The Preparation and Properties of Human Fibrinogen of Relatively High Solubility*

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ABSTRACT: Most of the methods now employed for the isolation and purification of human fibrinogen tend to exclude material, both clottable and nonclottable, of relatively higher and lower solubility during the preparation of the final product (*e.g.*, Blombäck fraction I-4).

A method for the purification and isolation of human fibrinogen of high solubility was developed which employed glycine precipitation of plasma with 2.1 M glycine at 5° for the initial isolation. Subsequent to glycine reprecipitation of the fibrinogen fraction, and removal of a cold-insoluble fraction (I-1), fractionation between 8 and 16% ethanol concentration yielded fibrinogen (I-5) which was more soluble than the fibrinogen precipitable at 8% ethanol concentration (I-2) and which averaged 91% clottable. Further subfractionation of I-5 with ethanol served to isolate more completely the high solubility fibrinogen from that of lower solubility, the resultant fraction being designated I-7 and having a clottability of about 95%. Reprecipitation of I-7 with glycine was far less complete than anticipated, but yielded material consistently 97% or more clottable. This observed change in glycine precipitability of I-7 was attributable to its relative isolation from fibrinogen of lower solubility with which it appeared to interact. Solubility studies of isolated fibrinogen subfractions (I-3 to I-8) demonstrated clearly the differences between these fractions, though all fractions were heterogeneous. The biologic reactivity to thrombin of the various subfractions was directly related to their solubility; the higher the solubility, the longer the thrombin time.

Since fibrinogen is among the least soluble of the plasma proteins, it is the major component of the material first precipitated from plasma by a variety of techniques. Most of the methods now employed for the preparation and purification of fibrinogen use fraction I prepared by cold ethanol fractionation of plasma (Cohn *et al.*, 1946) or a similar fraction as starting material. It can be estimated that fraction I prepared by method 6 of Cohn *et al.* (1946) includes approximately 65–80% of the plasma fibrinogen (Blombäck and Blombäck, 1956; Cohn *et al.*, 1946), whereas higher amounts (88–89%) are precipitated initially by the ether method of Kekwick *et al.* (1955). However, the procedures employed during the purification of the fibrinogen of these fractions remove not only clottable material with lower solubility, but also that of higher solubility (Blombäck and Blombäck, 1956; Kekwick *et al.*, 1955). In fact, Kekwick *et al.* (1955) noted that final purification by their method could only be brought about by removal of higher solubility material containing nonclottable impurities. Therefore, a sizeable portion of plasma fibrinogen of relatively high solubility is probably removed, along with contaminating nonclottable substances, by most of the commonly employed methods for isolation and purification of fibrinogen.

On the other hand, Kazal *et al.* (1963) reported that human plasma fibrinogen was completely precipitated by saturation with glycine, and that a highly clottable preparation could be obtained by reprecipitation of the fibrinogen fraction with saturated or near-saturated glycine solutions. Since the procedure entailed little loss of clottable material, fibrinogen purified by this method may be expected to contain a higher proportion of the more soluble fibrinogen removed by other methods (*vide supra*).

The following studies were undertaken to characterize the solubility of the human plasma fibrinogen not precipitated in Cohn fraction I by method 6 (Cohn *et al.*, 1946), and to isolate and purify fibrinogen(s) of relatively high solubility. To our knowledge, purification, isolation, and characterization of plasma fibrinogen of higher solubility than that derived from fraction I has not been reported.

Materials and Methods

Outdated ACD bank plasma, usually less than 4-weeks old when pooled, was used in batches of 1–4 l. Centrifugation at 4500*g* at 2–5° for 10 min was performed to remove cellular particles. Some plasma lots were stored frozen at −15 to −20° before use. Thrombin was Parke-Davis bovine thrombin stored as a stock solution in 50% glycerol at −15 to −20°

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at a concentration of 100 U. S. standard[1] units/ml.

Fibrinogen content was measured as fibrin by the method of Ratnoff and Menzie (1951). Duplicate 0.2-ml samples of whole plasma were routinely analyzed; for certain plasma supernatant fractions (*e.g.*, supernatant fraction I) of which the fibrinogen content was less than 1.0 mg/ml, 1.0-ml samples were analyzed. Clots were incubated for 3 hr or longer before analysis.

