APPENDIX

Determination of Free Non-volatile Fatty Acids

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For investigations concerning the mode of action of the clearing factor, estimations of free fatty acids in mixtures of normal rat plasma and lipaemic dog plasma were performed using a modification of van de Kamer's (1948) method as described for faeces. Because the method developed may be of value for similar or other uses, it is given here. In a 100 ml. flask 2 ml. of the plasma-mixture, containing either heparin or citrate as an anticoagulant, are run into 6 ml. of phosphoric acid solution (metaphosphoric acid, 50 g., NaCl, 250 g. water to 1 l.). After a few minutes 16 ml. of ethanol, containing 0·4 % amyl alcohol, are added, followed by 20 ml. of distilled light petroleum (b.p. 40–60°). The flask is stoppered and the mixture shaken by hand for 1 min. After separation, the upper layer is filtered through dry paper and the funnel covered in order to minimize evaporation. Care should be taken to avoid contamination with traces of the other phase. Ten ml. of the light petroleum extract is evaporated on a boiling-water bath and the residue dried *in vacuo*. Neutralized ethanol (5 ml.) containing 0·6 mg. of thymol blue per 100 ml. are added and the residue dissolved by boiling gently under reflux for 3 min. Finally, the fatty acids are titrated under a stream of N_2 with 0·01 N-NaOH. The results are corrected for a blank (usually about 0·060 ml. of 0·01 N-NaOH), obtained by running 2 ml. of water through the same procedure.

The reproducibility of the method suffices for many purposes. In twenty-four estimations giving results ranging from 0·21 to 21·0 μ-equiv. of fatty acids per 2 ml. of plasma tested, the differences between duplicate determinations were found to have a standard deviation of $\pm 0{\cdot}14$ μ-equiv.

Added amounts of stearic acid were recovered satisfactorily (Table 1).

Table 1. *Recovery of stearic acid added to plasma*

Stearic acid added (mg.)	Excess stearic acid found (mg.)	Recovery (%)
12·00	12·84	107
6·00	6·16	103
4·00	4·17	104
3·00	3·21	107
2·00	1·94	97
1·00	0·96	96

The presence of sodium citrate in plasma in amounts up to 7·6 mg./ml. did not interfere with the determination.

REFERENCE

Kamer, J. H., van de (1948). Thesis, Utrecht.

The Purification of Human Fibrinogen

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The principles underlying the use of water-miscible solvents for the separation of proteins from aqueous solutions under controlled conditions of pH, ionic strength and temperature have been described by Cohn and his colleagues (Cohn *et al.* 1946). These principles were exploited to develop systems for the serial fractionation of human plasma proteins with ethanol (Cohn *et al.* 1946; Cohn *et al.* 1950).

The possibility that ether might similarly be employed arose from experimental observations made when extending the method described by McFarlane (1942) for the removal of lipid from human serum, to the treatment of human plasma. Not only was lipid extracted from combination

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with plasma proteins, but the fibrinogen was precipitated though in an intractable form. Arising from this, methods have been described for the successive separation of fibrinogen, prothrombin, γ-globulin and albumin fractions from human plasma under rigidly aseptic conditions for clinical purposes, using defined quantities of ether with suitable adjustment of pH, ionic strength and temperature (Kekwick, Mackay & Record, 1946; Kekwick & Mackay, 1949; Kekwick & Mackay, 1954).

This paper describes the development of a method using ether in the further purification of human fibrinogen. The purity of intermediate fractions and of the final product was assessed by electrophoresis and ultracentrifuge measurements, by the determination of the proportion of the total protein nitrogen which was recoverable in the clot formed with thrombin, and by tests designed to establish the degree of contamination with, or freedom from, plasminogen and plasmin.

EXPERIMENTAL

Materials

All the chemicals used were of analytical reagent quality. Aqueous solutions and buffers were made up in sterile pyrogen-free water and, where necessary, were sterilized by passage through a Seitz filter, or by autoclaving at 20 lb. pressure for 30 min.

Diethyl ether. The ether used in the precipitations was of anaesthetic grade but free from antiperoxidants. It was stored at 2°, and freedom from peroxides was established by the standard British Pharmacopoeia test using KI solution.

Citrate–saline. Composition: Trisodium citrate dihydrate: 3·7 g./l.; NaCl: 8·5 g./l.

It was convenient to make up a stock solution containing 10 times these quantities, and to dilute this accurately to the above concentration. The solution was immediately sterilized in M.R.C. transfusion bottles by autoclaving for 30 min. at 20 lb. pressure.

Ether–citrate–saline. To 1 l. citrate–saline cooled to 2°, 80 ml. ether were added and dissolved by shaking; the solution was stored at 2°.

Citrate buffers. pH 6·1: 0·169 M trisodium citrate, 0·014 M citric acid. pH 6·5: 0·164 M trisodium citrate, 0·006 M citric acid. These buffers were considered to have an ionic strength $I = 1·0$; lower ionic strength buffers were prepared by suitable dilution from them and were sterilized by Seitz filtration.

Veronal buffer (pH 7·3). Sodium diethyl barbiturate: 5·87 g./l.; NaCl 7·21 g./l.; 0·2 M-HCl: 107·5 ml./l.

Streptokinase. Initially a simple filtrate from a culture of a beta haemolytic streptococcus (N.C.T.C., H 53 A) was used as a source of streptokinase, but later tests were made with a purified preparation (Streptodornase, Lederle). Both of these preparations were stored freeze-dried, and small quantities dissolved in appropriate buffers and concentrations as required.

Thrombin. Human thrombin was prepared by the method described by Lyttleton (1954), and had an activity of about 100 units/mg. protein N (Kekwick & Mackay, 1954). The plasminogen and plasmin content of these preparations was so low as to have a negligible influence on the experimental results obtained.

Analytical methods and assays

pH *Determination.* Values of pH were determined with a MacInnes type glass electrode, the outer chamber of which was filled with 0·05 M potassium hydrogen phthalate. The reference electrodes were 3·5 M calomel cells, and for calibration a solution containing 0·09 M-KCl plus 0·01 M-HCl was assumed to have a standard pH = 2·10. Measurements were made at room temperature with a valve potentiometer, the accuracy being ±0·01 pH unit. In systems containing solvent the pH values given are apparent, and no attempt has been made to correct for the effect of solvent.

Electrophoretic measurements. Samples were dialysed to equilibrium at 2° against phosphate buffer, pH 8·0, $I = 0·2$; (Na_2HPO_4, 0·0652 M; KH_2PO_4, 0·0044 M) and diluted with buffer to give a refractive increment $n_1 - n_0 = 0·00350$, where n_0 is the refractive index of the buffer and n_1 that of the protein solution. Refractive index determinations were made with a dipping refractometer using the Hg arc green line 546 mμ.

