protein preparation and from non-uniform distribution of mass along the length of the myosin rod. It is also possible that as yet incompletely understood charge and salt effects (*e.g.*, Pedersen, 1958; Wallis and Record, 1962), which may affect sedimentation and light scattering measurements differently, play a role.

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Heterogeneity of Human Fibrinogen*

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Chromatographic fractionation of human fibrinogen on DEAE cellulose was carried out with use of a continuous salt and *p*H gradient. The elution patterns revealed two major peaks and, generally, a small third peak, all of which contained clottable protein. This heterogeneity was confirmed by rechromatography and was observed in Blombäck fraction I-4, in fibrinogen prepared by the method of Laki, in Cohn fraction I, in any of these preparations after lysine treatment to remove plasminogen, as well as in whole human plasma. The chromatographically separated components did not differ ultracentrifugally (all sedimenting as single boundaries of $s^0_{20,buffer} = 7.5$ *S*) or immunologically, nor were they significantly different with respect to solubility in ethanol solutions, clotting times, $A^{1\%}_{1cm}$ at 280 mμ, ultraviolet spectrum, or *N*-terminal amino acids. They did show small differences in electrophoretic mobility at *p*H 5.5 or 8.6, the component of lowest anionic binding capacity, which amounted to 85 ± 2% of the fibrinogen of various preparations, having the least negative charge.

With the availability of a method for the preparation of human fibrinogen free of plasminogen (Mosesson, 1962), it became important to learn whether the lysine treatment involved in this purification induced any permanent changes in the fibrinogen. Chromatographic studies revealed that, although the elution patterns of treated and untreated fibrinogens were identical, both contained two major peaks of clottable protein (Mosesson and Finlayson, 1962). The present work was carried out to confirm this apparent heterogeneity and was extended to include studies of the nature of the components.

MATERIALS AND METHODS

Materials.—Single donor or pooled human plasma was frozen within 24 hours after blood collection into ACD.[1] Fraction I was prepared according to method 6 of Cohn *et al.* (1946). From this, purified fibrinogen was prepared by the method of Laki (1951) or that of Blombäck (Blombäck and Blombäck, 1956) (fraction I-4). Plasminogen was removed from these preparations by precipitation of the fibrinogen thrice in the presence of 0.1 M lysine (*p*H 7.0, ionic strength 0.15) at an ethanol concentration of 7% (Mosesson, 1962).

For certain studies fibrinogen was freeze dried from 0.3 M NaCl and reconstituted with water to the original volume before use. Purified fibrinogen was usually kept as a 1–2% solution in 0.3 M NaCl. All samples were stored at −20° until used.

The clottability of Blombäck fraction I-4 was greater than 95%; that of fibrinogen prepared by the method

* A summary of these results has been presented at the Ninth Congress of The International Society of Hematology.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; ACD, acid citrate dextrose anticoagulant, NIH solution A; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

of Laki was 90–92%. The values were 1–5% higher for the lysine-treated preparations. Cohn fraction I was 60–70% clottable, whereas lysine-treated fraction I was approximately 95% clottable.

Chromatography.—Protein samples were dialyzed at 5° against "starting buffer" for at least 24 hours. At the end of this time they were subjected to column chromatography on DEAE cellulose by the method previously described (Finlayson *et al.*, 1960), utilizing a deep concave gradient from starting buffer (0.005 M H_3PO_4, 0.039 M Tris, *p*H 8.6) to final buffer (0.5 M H_3PO_4, 0.5 M Tris, *p*H 4.1). The small amount of material which precipitated during the dialysis of some preparations was suspended in the supernate before the sample was placed on the column. All chromatography was carried out at 6°; the fractions obtained were read at 280 mμ in a Beckman DU spectrophotometer.

