Heterogeneity of Bovine Fibrinogen and Fibrin*

DEANE F. MOSHER* AND ELKAN R. BLOUT

From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

We have attempted to relate the heterogeneity found when native bovine fibrinogen, which is known to have the subunit structure \( \alpha\alpha\beta\beta\gamma\gamma \), was chromatographed on DEAE-Sephadex A-50, to electrophoretic differences among the constituent polypeptide chains and differences in the content of charged groups.

Bovine fibrinogen, purified from the blood of individual animals, eluted from DEAE-Sephadex A-50 as three major peaks, each of which appeared heterogeneous. Two \( \gamma \) chains were distinguished by polycrylamide gel electrophoresis of the reduced, \( S \)-carboxymethylated derivative of unchromatographed fibrinogen in \( 8 \) M urea at pH 8.6. Fibrin from the first major chromatographic peak contained only the more cationic \( \gamma \) chain. Fibrin from the second peak contained equal amounts of the two \( \gamma \) chains as judged by densitometry of stained gels. Fibrin from the third peak contained predominantly the more anionic \( \gamma \) chain. These findings indicate that separation into the three major peaks is due to the three possible combinations of two different \( \gamma \) chains in native fibrinogen. Since a Ferguson plot of the relative mobilities of the two \( \gamma \) chains versus gel concentration in \( 8 \) M urea at pH 9.5 yielded parallel lines, and since the two \( \gamma \) chains were not separated by polycrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate at pH 7.0, it is likely that the two \( \gamma \) chains differ in charge but not in size. Analyses of fractions within each of the major peaks suggest that heterogeneity within the peaks is due to differences in phosphate content, which varied from 0.6 to 3.8 moles of phosphate per mole of fibrinogen.

These observations indicate that within an individual animal there are as many as 30 different fibrinogen molecules, differing either in the composition of \( \gamma \) chains or in the content of bound phosphate.

---

* This research was supported in part by United States Public Health Service, National Institutes of Health Research Grants AM07300 and HL 48216.02.

† Present address, United States Army Medical Research Institute of Infectious Diseases, Frederick, Md. 21701.

‡ This nomenclature for the three chains was recommended by the Committee on Nomenclature of the International Society on Hemostasis and Thrombosis (Oslo, 1971).
further purified using the method of Glover and Shaw (18). U.S. standard thrombin, Lot B3, was used as a standard of activity (19). Chromatography columns were constructed from polymethacrylate tubes in which the resin beds were supported by porous polyethylene discs.

**Purification of Fibrinogen**—Fibrinogen from the plasma of individual cows was prepared using a modification of the method of Capet-Antonini (20). Eight parts of blood, spurting from eight cows, was prepared using a modification of the method of Mulloy and Shaw (18). Polymethylacrylate tubes in which the resin beds were supported by porous polyethylene discs.

The great vessels of a freshly killed animal, were mixed with 1 part of 0.1% of Capet-Antonini (20). Eight parts of blood, spurting from eight individual cows, was prepared using a modification of the method of Capet-Antonini (20). Polymethylacrylate tubes in which the resin beds were supported by porous polyethylene discs.

The resin beds were washed several times with 25% saturated ammonium sulfate, and dissolved in 0.05 M sodium phosphate, 0.01 M sodium-EDTA, pH 6.6. A second, identical ammonium sulfate precipitation yielded a tenacious, white precipitate which was dissolved in 0.01 M Tris, 0.1 M sodium chloride, pH 7.4, and dialyzed at 4° against 5 volumes of the same buffer. A small amount of precipitate was removed by centrifugation. The solution was cooled to 0°-10°, and c-ACA and ethanol were added to give 0.1 M and 7% solutions respectively. The precipitate was collected by centrifugation and dissolved in 0.01 M Tris, 0.01 M sodium-EDTA, 0.1 M sodium chloride, pH 7.4. The fibrinogen solution was frozen and stored at -30°.

Centrifugation was done in a Sorvall RC-2B refrigerated centrifuge using GSA or GS3 rotors. Protein precipitates were collected by centrifugation at 5000 × g for 20 min at 4°. Protein solutions were handled in polycarbonate or polyethylene containers. Dialysis tubing was prepared by heating the tubing successively at 80° in 0.1 M sodium carbonate, 0.1 M sodium-EDTA, 0.1 M sodium chloride, and distilled water. pH was measured at 25° using a Radiometer pHm 29 pH-Meter. The pH values quoted for the various buffers are not corrected to the temperature at which the buffers were used.