Electrophoretic measurements were made in the Tiselius (1937) apparatus at 0°, using a potential gradient of 6 v/cm. Optical observations by the diagonal schlieren method (Philpot, 1938) were photographically recorded on Ilford half-tone panchromatic plates, using a high pressure Hg arc as a light source from which monochromatic light 546 mμ. was isolated by using a suitable filter.

By optical projection of the recorded photographs with an enlargement of eight diameters on to mm. graph paper, tracings were made. The curves were analysed by reflexion across median lines to determine the quantities of the components present. The results are expressed as the percentage of total protein in a mixture attributable to any single component. The values for both ascending and descending limb boundaries are given because there is evidence of interaction between fibrinogen and another protein constituent in some of the fractions.

Ultracentrifuge measurements. Dialysed samples were diluted to $n_1 - n_0 = 0·00200$ so that the solution contained phosphate buffer pH 8, $I = 0·2$, +0·15 M-NaCl. The solutions were subjected to 250 000 g in the Svedberg oil turbine ultracentrifuge (Svedberg & Pedersen, 1940), using a 12 mm. cell, the optical recording being as described under electrophoresis.

Nitrogen estimations. These were made in triplicate by a micro-Kjeldahl procedure requiring about 3 mg. N for each estimation. The protein was digested in 5 ml. sulphuric acid (microanalytical reagent) and selenium oxychloride was used as a catalyst. The NH_3 was steam distilled into 0·02 M-HCl and 0·02 M-NaOH used for back titrating, with methyl red as an indicator. The titrations were carried out in a stream of CO_2-free air.

The estimation of fibrinogen by conversion into fibrin. If fibrinogen is clotted with thrombin under suitable conditions, the fibrin clot formed can readily be reduced to a compact film, the volume of which is less than 1% of that of the original clot. By this means most of the extraneous protein which may be present with the fibrinogen is removed, and the compact film can easily be washed before the estimation of the fibrin nitrogen by the micro-Kjeldahl method.

The fibrinogen solution containing approximately 0·1 g./100 ml. was adjusted colorimetrically to pH 7·2, and 25 ml. were pipetted into a Pyrex test tube 8 × 1 in. To this 2·5 units of thrombin were added, the mixture was stirred thoroughly with a fine glass rod (3 mm. diameter) and allowed to stand at room temperature with the stirring rod in the tube. Clotting should commence within 10 min. of the addition of thrombin. After 1 hr. the clot was loosened from the wall of the test tube, and by gentle pressing and rotating by means of the rod against the walls of the test tube, it was compressed to a thin sheath adhering to the stirring rod. A further 2·5 units of thrombin were then added as before, and after another hour at room temperature any additional clot which formed was wound on to the rod. The compacted clot was washed on the rod by immersion in citrate saline and then twice similarly in distilled water. The washed clot was then digested off the rod by the standard Kjeldahl procedure, and the nitrogen content determined as described.

The supernatant solution from the clot was usually stored overnight at 2° to ensure that clotting had been complete. If further clotting occurred the fibrin was either added to the original digest or the estimation repeated.

In estimating fibrinogen in plasma or in any fraction having marked antithrombin activity, rather more thrombin was required to induce clotting in 10 min.

The ratio of clottable to total nitrogen of fibrinogen preparations. This value was determined by estimating the fibrinogen nitrogen as described above, and also the total nitrogen of an equal volume of the same fibrinogen solution by the standard micro-Kjeldahl method. The ratio is usually expressed as the percentage of the total nitrogen which appears in the clot (% nitrogen clottable). It should be noted that there is evidence that a peptide is split from fibrinogen during the clotting process (Bailey, Bettelheim, Lorand & Middlebrook, 1951; Lorand, 1952). For bovine fibrinogen the peptide nitrogen split off is close to 3·0–3·5% of the fibrinogen nitrogen. This implies that the clottability of 100% fibrinogen would be assessed as 96·5–97% of the total nitrogen by the above method.

Detection of plasmin and plasminogen. In this paper plasmin refers to the active proteolytic enzyme of human plasma which exhibits optimum activity in the region of pH 7·2–7·6; the precursor of plasmin, plasminogen may be activated to give a maximum yield of plasmin by interaction with streptokinase (see Christensen & MacCleod, 1945). In order to determine the degree of contamination with plasminogen or plasmin of fibrinogen-containing fractions, a method depending on fibrinogenolysis was used. The end point of the reaction was judged by the loss of the ability of a solution of the fraction under test to form a clot when thrombin was added to a sample. Under standardized conditions the time required for the fibrinogen to lose the ability to clot, referred to as the 'life', is inversely related to the amount of lytic enzyme present.

Plasmin test. Sterile samples of fibrinogen, freeze-dried from solution in citrate–saline, were reconstituted to their original volume with sterile distilled water, and diluted with sterile veronal buffer pH 7·3 to give a solution containing 0·2 g. fibrinogen/100 ml. This solution was incubated at 37°. Samples (0·2 ml.) were removed aseptically at suitable intervals and to them was added an equal volume of a thrombin solution containing 2 thrombin units/ml. The 'plasmin life' is the time elapsing from the beginning of the incubation period until the last occasion on which any clot formation occurs.

Plasminogen test. Sterile freeze-dried samples of fibrinogen were reconstituted as described above and diluted with veronal buffer pH 7·3 to give a solution containing 0·25 g. fibrinogen/100 ml. A 4·0 ml. sample was pipetted into a test tube and placed in a thermostat bath at 37·0 ± 0·1°, and after allowing a short time for temperature equilibration, 1·0 ml. of a solution of streptokinase in veronal buffer pH 7·3, previously brought to temperature in the bath, was added. At suitable intervals, 0·2 ml. samples of the mixture were measured into 2 × ⅜ in. test tubes also in the bath, and 0·2 ml. thrombin solution (10 units/ml.) was added to these.

During the incubation period, measured from the time of addition of the streptokinase, and the clotting time of samples progressively lengthened as the fibrinogen was digested, and the amount of fibrin in the clot decreased. The end point was deemed to have been reached when no indication of clot formation could be detected 3 min. after the addition of thrombin to the test sample.

The 'plasminogen life' is the period from the beginning of incubation until the first occasion on which no clot formation is detected in the test sample.

Streptokinase standardization. To obtain comparable results with different batches of streptokinase, these were standardized against a single preparation of freeze-dried fibrinogen fraction F.1W. (see below). Plasminogen life tests were carried out as described above, and the amount of streptokinase added was varied by varying the concentration in the 1·0 ml. of solution added. The lowest concentration of streptokinase giving the shortest plasminogen life was then used for tests with other fractions.