Clottability was determined by a modification of the method of Laki (1951). Samples of 0.1 or 0.2 ml of fibrinogen solution were added to 1.9 or 1.8 ml of 0.05 M sodium phosphate buffer, pH 6.4, respectively, and the mixture clotted by the addition of 1.0 ml of distilled water containing 0.5 to 1 U. S. standard unit of thrombin/ml. After 3 hr or more, the clot was removed with a nichrome wire loop, allowed to synerize, dipped several times into the supernatant clot liquor, and discarded. The absorbancy of the clot liquor was read at 280 mμ (in a Beckman DU spectrophotometer) against an appropriate blank and compared to the absorbancy of an unclotted sample of fibrinogen diluted to 3.0 ml with 0.3 M NaCl. Clottability was expressed as the per cent of total absorbancy removed with the clot.

Concentration of purified dialyzed fibrinogen solutions was determined in a Brice–Phoenix differential refractometer. A specific refractive increment of 0.188 ml/g at 546 mμ was assumed (Armstrong *et al.*, 1947). The concentration of those fractions which had not been dialyzed or whose concentrations were too low to be determined with the refractometer were determined by assuming an absorbancy coefficient ($A^{1\%}_{1cm}$) of 15.5 at 280 mμ (or by using the calculated absorbancy coefficient for the particular sample concerned).

Thrombin clotting times were determined at pH 7.0 in a medium which was 0.01 M in phosphate and 0.135 M NaCl ($\Gamma/2 = 0.15$). Fibrinogen concentration was expressed as the final concentration after the addition of thrombin. The end point was the first appearance of fibrin in the clotting mixture after the addition of thrombin.

Fractionation of plasma to obtain fractions I and II + III was performed according to method 6 of Cohn *et al.* (1946). Subfractions of II + III were obtained by first extracting II + III (Oncley *et al.*, 1949) according to method 9. Fraction II was then extracted according to method 10 (Cohn *et al.*, 1950) and reprecipitated from solution by adjusting the pH to 7.1 and raising the ethanol concentration to 25% at −5°. Nomenclature was that used in methods 9 and 10. Centrifugation was carried out at 4500–6000*g* unless otherwise noted.

The nomenclature for fibrinogen subfractions was an extension of that introduced in method 6 (Cohn *et al.*, 1946) and developed by Morrison *et al.* (1948) and Blombäck and Blombäck (1956). For ease of presentation, each significant subfractionation step was assigned a different number. The molar concentrations of the various glycine solutions were assumed (Kazal *et al.*, 1963).

Cellulose acetate electrophoresis was performed in a Shandon apparatus[2] using Sepraphore III[3] cellulose acetate strips. Barbital buffer (pH 8.6, $\Gamma/2 = 0.05$) was used in these studies. Immunoelectrophoresis was performed according to the micromethod of Scheiddeger (1955) in the Shandon apparatus in a 1% agar medium. Electrophoresis was carried out for 2–3 hr at 110 v. Antisera used were commercially available horse,[4] goat,[5] and rabbit[6] antihuman serum (none of which had detectable antifibrinogen antibodies) and rabbit antihuman fibrinogen prepared in this laboratory.

Results

1. Studies with Human Plasma and Plasma Subfractions. Four samples of pooled bank plasma were examined for their fibrinogen content before and after removal of fraction I at 8% ethanol concentration (v/v) and 0 to −3°. The average whole plasma fibrinogen concentration (corrected for dilution by anticoagulant) was 2.55 mg/ml. The supernatant plasma of fraction I (uncorrected for anticoagulant or ethanol dilution) contained 0.50 mg/ml (range 0.48–0.53), an amount which represented an average of 26.1% of the total fibrinogen (range 20.8–32.7%).[7]

The effect of fibrinogen concentration on the observed distribution was studied in one of the samples (Table I). Dilutions of plasma were made with 0.135 M NaCl–0.01 M sodium phosphate buffer, pH 7.0 ($\Gamma/2 = 0.15$). Fractionation was carried out as above at 8% ethanol concentration. Total plasma fibrinogen or supernatant plasma fibrinogen determinations were made on samples calculated to contain the same amount of protein as the undiluted specimen. When corrected for dilution, no major variations in the distribution of fibrinogen were noted although the supernatant fibrinogens were slightly higher in both the 1:2 and 1:4 dilutions; thus the observed distribution of plasma fibrinogen in 8% ethanol was essentially independent of protein concentration within the range examined.

