[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL[1]]

# Preparation and Properties of Serum and Plasma Proteins. XVIII. The Separation of Purified Fibrinogen from Fraction I of Human Plasma.

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Fibrinogen is the plasma protein which forms fibrin under the action of thrombin; hence, its properties are of importance in any consideration of blood coagulation. It was early identified in plasma by virtue of its insolubility in concentrated salt solutions[3] and by its precipitation on moderate heating.[4] In 1879 Hammarsten[5] separated and purified this protein by a simple salting-out with half-saturated sodium chloride and this has been the classical method for its separation[6,7] although other salts such as ammonium sulfate[8] and potassium phosphate[9] have also been used. More recently fibrinogen has been shown also to comprise the largest component of the most insoluble major fraction separated from plasma by ethanol precipitation near *p*H 7 at low temperatures.[10,11,12]

Although the initial separation of fibrinogen is readily accomplished, its further purification is made difficult on the one hand by the ever present danger of clotting[13] and on the other hand by its high inherent lability. Procedures for its purification should, therefore, comprise as few steps and require as little time as possible and should avoid conditions which may promote clotting or denaturation.

In earlier studies there is some confusion regarding the characterization of fibrinogen, and this characterization has sometimes been based on the same chemical properties utilized in its separation. Thus, for example, it has often tacitly assumed that no account need be taken of components of lower solubility than fibrinogen, or others which may interact with it under the conditions of precipitation. Preparations repeatedly precipitated, as, for instance, by half-saturated sodium chloride, have often been considered pure without further verification. It seems clear, however, that fibrinogen must be characterized by its physiological activity, namely, the ability to form fibrin under the action of thrombin, and some of the analytical aspects of this reaction have been considered elsewhere.[14] The present study considers the solubility of fibrinogen in ethanol–water mixtures of controlled *p*H and ionic strength. It then describes the further fractionation of Fraction I of human plasma using this more convenient and easily controlled system to yield purified fibrinogen together with a hitherto unrecognized component of normal pooled plasma chiefly characterized by its insolubility at low temperatures.

Another component of Fraction I, probably very small in absolute concentration but of great physiological importance, is the so-called "antihemophilic globulin,"[11,15] which shortens the clotting time of hemophilic blood without affecting that of normal blood. Although this component is far more heavily concentrated in Fraction I than in any of the other plasma fractions,[16] we have not yet achieved its separation from fibrinogen in active form. Indeed the precipitation at *p*H 5, described in this paper as a step in the purification of fibrinogen, may possibly inactivate the antihemophilic factor. The procedure here presented, therefore, is irrelevant to the problem of concentrating the latter factor; and this problem will not be further discussed here.

**Materials and Methods.**—Fraction I of human plasma[10-12] was the starting material in all of these studies. This was preferably used immediately after separation, although frozen or dried materials can often be used with success. Fraction I normally contains 60% of fibrinogen. Av-

(1) This paper is Number 69 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross. This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Inquiries concerning the work reported in this paper should be addressed to Dr. J. T. Edsall.

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(3) P. Denis, "Mémoires sur le sang," Paris, 1859.

(4) L. Fredericq, *Bull. acad. roy. méd. Belg.*, **44**, 56 (1877).

(5) O. Hammarsten, *Arch. ges. Physiol.*, **19**, 563 (1879).

(6) M. Florkin, *J. Biol. Chem.*, **87**, 629 (1930).

(7) W. H. Howell, *Am. J. Physiol.*, **26**, 453 (1910); **35**, 143 (1914); H. Eagle, *J. Gen. Physiol.*, **18**, 531 (1935); E. Chargaff and A. Bendich, *J. Biol. Chem.*, **149**, 93 (1943).

(8) A. J. Quick, *J. Immunol.*, **29**, 87 (1935); H. Neurath, J. E. Dees and H. Fox, *J. Urol.*, **49**, 497 (1943).

(9) L. B. Jaques, *Biochem. J.*, **37**, 344 (1943).

(10) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(11) J. T. Edsall, R. M. Ferry and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 557 (1944).

(12) J. T. Edsall, J. D. Ferry and P. R. Morrison (in preparation).

(13) This applies particularly to the large scale collection and processing of human blood and plasma, where delays in cooling and separation can occur. These problems are discussed in reference 12, together with a consideration of the optimal conditions for the separation of Fraction I from plasma.

(14) P. R. Morrison, THIS JOURNAL, **69**, 2723 (1947).

(15) F. H. L. Taylor, C. S. Davidson, H. J. Tagnon, M. A. Adams, A. H. MacDonald and G. R. Minot, *J. Clin. Invest.*, **24**, 698 (1945); G. R. Minot, C. S. Davidson, J. H. Lewis, H. J. Tagnon and F. H. L. Taylor, *ibid.*, **24**, 704 (1945).