Chromatography—Fibrinogen to be chromatographed was thawed and dialyzed at 4° against 0.05 M sodium phosphate, 0.01 M sodium-EDTA, pH 7.4. The solution was cleared of a small amount of precipitate by centrifugation and charged onto DEAE-Sephadex A-50 equilibrated with phosphate-EDTA buffer. The protein was eluted with a linear 0 to 0.20 M sodium chloride gradient made up in phosphate-EDTA buffer. Chromatography of large amounts of protein was done in a jacketed column, 5 × 80 cm, cooled to 3° with a Lo-Temptrol 154 constant temperature bath (Precision Scientific Co.). Smaller samples were chromatographed in columns, 1.2 × 25 cm, constructed so that four columns could be developed in parallel from the same gradient reservoirs. The tops of these columns opened into a common chamber with a volume of approximately 5 ml, and the chamber was connected to the gradient reservoirs. It was found that if the columns were packed simultaneously to the same height using the same batch of resin, the flow rates and the conductivities of the effluents during gradient elution were identical, and duplicate samples of purified fibrinogen from single animals gave identical elution profiles. The A280 was measured with a Beckman DB spectrophotometer and conductivity with a Seriess conductivity bridge, model RCM 15B1. Fractions were pooled and concentrated by precipitating the protein with 33% saturated ammonium sulfate at 4°.

**Carboxymethylation of Fibrin**—To remove thrombin and non-clottable proteins, fibrin was formed and eluted in 10- or 20-m1 plastic syringes. A polyethylene disc cut to the internal diameter of the syringe was placed in the bottom of the barrel, and polyethylene tubing was fitted over the hub so that flow could be controlled with a clamp. The syringe was placed upright and partially filled with 1 mM Tris, 0.14 M sodium chloride, pH 6.9. The buffer was allowed to flow until the level was just above the disc. The syringe was then filled with fibrinogen solution in Tris-sodium chloride buffer, and the fibrinogen was clotted with thrombin, 1 U.S. unit per ml. After 4 hours, Tris-sodium chloride buffer was layered on top of the clot, the top of the syringe was closed with a rubber stopper equipped with a plastic fitting and inflow tubing, and the clot was eluted with buffer from a reservoir until the thrombin concentration of the effluent fell to 10-3 to 10-4 U.S. units per ml. At room temperature and fibrin concentrations above 2 mg per ml, the bed of fibrin remained intact while buffer was passed through it at flow rates of 0.1 to 0.5 ml per min and pressure heads of 10 to 20 cm of water.

Synecmosis of the eluted clot was induced by disturbing the clot with a spatula, the fibrin was dissolved in 1 M sodium bromide, 0.07 M sodium acetate, pH 5.0, and the soluble fibrin was dialyzed for 72 hours against at least six changes of 700 to 1000 volumes of 2 M acetic acid. The clear fibrin solution was lyophilized. Fibrin was reduced and carboxymethylated using the method of Murano et al. (13).

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate at pH 7.0 was performed in 5% gels using the method of Weber and Osborn (21). Prior to electrophoresis, samples were incubated for 10 min at 100° in 4 M urea, 1% sodium dodecyl sulfate, and 1% β-mercaptoethanol. Electrophoresis in 8 M urea was performed in gels of the following composition: 5% (w/v) acrylamide, 0.17% (w/v) N,N'-methylenebisacrylamide, 0.04% (v/v) N,N',N'-tetramethylethyenediane, 0.25 mg per ml of ammonium persulfate, 8 M urea, and 0.40 M Tris-chloride, pH 8.6. Samples were dissolved in 10 M urea, 0.01 M sodium-EDTA, 0.1 M glycerol, pH 9.6 (22), and subjected to electrophoresis for 135 min at 4 mA per gel. The reservoir buffer contained Tris, 0.6 g per liter, and glycerine, 2.88 g per liter. To determine relative mobility as a function of gel concentration, multiphasic Buffer System A of Rodbard and Chrambach (23) was used, the upper and lower gels being made 8 M in urea. Gels were stained with Coomassie brilliant blue and destained by diffusion (21). Densitometer tracings were made on a Photovolt scanner.