Glycine, at a concentration of 2.1 M (2.2 moles of glycine added/l.), precipitated a greater proportion of plasma fibrinogen than did 8% ethanol. Six samples of plasma in which glycine was dissolved to a concentration of 2.1 M were stirred for 30 min or more at

[1] Commonly designated "NIH Unit."

[2] Available from Colab, Chicago Heights, Ill.

[3] Gelman Instrument Co., Ann Arbor, Mich.

[4] Lloyd Brothers, Inc., Cincinnati, Ohio.

[5] Colorado Serum Co., Denver, Colo.

[6] Immunology, Inc., Chicago, Ill.

[7] The distribution of plasma fibrinogen in ethanol has also been examined in a number of single-donor specimens, collected in various anticoagulants. In all normal human donors examined, whether the plasma was collected and processed in nonwettable equipment or whether it was fractionated while fresh or not, a significant proportion of the total fibrinogen was always found to be soluble in 8% ethanol.

TABLE I: The Precipitation of Plasma Fibrinogen with 8% Ethanol as a Function of Fibrinogen Concentration.

Diln with Saline Buffer ($\Gamma/2$ = 0.15)	Total Plasma Fibrinogen		Fibrinogen in Supernatant of Fraction I		
	Uncorrected[a] Fibrinogen (mg/ml)	Corrected[b] Fibrinogen (mg/ml)	Uncorrected[a] Fibrinogen (mg/ml)	Corrected[b] Fibrinogen (mg/ml)	% Total[c]
0	2.49	2.74	0.50	0.60	21.9
1:2	1.28	2.82	0.31	0.75	26.6
1:4	0.62	2.70	0.14	0.67	24.8

[a] Actual concentration. [b] Corrected for plasma dilution with saline, anticoagulant, and/or ethanol. [c] Based upon corrected values.

5° before centrifugation at 5000*g* for 20 min. The fibrinogen content of the supernatant plasma averaged 0.23 mg/ml (range 0.21–0.27) which, when corrected for dilution due to glycine addition, was found to represent approximately 9% (8.5–10%) of the plasma fibrinogen. A single plasma sample which had been precipitated with 2.1 M glycine at 5° as above was centrifuged at a higher revolution per minute (23,000*g*) to ascertain if there was a significant increase in yield; the supernatant plasma fibrinogen was found to be 0.21 mg/ml or 9% of the total fibrinogen. Even the addition of glycine to 2.7 M (2.83 moles of glycine added/l.) at 20° (an amount calculated to saturate plasma at that temperature) and centrifugation at 23,000*g* did not bring about complete precipitation of plasma fibrinogen, the supernatant fibrinogen (0.15 mg/ml) amounting to 7% of the total plasma fibrinogen of that sample. The further addition of glycine to this supernatant solution left undissolved glycine in the plasma, and a small additional precipitate was obtained at 20° and 23,000*g* but the amount of supernatant fibrinogen remained unchanged at 0.15 mg/ml. It was concluded that 91–93% of plasma fibrinogen was precipitable by 2.1 M glycine at 5° or 2.7 M glycine at 20°.

No detectable fibrinogen remained in the plasma after precipitation of fraction II + III at 25% ethanol concentration; in fact, only trace amounts of clottable protein were detectable in the supernatant remaining after a 15% ethanol precipitate of plasma. The fibrinogen associated with freshly prepared fraction II + III could be virtually quantitatively extracted, along with nonfibrinogen components, with the Blomback and Blombäck (1956) extraction buffer (0.055 M citrate, 1 M glycine, 6.5% ethanol, pH 6.0) at 0°; glycine precipitation of fraction II + III fibrinogen (2.1 M glycine, 0.1 M phosphate, pH 7.0, $\Gamma/2$ = 0.19, fibrinogen concentration = 3.0–5.0 mg/ml, T = 5°) was incomplete (approximately 50 to 60%) regardless of the presence or absence of the γ-globulin (II) and/or β-lipoprotein (III-0)[8] fractions. In contrast, extraction of ethanol fraction I or I-2 with the Blomback buffer removed only small quantities of the clottable protein of these fractions (5% or less). Furthermore, fraction I-2 fibrinogen prepared from the same plasma as the II + III fibrinogen was almost quantitatively precipitated by 2.1 M glycine under the conditions stated above. The admixture of fraction I-2 fibrinogen with the γ-globulin (II) or β-lipoprotein (III-0) fractions (concentrated 5–10 times relative to plasma) caused no appreciable change in glycine precipitability of the fibrinogen present. These results indicated that there were significant solubility differences between the fibrinogen of fraction I and that of II + III and, as far as the bulk of the fibrinogen under examination was concerned, the observed solubility behavior was independent of the presence of fraction II or III-0.