(16) A small amount of antihemophilic globulin passes into Fraction II + III, and on further subfractionation is concentrated in the prothrombin-rich Fraction III-2.[11,17]

(17) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., (in preparation).

erage values for the other electrophoretic components present are albumin, 7%; $\alpha$-globulin, 8%; $\beta$-globulin, 15%; and $\gamma$-globulin, 9%. The presence of most of these impurities can be accounted for by occlusion; but there is at least one importance of Fraction I which is actually lesss oluble than fibrinogen.

In the ultracentrifuge,[18] the major component, representing some 50% and sedimenting at about 8.5 to 9 S, is identified as fibrinogen. Fibrinogen is a highly asymmetrical molecule, approximately 700 Å. long by 38 Å. in cross section.[18,19]

**Preliminary Solubility Studies.**—In the small-scale experiments, designed to measure the solubility of the fibrinogen in Fraction I under different conditions, a dried preparation, containing some sodium citrate to inhibit clotting, was used to insure uniformity. This was dissolved to give a fibrinogen concentration of 20 g./l. and clarified by filtration. After cooling, the aliquots were mixed with cold solutions containing phosphate or acetate buffers and ethanol in such amounts that the desired final conditions of *p*H, ionic strength, ethanol and fibrinogen concentrations were attained. Acetate buffers were employed below *p*H 6; phosphate buffers at higher *p*H values. After equilibration at 0° for sixteen to twenty hours the solid phase was separated by centrifugation at this temperature. The precipitate was then dissolved in the initial volume of 0.15 *M* sodium chloride at room temperature,

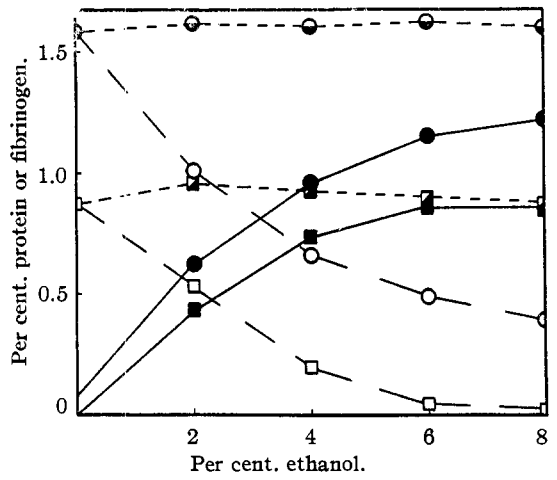


Fig. 1.—The solubility of fibrinogen and Fraction I as a function of ethanol concentration at constant *p*H and ionic strength. Ordinate, fibrinogen or total protein in per cent. referred to the initial volume; temperature, 0°; *p*H, 6.4; ionic strength, 0.30, sodium phosphate. Squares, fibrinogen; circles, total protein; open symbols, supernatant phase; closed symbols, precipitated phase; half-closed symbols, sum of the two phases (this should be constant).

(18) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. and Coll. Chem.*, **51**, 184 (1947).

(19) J. T. Edsall, J. F. Foster and H. Scheinberg, THIS JOURNAL, **69**, 2731 (1947).

and both this solution, and the supernatant, were analyzed for fibrinogen and total protein, correction being made for the volume of the precipitate. The concentration of fibrinogen was determined by gravimetric analysis[14] by forming a dilute clot under specified conditions (fibrinogen concentration, 1 g./l.; thrombin concentration, 0.1 unit/cc.; *p*H, 6.2–6.4); the clot was then synerized, washed, dried and weighed. Total protein was determined by biuret analysis,[20] using a concentrated serum albumin solution, previously standardized against solutions of Fraction I, as a standard.

Subsequent large scale fractionations utilized the Fraction I precipitate from 25–60 liters of fresh plasma, which contained 35–80 g. of fibrinogen. The material was taken from the centrifuge bowl and dissolved at once in a citrate buffer. The citrate, by combining with ionized calcium, retarded the conversion of any prothrombin to thrombin and the subsequent clotting of the fibrinogen. The low *p*H (near 6.2) of the buffer employed also retards clotting.[21] Further details of technique are referred to in the Appendix.