**Analytical Procedures**—Fibrinogen concentration was determined spectrophotometrically, using an absorption coefficient at 280 nm of A280 = 1.523 (24), or by the method of Lowry et al. (25), using unchromatographed fibrinogen as a standard. Clottable protein was estimated using the method of Laki (26). Phosphate and sialic acid assays were performed on protein solutions which had been dialyzed for 72 hours against at least six changes, 200 to 1000 volumes each, of 2 M acetic acid. Phosphate was analyzed by the method of Warin (28) after hydrolysis in 0.1 N sulfuric acid at 80° for 1 hour, using N-acetylneuraminic acid as a standard.

**RESULTS**

Ten liters of blood, obtained from a single cow, yielded 8 to 10 g of 98 to 99% clottable protein. Polyacrylamide gel electrophoresis was performed in 8 M urea. The clear fibrin solution was lyophilized. Fibrin was reduced and carboxymethylated using the method of Murano et al. (13).
phoresis in sodium dodecyl sulfate (Fig. 1A) showed that 99% of the stain was in the triplet characteristic of the Aα, Bβ, and γ chains of fibrinogen (29). In addition, there were two minor bands of lesser mobility (Components I and II) which were not completely eluted from the fibrin clot, and a doublet of very low mobility (Component III) which appeared to be the major nonclottable component. On prolonged storage at -30°C a small amount of material of greater mobility than the fibrinogen triplet (Component IV) appeared, indicating degradation. The molecular weights of the four minor components were determined on a plot of electrophoretic mobility (relative to bromphenol blue) versus the logarithm of the known molecular weights of reduced albumin, immunoglobulin light chain, and immunoglobulin heavy chain. The molecular weights were: I, 108,000; II, 155,000; III, 280,000; and IV, 35,000. Because molecular weight standards larger than 68,000 were not used, the estimated molecular weights of Components I, II, and III are subject to more than the usual 10% uncertainty of this method (21).

Polyacrylamide gel electrophoresis of reduced and carboxymethylated subunits in 8 M urea, pH 8.6, revealed that the Aα and Bβ chains moved as diffuse bands, whereas the γ chain appeared as a doublet (Fig. 1B). In the fibrinogen preparation on which the chromatographic studies reported in this paper were performed, the proportion of the more anionic to the more cationic γ chain (hereafter called γ1 and γ2, respectively, as suggested by the IUPAC-IUB Commission of Biochemical Nomenclature (30)) was estimated by densitometry to be 0.36: 0.64. In this fibrinogen preparation there were 2.6 moles of phosphate and 7.2 moles of sialic acid per 340,000 g of protein.

The elution profile for this single animal fibrinogen preparation on DEAE-Sephadex A-50 is shown in Fig. 2. Three main peaks, each with several shoulders, were seen. The effluent was pooled and concentrated as indicated. Of the protein, 38.6% was in Fractions 1 to 8, 41.3% was in Fractions 9 to 16, and 14.1% was in Fractions 17 to 21. Upon rechromatography, protein from each of the main peaks eluted at the same position in the gradient as originally (Fig. 3A). Adjacent fractions from the same peak did not elute coincidentally (Fig. 3B), suggesting that the shoulders seen within the main peaks represent heterogeneity within the peaks. During these studies, pH, ionic strength, and temperature were monitored closely. It was found that temperature variations of 3°C caused dramatic artifacts in the elution patterns when shallow gradients were run. The scatter of the data in Fig. 3, A and B, corresponds to the temperature cycle of the cold room in which chromatography was done.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the reduced fractions from DEAE-Sephadex chromatography indicated that in all fractions intact Aα, Bβ, and γ chains predominated (Fig. 4A). The small amount of degraded material (Component IV) eluted in Fractions 1 to 4. The high molecular weight, nonclottable contaminant (Component III) eluted with the fibrinogen in Fractions 16 to 21. Component III was estimated by densitometry to be 1.1, 5.5, 10.6, and 18.8% of the protein in Fractions 16, 18, 20, and 21, respectively. The two minor components (I and II) which could not be eluted from fibrin were seen throughout.