Paper Electrophoresis.—Fractions from the column were concentrated by negative pressure dialysis at 2° against either 0.3 M NaCl or Tris-acetate-NaCl buffer (*p*H 8.0).[2] These concentrated fractions, as well as appropriate aliquots of unchromatographed preparations, were subjected to paper electrophoresis in a Spinco model R cell with either barbital buffer, *p*H 8.6, ionic strength 0.075, or Tris acetate buffer, *p*H 5.5, ionic strength 0.05 or 0.075. Electrophoresis was carried out at room temperature on Whatman 3MM filter paper; a potential of 75 or 100 V was applied for 18 hours, after which the strips were stained with bromphenol blue and densitometry done with a Spinco Analytrol.

Ultracentrifugation.—Solutions of unchromatographed fibrinogen dialyzed against Tris-acetate-NaCl buffer or chromatographic fractions concentrated by negative pressure dialysis against this buffer were centrifuged at 20° in a Spinco model E ultracentrifuge. Protein concentrations ranged from 0.03 to 1.5%.

Moving Boundary Electrophoresis.—Appropriate chromatographic fractions were pooled, the fibrinogen was precipitated with ethanol at 0° to −2°, and the precipitated protein was redissolved in 0.3 M NaCl. These solutions were dialyzed against barbital buffer, *p*H 8.6, ionic strength 0.10, and subjected to moving boundary electrophoresis at 1° in an Aminco model B apparatus. Solutions of unchromatographed fibrinogen were analyzed in a similar manner.

N-Terminal Amino Acid Analysis.—Analyses of the *N*-terminal amino acids present were done by the dinitrofluorobenzene method (Fraenkel-Conrat *et al.*, 1955). Paper chromatography of the dinitrophenyl amino acids was performed in a toluene-2-chloroethanol-pyridine system. When a second dimension was run, the 1.5 M phosphate (*p*H 6) system was utilized.

Clottable Protein.—Two methods of estimating clottable protein were employed, both modifications of the spectrophotometric method used by Laki (1951). Method A was used when the clottable protein concentration was sufficiently high that dilution with buffer to achieve the desired conditions of *p*H and ionic strength for clotting did not reduce the final concentration below 0.05%. This method entailed the dilution of the sample with 0.05 M sodium phosphate buffer (*p*H 6.1), coagulation of 0.5-ml aliquots with human thrombin (0.1 ml; 20 NIH units/ml),[3] incubation at room temperature for at least 1½ hours, and subsequent removal of the synerized clot by means of a nichrome wire loop. The clot supernates were further diluted with saline (2.4 ml) which was pipetted in such a way as to wash the clots. The supernates were read at 280 mμ in a Beckman DU spectrophotometer, and their absorbances were compared with those of suitably diluted unclotted aliquots. Corrections for the thrombin used were negligible, owing to the high specific activity of the preparation.

Method A was the method of choice wherever applicable, but it was often difficult to apply to the eluate from a chromatographic column, since the concentration of clottable protein was relatively low and the *p*H and ionic strength varied throughout the elution scheme. Dialysis of individual fractions against buffer was sometimes employed, but proved to be a tedious process in view of the number of determinations to be made; even then low protein concentration posed difficulties. Negative pressure dialysis for concentrating fractions was impractical except for early comparative studies when method B was being developed. Concentration by precipitation with ethanol carried with it the possibilities of incomplete precipitation and/or recovery.

Accordingly, method B was developed. It entailed the dilution of the samples to the same conditions of *p*H and ionic strength as in method A plus the addition of a highly purified fibrinogen preparation (clottable protein by method A greater than 97%) to bring the clottable protein to the optimal concentration range. Appropriate corrections were applied to eliminate the absorbance in the supernate due to the non-clottable protein of the added fibrinogen. A series of samples was analyzed by method B; method A was then used to analyze aliquots of these same samples after concentration by negative pressure dialysis. Results obtained by the two methods agreed within 2%. Values are reported to the nearest 0.1%.

The advantages of method B were the rapidity with which a column could be screened and the sensitivity which enabled precise determinations on eluates whose absorbances were as low as 0.030. When the absorbance of the clottable protein in a chromatographic fraction was so low (*e.g.*, in the case of plasma or in the latter part of an elution pattern) that dilution precluded measurements, samples were dialyzed rather than diluted and were then determined by method B.