## Experimental

Solubility.—The solubility of fibrinogen in Fraction I was measured at five *p*H values ranging from 5.0 to 7.0, at ethanol concentrations from 0 to 8% and at ionic strengths of 0.30, 0.15 and 0.075. The temperature was 0 to −1° in all cases. The total concentration of fibrinogen was 10 g./l. in two series at 0.30 and 0.15 ionic strength, and 5 g./l. in two series at 0.15 and 0.075 ionic strength. The effect of ethanol on the solubility of fibrinogen and Fraction I at a single *p*H and ionic strength is shown in Fig. 1. The total amounts of both fibrinogen and total protein, derived from the sum of the respective concentrations in the two phases, should be constant. Totals obtained in most cases were quite satisfactory. However, under some circumstances, notably at higher ethanol concentrations, a falling off in these values was sometimes observed, which seemed to reflect an interference in the analysis by this reagent.

The solubility of fibrinogen falls off sharply as the ethanol concentration is increased. At a *p*H value near 7.0 fibrinogen is largely precipitated at ethanol concentrations above 6%; at low *p*H values near 5.0, which is in the region of its isoelectric point, the solubility of fibrinogen in ethanol concentrations greater than 1% is negligible. The effect of these variables on the solubility of fibrinogen is shown in Figs. 2, 3 and 4, in each of which a solubility surface is shown as determined by *p*H and ethanol concentration at a single ionic strength.

Contrasted to the behavior of fibrinogen, the solubility of the non-clottable protein of Fraction I shows little sensitivity to ethanol in these concentrations. It is similarly reduced at low *p*H values, but to a much lesser extent than that of the fibrinogen. These relative solubility relations indicate the conditions under which maximum purification can be achieved by a single precipitation. The *p*H is the most important variable and the best results were achieved at the lowest *p*H value. Under these conditions yields near 80% were attained at each of the concentrations employed.

Further study of the data indicated the presence of a non-clottable component whose solubility is less than that of fibrinogen. This was deduced by comparing the yields and purities of the precipitates removed with

(20) J. W. Mehl, *J. Biol. Chem.*, **157**, 173 (1945).

(21) J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 388 (1947).

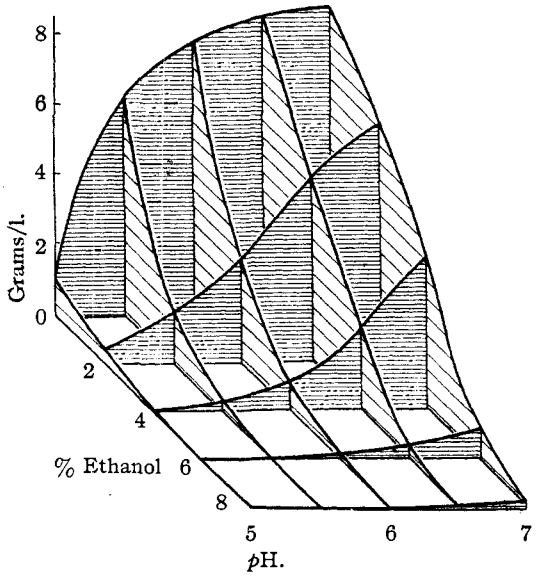


Fig. 2.—The solubility of fibrinogen (from Fraction I) as a function of *p*H and ethanol concentration; ionic strength, 0.30. Ordinate, fibrinogen concentration in g./l.; abscissa, *p*H; Z axis, ethanol concentration in per cent. by volume; temperature, 0°. The values in this figure were interpolated from two dimensional charts of the solubility as a function of *p*H at constant ethanol concentration. $\Gamma/2 = 0.30$.

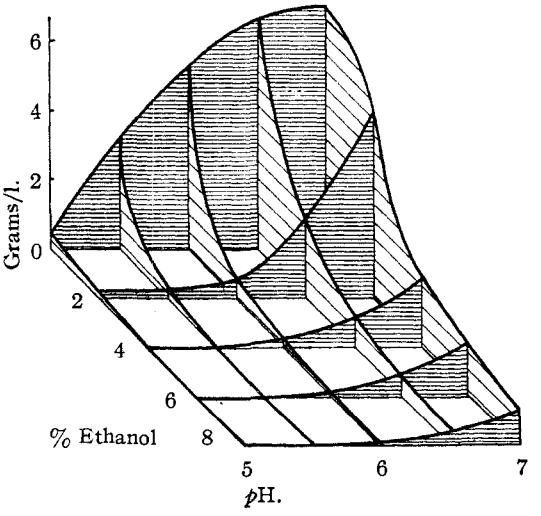


Fig. 3.—The solubility of fibrinogen (from Fraction I) as a function of *p*H and ethanol concentration; ionic strength, 0.15. Coördinates as in Fig. 2. $\Gamma/2 = 0.15$.

increasing ethanol concentrations. As the ethanol concentration was increased, a greater yield was obtained as expected, but the purity also increased. This was an unexpected finding, since fibrinogen has generally been considered the component of lowest solubility in plasma, and earlier procedures for purification were based on this assumption. It is, however, in accord with the observation that after one or two precipitations, repeated further ethanol precipitations near *p*H 7 do not increase the purity to more than 85–90%.[11] To check this deduction of the presence of a very insoluble component the composition of fractions removed successively under conditions