Because the nonclottable component constituted up to 18.8% of the protein in the Peak III fractions, as judged by densitometer tracings of sodium dodecyl sulfate gels, we chose to study fibrin rather than fibrinogen when comparing the major peaks to one another. Fractions from the three major peaks were pooled as in Fig. 2 and clotted. Elution of the fibrin clots com-
single animal preparations have been determined. It is the data phosphate content and $\gamma$ chain composition of fractions of two of and sialic acid. In these experiments fibrinogen was not con-
pverted to fibrin. Therefore, the high molecular weight protein
(Component III in the sodium dodecyl sulfate gels) contaminated fibrinogen in fractions from Peak III. The sialic acid content of from 0.6 to 3.8 males/340,000 g, and fibrinogen from the ascend-
ing portions of the major peaks contained less phosphate than that from the descending portions (Table I).

Elution profiles similar to that shown in Fig. 2 have been found for fibrinogen preparations purified from five different cows and also for a preparation purified from commercially ob-
tained bovine Fraction I using Laki's method (26). The phos-
phate content and $\gamma$ chain composition of fractions of two of the single animal preparations have been determined. It is the data from one of these single animal preparations that is presented in Fig. 4B and Table I. The data from the other single animal preparation are similar.

To account for the heterogeneity seen within each of the major peaks, fractions within the peaks were analyzed for phosphate and sialic acid. In these experiments fibrinogen was not converted to fibrin. Therefore, the high molecular weight protein (Component III in the sodium dodecyl sulfate gels) contaminated fibrinogen in fractions from Peak III. The sialic acid content of the various fractions ranged between 6.7 and 9.0 moles/340,000 g of protein and did not vary in a systematic manner from fraction to fraction (Table I). In contrast, the phosphate content varied from 0.6 to 3.8 moles/340,000 g, and fibrinogen from the ascending portions of the major peaks contained less phosphate than that from the descending portions (Table I).

Elution profiles similar to that shown in Fig. 2 have been found for fibrinogen preparations purified from five different cows and also for a preparation purified from commercially ob-
tained bovine Fraction I using Laki's method (26). The phos-
phate content and $\gamma$ chain composition of fractions of two of the single animal preparations have been determined. It is the data from one of these single animal preparations that is presented in Fig. 4B and Table I. The data from the other single animal preparation are similar.

**Discussion**

Our studies indicate that within an individual cow there are many different types of fibrinogen molecules which can be partially separated chromatographically, and that these different molecules are a result of two types of heterogeneity, hetero-
genicity of $\gamma$ chains and heterogeneity of phosphorylation. A model is presented in Fig. 6 relating the elution profile to $\gamma$ chain heterogeneity and phosphate content. It is based on the as-
sumptions: (a) that there are two sites for phosphorylation per set of three polypeptide chains, or four sites per fibrinogen mole-
cule; (b) that these sites are phosphorylated independently of one another; (c) that phosphorylation at the first site cannot be distinguished chromatographically from phosphorylation at the second; (d) that the two types of $\gamma$ chains randomly combine in forming the dimeric structure; (e) that the chromatographic difference between the two types of $\gamma$ chains is approximately 5 times greater than the difference between a phosphorylated and nonphosphorylated molecule; and (f) that the separation effected by an incremental increase in chloride ion concentration is not constant throughout the linear gradient. The values for $\gamma_1$:$\gamma_2$ (0.36:0.64) and phosphate content (2.6 moles/340,000 g) were determined analytically. In this model there are eight different sets of three polypeptide chains of six different chromatographic valences. These combine to give 36 different kinds of fibrinogen molecules of 15 different chromatographic valences. The model agrees well with the elution profile in Fig. 2, suggest-
ing that heterogeneity of $\gamma$ chains and of phosphorylation account for all of the chromatographic heterogeneity and that these two types of determinants randomly combine. However, more complex models, in which there are more than two sites for phosphorylation, in which chromatographic determinants do not randomly combine, or in which there are other, as yet undetected, sources of heterogeneity (31), can be formulated. To decide among such models, additional information is needed. One must know: (a) how the two $\gamma$ chains differ from one another; (b) at what point in the lifetime of the fibrinogen molecules examined, synthesis, secretion, circulation, or purification, the combination of determinants occurs; and (c) if one component is selectively purified relative to another during the isolation procedure. Fibrinogen preparations from a number of animals should be examined.