2. The Isolation and Purification of Fibrinogens of Varying Solubility. Because of the high percentage of the total plasma fibrinogen which was precipitated by glycine (*vide supra*) and because of the high degree of subsequent purification which could be obtained without appreciable loss, the glycine precipitate of plasma was chosen as the starting material for studies related to the isolation of high solubility fibrinogen. Glycine (2.1 M) at 5° was selected for the initial precipitation because the lower temperature was more favorable to the stability of the preparation, and no appreciable increase in yield was obtained at 20° and 2.7 M glycine. Fibrinogen precipitated by glycine from outdated plasma proved to be stable and did not require adsorption with barium or analogous substances as suggested by Kazal *et al.* (1963) for fresh plasma. Prior adsorption of plasma with barium chloride did not, however, influence the solubility of fibrinogen. A method, utilizing glycine precipitation and ethanol subfractionation, was developed for purifying and isolating fibrinogens of high solubility. The details are presented below and outlined in Figure 1.

After initial glycine precipitation of plasma, the precipitate is dissolved in 0.15 M NaCl, 0.01 M sodium

[8] Small amounts of clottable protein were found in both the γ-globulin and β-lipoprotein fractions and together were estimated to amount to 15–20% of the fibrinogen associated with fraction II + III. Quantitation of the fibrinogen content of fractions containing high amounts of lipoproteins was difficult owing to their high occlusive tendencies (Morrison, 1947).