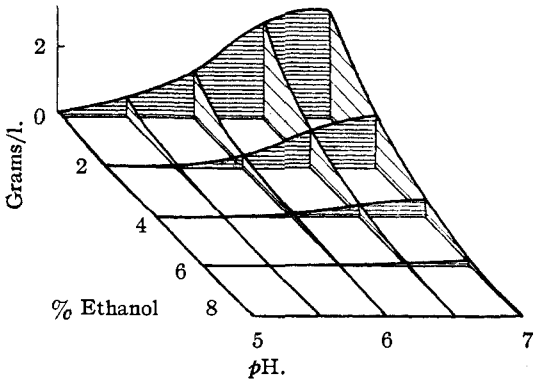


Fig. 4.—The solubility of fibrinogen (from Fraction I) as a function of *p*H and ethanol concentration; ionic strength, 0.075. Coördinates as in Fig. 2. $\Gamma/2 = 0.075$.

of increasing ethanol concentration was examined; results of two such experiments are presented in Table I. The amount of non-clottable protein does indeed decline as the ethanol is increased, as would be required of a less soluble component or complex. At higher ethanol concentrations the non-clottable moiety again increases as the more soluble components begin to precipitate.

TABLE I

THE COMPOSITION OF PRECIPITATE REMOVED WITH SUCCESSIVELY HIGHER ETHANOL CONCENTRATIONS[a]

| Conditions of precipitation | Per cent. clottable protein A | B |
|---|---|---|
| Cooled to 0°, no ethanol | 49 | 62 |
| Ethanol to 0.5% | 64 | 83 |
| Ethanol to 1.0% | 77 | |
| Ethanol to 2.0% | 82 | 92 |
| Ethanol to 3.0% | 96 | 97 |
| Ethanol to 6.0% | | 68 |
| Ethanol to 8.0% | | 55 |

[a] Ethanol concentrations given as per cent. refer to the mixture at 25°.

**Fractionation.**—The conclusions drawn from these solubility measurements—namely, that fibrinogen can be most readily separated from the bulk of the non-clottable proteins by precipitation at a low *p*H, and that there exists a component with lower solubility than fibrinogen—were applied in the following large-scale fractionations. Fresh Fraction I dissolved in citrate buffer at a *p*H of 6.2 to 6.3 was cooled and brought to a low *p*H (4.9 to 5.5)[22] and a low ethanol concentration (0 to 4%). After equilibration, the precipitate (Fraction IA) was separated by Sharples centrifugation. This precipitate was dissolved at a *p*H of 6.2 to 6.4 and brought to 0.5% ethanol at 0°. Under these conditions a precipitate, Fraction I-1, containing the material of lower solubility than fibrinogen, separated out. In the absence of a specific name this insoluble component will be denoted simply as cold-insoluble globulin. After removing this precipitate, the supernatant was brought to 8% ethanol and another precipitate, Fraction I-2, was removed.

(22) It has been suggested that fibrinogen, like prothrombin[23] and some other proteins, may be sensitive to low *p*H values acid to its isoelectric point. Our evidence bearing on this point is not conclusive, but under the conditions employed here the solubility, clottability and stability of the preparations obtained was highly satisfactory. Moreover, ultracentrifuge measurements, and studies of double refraction of flow indicated no denaturation as compared with the original Fraction I. However, as a precaution, the duration of exposure to *p*H 5 has been kept as short as possible.

(23) W. H. Seegers, *J. Biol. Chem.*, **136**, 103 (1940).

This contained fibrinogen in a high state of purity. The supernatant solution, containing small amounts of fibrinogen and nonclottable protein, was discarded. The results of four such experiments[24] are summarized in Table II.

TABLE II

THE COMPOSITION OF SUBFRACTIONS OF FRACTION I

| Fr. | *p*H | Ethanol, % | Fibrinogen in % | 166a | 167a | 166b | 167b |
|---|---|---|---|---|---|---|---|
| I | 7.2 | 8 | Purity | 64 | 62 | 64 | 62 |
| | | | Yield | (100) | (100) | (100) | (100) |
| I-A | *a* = 5.0 | 1 | Purity | 85 | 86 | | |
| | | | Yield | 73 | 73 | | |
| | *b* = 5.5 | 4 | Purity | | | 72 | 82 |
| | | | Yield | | | 67 | 62 |
| I-1 | 6.2 | 0.5 | Purity | 55 | 66 | 40 | 60 |
| | | | Yield | 6 | 17 | 10 | 15 |
| I-2 | 6.2 | 8 | Purity | 98 | 98 | 94 | 95 |
| | | | Yield | 37 | 53 | 40 | 48 |