Several reports in the literature support the conclusions of this investigation and suggest that our results, obtained with bovine fibrinogen, may also apply to humans and other species. Brada (4), Prager et al. (5), and Tsyndakovskaya et al. (6) reported that bovine fibrinogen was heterogeneous when examined by chromato-
genography on anion exchangers. In contrast, Finlayson and Mosesson (9) reported that bovine fibrinogen eluted from DEAE-
cellulose as a single peak; however, the peak they observed was asymmetrical. Godal and Lüscher (7) and Finlayson and Mo-

![Fig. 2. Chromatography of purified bovine fibrinogen on DEAE-Sephadex A-50. Protein (2660 mg) was applied to a column (5 x 80 cm) and eluted with an 8-liter linear gradient at 3°. The flow rate was 44 ml per hour, and 11-ml aliquots were collected. The absorbance at 280 nm (●—●) and the molarity of sodium chloride (O—O), as estimated by conductance measurements, are plotted. Tubes were pooled into 23 fractions as indicated and concentrated for sodium dodecyl sulfate gel elec-
rophoresis, sialic acid determination, and phosphate determina-
tion. For conversion to fibrin and subsequent carboxymethylation, protein was further pooled into Fractions I, II, and III, representing each of the major peaks. The increase in sodium chloride concentration at the first arrow represents 0.2 m sodium chloride applied when the gradient was 90% completed. The column was stripped with 0.5 m sodium hydroxide, which broke through at the second arrow. The yield of protein was 95.4%.](http://www.jbc.org/doi/suppl/10.1074/jbc.266045619/suppl_file/537437174.png)
Heterogeneity has also been described at the level of the subunit polypeptide chains. Gerbeck et al. (10) separated the reduced, S-sulfo γ chains of bovine fibrinogen into two fractions by chromatography in 8 M urea on carboxymethylcellulose. The two fractions could be distinguished electrophoretically at pH 4.8. Heterogeneity of γ chains has also been shown chromatographically for human S-sulfo (11) and S-carboxymethyl (12) fibrinogens. Two S-sulfo γ chains from ovine (14, 15), porcine (14), and human (11) fibrinogens have been distinguished electrophoretically.

The relationship between γ chain heterogeneity reported from other laboratories and that presented in the present paper is unclear. Mosesson et al. (11) examined human fibrinogen, which could be separated into two peaks on DEAE-cellulose. S-sulfo γ chains derived from the first peak produced a single band when subjected to electrophoresis in 8 M urea at pH 2.7 and 8.6, while S-sulfo γ chains derived from the second peak produced two bands of approximately equal density. It was estimated that the more anionic γ chain accounted for 7 to 8% of the total. One can calculate that if 93% of human γ chains are more cationic and 7% are more anionic, random combination of γ chains would result in 13% of human fibrinogen having both types. This figure agrees with the proportion of human fibrinogen (15%) estimated by Finlayson and Mosesson (8) to be in their Peak II. The third peak for human fibrinogen, containing only the more anionic chain and homologous to our Peak III, would account for less than 1% of the fibrinogen. Mosesson et al. (11) also reported chromatographic heterogeneity on carboxymethylcellulose in 8 M urea for S-sulfo γ chains from both their first and second peaks. They suggested that the chromatographic heterogeneity reported by Gerbeck et al. (10) for bovine fibrinogen was homologous to this second type of heterogeneity and not to the type of heterogeneity which accounted for the charge difference between Peaks 1 and 2 of human fibrinogen. We believe the type of heterogeneity of γ chains that we have found is homologous to the γ,γ' heterogeneity of Mosesson et al. (11) which accounted for the difference between Peaks 1 and 2 of human fibrinogen. We do not know how our findings are related to those of Gerbeck et al. (10).

The finding that the relative mobilities of the two γ chains
Fig. 5. Ferguson plot of S-carboxymethyl fibrin. A discontinuous buffer system was used as described in the "Experimental Procedures." The concentration of the stacking gel was 2.5% (w/v), and the ratio of monomer to cross-linker was 20:1. The pH of the separating gel was 9.5. The data plotted are from an experiment using S-carboxymethyl fibrin from Peak II. Similar results were obtained using S-carboxymethyl fibrinogen derived from unchromatographed protein. • and ○ represent γ1 and γ2, respectively. ■ represents the midpoint of the broad band containing α and β chains, which were not separated well in this system.