Preparative procedures

Technique of precipitation. All solutions were kept in a low temperature laboratory at 2°, where most of the routine operations were carried out using low temperature thermostat tanks and refrigerated bucket centrifuges controlled to any required temperature ±0·2°.

Precipitations were carried out in closed glass vessels, the metal caps of which (Fig. 1) provided for the passage of a stirrer (*D*), capillary jet (*B*), adjustable siphon (*C*), thermometer pocket (*A*) and a bacteriologically protected air inlet (*E*). Various sizes of this type of apparatus were available and all could be sterilized in an autoclave. Solutions were transferred from one vessel to another by the use of sterile compressed air.

The solution to be treated was passed through the siphon tube into the precipitation vessel which was placed in the low-temperature thermostat tank adjusted to the required temperature. Sterile buffer solutions and ether were added, at speeds controlled by small needle valves, through the capillary jet, whilst efficient stirring was maintained but with avoidance of frothing. The temperature of mixtures was measured with a thermometer placed in the thermometer pocket, which was partially filled with ethanol to facilitate heat transfer. Heat evolved during the addition of ether was controlled by adjusting the rate of flow of ether so that specified temperatures were not exceeded.

When the desired conditions had been attained, mixtures were allowed to equilibrate without stirring, usually overnight (16 hr.), by which time the precipitate had usually

settled to a sludge, occupying 10–20% of the total fluid volume. The clear supernatant was siphoned off, the precipitate spun down at controlled temperature in screw-capped bottles in the refrigerated bucket centrifuge and the residual supernatant added to the main bulk. In experiments of a quantitative nature the whole mixture was centrifuged.

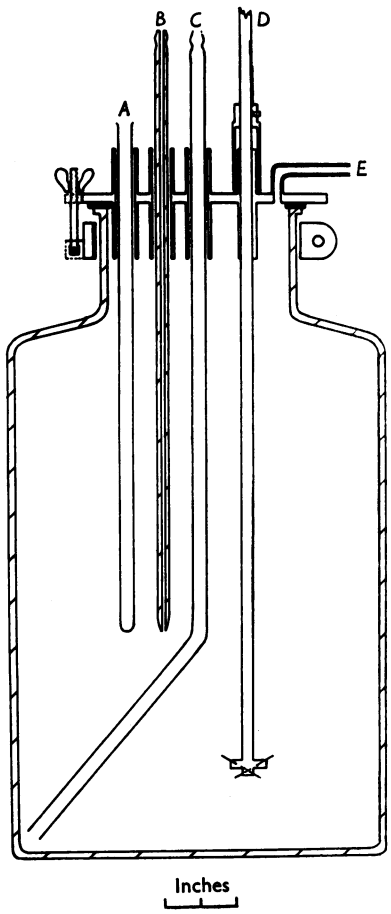

Fig. 1. Diagrammatic scale drawing showing detail of Pyrex glass precipitation vessel and fittings. Metal parts clear. Rubber parts filled in black. Glass parts cross hatched as far as possible. Scale indicates inches. *A*, thermometer pocket; *B*, capillary jet; *C*, siphon tube; *D*, stainless steel stirrer assembly; *E*, air inlet. *A*, *B* and *C* are secured to the tubes in the metal top plate by rubber sleeves, which are not shown. To *E* is attached a sterile cotton-wool air filter when the vessel is in use.

Technique of extracting a precipitate. In order to compare the relative efficacy of various buffers in the extraction of precipitated protein, it is necessary to control within relatively narrow limits the mass of protein and volume of buffer used to extract it. The extraction procedure was utilized here only for fractionating the product designated F.1W.; though the following description refers specifically to this fraction the method is applicable to other materials. The operations were carried out aseptically.

To obtain approximately equal amounts of the precipitate F.1W., the preceding precipitate F.1 was evenly suspended by mechanical stirring in 30% of the plasma volume of 8 vol. % (i.e. 8 vol./100 vol.) ether–citrate–saline at 2°, and equal volumes of this suspension were transferred into transfusion bottles. The suspension was centrifuged 30 min. at 2° and 1500 rev./min. and the supernatants poured off, leaving approximately equal samples of the washed precipitate F.1W.

At 2°, a volume of buffer, calculated in relation to the volume of plasma from which the precipitate samples were derived, was added to the packed F.1W. precipitate, which was then thoroughly dispersed by brisk mechanical stirring to give a uniform suspension. The rate of stirring was then reduced to a gentle speed and continued at a controlled temperature (0–2°) until the protein content of the extract became constant, this being established by the constancy of the refractive index of samples of extract removed from time to time, suspended precipitate being centrifuged down in these samples before the measurements were made. Usually extraction for 3 hr. was sufficient, and following this the undissolved residue was centrifuged down at 2°, the supernatant removed and the residue dissolved at room temperature in citrate–saline to provide a suitable solution for analytical measurements.

RESULTS

Preparation of plasma

Blood was drawn into 120 ml. trisodium citrate solution (3 g./100 ml.) in a standard blood transfusion bottle to make a final volume of 540 ml. The blood, cooled to 2°, was centrifuged in the original bottles for 30 min. at 1450 rev./min. and 2°, within 24 hr. of being taken. The supernatant plasma was siphoned off and passed through a paper-pulp filter at 2° under a pressure of 30 cm. Hg, to remove the residual cellular material and particulate lipid.

The clear yellow plasma obtained had an average protein content of 4·5–4·6 g./100 ml. and a pH value of 7·7.

Conditions for the precipitation of fibrinogen from plasma with ether

The optimum conditions for the precipitation of fibrinogen from plasma were determined by dissolving ether in a standard volume of filtered plasma, the temperature throughout the addition being maintained at $0{\cdot}0 \pm 0{\cdot}5°$. The amounts of ether added covered the range 3–14 ml./100 ml. plasma (3–14 vol. %). The mixtures were allowed to equilibrate in closed vessels at $0{\cdot}0 \pm 0{\cdot}2°$ for 16 hr., after which the precipitates which formed were spun down for 30 min. at 1450 rev./min. and $0{\cdot}0 \pm 0{\cdot}2°$. The clear supernatants were poured off quantitatively and made up to a standard volume with citrate–saline. The precipitates were dissolved in citrate–saline and also made up to a standard volume. These solutions and the original plasma were assayed for fibrinogen by determining the protein

nitrogen recovered in the clot formed with thrombin. The results of these assays, in which the amount of fibrinogen is expressed as a percentage of the total initial fibrinogen, are given in Table 1.

The results show that the proportion of fibrinogen precipitated steadily increases until the amount of ether added reaches 10 vol. %, at which point 88–89 % of the plasma fibrinogen is precipitated. Further increase in the amount of ether added up to 14 vol. % results in no significant increase in the amount of fibrinogen precipitated. The results of Table 1 also show that within the limits of error the fibrinogen of the plasma can be quantitatively accounted for in the precipitate and supernatant fluid.