**Fraction I-A.**—The separation of this fraction at *p*H 5.0 and 1% ethanol is more effective than at *p*H 5.5 and 4% ethanol, since both the percentage and the total yield of clottable protein are higher under the former conditions. The material remaining in solution, after Fraction I-A is precipitated, includes all the principal electrophoretic components and undoubtedly largely derives from their mechanical occlusion in the initial Fraction I precipitate, rather than from their insolubility under those conditions of separation. Table III shows the relative and absolute amounts of these components, in one run, for this solution as compared to the initial Fraction I. The β-globulin is the only sizable component remaining in the precipitate and even this has been considerably reduced. In this particular case 70% of the non-clottable material was removed together with less than 5% of the fibrinogen.

TABLE III

ELECTROPHORETIC ANALYSIS OF FRACTION I AND OF THE SOLUTION REMAINING AFTER PRECIPITATION OF FRACTION I-A

| Run 176 | alb. | α | β | ø | γ | Clottable | Nonclottable |
|---|---|---|---|---|---|---|---|
| Fraction I | | | | | | | |
| % | 5 | 6 | 14 | 65 | 10 | 66 | 34 |
| g. | 2.9 | 3.5 | 8.2 | 39 | 5.8 | 39 | 20.4 |
| Solution[a] | | | | | | | |
| % | 17 | 14 | 19 | 7 | 33 | 7 | 93 |
| g. | 2.9 | 2.4 | 3.3 | 1.3 | 5.7 | 1.3 | 14.3 |

[a] It was necessary to concentrate this material by drying from the frozen state.

**Fraction I-2.**—As separated by this procedure, Fraction I-2 contained about 50% of the initial fibrinogen in a purity of 93 to 98%.[25] However, upon drying this material from the frozen state a loss in clottability of 5 to 8% was always observed. Since in experiments on whole Fraction I[14] successive drying steps of this sort did not result in any further diminution in fibrinogen, the loss observed here may define a portion of the fibrinogen which had become partially denatured. The dry fraction dissolves rapidly and completely, indeed much more readily than the original Fraction I probably because of the removal of the cold-insoluble component in Fraction I-1. That fraction in contrast dissolves very slowly from the dry state. In ordinary concentrations the solutions of Fraction I-2 are clear and colorless, having been freed of traces of yellowish pigment which sometimes color the original fraction. More concentrated solutions, however, appear yellowish in transmitted light due to the selective Tyndall scattering of the shorter wave lengths. Solutions as concentrated as 100 to 110 g./l. have been prepared but these were extremely viscous even at room temperature, and gelatinous at 0°. With the addition of further protein gelation occurred even at 20–25°.

An electrophoretic diagram[26] of fraction I-2 is shown in Fig. 5. This particular fraction had been dried and redissolved and was 92% clottable; before drying the value had been 98%. Of this material 95 to 98% migrated with a mobility characteristic of fibrinogen in whole plasma. However, after an extended run as in Fig. 5, 5 to 8% of a slightly faster component could be seen separating from the main peak. The amount of this material corresponds to the loss in clottability upon drying. This, and the closeness of its electrophoretic behavior to that of fibrinogen, suggest that it was derived from fibrinogen during the drying procedure. A similar picture of Fraction I-2 was seen in the ultracentrifuge[27] (Fig. 6). Here the main component comprising some 87% of the fraction sedimented at 8.5 S. Most of the residual material sedimented at $s_{20,w}$ = 12–14 S, but small amounts sedimenting at $s_{20,w}$ = 2–3 S and $s_{20,w}$ = 18–20 S were sometimes observed. Flow birefringence measurements[19] place the average length at approximately 700 Å. and this, together with the data for sedimentation and viscosity,[18] indicates a molecular weight near 500,000. Osmotic pressure measurements of the molecular weight[18] gave 580,000 but since these measurements involved determination of very low pressures in solutions of a somewhat unstable protein, the value must be considered as approximate.

