ala	0.167	36.0
Val	0.172	37.1
Cys*	0.089	19.2
Met	0.038	8.2
Ile	0.122	26.3
Leu	0.193	41.6
Tyr	0.108	23.2
Phe	0.103	22.2
His	0.117	25.2
Arg	0.098	21.1
Trp†	0.054	11.6
NH ₃	0.397	85.5

Total wt. of amino acid residues recovered (μ g.) ... 337.6 (72.8%)

Total wt. of material applied to the column (μ g.) ... 464

Total wt. of nitrogen recovered (μ g.) ... 58.1 (94.3%)

Total wt. of nitrogen applied (μ g.) ... 61.6

* Determined as cysteic acid after hydrolysis of oxidized protein.

† Determined by the colorimetric procedure of Spies & Chambers (1948).

been made to investigate the reason for its instability, some of the observations that have been made are relevant. The stability of the TEAE-cellulose extracts depended partly on the removal of foam from the blood as it was collected from the animal. It may be that the presence of foam activates an enzyme in the plasma, which subsequently destroys the factor V.

Factor V appears to undergo slow reversible changes, as illustrated by the reactivation of preparations which have become inactive by rechromatography on phosphorylated cellulose, or by the reversible inactivation caused by the storage of the protein in 0.5M-sodium chloride.

The instability of the protein in the presence of chelating agents seems to imply that the structural integrity depends on the presence of a bivalent metal ion. Similar observations have been made by Blombäck & Blombäck (1963), who found that partially purified preparations of human factor V are best stabilized with Mg^{2+} , Ca^{2+} or Sr^{2+} ions, whereas other bivalent metal ions were less effective.

The failure to reactivate factor V with Ca^{2+} or

Mg²⁺ which has been inactivated by chelating agents supports the idea that the removal of the metal ion results in a structural change in the protein.

The results of the amino acid analysis show that factor V contains a large proportion of hydrophilic residues, as would be expected since the protein is soluble in distilled water. There is not good agreement between the analysis published by Aoki *et al.* (1963) and that obtained here, but this is likely to be due to contamination since their material was considerably less pure. The most noteworthy feature of the analysis is that, though amino acid and ammonia nitrogen recovered from the columns accounts for 95% of the total nitrogen applied, the total weight of the amino acid residues recovered corresponds to only 75% of the starting material. It may be that the affinity of factor V for hydrophobic surfaces is determined by this residual material. This residual material may also be the reason why factor V, which is an anion at neutral pH, is firmly bound to a cation-exchanger.

A further anomalous property of this preparation was demonstrated by its behaviour on columns of Sephadex G-100 or G-200. With the same procedure as Papahadjopoulos *et al.* (1964), the protein was not readily eluted from Sephadex, as would have been expected from its molecular weight, but was only slowly eluted over a period of days, which suggests some form of interaction with the gel.

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