Thrombin Clotting Times.—Thrombin clotting times were determined at *p*H 7.0 in a medium which was 0.01 M in phosphate and 0.135 M in NaCl. For each series of determinations, a thrombin concentration which gave a clotting time of 13–17 seconds was chosen. Several concentrations of each sample of fibrinogen were then clotted with this level of thrombin. The time necessary for the formation of a visible fibrin film on a nichrome wire loop was taken as the clotting time. Each determination was done in triplicate.

Experimental and Results

Chromatographic Separations.– The chromatography of Blombäck fraction I-4 revealed two major peaks and a small shoulder, all containing clottable protein (Fig. 1). Neither freeze drying nor treatment with lysine or ε-aminocaproic acid brought about any change in the elution pattern. Rechromatography of peaks 1 and 2 (Fig. 1) after dialysis against starting buffer demonstrated that this heterogeneity was real and not a function of the gradient employed. The elution pattern of rechromatographed peak 1 was a single symmetrical peak, whereas that of peak 2 exhibited two peaks in the positions of those in the original chromatogram (Fig. 1).

[2] Tris-acetate-NaCl buffer = 0.05 M Tris in 0.275 M NaCl, of which the *p*H had been adjusted to 8.0 with acetic acid; ionic strength = 0.3.

[3] The human thrombin was prepared by Dr. D. L. Aronson of this Division (unpublished method).

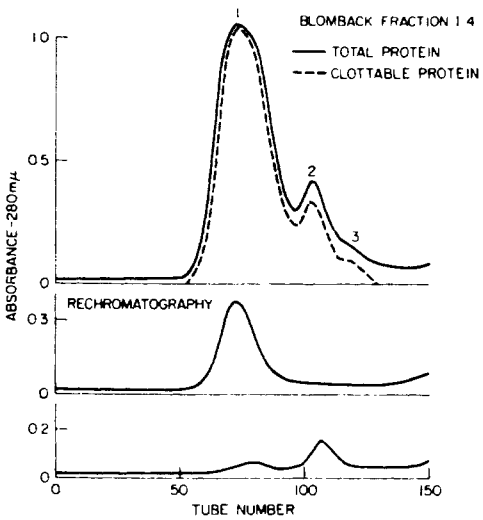

FIG. 1.—Chromatography of Blombäck fraction I-4. Each tube contained 12 ml of eluate. Horizontal lines indicate the tubes pooled for rechromatography.

Peak 1 constituted 77% of the total absorbance measured in the elution pattern of fraction I-4. Rechromatography of peak 2 showed that 34% of this material was fibrinogen of low anionic binding capacity (*viz.*, peak 1). Thus it could be calculated that peak 1 amounted not to 77%, but rather to 77 + (0.34)(23) or 85% of the total fibrinogen. The variation among all samples investigated in this manner was ±2%. This calculation is believed to be correct since the values of $A_{1\,cm}^{1\%}$ at 280 mμ, determined as described below, agreed closely for peaks 1 and 2. Moreover, the ultraviolet spectra of peaks 1 and 2 were identical. The chromatographically separated components were precipitated with ethanol, redissolved in 0.3 M NaCl, and dialyzed against this solution. Protein concentrations were then determined with the aid of a Brice-Phoenix differential refractometer, the final dialysate being used as the blank. Absorbance was measured in 1-cm cuvets at 280 mμ in a Beckman DU spectrophotometer. In view of the good agreement of the $A_{1\,cm}^{1\%}$ values of various preparations, the average figure of 15.5 was accepted for all further computations.

Fibrinogen prepared according to Laki exhibited an elution pattern very similar to that of fraction I-4 (Fig. 1) except for a small amount of non-clottable material in the early portion of the pattern (γ-globulin) and in peaks 2 and 3. Lysine treatment removed the non-clottable protein which had appeared in the early part of the pattern. The elution pattern of lysine-treated fraction I closely resembled that of lysine-treated Laki fibrinogen and, except for a somewhat higher amount of non-clottable protein in peaks 2 and 3, did not differ greatly from that of fraction I-4.