Table 1. *The precipitation of fibrinogen from plasma with ether*

pH 7·7, temp. = 0·0°.

Ether (vol. %)	% total fibrinogen	
	Precipitate	Supernatant
0	—	100
3	25·1	—
4	48·8	47·9
6	73·3	—
8	84·7	15·7
10	88·4	—
11	88·2	11·2
12	88·3	—
14	90·2	—

On the basis of these results the precipitation of fibrinogen from plasma by the addition of 11 vol. % ether at 0·0° has been chosen as a standard procedure, providing a yield of about 90 % of the total plasma fibrinogen. This material is designated F. 1.

Purification experiments using citrate–saline solutions

Comparison of reprecipitating and washing the crude fibrinogen (F. 1). The crude fibrinogen F. 1 is contaminated with plasma proteins occluded in the precipitate, fibrinogen accounting for 50 % of the total protein present on the basis of electrophoretic analysis and determinations of the total and clottable protein nitrogen (Table 2). Though albumin is the main contaminant the fraction contains in particular much of the plasma antihaemophilic globulin and substantial quantities of plasminogen.

To explore possibilities of further purification, a quantity of the precipitate F. 1 was dissolved in citrate–saline to give a total volume equivalent to 30 % of that of the plasma from which it was obtained. In order to effect complete solution it was necessary to warm the mixture to 20°. The reprecipitation of the fibrinogen was examined relative to the amount of ether added in a similar manner to that described for plasma, and the precipitation curve obtained indicated that at 0·0° and 8 vol. % ether the recovery of fibrinogen was almost quantitative, less than 5 mg. fibrinogen/100 ml. solution remaining dissolved.

The reprecipitation of fibrinogen from citrate–saline solution at 0·0° with 8 vol. % ether to give F. 2, resulted in a marked increase in purity (Table 2, Fig. 2), but in the course of the procedure some of the plasminogen was activated to plasmin, or plasmin inhibitor removed, so that the plasmin 'life' of the fibrinogen was reduced. The solubility results indicated that a substantial purification of the fibrinogen might alternatively be attained by suspending the precipitate F. 1 at 0·0° in 30 % of the plasma volume of citrate–saline to which had been added 8 ml. ether/100 ml., and spinning down the precipitate at 0·0° for 30 min. at 1450 rev./min. The analytical figures for the washed fibrinogen (F. 1 W.) are included in Table 2. These indicate that the washing procedure is quite as effective for purification at this stage as solution and reprecipitation, and furthermore, less activation of plasminogen to plasmin occurs, the product F. 1 W. having a longer plasmin 'life' than F. 2. The washing procedure was accordingly adopted as the second step in the purification.

Table 2. *Fibrinogen fractions purified from citrate–saline solution*

Fraction		Electrophoretic analysis				Clottable N/total N (%)	Plasmin life (hr.)
		Albumin	Globulins		Fibrinogen		
			$\alpha+\beta$	γ			
F. 1	A	23·2	15·4	10·1	51·3	49·8	1000
	D	26·3	9·3	4·6	59·8		
F. 2	A	3·2	8·7	6·1	82·0	74·9	260
	D	5·4	4·1	5·4	85·2		
F. 1 W.	A	2·3	11·2	4·2	82·2	83·7	380
	D	5·0	3·6	7·5	83·8		
F. W 3	A	0·0	6·7	2·8	90·6	90·4	120
	D	0·0	1·4	0·0	98·6		

A, ascending limb analysis (anode); D, descending limb analysis (cathode).

There is obviously a limit to the effectiveness of repeatedly washing a protein precipitate with samples of the same fluid in order to improve the purity of one of its constituents. Accordingly, in an attempt to purify the washed precipitate (F.1W.) further, it was dissolved in 30% of the plasma volume of citrate–saline and reprecipitated twice at 0·0° with 8 vol. % ether. The analytical data for the product obtained (F.W3) are also included in Table 2.

A comparison with F.1W. shows that all the albumin and some of the globulins were removed. The fibrinogen content, from the nitrogen determinations, was raised only to 90·4%, though the electrophoretic analysis gave a slightly higher value. Simultaneously, there was a further activation of plasminogen to plasmin, indicated by the marked decrease in the plasmin 'life' of the fibrinogen.

Fractional precipitation from solutions of F.1W. *with ether at* 0·0°. The fact that the clottable protein reached a value of only 90% in the product F.W3, indicated that at some level of ether concentration non-clottable protein must have been precipitated with the fibrinogen. In order to examine this further, a solution of F.1W. was fractionally precipitated at 0·0° by adding ether to give a concentration of 2 vol. % and collecting the precipitate, then raising the ether concentration of the supernatant to 4 vol. % and finally 8 vol. %, thus collecting two more fractions.

Table 3. *Fractional precipitation of fibrinogen from a citrate–saline solution of* F.1W. *at* 0·0°

Fraction, ether (vol. %)	Clottable N/total N (%)	Plasmin life (hr.)	Plasminogen life (min.)
0–2	81·3	160	75
2–4	93·2	165	75
4–8	89·6	320	65
Control F.1W.	—	500	25

It is clear from Table 3 that at both low and high ether concentrations non-clottable protein was precipitated, the intermediate 2–4 vol. % ether fraction consisting of fibrinogen of the highest purity attained. However, the plasmin and plasminogen 'life' of this fraction indicated that it was still seriously contaminated with plasmin and plasminogen. Many other similar variations designed to improve the purity by fractional precipitation from citrate–saline with ether were no more satisfactory.

Extraction of F.1W. *with buffers at* 0–2°

On the basis of the results already presented it can be concluded that it is desirable to maintain fibrinogen fractions containing ether, plasminogen and plasmin at temperatures close to 0° during precipitation procedures, and also that citrate–saline (pH 7·7, $I = 0·225$) is not a solvent likely to facilitate the separation of fibrinogen from plasminogen and plasmin. The problem, therefore, was to find a buffer capable of dissolving fibrinogen and not plasmin or plasminogen, at temperatures close to 0°.

Ratnoff (1948) observed that when samples of human plasma were diluted with 19 vol. of distilled water with simultaneous adjustment of the pH over the range 4·80–6·30, the precipitate which formed at pH 5·3–5·4 contained the maximum amount of plasminogen. However, when the plasminogen was estimated by conversion into plasmin with streptokinase, the curve showing the amount of plasminogen precipitated as a function of pH was relatively flat from pH 5·4 to 6·3. This indicated that at relatively low ionic strengths the solubility of plasminogen is low even at pH 6·3. The isoelectric point of fibrinogen is close to pH 5·4, and though its solubility is minimal in this region it increases markedly as the pH is raised. Consequently, by choosing buffers of suitable ionic strength in the pH range 6·0–6·5 it appeared possible that fibrinogen might be extracted from the F.1W. precipitate, leaving much of the plasminogen and plasmin undissolved.