**Fraction I-1.**—This fraction possesses some remarkable properties. As can be seen from the conditions of its separation, it is highly insoluble under circumstances where the other plasma proteins are quite soluble. Lerner and Greenberg[28] have recently described a "cold-insoluble" protein which separated from a pathological plasma at 0° and they cited several other reports of the presence of such components in pathological plasmas. Lerner and Greenberg report the proteins studied by them to have a molecular weight of 190,000, whereas studies from this laboratory indicate that the globulin of Fraction I-1 is much larger than this.[29]

The work of I. R. Morrison[30] indicates the presence of a component in plasma which may be identical with our cold-insoluble globulin. Employing fractionation with ammonium sulfate, he removed an initial fraction, at 1/6 saturation, which had distinctly different properties from other fibrinogen containing fractions separated between 1/6 and 1/4 saturation with ammonium sulfate. The initial fraction separated as a "tenacious, rubbery mass"; it precipitated on cooling and redissolved on warming. The amount varied markedly from one individual patient to another, and it was found to be particularly high during the last trimester of pregnancy. This fraction (denoted by I. R. Morrison as "contractinogen") formed clots which differed from those of the usual fibrinogen fraction, and showed a great tendency to synere-

(24) Detailed technical instructions for the preparation of purified fibrinogen are given in the Appendix. It should be noted that this system was designed to separate several components of Fraction I without loss and in as few steps as possible, both for economy in preparation and to minimize the exposure of the protein to handling procedures. It can clearly be modified in the light of the solubility relations described above should considerations of either yield or purity be more important; or the several subfractions may be further purified or concentrated.

(25) A. G. Ware, M. M. Guest and W. H. Seegers, *Arch. Biochem.*, **13**, 231 (1947), have recently described a method for the purification of bovine fibrinogen which depends on its remaining as a precipitate after slowly melting frozen plasma. Excellent purities were reported, but the yields appeared to be very low, of the order of 5%.

(26) Electrophoretic analyses were carried out by M. J. E. Budka and K. C. Morrison.

(27) Ultracentrifugal analyses were carried out by C. G. Gordon under the direction of Dr. J. L. Oncley.

(28) A. B. Lerner and G. R. Greenberg, *J. Biol. Chem.*, **162**, 429 (1946).

(29) Unpublished work by J. T. Edsall, G. A. Gilbert and H. A. Scheraga in this laboratory.

(30) I. R. Morrison, *Am. J. Med. Sci.*, **211**, 325 (1946).

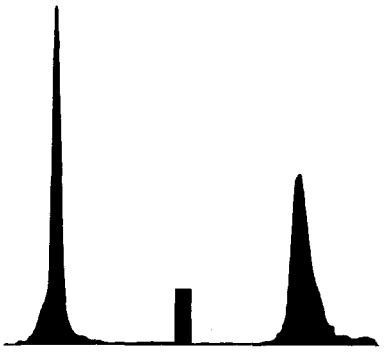

Fig. 5.—Electrophoretic schlieren diagram of a Fraction I-2 (Lot 167). Ionic strength, 0.20 citrate and barbiturate; conductivity, 0.00377 ohm$^{-1}$ cm.$^{-1}$; *p*H 8.5. These pictures were taken after 300 minutes at a current of 30 m. amp. The starting boundary is represented by the center bar. Ascending side, left; descending side, right. Note the slightly faster component which is just beginning to separate from the main peak.

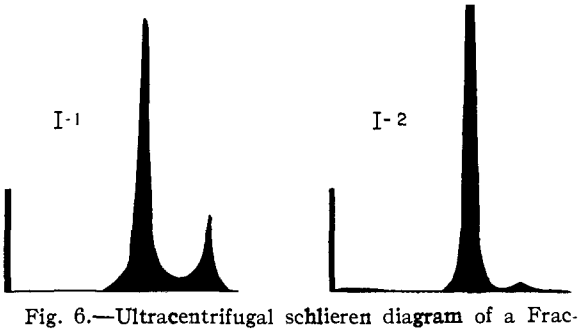


Fig. 6.—Ultracentrifugal schlieren diagram of a Fraction I-1 and I-2 (Lot 167). The vertical bar represents the meniscus and these diagrams have been corrected for the base line. These pictures were taken after 60 minutes at 280,000 times gravity. The peak of the main component in Fraction I-2 has not yet descended. Protein concentration, 10 g./l.; *p*H, 6.3; ionic strength, 0.30, sodium citrate.

sis. The same preparations also had a marked effect on the red cell sedimentation velocity. The latter point has not been tested by us, but it appears probable that Morrison's "contractinogen" fraction contains a component identical with our cold-insoluble globulin.

In the light of these findings, it seems probable that the cold-insoluble globulin in preparations from pooled plasma is largely derived from a small fraction of the donors. The amount recovered appears to vary significantly from run to run and this supports the view that this globulin is not a normal plasma component. The amounts recovered from Fraction I corresponded to a plasma concentration of 0.15 to 0.45 g./l. or only 0.3 to 0.7% of the total pooled plasma protein.

The form of the Fraction I-1 precipitate differs strikingly from that of other plasma components. If the solution is cooled without stirring or other agitation, the precipitate appears identical in form to a coarse-structured fibrin clot.[21] The gel so formed occupies the whole volume of the solution, it is opaque and, like the fibrin clot, it will synerize under mechanical pressure to yield a compacted precipitate. It differs from the fibrin clot, however, in that the gelation is completely reversible and upon warming the gel dissolves. This sequence may be repeated, although a small amount appears to become insoluble at each cycle.