Fraction I (before lysine treatment) yielded many chromatographic peaks, but the distribution of clottable protein showed only the two major components with a third small peak following (Fig. 2). It is interesting to note that when the proportions of fibrinogen in peaks 1–3 were determined by both clottability and densitometry after paper electrophoresis, the figures agreed within 6%. These results were obtained irrespective of whether the fraction I was prepared from single donor or pooled plasma.

Peak 1A (Fig. 2) contained relatively little clottable protein. Most of this non-clottable material (largely albumin) as well as some of the protein eluted by the early part of the gradient could be removed by washing

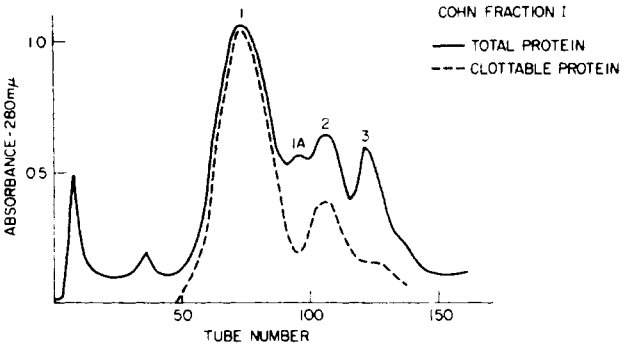

FIG. 2.—Chromatography of Cohn fraction I. Each tube contained 12 ml of eluate.

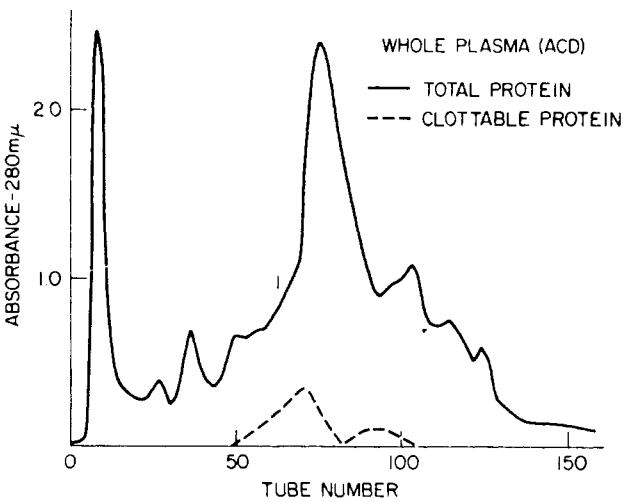

FIG. 3.—Chromatography of whole human plasma. Each tube contained 12 ml of eluate.

with glycine or citrate. The appearance of the elution pattern of glycine- or citrate-washed fraction I was therefore intermediate between those of fractions I and I-4.

Since the two major peaks of fibrinogen were seen in all preparations, it became of interest to determine whether they could be detected in whole plasma. Twenty-five ml samples of plasma were chromatographed. The elution patterns, as expected, did not differ appreciably from that of serum separated with the same gradient (Yokoyama *et al.*, 1961) except for the higher absorbance in the region designated 1 (Fig. 3). Clottability measurements showed that the two components were indeed present in whole plasma, evidently in the same relative proportions observed in purified fibrinogen preparations (Fig. 3). This was true for both single donor and pooled plasma.

When purified fibrinogen samples were subjected to chromatography, recoveries ranged from 50% to greater than 92% and averaged about 75%. The shape of the elution pattern and the calculated proportions of the components were the same regardless of the recovery. After gradient elution had been completed, an additional 5% of the fibrinogen could be eluted simply by passing starting buffer through the column. When this material was dialyzed and subsequently rechromatographed, it gave an elution pattern indistinguishable from that of fraction I-4.

Other gradients, employing Tris phosphate, Tris acetate, or NaCl buffered with these solutions, were used for the chromatography of fraction I-4. Elution patterns similar to that shown in Figure 1 (*i.e.*, two

peaks or two peaks plus a small shoulder) were obtained.