Some preliminary experiments using phosphate buffers showed that at pH 6·0 and at ionic strengths up to 0·4, extracts of F.1W. contained relatively less plasminogen and plasmin than the material which remained undissolved at 2°. However, the extracted fibrinogen tended to become insoluble in these buffers on standing. In addition, it was difficult to estimate the amount of fibrinogen in the extracts, because clots formed in the presence of phosphate ion were rather friable and would not readily synerese. Citrate buffers proved to be much more satisfactory.

Effect of variation in ionic strength in citrate buffer, pH 6·1. From the stock citrate buffer (pH 6·1, $I = 1·0$) a series of dilutions was made to give ionic strengths 0·05, 0·15, 0·30, 0·45. Equal quantities of F.1W. precipitate were extracted, as described under 'Procedures', with these buffers at 2°, the volume of the buffer being adjusted so that the total amount of buffer salt was the same in each extraction. For the buffer of $I = 0·30$ the volume taken was equivalent to 40% of the plasma volume, for $I = 0·15$, 80% of the plasma volume, and so forth.

It will be seen from Table 4 that as the original buffer mixture was diluted the measured pH increased significantly, but this could not be avoided. The extract at $I = 0·05$ had a much longer plasminogen life than those at higher ionic strengths, but despite the large volume of buffer used only

26% of the original fibrinogen was dissolved. The most advantageous buffer appeared to be that of $I=0{\cdot}3$, since the plasmin was least soluble at this ionic strength and 50% of the fibrinogen was recovered in the extract.

There was no increase in the amount of fibrinogen extracted when the buffer volume at $I=0{\cdot}3$ was increased from 40 to 60% of the plasma volume. In later experiments the extract with this buffer contained between 60 and 65% of the F.1W. fibrinogen.

Effect of variation of pH *of citrate buffer at* $I=0{\cdot}3$. Using citrate buffer pH 6·5, $I=0{\cdot}3$, improved the yield of fibrinogen in the extract (Table 5). The results in this table also show that the extracts in each instance contain rather less plasminogen and plasmin than the residues.

possibilities of the extraction at low ionic strength (Table 4, $I=0{\cdot}05$) which had been passed by temporarily on account of the poor recovery of fibrinogen. The problem was attacked in another manner by diluting the citrate buffer extracts $I=0{\cdot}3$ with 5 vol. distilled water, to provide solutions of ionic strength 0·05 for fractional precipitation with ether at 0°.

In Table 6 the results are given for two fractionations of the pH 6·1 extract and one for the pH 6·5 extract, all the material coming from a single preparation of F.1W. As might be anticipated, a precipitate formed on diluting the extracts from $I=0{\cdot}3$ to $I=0{\cdot}05$. The precipitate from the pH 6·1 extract contained 11·6% of the initial fibrinogen, leaving 50% in solution, which was about twice the quantity obtained by extracting the F.1W.

Table 4. *Characteristics of extracts of* F.1W. *obtained with citrate buffers of differing ionic strength at* 2°

Buffers made by diluting a stock solution pH 6·1, $I=1{\cdot}0$, containing 0·169M-Na citrate + 0·014M citric acid.

Ionic strength	pH	F.1W. fibrinogen in extract (%)	Clottable N/total N (%)	Plasmin life (days)	Plasminogen life (min.)
0·05	6·49	25·9	60·4	15	57
0·15	6·31	40·7	70·2	18	17
0·30	6·18	50·2	72·5	26	11
0·45	6·09	62·3	71·9	8	13

Table 5. *The extraction of* F.1W. *with citrate buffers* pH 6·1 *and* 6·5, $I=0{\cdot}3$ *at* 2°

Buffer vol. equivalent to 40% plasma vol.

pH	% F.1W. fibrinogen	Clottable N/total N (%)	Plasmin life (days)	Plasminogen life (min.)
6·1 extract	61·6	78·0	16	14
6·1 residue	38·4	78·5	9	11
6·5 extract	67·1	76·2	21	13
6·5 residue	32·9	78·3	9	9
Control F.1W.	—	78·0	7	9

Fractionation of citrate buffer extracts of F.1W.

The fractional precipitation with ether at 0° of the extracts obtained at pH 6·1 and 6·5, $I=0{\cdot}3$, was examined in a manner similar to that shown in Table 3 for citrate–saline solutions, since there was a possibility that at these lower pH values a separation of plasminogen and plasmin from fibrinogen might be effected. Again no useful separation was achieved and the results showed that the extracts also contained non-clottable protein of lower and higher solubility than fibrinogen (cf. Table 3).

Fractional precipitation of $I=0{\cdot}3$ *extracts with ether at* 0° *after diluting to* $I=0{\cdot}05$. The failure to separate plasminogen and plasmin from fibrinogen with ether at $I=0{\cdot}3$ refocused attention on the

directly with the same volume of pH 6·1, $I=0{\cdot}05$ buffer (Table 4). The precipitate also contained a large proportion of the plasminogen and plasmin of the whole extract, which is reflected in the increase, especially in the plasminogen life, of the fractions subsequently separated at 0–5 and 5–8 vol. % ether. The preliminary removal of a fraction at 1 vol. % ether decreased the enzyme content of the subsequent 1–5 vol. % ether fraction, though this entailed the loss of 22% of the fibrinogen. The 5–8 vol. % ether fraction was unaffected by the initial 1 vol. % step.

A comparison with the similar fractionation of the pH 6·5 extract indicates that pH 6·1 is the more advantageous. At pH 6·1 more of the plasminogen and plasmin are removed at 1 vol. % ether, and the subsequent 1–5 vol. % ether fraction

consequently contains less of these than the corresponding pH 6·5 fraction. For these reasons the use of the pH 6·5 buffer was discontinued at this stage of the work.

Another point of interest in the pH 6·1 fractionation (Table 6) is that though much of the plasmin is precipitated by 1 vol. % ether, a further precipitation occurs in the 5–8 vol. % ether fraction, together with non-clottable proteins. The electrophoretic analysis of F.1W. (Table 2) shows that a small amount of albumin is present, and this would undoubtedly be extracted by the pH 6·1, $I = 0·3$ buffer. On reducing the ionic strength to 0·05 it seemed possible that some of this albumin might co-precipitate with fibrinogen at 8 vol. % ether. This co-precipitation might be prevented by first removing the enzyme rich fraction at $I = 0·05$ and 0 to 1 vol. % ether, and then increasing the onic strength again before precipitating the main bulk of fibrinogen.