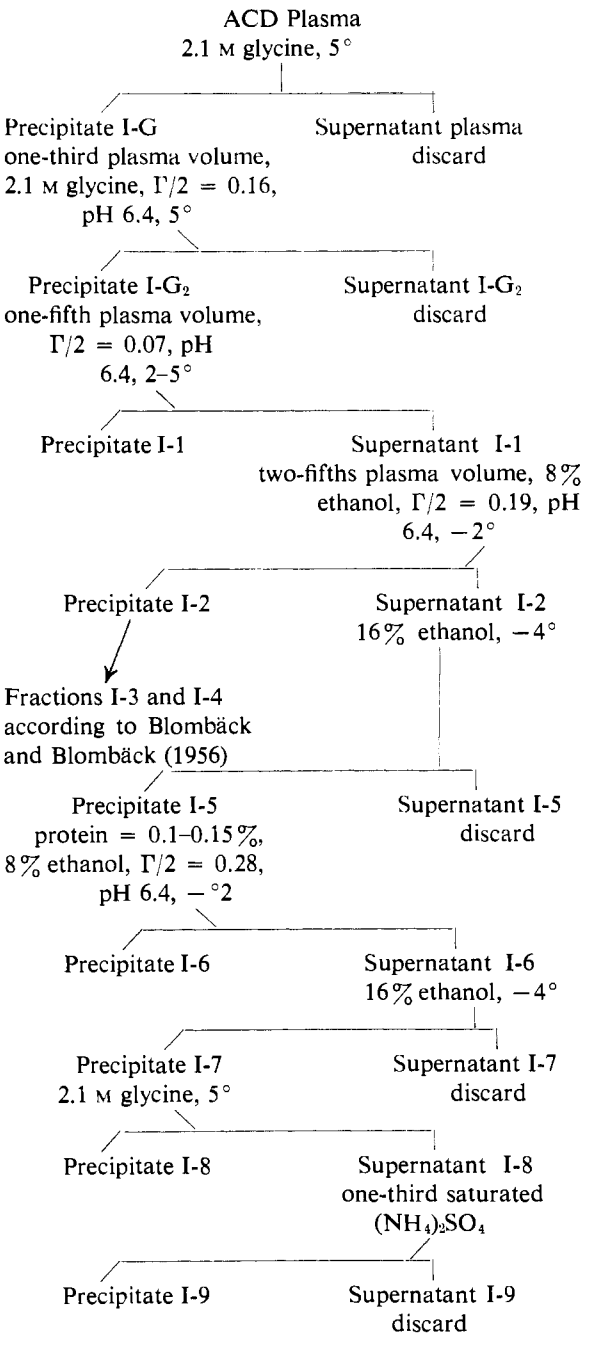

FIGURE 1: Diagrammatic representation of fractionation procedures.

phosphate, pH 6.4 buffer (Γ/2 = 0.16), brought to one-third of the original plasma volume and reprecipitation with 2.1 M glycine at 5° carried out.[9] The second glycine precipitate (I-G2) is dissolved and brought to one-tenth of the original plasma volume with 0.10 M sodium phosphate pH 6.4 buffer (Γ/2 = 0.14). Fraction I-1 is removed according to Laki (1951) by further diluting with an equal volume of water and allowing the mixture to stand for at least 6 hr at 2–5° before removal of the cold insoluble material (I-1). To the supernatant solution is added an equal volume of 0.3 M NaCl (resultant Γ/2 = 0.19) and the solution cooled to −2 to −3° during the addition of 95% ethanol to a final concentration of 8% (v/v). The precipitate (I-2) is removed by centrifugation at −3°, dissolved in 0.3 M NaCl, and stored at −15 to −20°, or further processed[10] according to the method of Blomback and Blombäck (1956) to yield fractions I-3 and I-4. The fibrinogen soluble in 8% ethanol (supernatant I-2) is then quantitatively precipitated by raising the ethanol concentration to 16% and lowering the temperature to −4°. The resulting fraction (I-5), collected by centrifugation at −4°, amounts to 5–10% of the original plasma fibrinogen. In order to achieve more complete isolation of high solubility fibrinogen from that of lower solubility, the following procedures are carried out. Fraction I-5 is dissolved in 0.27 M NaCl, 0.01 M sodium phosphate pH 6.4 buffer (Γ/2 = 0.28) and brought to a protein concentration of 0.1–0.15%. Ethanol (95%) is added to 8% concentration while the solution is cooled to −2 to −3° and the resulting precipitate (I-6) centrifuged at −3°.[11] The fibrinogen remaining in solution is again quantitatively precipitated by raising the ethanol concentration to 16% at −4° and centrifugation of the resultant precipitate (I-7) at −4°.

Fraction I-5 obtained from six such preparations averaged 91% clottable (range 85–95%); fractions I-6 and I-7 (three preparations examined) averaged 92% (90–96%) and 95% (94–97%) clottable, respectively. Reprecipitation of fraction I-7 with 2.1 M glycine consistently yielded preparations with greater than 97% clottability. However, in contrast to the high yield obtained in earlier steps (*vide supra*) a far less complete fibrinogen precipitation was accomplished. For example, fraction I-7 from a single preparation was reprecipitated with 2.1 M glycine at 5° from a medium containing 0.1 M NaCl–0.01 M sodium phosphate pH 6.4 (Γ/2 = 0.11) and 0.5% fibrinogen. Only 60% of the fibrinogen was precipitated under these conditions, the supernatant fibrinogen being recovered by the addition of saturated ammonium sulfate solution to 33% saturation. The ammonium sulfate fraction (I-9) was 91.5% clottable whereas the glycine-precipitated fraction (I-8) was 98.7% clottable. An understanding of the nature of the change in solubility was gained from recombination studies of various fibrinogen fractions (*vide infra*).

[9] Although the same approximate concentration of fibrinogen remains in this glycine supernatant, recovery is high owing to the lower volume, and exceeds 95%.

[10] Extraction of this fraction (I-2) according to Blombäck and Blombäck (1956) removes less than 5% of the clottable material and is usually omitted.

[11] The higher the initial fibrinogen concentration, the larger the amount precipitated in this fraction. At the stated initial protein concentrations, I-6 amounts to about 35% (20–50%) of the fibrinogen of I-5.