Examination of Chromatographically Separated Components.—Ultracentrifugation of chromatographic peaks 1–3 from fraction I-4 revealed a single boundary, as did ultracentrifugation of fraction I-4 itself. Furthermore, the chromatographically separated fibrinogens all sedimented at the same rate as the unchromatographed preparation, the extrapolated value, $s^0_{20,\text{buffer}}$ being 7.5 *S*. The value for $s^0_{20,w}$ was then estimated to be 8.0 *S*.

The chromatographic fractions of Blombäck fibrinogen were immunologically identical. In Ouchterlony agar-diffusion experiments[4] using rabbit antiserum prepared against fraction I-4, the precipitin lines formed by peaks 1 and 2 and unchromatographed fraction I-4 fused. Moreover, antiserum which had been absorbed with peak 1 gave no reaction against peak 2.

It was thought that the chromatographically distinguishable components might exhibit solubility differences in ethanol solutions. Fraction I-4 was therefore dissolved in phosphate-buffered saline (*p*H 7, ionic strength 0.15) and reprecipitated at −2° with ethanol in a stepwise fashion. When, as determined by absorbance at 280 mμ, 50% of the protein had been precipitated (ethanol concentration approximately 2.5%), that fibrinogen which remained in solution ("late-precipitating I-4") was precipitated as a single fraction by raising the ethanol concentration to 10%. The chromatographic elution pattern of the "late-precipitating I-4," however, was identical to that of Blombäck fibrinogen (Fig. 1). This finding was borne out by the fact that solubility curves (cumulative protein precipitated versus ethanol concentration) of peaks 1–3 were not significantly different, whether compared with each other or with that of fraction I-4.

Thrombin clotting times were determined for various samples from several chromatograms of plasminogen-free fibrinogen. Pooled fractions were concentrated by precipitation from a 10% ethanol solution at 0° to −2° (*p*H 6.0–7.0), reconstituted in 0.3 M NaCl, and dialyzed against three changes of this solution before assay. These samples were compared with unchromatographed fibrinogen which had been similarly dialyzed and diluted to the conditions described under Methods. No significant differences in clotting times were observed.

N-terminal amino acids of peaks 1 and 2 were compared with those of unchromatographed fibrinogen. No differences were detected. Although the major *N*-terminal amino acids identified were alanine and tyrosine (thus confirming the work of Blombäck and Yamashina, 1958), small amounts of glycine were found in all samples, as well as even smaller amounts of other amino acids. Bergström and Wallen (1961), using the phenylisothiocyanate method, have reported small amounts of glycine among the *N*-terminal amino acids of bovine fibrinogen.

Paper electrophoresis at *p*H 8.6 indicated slight differences in mobilities of the chromatographically separated fibrinogens. Fractions appearing progressively later in the elution pattern showed successively slightly greater mobilities toward the anode, though even the fastest (peak 3) moved at only half the rate of the β-globulin in whole plasma. Paper electrophoresis was then carried out at *p*H 5.5. Under this condition the protein showed considerable tailing, but the differences between the chromatographic peaks could be demonstrated consistently. Migration of the fibrinogens was toward the cathode (probably largely under the influence of endosmosis), peak 1 moving the greatest distance and peak 3 remaining near the origin. These observations were extended by performing immunoelectrophoresis[4] at *p*H 8.6; the differences seen in experiments at this *p*H on paper were confirmed.

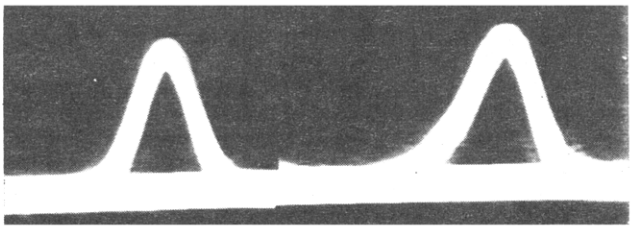

FIG. 4.—Electrophoresis of fibrinogen preparations. *Right*, Blombäck fraction I-4. *Left*, a pool of fractions from the center of peak 1. Protein concentration was 0.6–0.7%. Photographs show descending boundaries after 300 minutes of migration. Migration was toward the left (anode).