Therefore in a further experiment, the effects of 0·5 and 1·0 vol. % ether were examined at pH 6·1, $I = 0·05$ in order to find conditions for removing as much plasminogen and plasmin as possible with the least loss of fibrinogen (Table 7). Whereas the subsequent 1·0–8·0 vol. % ether fraction had slightly less enzyme contaminant, the 0·5–8·0 fraction had a significantly higher proportion of clottable protein and the fibrinogen recovery was greater.

In another sample, after removing the enzyme-rich fraction with 1·0 vol. % ether, the ionic strength of the supernatant solution was raised to 0·20 by the addition of 0·066 vol. of stock concentrated citrate–saline, which also raised the pH to 6·50, and the ether was then brought to 8 vol. %. Though the recovery of fibrinogen in the precipitate was only 22 %, the product was remarkably free from plasminogen and plasmin and the proportion of clottable protein very high (Table 7).

Fractionation of pH 6·1, $I = 0·3$ *extracts with ether at* 0° *by successive low and high ionic strength stages.* An improvement in the fibrinogen recovery at $I = 0·20$ could be expected on raising the ether concentration to 11 vol. %, and the results of such an experiment are given in Table 8, the enzyme-rich fraction having been removed with 0·5 vol. % ether at $I = 0·05$. Though the enzyme content of the 0·5–11 % ether fraction was extremely low, the clottable N/total N at 96·0 % had been exceeded previously, and the value for the 8–11 vol. % ether subfraction indicated that non-clottable protein was precipitated in this region.

Table 6. *Fractionation of* pH 6·1 *and* 6·5, $I = 0·3$ *citrate buffer extracts with ether at* 0° *after diluting to* $I = 0·05$

pH	Fraction, ether (vol. %)	% F.1W. fibrinogen	Clottable N/total N (%)	Plasmin life (days)	Plasminogen life (min.)
6·1	0	11·6	60·6	<1	5
	0–5	39·1	94·3	10	120
	5–8	5·3	88·2	7	>1320
	0–1	22·2	73·4	1	11
	1–5	27·4	92·3	16	180
	5–8	5·5	89·2	7	>1320
	Whole extract	61·7	78·0	16	11
	Residue	38·5	78·5	7	9
6·5	0–1	14·4	67·8	1	7
	1–5	35·7	94·3	7	25
	5–8	9·3	90·4	7	>240
	Whole extract	67·8	76·2	19	11
	Residue	32·9	78·3	7	9
	Control F.1W.	—	78·0	7	9

Table 7. *Fractionation of* pH 6·1, $I = 0·3$ *citrate buffer extract with ether at* 0° *after diluting to* $I = 0·05$

Fraction, ether (vol. %)	Ionic strength	% F.1W. fibrinogen	Clottable N/total N (%)	Plasmin life (days)	Plasminogen life (min.)
0–0·5	0·05	10·7	59·8	<1	11
0–1·0	0·05	15·7	67·1	<1	11
0·5–8·0	0·05	41·3	97·8	12	200
1·0–8·0	0·05	37·2	93·8	16	300
*1·0–8·0	0·20	22·1	98·8	59	>1440
Whole extract	0·30	60·5	74·1	16	15
Control F.1W.	—	—	74·2	7	7

* 0–1 vol. % ether fraction removed at $I = 0·05$ and supernatant then raised to $I = 0·20$ before bringing ether to 8 vol. %.

Table 8. *Fractionation of* pH 6·1, $I = 0·3$ *citrate buffer extract with ether at* 0° *by successive low and high ionic strength stages*

pH	Ionic strength	Fraction, ether (vol. %)	% F.1W. fibrinogen	Clottable N/total N (%)	Plasmin life (days)	Plasminogen life (min.)
6·1	0·05	0·0–0·5	9·5	51·0	<1	5
6·5	0·20	0·5–8·0	38·7	95·6	40	>360
6·5	0·20	8·0–11·0	9·6	92·3	38	>180
6·5	0·20	0·5–11·0	48·2	96·0	43	>1800
Whole extract	—	—	67·0	77·5	8	11
Control F.1W.	—	—	—	76·1	5	11

Table 9. *Fractionation of* pH 6·1, $I = 0·3$ *citrate buffer extract. Effect of* pH *variation at the final high ionic strength precipitation stage*

pH	Ionic strength	Fraction, ether (vol. %)	% F.1W. fibrinogen	Clottable N/total N (%)	Plasmin life (days)	Plasminogen life (min.)
6·1	0·05	0·5	13·4	69·1	<1	9
6·5	0·20	0·5–11	34·3	97·7	15	>420
7·0	0·20	0·5–11	35·2	98·4	45	>420
7·3	0·20	0·5–11	34·9	97·2	22	>240
7·6	0·20	0·5–11	35·1	97·7	4	>420
Whole extract	—	—	52·5	75·7	8	10
Control F.1W.	—	—	—	80·4	6	12

In order to overcome this, a final experiment was carried out (Table 9). After removing the enzyme-rich fraction at 0·5 vol. % ether $I = 0·05$, and then raising the ionic strength as before to 0·20, samples of supernatant were adjusted with 0·2 M-Na_2HPO_4 to the pH values shown, the amounts required having been determined electrometrically, and the ether concentration was then brought to 11 vol. %. It is clear from Table 10 that pH 7·0 provided optimum conditions for recovery, high proportion of clottable protein and freedom from enzyme contamination.

Summary of the purification procedure

On the basis of the experimental results presented, the following procedure was chosen to provide the best yield of fibrinogen of the highest purity.

(1) The fibrinogen is precipitated from plasma by adding 11 vol. % ether at 0·0°, and after allowing the mixture to equilibrate for 16 hr., as much as possible of the clear supernatant is siphoned off; the sludge is then centrifuged for 30 min. at 0·0° and 1450 rev./min. The centrifuged precipitate F.1 contains 90 % of the plasma fibrinogen.

(2) The precipitate F.1 is washed by suspending it in 30 % of the plasma volume of 8 vol. % ether–citrate–saline at 0–2° and centrifuged as before to give the product F.1W. The recovery of fibrinogen at this stage is almost quantitative.

(3) The F.1W. precipitate is extracted with 40 % of the plasma volume of citrate buffer (pH 6·1, $I = 0·3$) at 0–2° for 3 hr. and the undissolved residue is centrifuged down. The extract usually contains between 55 and 65 % of the F.1W. fibrinogen.

(4) The citrate buffer extract (pH 6·1, $I = 0·3$) is diluted with 5 vol. distilled water at 0–2° and the ether concentration brought to 0·5 vol. % at 0·0°. After 16 hr. equilibration the enzyme-rich precipitate, which also contains 9–14 % of the F.1W. fibrinogen, is removed.