Table I

Phosphate and sialic acid content of chromatographic fractions

Phosphate and sialic acid content of the pooled and concentrated fractions were determined as described under "Experimental Procedures" and are expressed as moles per 340,000 g of protein. Phosphate assays were performed in duplicate, and sialic acid assays in quadruplicate. The average range of the sialic acid analyses was 2.3 moles/340,000 g of protein.

| Fraction | Amount phosphate/mole fibrinogen* (moles) | Amount sialic acid/mole fibrinogen* (moles) |
|----------|------------------------------------------|-------------------------------------------|
| 2        | 1.6                                      | 7.9                                       |
| 3        | 1.2                                      | 7.0                                       |
| 4        | 1.6                                      | 7.0                                       |
| 5        | 2.3                                      | 6.7                                       |
| 6        | 2.9                                      | 6.9                                       |
| 7        | 3.0                                      | 6.7                                       |
| 8        | 3.8                                      | 7.0                                       |
| 9        | 2.9                                      | 7.6                                       |
| 10       | 2.0                                      | 7.1                                       |
| 11       | 1.5                                      | 7.4                                       |
| 12       | 1.5                                      | 7.6                                       |
| 13       | 2.8                                      | 7.7                                       |
| 14       | 3.7                                      | 8.4                                       |
| 15       | 2.6                                      | 6.9                                       |
| 16       | 2.0                                      | 8.9                                       |
| 17       | 1.5                                      | 9.9                                       |
| 18       | 1.7                                      | 7.0                                       |
| 19       | 2.5                                      | 8.1                                       |

* The molecular weight of fibrinogen is assumed to be 340,000.

* These values are not corrected for Component III contaminating these fractions.

Fig. 4. Polyacrylamide gel electrophoresis of chromatographically separated fibrinogen and fibrin. A, sodium dodecyl sulfate gels: the fraction (see Fig. 2) is indicated below each gel; 5 to 25 μg of protein were electrophoresed; the band assignments are the same as in Fig. 1A. B, alkaline urea gels: fibrin from Peak I, 15 μg; fibrin from Peak II, 17 μg; and fibrin from Peak III, 15 μg. The anode is towards the right; the direction of electrophoresis was from cathode to anode. The densitometer tracings are reduced to the dimensions of the gels. Band assignments are the same as in Fig. 1B.

are parallel over a range of polyacrylamide gel concentrations in 8 m urea, pH 9.5, indicates that the two chains differ in charge but do not differ grossly in size (23). The structural basis for the charge differences is not known. Gerbeck et al. (10) found that the histidine, glycine, isoleucine, tryptophan, and sialic acid content of their two γ chain fractions differed. These investigators also reported that the fractions persisted after mild acid hydrolysis and concluded that differences in sialic acid content were not sufficient to explain the separation. We did not see
significant differences in the sialic acid content of chromatographic fractions of native fibrinogen. However, there was considerable scatter in these data, and analyses of isolated γ chains need to be done. Such studies are currently underway.

Prager et al. (23) and Tsynda et al. (34) used bovine fibrinogen, and Finlayson and Mosesson (35), using human fibrinogen, reported that on rechromatography one of their fractions behaved like the starting material and chromatographed as sevaphorylated fibrinopeptide A. The three fractions were usually obtained in original fractions were observed (Fig. 3A). This is in accord with reports of Capet-Antonini and Guinand (32), and that if these subunits were nonidentical, they could randomly reassociate to give several species upon rechromatography. We noted that late eluting fractions were contaminated with protein from earlier fractions, as in Fig. 3A. We also considered the possibility that purified fibrinogen could dissociate into subunits of molecular weight 180,000 as described by Capet-Antonini and Guinand (32), and that if these subunits were nonidentical, they could randomly reassociate to give several species upon rechromatography. However, when we mixed fractions from Peaks I and II that should have been able to give rise to Peak II material by such hybridization, only the earlier fractions were observed (Fig. 3A). This is in accord with recent reports (33, 34) in which subunits of molecular weight 180,000 were not found and with the report that the Aα and γ chains are held together by disulfide bonds (36).