The fraction is composed of fibrinogen and the nonclottable component, which we have termed cold-insoluble globulin.[29] Roughly one-half of the fraction is clottable under the conditions of assay. The components appear to form a definite complex on precipitation, since reprecipitation under identical conditions yielded a product with the same clottability (*i. e.*, the precipitated and supernatant phases were identical). Ultracentrifugal analysis of this fraction shows two well-defined peaks (Fig. 6). The larger component sediments at a rate characteristic of fibrinogen ($s_{20,w}$ = 7–9 S) and, indeed, so closely parallels the behavior of purified fibrinogen that they are presumably identical (Table IV).

TABLE IV

SEDIMENTATION CONSTANTS FOR FRACTIONS I-1 AND I-2[a]

| Concentration, g./l. | Fraction I-2 | Fraction I-1 Major[b] | Fraction I-1 Minor |
|---|---|---|---|
| 20 | 6.99[c] | 7.02[c] | 9.76[c] |
| 10 | 8.28[c] | 8.31[c] | 11.8 |
| 5 | 8.39[c] | 8.44[c] | 12.2 |

[a] As $s_{20,w}$ in Svedberg units. [b] Note the exact correspondence at each concentration between the sedimentation constants of the major components of both fractions. [c] The second decimal place in these values for *s* is uncertain.

Since the cold-insoluble globulin begins to precipitate even from moderately dilute solutions (5 g./l.) of this fraction at temperatures of 4 to 8°, it is difficult to make electrophoretic analysis under the usual conditions (*i. e.*, at 4°). However, such a run[28] is shown in Fig. 7. Some precipitation has occurred, but two distinct components can be distinguished. The slower and larger component must be fibrinogen and the relative area of this peak corresponds to the clottability of the fraction. The smaller component (*i. e.*, the cold-insoluble globulin) has a higher mobility apparently corresponding to that of a $\beta_1$-globulin. This was to be expected from the composition of the solution remaining after the precipitation of Fraction I-A (*cf.* Table III) which pointed to a $\beta_1$-globulin as being the major remaining impurity.

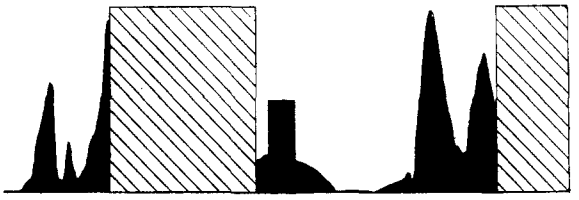

Fig. 7.—Electrophoretic schlieren diagram of a Fraction I-1 (Lot 167). The center bar represents the starting boundary and the lined area shows precipitation. Ascending side, left; descending side, right; *p*H, 7.5; ionic strength, 0.20, phosphate and citrate, conductivity 0.00524 ohm$^{-1}$ cm$^{-1}$. These pictures were taken after three hundred minutes at a current of 30 m. amp.

In Fig. 7 precipitation is seen to begin at the fibrinogen gradient on the ascending side and at the cold-insoluble globulin gradient on the descending side. In other words, precipitation occurred only in the presence of both electrophoretic components. Since interaction between the two components is so strongly indicated, the smaller peak with an intermediate mobility may represent a small amount of the dissolved complex. This material might in turn constitute the heavy component ($s_{20,w}$ = 18–20 S) in the sedimentation diagram.

The various components revealed by electrophoretic, ultracentrifugal and clotting analyses have been arranged in Table V to show their suggested interrelationships.

TABLE V

COMPARISON OF THE COMPONENTS IN FRACTIONS I-1 AND I-2

| Fraction | Electrophoresis peak | % | Sedimentation $s_{20,w}$ S | % | Clottability, % | Component |
|---|---|---|---|---|---|---|
| 167 I-1 | $\phi$ | 63 | 8–9 | 65 | 60 c. | Fibrinogen |
| | ($\beta_2$) | 6 | 20 | 5 | | (Complex) |
| | | | | | 40 n. c. | |
| | $\beta_1$ | 31 | 13 | 25 | | Cold insoluble |
| 167 I-2 | $\phi$ | 89 | 8–9 | 88 | 92 c. | Fibrinogen |
| | $\phi$ | 6–8 | 20 | 5 | | (Modified |
| | | | | | 8 n. c. | fibrinogen) |
| | $\beta_1$ | 3–5 | 13 | 5 | | (Cold insoluble) |

## Appendix

On the basis of the studies outlined in this paper, the following procedure has been adopted for the separation of purified fibrinogen from Fraction I.