3. Studies of Purified Fibrinogen Subfractions. Fractions I-4 and I-8 prepared from the same plasma and more than 98% clottable were combined in approximate 1:1 ratios and precipitated by 2.1 M glycine at 5° (Table II). Solutions were centrifuged at 23,000*g*

TABLE II: The Effect of Recombination of Fraction I-4 with I-8 on the Fibrinogen Precipitated by 2.1 M Glycine.

Soln	Relative Contribution to the Soln[a] I-4	I-8	Final Fibrinogen Concn (mg/ml) before Glycine Addn	Supernatant Fibrinogen after Glycine Pptn (mg/ml)
1	1	0	1.87	0.16
2	0.5	0	0.83	0.20
3	0	1	1.87	0.82
4	0	0.5	0.91	0.58
5	1	1	3.57	0.69
6	0.5	0.5	1.79	0.49

[a] Solutions were all made to a constant volume (6.0 ml) by the appropriate additions of calculated amounts of protein material and buffers so that the final ionic environment was 0.15 M NaCl, 0.01 M sodium phosphate pH 6.4 ($\Gamma/2$ = 0.16). The relative amount of protein added to each mixture is represented by the number 1, 0.5, or 0.

for 20 min and the clear supernatants were examined for their content of fibrinogen. The amount of fibrinogen in the supernatant solution of the combined fibrinogen fractions (solution 5 or 6, Table II) was less than the total amount in the supernatant solutions of I-4 and I-8 fractionated separately at an initial concentration approximately equal to that of the mixture (*i.e.*, solutions 1, 3, and 6, Table II) or at a concentration equivalent to the contribution of that particular fraction to the mixture (*i.e.*, solutions 2, 4, 6, and 1, 3, 5, Table II). Thus, the copresence of fibrinogen of high solubility with that of lower solubility appeared to account for the variable precipitability of the former in the presence of glycine.

In another form of solubility experiment, varying concentrations of fibrinogen subfractions which had been prepared from a single plasma batch were dialyzed against a 0.01 M phosphate buffer pH 6.6 ($\Gamma/2$ = 0.015) at 5 to 8° (Figure 2). After 48 hr the dialysis bags were removed and carefully dried, and the contents centrifuged at 5°. The concentration of the clear supernatant solutions was determined and plotted against the fibrinogen concentration of the undialyzed sample (saturating body). There were distinct differences in the solubilities of the fractions involved, consistent with their behavior on ethanol or glycine fractionation.

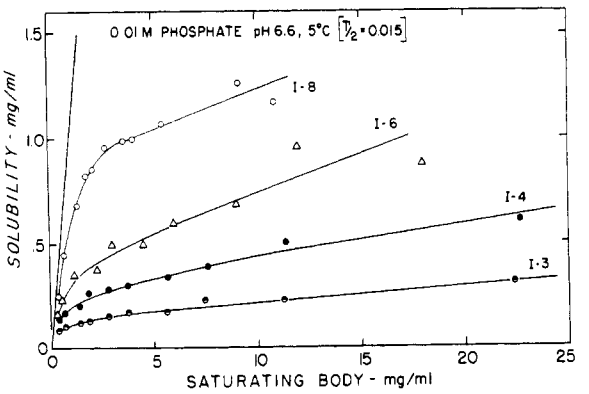

FIGURE 2: Solubility of fibrinogen subfractions. The concentration of the supernatant fibrinogen after dialysis (ordinate) is plotted against the concentration of the undialyzed solution (saturating body). All subfractions were more than 98% clottable. The solid line through the origin represents the initial theoretical solubility curve of a pure component. I-3, ◓; I-4, ●; I-6, Δ; I-8, O.

Furthermore, in no instance was the phase rule solubility criterion for homogeneity met.