These differences in mobility during zone electrophoresis are consistent with the consideration that the main component (peak 1) bears the least negative charge, manifested in ion exchange chromatography by the lowest anionic binding capacity. Thus the fact that 15 ± 2% of the fibrinogen bears a greater negative charge than does peak 1 offered an explanation for the skewing of the schlieren pattern of fibrinogen toward the anode during moving boundary electrophoresis (Blombäck and Blombäck, 1956; Caspary and Kekwick, 1957). To test this, fraction I-4 and peak 1 were subjected to moving boundary electrophoresis for 5 hours, with a current of 12.1 ma. Although peak 1 exhibited a slight degree of skewness, it was far more symmetrical than fraction I-4. Moreover, a narrow pool of fractions taken from the center of peak 1 (such as that used for rechromatography in Figure 1) showed even less asymmetry (Fig. 4).

Bovine Fibrinogen.—For comparison, a sample of bovine fibrinogen prepared by the method of Laki (1951) from bovine fraction I[5] was chromatographed. At least 93% of the protein was recovered. Unlike that of human fibrinogen, the elution pattern consisted of a single peak (emerging somewhat later than peak 1 of human fibrinogen) with a small shoulder on the descending side; the distribution of clottable protein showed only a single peak with no shoulder.

DISCUSSION

Several investigators have subjected fibrinogen to chromatography. Brada (1957) obtained subfractions of bovine fraction I by stepwise elution from calcium phosphate columns. Prager *et al.* (1960) chromatographed Blombäck fibrinogen on DEAE cellulose using gradient elution in the presence of 0.1 M urea. The fact that a single broad peak emerged under these conditions led them to suggest that the heterogeneity seen in fraction I (coagulable protein in 3 to 5 peaks) was due to the interaction of fibrinogen with non-clottable proteins.

Godal and Lüscher (1960) studied the chromatographic behavior of human fibrinogen preparations under various conditions. Although certain of our results agree with observations of these workers, the present findings do not substantiate their conclusions. They stated that it was impossible to chromatograph

[4] These experiments were carried out in association with Dr. Mitsuo Yokoyama of the National Institute of Neurological Diseases and Blindness, for whose cooperation the authors express their gratitude.

[5] Pentex Lot No. 20616.

fibrinogen with good yields at temperatures lower than 10–12°. Our chromatography was carried out at 6°, but nevertheless the average recovery (75%) was equal to that obtained by Godal and Lüscher when chromatography was done (presumably at 10–12°) without EDTA. Moreover, in many cases our yields were in excess of 85%. Secondly, these authors concluded that human fibrinogen was a homogeneous substance, despite the fact that they obtained two peaks of clottable protein by gradient elution of fraction I from DEAE cellulose.

Hartley and Waugh (1960) have demonstrated that bovine fibrinogen is heterogeneous; however, it is uncertain how heterogeneity revealed by chromatography is related to that shown by classical solubility studies. The slightly asymmetrical electrophoretic pattern of peak 1 as well as the presence of several *N*-terminal amino acids indicates that the material in a single chromatographic peak does not behave as a completely homogeneous protein. In fact, even chromatography itself might be capable of revealing further heterogeneity. Single chromatographic peaks of bovine (Hartley *et al.*, 1962) and human[6] serum albumin can represent summations of chromatographically distinguishable components.

On the other hand, the possibility must be considered that the heterogeneity demonstrated in the present work might reflect the properties of one type of fibrinogen molecule to which compounds with various numbers of charged groups are bound. For example, recent work by Laki and Mester (1962) indicates that the carbohydrate moiety is not only an integral part of the bovine fibrinogen molecule but is apparently necessary for proper clotting. Measurements of the sialic acid content[7] of peaks 1 and 2 were therefore made, but no consistent, significant differences were found. As stated above, any such difference between the two peaks was not sufficient to cause a difference in their thrombin clotting times.

[6] Finlayson, J. S., and Suchinsky, R. T., unpublished data.

[7] Sialic acid analyses were kindly done by Dr. Leonard Warren of the National Institute of Arthritis and Metabolic Diseases using the thiobarbituric acid method (Warren, 1959)

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