Blombäck et al. (16) demonstrated that a serine in human fibrinopeptide A was incompletely phosphorylated. It has been suggested (17) that the phosphorylated and nonphosphorylated Aα chains may have a random distribution in the fibrinogen dimer. Shainoff et al. (35) reported that native human fibrinogen could be chromatographically separated into three fractions, the first containing only nonphosphorylated fibrinopeptide A, the second equal amounts of phosphorylated and nonphosphorylated fibrinopeptide A, and the third only phosphorylated fibrinopeptide A. The three fractions were usually obtained in relative proportions of 5:5:1. These proportions are close to those (0.46:0.44:0.10) which can be calculated assuming random combination of nonphosphorylated and phosphorylated A peptides, if the former accounts for 1/5 of the total A peptides.

In bovine fibrinogen the phosphate is confined to the parts of the molecule which remain after thrombin proteolysis (36, 37). Krajewski and Cierciwieski (38) reported that the phosphorus was located on the Aα and Bβ chains and occurred as phosphoserine. Their findings support our assumption that there are two sites of phosphorylation per set of Aα, Bβ, γ, and γ chains, and that these sites are incompletely phosphorylated.

The functional and physiological significance of the chemical heterogeneity described here is not known. We have seen the same populations of molecules in approximately the same proportions in studies of fibrinogen purified from five different cows and also in studies of fibrinogen purified from commercially obtained Fraction I. It may be that formation of a complex mixture of molecules is regulated during synthesis, secretion, and circulation and that the mixture is important for homeostasis. The ratio of the more anionic and more cationic γ chains probably differs from species to species. As we have discussed,
proteins probably reflects post-translational modification of these molecules (39). Several clinical reports suggest that the amount of phosphate in secretory adults. A detailed study of fibrinogen purified from a patient contained significantly more phosphate than that purified from human fibrinogen, whereas we have observed a ratio of 0.36:0.64 for bovine fibrinogen. The finding of phosphate in secretory fibrinogen (41). Shainoff et al. (40) found that fetal fibrinogen purified from human cord blood permitted us to refer to his unpublished results. the physiological and pathophysiological importance of fibrinogen phosphate content, should facilitate further investigations into method by which fibrinogen may be separated into several fractions and relating the heterogeneity noted in native fibrinogen to the occurrence of two different γ chains and to differences in hemangioma and low blood levels of fibrinogen. The same authors also reported a decrease in phosphorylated A peptide and an increase in nonphosphorylated A peptide during incubation of blood and plasma and suggested that fibrinogen may be fully phosphorylated when secreted and progressively dephosphorylated in the circulation. Our studies, by describing a method by which fibrinogen may be separated into several fractions and relating the heterogeneity noted in native fibrinogen to the occurrence of two different γ chains and to differences in phosphate content, should facilitate further investigations into the physiological and pathophysiological importance of fibrinogen heterogeneity.

Acknowledgments—We thank Mr. Fred Gilchrist for helping design and construct some of the equipment used in this study, Mr. Will Veach for many helpful discussions, and Dr. John Finlayson for reviewing the manuscript prior to submission and permitting us to refer to his unpublished results.