(5) The ionic strength of the supernatant from (4) is raised to 0·20 by the addition of 0·066 vol. of citrate–saline concentrated 10 times. The pH is adjusted to 7·0 with the requisite amount of 0·2 M-Na_2HPO_4 and the ether concentration raised to 11 vol. % at 0°; the mixture is allowed to equilibrate for 16 hr.

The precipitate which forms is the purified fibrinogen, the yield being 30–40 % of the F.1W. fibrinogen. It is convenient to dissolve it in citrate–saline to give solutions containing 0·5–1·0 g. protein/100 ml. These are Seitz filtered before use or before freeze-drying for storage.

When preparations are made from 5 l. or more of plasma, it is convenient to ensure complete removal of the enzyme-rich fraction separated at pH 6·1 and $I = 0·05$ by passing the supernatant under 30 cm. Hg air pressure through a clarifying filter pad (Ford FCB) at 0–2°, on account of the large volume of solution involved. This step on the small scale had been accomplished by centrifuging the whole supernatant, since this precipitate did not settle out well. Complete removal is essential if fibrinogen with clottable N/total N ratios of 97–98 % and good stability is to be obtained.

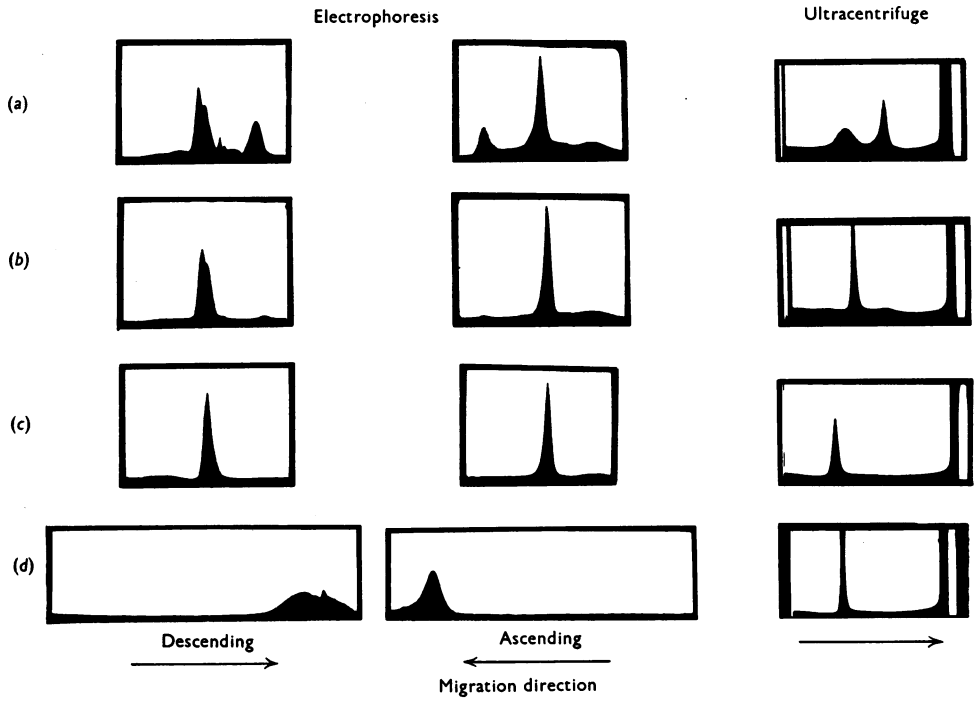

Fig. 2. Electrophoresis and ultracentrifuge diagrams of the main fractions obtained during the course of preparing purified fibrinogen. (*a*) Crude fibrinogen, F.1. (*b*) Washed fibrinogen, F.1W. (*c*) and (*d*) Final purified fibrinogen.

Electrophoresis photographs. Phosphate buffer (pH 8·0, $I=0·2$) temp. $=0·2°$. Protein concentrations: (*a*) 1·5; (*b*), (*c*) and (*d*), 1·0 g./100 ml. Exposures: (*a*), (*b*) and (*c*) after 3 hr. and (*d*) after 20 hr. at 15 mA.

Ultracentrifuge photographs. Phosphate–NaCl buffer: phosphate pH 8·0, $I=0·2$; 0·15M-NaCl. Protein concentrations: (*a*) 1·5; (*b*) 1·0; (*c*) 0·60, (*d*) 1·0 g./100 ml. Speed: (*a*) and (*b*) 60000 rev./min. (*c*) and (*d*) 54000 rev./min. All exposures 50 min. after reaching full speed.

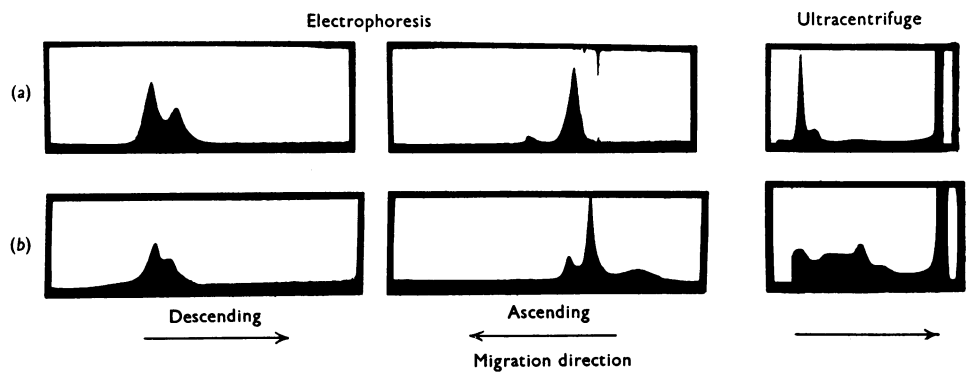

Fig. 3. Electrophoresis and ultracentrifuge diagrams of subfractions obtained during the course of preparing purified fibrinogen. (*a*) Fraction insoluble at pH 6·1, $I=0·3$, temp. $=0°$. (*b*) Fraction insoluble at pH 6·1, $I=0·05$, temp. $=0°$, ether 0·5 vol. %.

Electrophoresis photographs. Phosphate buffer (pH 8·0, $I=0·2$) temp. $=10·0\pm0·2°$. Protein concentrations (*a*) 1·5; (*b*) 2·0 g./100 ml. Exposures (*a*) after 20 hr. at 5 mA, (*b*) after 5 hr. at 10 mA.

Ultracentrifuge photographs. Phosphate–NaCl buffer: phosphate pH 8·0, $I=0·2$; 0·15M-NaCl. Protein concentration: (*a*) 1·0, (*b*) 1·5 g./100 ml. Speed: 54000 rev./min. Exposures: (*a*) 30 min., (*b*) 40 min. after reaching full speed.