Upon the addition of thrombin, all subfractions tested yielded clots which were indistinguishable from one another in gross appearance. The thrombin clotting times of various fibrinogen subfractions, however, were directly related to solubility, *i.e.*, the lower the solubility of the fibrinogen, the shorter the clotting time (Figure 3). This relationship was valid as well in other studies of the recombination of fractions I-5 and I-2 in which a thrombin time intermediate between that of I-2 and I-5 was found, or in the comparison of fraction I-3 to fraction I-4, in which the thrombin time of I-3 was found to be shorter than that of I-4. Moreover, subfractionation of fraction I-4 with ethanol was performed to yield two fractions and these subfractions were compared at equivalent concentrations. The first precipitated fraction was found to have a thrombin time which was shorter than I-4 itself; the second fraction, one which was longer than the parent preparation (I-4).

There were no differences in mobility between any fractions of I-5 and/or I-2 when subjected to electrophoresis on cellulose acetate strips nor were any differences noted upon immunoelectrophoresis in agar gel; the precipitin lines forming in the latter study against rabbit antihuman fibrinogen were indistinguishable from the fibrinogen line of normal human plasma. On the other hand, no precipitin lines formed with these fibrinogens against antihuman serum prepared in rabbits, goats, or horses. In an immunodiffusion experiment against rabbit antihuman fibrinogen, the precipitin lines formed by fractions I-4 and I-8 fused with each other and with the fibrinogen line of normal human plasma.

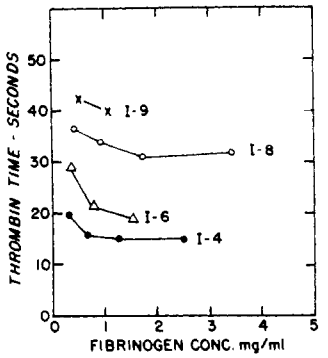

FIGURE 3: Thrombin clotting times of certain fibrinogen subfractions prepared from a single plasma batch; $\Gamma/2 = 0.15$; temperature 37°. I-4, ●; I-6, Δ; I-8, ○; I-9, ×.

Discussion

The dual effect of glycine on fibrinogen, demonstrated by Blombäck and Blombäck (1956), appears to be a general one (Cohn and Edsall, 1943) which has been demonstrated for other proteins (Grönwall, 1942; Richards, 1937) as well. Its property of increasing the solubility or activity of amino acids or proteins in aqueous solution is attributable to its effect of raising the dielectric constant of the medium; its ability to decrease solubility at higher concentrations is attributable to a "salting-out" effect. Although the ability of glycine to precipitate fibrinogen of Cohn fraction I was first recorded by Edsall and Lever (1951), the observation that saturated glycine solutions favor the precipitation of plasma fibrinogen under conditions in which other plasma proteins remain soluble was first reported in detail by Kazal *et al.* (1963) who applied this to the preparation of highly purified fibrinogen. This technique, because of the initial high recovery from plasma, and because of the simplicity with which a high degree of purification was readily obtained, was used in our studies for the initial precipitation and subsequent purification of fibrinogen of relatively high solubility. However, in contrast to the results reported by Kazal *et al.* (1963), a complete precipitation of human plasma fibrinogen could not be obtained even with fresh single donor plasma, and it must be concluded that a small amount of plasma fibrinogen exists which may have a solubility even higher than any of the material which we were able to isolate and purify.

There is the possibility that binding of fibrinogen to other nonfibrinogen proteins or plasma components may account for some of the solubility characteristics observed; the small amount of fibrinogen extracted with the β-lipoprotein fraction (III-0) or the γ-globulin fraction (II) from fraction II + III could be bound to constituents of these fractions and manifest an increase in solubility. However, with regard to the plasma glycine fibrinogen fractions studied, it could be concluded from the available evidence that the observed differences in solubility were intrinsic to the fibrinogen itself: recombination of concentrated solutions of fraction II or fraction III-0 with fraction I-2 fibrinogen caused no appreciable change in the glycine solubility of the fibrinogen; no differences in electrophoretic mobility on cellulose acetate strips or in agar gel between fibrinogens of different fractions (*e.g.*, I-4 and I-8) were demonstrable; immunodiffusion and immunoelectrophoretic studies of these fibrinogens against several antihuman sera failed to reveal any other antigenic moieties; and ultracentrifugation of fraction I-8 in this laboratory revealed a single peak with a sedimentation velocity ($s^0_{20,w}$) only slightly less than that of fraction I-4 fibrinogen (M. W. Mosesson, unpublished data).