REFERENCES
1. Blomback, B. (1967) in Blood Clotting Enzymology (Seegers, W. H., ed) pp. 145-216, Academic Press, New York.
2. Blomback, B., Blomback, M., Henschen, A., Hessel, B., Iwanaga, S. & Woods, K. R. (1968) Nature 218, 130–134.
3. Blomback, B. (1970) Symp. Zool. Soc. London 27, 167–187.
4. Beada, Z. (1967) Naturwissenschaften 44, 561.
5. Prager, M. D., Speer, R. J. & Work, P. S. (1960) Fed. Proc. 19, 93.
6. Tsyanskovska, S. N., Kotkova, K. I. & Galanova, T. F. (1964) Ukrainsk’kyi Biohim. Zh. 36, 445–452.
7. Godal, H. C. & Lüšcher, E. F. (1960) Scand. J. Clin. Lab. Invest. 12, 47–55.
8. Finlayson, J. S. & Mosesson, M. W. (1963) Biochemistry 2, 42–46.
9. Finlayson, J. S. & Mosesson, M. W. (1964) Biochim. Biophys. Acta 82, 415–417.
10. Gerbeck, C. M., Yoshikawa, T. & Montgomery, R. (1969) Arch. Biochem. Biophys. 124, 67–75.
11. Mosesson, M. W., Finlayson, J. S. & Umfleet, R. A. (1972) J. Biol. Chem. 247, 5223–5227.
12. Henschen, A. & Edman, P. (1972) Biochim. Biophys. Acta 263, 381–386.
13. Murano, G., Wiman, B., Blomback, M. & Blomback, B. (1971) Fed. Eur. Biochem. Soc. Lett. 14, 37–41.
14. Cartwright, T. & Kekwick, R. G. O. (1971) Biochim. Biophys. Acta 236, 550–562.
15. Lott, W., Israel, L. G., Bishop, A. J. & Israel, E. D. (1971) Thromb. Diath. Haemorrh. 26, 528–540.
16. Blomback, B., Blomback, M., Edman, P. & Hessel, B. (1966) Biochim. Biophys. Acta 115, 371–396.
17. Blomback, B., Hessel, B., Iwanaga, S., Reuterby, J. & Blomback, M. (1972) J. Biol. Chem. 247, 1496–1512.
18. Gloyer, G. & Shaw, E. (1971) J. Biol. Chem. 246, 4904–4905.
19. Baughman, D. J. (1970) Methods Enzymol. 15, 157–158.
20. Capet-antonini, F. (1966) Ball. Soc. Chim. Biol. 48, 989–994.
21. Weber, K. & Oshorn, M. (1969) J. Biol. Chem. 244, 4000–4012.
22. Takagi, T. & Iwanaga, S. (1969) Biochim. Biophys. Acta 194, 584–596.
23. Rodbard, D. & Chrambach, A. (1971) Anal. Biochem. 40, 15–134.
24. Mihalyi, E. (1968) Biochemistry 7, 208–223.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
26. Laki, K. (1951) Arch. Biochem. Biophys. 32, 317–324.
27. Bartlett, G. R. (1965) J. Biol. Chem. 230, 469–466.
28. Warren, L. (1959) J. Biol. Chem. 234, 1971–1975.
29. McKee, P. A., Mattock, P. & Hill, R. L. (1970) Proc. Nat. Acad. Sci. U. S. A. 66, 738–744.
30. IUPEC-IUB Commission on Biochemical Nomenclature (1971) J. Biol. Chem. 246, 6127–6128.
31. Gaffney, P. J. (1971) Nature New Biol. 230, 54–56.
32. Capet-Antonini, F. C. & Guinand, S. (1970) Biochim. Biophys. Acta 230, 486–496.
33. Endres, G. F. & Scheraga, H. A. (1971) Arch. Biochem. Biophys. 144, 519–528.
34. Demchenko, O. P. & Zhuravski, H. H. (1971) Ukrainsk’kyi Biohim. Zh. 43, 173–177.
35. Shainoff, J. R., Bumpus, F. M. & Finlayson, J. S. (1971) Biochim. Biophys. Soc. Annu. Meet. Abstr. 11, 69a.
36. Fantl, P. & Ward, H. A. (1962) Biochim. Biophys. Acta 64, 568–570.
37. Blomback, B., Blomback, M. & Starelle, J. (1963) Biochim. Biophys. Acta 74, 148–151.
38. Krajewski, T. & Cieniewski, C. (1972) Biochim. Biophys. Acta 271, 174–181.
39. Turkington, R. W. & Topper, Y. J. (1966) Biochim. Biophys. Acta 127, 366–372.
40. Witt, I. & Muller, H. (1970) Biochim. Biophys. Acta 221, 402–404.
41. Roy, F. A., Shainoff, J. R., Vogel, A. & Jackson, D. P. (1971) J. Clin. Invest. 50, 1874–1884.
42. Shainoff, J. R., Dyment, P. G., Hoffman, G. C. & Bumpus, F. M. (1972) Circulation 46, Suppl. II, 52.
Heterogeneity of Bovine Fibrinogen and Fibrin
Deane F. Mosher and Elkan R. Blout

J. Biol. Chem. 1973, 248:6896-6903.

Access the most updated version of this article at http://www.jbc.org/content/248/19/6896

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/19/6896.full.html#ref-list-1