Precipitate I from normal human citrated plasma is used as the starting material for this preparation. Each kilogram of this precipitate is suspended[31] in 4 liters of citrate buffer[32] of *p*H 5.90 ± and ionic strength 0.30, at a temperature of 10° ± 2°. This suspension should be warmed to 20–25° and gently stirred for thirty minutes. After the addition of an appropriate filter aid[33] the solution is filtered with suction through a very porous paper[34] on a Buchner funnel. After further clarification[35] the solution is cooled to about 7°[36] and then brought to a *p*H of 5.00–5.10 by the slow addition with a stirring of one and one-half volumes of 0° citrate buffer of ionic strength 0.30 to which the appropriate amount of hydrochloric acid and 2% of ethanol had been added. Stirring is continued while the suspension is cooled to 0°.

**Precipitate I-A** should contain roughly 80% of the initial fibrinogen at a purity of about 85%. It is removed by centrifuging the above suspension with a high rate of flow.[37] Each kilogram of Fraction I-A is suspended[31] in 6 liters of 10 ± 2° "standard" sodium citrate buffer, to which has been added sufficient sodium hydroxide to bring the *p*H of the suspension to 6.35 ± 0.05. After warming to 20–25° and stirring gently for twenty minutes, the solution is filtered[34] and cooled to 10°.[36] An equal volume of 0° citrate buffer[32] containing 1% ethanol is added with slow stirring which is continued as the solution is slowly cooled to 0°.

**Precipitate I-1,** which is dense, stringy and clot-like, may be removed without centrifugation. It contains roughly 20% of the initial protein of which two-thirds or less is fibrinogen and the rest is a β-globulin which is insoluble at low temperature.

The supernatant solution from precipitate I-1 is brought to an ethanol concentration of 8% by the addition of an equal volume of −1°, 15% ethanol.

**Precipitate I-2** contains about 60% of the starting fibrinogen and should contain less than 5% of non-clottable protein. It is removed by centrifugation[37] at 0 to 1°. This precipitate is suspended[31] in 4 liters of 0.3 molar sodium chloride and after clarification[38] the solution is frozen and dried from the frozen state. Losses in clottable protein of the order of 5% are usually observed upon drying.

In view of the lability of fibrinogen, these steps should be carried through as quickly as possible. Difficulties may be encountered in the use of Fraction I which has been aged or exposed to unfavorable conditions.

## Summary

1. The solubility of fibrinogen, and of the non-clottable proteins of Fraction I of human plasma, has been investigated as a function of *p*H, ionic strength and ethanol concentration at 0°. The solubility of fibrinogen is very sensitive to all these variables.

2. Separation of purified fibrinogen from Fraction I has been accomplished in a process involving two major steps: (a) precipitation of fibrinogen near *p*H 5.1, leaving most of the non-clottable protein in solution; (b) precipitation of a non-clottable component (cold-insoluble globulin) which still remains associated with fibrinogen after the *p*H 5.1 precipitation by cooling the solution from about 20 to 0°, at *p*H near 6.3. Sodium citrate buffers are employed. The ionic strength is maintained at 0.30 throughout the process, and the ethanol concentration is near 0.5–1%.

3. The properties of the cold-insoluble globulin—notably its great insolubility at 0° and rapid increase of solubility with temperature—are briefly discussed. At 0°, and at ethanol concentrations near 3%, this component is definitely less soluble than fibrinogen.

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(31) Precipitates containing fibrinogen are tough, rubbery and difficult to suspend. A Waring mixer is used for this step but foaming should be prevented and a low temperature and minimal mixing time are recommended for such a vigorous procedure.

(32) This buffer contains 0.055 mole of sodium citrate and about 0.013 mole of acid in each liter. The exact amount of hydrochloric acid should be adjusted so that the dissolved Fraction I has a *p*H of 6.25–6.40. It is convenient to prepare a stock solution of 4 times this concentration.

(33) Fifty grams of standard "Super-Cel" for each liter of solution has proved satisfactory.

(34) Two Whatman no. 4 papers are effective.

(35) Pressure filtration through a Hormann D-2 pad usually suffices to remove most of the turbidity. Washing of the pads with citrate buffer to remove calcium is recommended. These filtrations will tend to remove traces of prothrombin or thrombin but may also eliminate wanted substances (*e. g.* antihemophilic globulin).

(36) If the solution is cooled too far a stringy clot-like precipitate will form.

(37) Sharples centrifugation packs the precipitate closely, occluding less of the supernatant phase, but bucket centrifugation may be used.

(38) For this final clarification pads as fine as Seitz (Republic) S-3 or Hormann D-10 may be used if desired.