On the other hand, the copresence of fibrinogen of low solubility with that of higher solubility has been shown to lower the over-all solubility of the mixture in the presence of glycine. Regardless of the explanation for this phenomenon, the high recovery of fibrinogen by glycine precipitation from plasma and during the initial glycine reprecipitation, contrasted with the relatively incomplete glycine precipitation of more highly soluble fibrinogen solutions (*e.g.*, I-7, fraction II + III fibrinogen), was attributable to this effect.

The heterogeneity of human fibrinogen derived from fraction I or its equivalent has previously been well established on the basis of its solubility characteristics (Morrison *et al.*, 1948; Blombäck and Blombäck, 1956), its chromatographic (Finlayson and Mosesson, 1963) and electrophoretic (Blombäck and Blombäck, 1956; Caspary and Kekwick, 1957; Finlayson and Mosesson, 1963) behavior, and even with regard to its biologic reactivity to plasmin (Mosesson and Finlayson, 1963). This work has demonstrated that the heterogeneity of human fibrinogen on the basis of its solubility includes species whose solubilities lie well beyond that of the fibrinogen(s) purified from fraction I, and which are distinguishable as well by their biologic reactivity to thrombin. Although the thrombin reactivity of the isolated fibrinogen subfractions bears a direct relationship to the solubility of the fibrinogen concerned and probably represents an intrinsic property of the molecule itself, the present data do not distinguish between retardation of the fibrinogen–fibrin conversion at the proteolytic phase and/or the polymerization phase (Scheraga and Laskowski, 1957) nor do they exclude the possibility that a nonfibrinogen contaminant may be responsible for the observed phenomenon. The subfractionation of purified bovine fibrinogen into fractions with varying solubility and reactivity to thrombin (Hartley and Waugh, 1960) is analogous to our findings with human fibrinogen, but in view of the fact that the solubility of bovine fibrinogen is somewhat different from human (Morrison *et al.*, 1951; Blombäck and Blombäck, 1956), these apparent similarities may not prove in the last analysis to be comparable in every respect.

It is unlikely that human fibrinogen purified by the more widely used procedures contains appreciable

amounts of fibrinogen of the solubility of I-7 or higher. Conversely, the fibrinogen(s) of "high solubility" (*e.g.*, I-7) isolated by the subfractionation procedures outlined appear to be relatively free of fibrinogen(s) of lower solubility. Since none of the fibrinogen subfractions studied was homogeneous (Figure 2), it is convenient to view the species under consideration as forming a continuous spectrum which can only be partially resolved by these techniques. Nevertheless, the "high-solubility" fibrinogen which has been isolated in highly clottable form has made available for biophysical and biological characterization[12] a species not previously studied.

It is attractive to consider that the heterogeneity of fibrinogen on the basis of its solubility is related, at least in part, to its partial degradation by plasma proteolytic enzymes. Indeed, there is evidence in support of the concept that fibrinogens of low solubility represent complex intermediates formed as a result of the action of thrombin and perhaps plasmin as well (Shainoff and Page, 1962; Bang *et al.*, 1962; Lipinski *et al.*, 1964). That plasma fibrinogen of high solubility may represent forms partially degraded by the enzyme plasmin is a reasonable but as yet unproven hypothesis. The fact that the degradation of human fibrinogen by plasmin is a complex one involving the cleavage of several bonds before the loss of biological identity[13] (Wallén and Bergström, 1957; Miles *et al.*, 1964) supports the notion that many forms of clottable fibrinogen are possible; the demonstration in this laboratory that fibrinogen clottable with thrombin and having solubility characteristics of fraction I-5[13] may be produced *in vitro* by plasmin digestion of fraction I-4 fibrinogen is entirely consistent with this view.

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[12] Further characterization and comparison of fibrinogen of high solubility with that of lower solubility is the subject of a future publication (M. W. Mosesson, N. Alkjaersig, A. P. Fletcher,and S. Sherry, in preparation).

[13] Recent studies in this laboratory have shown that approximately 17–20 bonds are titrated in the pH-Stat (pH 8.6) during the digestion of human fibrinogen (I-4) with plasmin before clottability is completely lost. Furthermore, plasmin digestion of Blombäck fraction I-4 produced clottable derivatives, soluble in % but precipitable with 16% ethanol.