Some properties of the main fractions separated

Values have already been given for the clottable N/total N ratio and enzyme content of the fractions F.1, F.1W. and the cold insoluble residue. In Figs. 2 and 3, anode and cathode electrophoresis diagrams and ultracentrifuge diagrams of these fractions are shown.

The electrophoresis diagrams for F.1 and F.1W. are those from which the analytical values given in Table 2 were obtained. In both these fractions the fibrinogen gives rise to a single slightly skewed peak in the anode limb, whereas in the cathode limb two components are apparent. Attempts to analyse the complex cathode fibrinogen peak led to the conclusion that both components must contain clottable protein. In conjunction with the unsymmetrical migration and accompanying divergences in the electrophoretic analyses for the anode and cathode limb boundaries, this implies that complex formation occurs between fibrinogen and some other contaminating plasma protein under the conditions obtaining in the cathode limb.

The cold insoluble residue which at 0–2° is obtained as a translucent, semi-elastic almost cartilaginous solid, has a very high temperature coefficient of solubility. The electrophoretic examination of this material was made at $10 \pm 0{\cdot}2°$ in order to obtain a clear 1·5% (w/v) solution in phosphate buffer (pH 8·0, $I = 0{\cdot}2$) these conditions having been found more satisfactory than a higher ionic strength at 0–2°. The electrophoresis diagrams (Fig. 3) show a much greater degree of complex formation than for the F.1 and F.1W. fractions. Two components only are present and in the anode limb these account for 91 and 9% and in the cathode 62 and 38% of the total protein respectively.

The electrophoretic characteristics of the fraction precipitated by 0·5 vol. % ether at $I = 0{\cdot}05$ resemble those of the cold insoluble residue, and in this instance also the experiments had to be conducted at 10°.

The ultracentrifuge diagrams of F.1 and F.1W. illustrate the removal of the albumin contaminating F.1, and also reveal the presence of a small proportion of a component with a higher sedimentation coefficient than the main peak of F.1W.; the main peak is due to fibrinogen and some globulin from which the fibrinogen would not be expected to resolve. In the cold insoluble residue the proportion of the heavier component ($S_{20} \simeq 11S$) is much higher than in F.1W. and there are trace amounts of a component with $S_{20} \simeq 17{\cdot}5S$. The 0·5% ether fraction is very complex in its behaviour in the ultracentrifuge and displays four components. The most slowly sedimenting component is apparently a breakdown product resulting from the action of plasmin on the fibrinogen present in the fraction.

The purified fibrinogen

Some characteristics of larger scale preparations of fibrinogen are given in Table 10. The clottable N/total N ratio varies between 97·0 and 98·7%, with a standard deviation in each instance of about ±0·2%. As a result of freeze-drying this value usually falls by about 1%.

With the large-scale preparations of purified fibrinogen, there was enough material for an exhaustive examination of the plasmin and plasminogen lives; this is the reason for the apparent difference in order to magnitude between the values of Table 10 and that of previous tables. The plasmin life indicates that the preparations were effectively uncontaminated with this enzyme. The plasminogen life indicates either that the enzyme precursor was absent, or that the activator precursor, with which, according to Müllerz & Lassen (1953), streptokinase reacts, was absent.

Table 10. *Characteristics of some larger batches of purified fibrinogen*

Batch	Clottable N/total N (%)	Plasmin life (days)	Plasminogen life
33	97·0	107	>20 hr.
36	97·2	>42	>42 days
37	98·7	>42	>42 days
38	97·1	>42	>42 days

In the ultracentrifuge (Fig. 2) a single component alone is present in fresh preparations, but after storage in solution at pH values between 7 and 8 for 3 weeks at 0–2° a component of higher sedimentation coefficient usually appears. At pH 8·0 in phosphate buffer a single component is apparent in both anode and cathode limbs of the electrophoresis apparatus after 3 hr. migration at 15 mA. After 300 mA-hr. the anode limb still shows a single peak but in the cathode limb the behaviour is complex.

Sedimentation and diffusion measurements on such purified fibrinogen preparations gave a molecular weight of $341\,000 \pm 10\,000$ (Caspary & Kekwick, 1954). There were indications at concentrations lower than 0·15 g./100 ml., that the molecules tended to dissociate.

DISCUSSION

There have been many attempts to purify fibrinogen, more especially from animal sera, in which salting out methods with sodium chloride, ammonium sulphate and latterly potassium phosphate

have been used. The accumulated experience showed the general desirability of fractionating in the presence of sufficient amounts of anticoagulant salts, such as citrate or oxalate, to prevent clotting during the course of purification, and also that it was advantageous to work at temperatures just above 0° on account of the lability of fibrinogen, though this apparent lability may partly have been due to the unrecognized effects of contamination with plasma proteases. Purification to the limit possible with the criteria available, was seldom attempted.

The renaissance of solvent fractionation procedures with their greater variety of conditions almost coincided with the elucidation of much of the basic nature of the plasminogen–plasmin system. However, solvents such as ethanol and ether are apparently able to dissociate a plasmin–inhibitor complex, or to some extent possibly to activate plasminogen to plasmin. Since the latter is fibrinogenolytic as well as fibrinolytic, fibrinogen solutions obtained by the use of organic solvents sometimes lose rapidly their characteristic ability to form a clot with thrombin. To determine the physicochemical characteristics of fibrinogen from such preparations is obviously open to criticism.

The preparation of purified human fibrinogen by ethanol fractionation has been described by Morrison, Edsall & Miller (1948). They showed that a non-clottable protein of lower solubility than fibrinogen was present in their crude concentrates, a finding which is confirmed by results presented here. After removing this cold-insoluble globulin they obtained preparations in which fibrinogen represented 95–98 % of the total protein as determined by gravimetric analysis (Morrison, 1947). These values are presumably subject to a correction of 1·5–2·0 % for ash and then would correspond with 93·5–96·5 % purity on the basis of the clottable N/total N ratio used in the present paper. In the ultracentrifuge such preparations showed two components with sedimentation coefficients 8·5 (87 %, main component) and 12–14*S* and occasional traces of components of 2–3 and 18–20*S*. In the electrophoresis apparatus 5–8 % of a faster component separated from the main peak after prolonged migration. No data were provided to indicate whether these preparations were free from plasminogen or plasmin, but our experience would suggest that the preparative procedure used would be unlikely to remove these contaminants.

The procedure described in this paper for the preparation of fibrinogen provides a yield of 30–40 % of the initial material. The product is demonstrably free from other components participating in the clotting mechanism such as prothrombin and antihaemophilic globulin. It is substantially free from plasmin and most probably from plasminogen.

In the ultracentrifuge a single component alone is observable, though a second more rapidly sedimenting component tends to form after storing solutions at 0–2° for some weeks. In the electrophoresis apparatus at pH 8·0 the anode boundary moves as one component even after prolonged migration but the cathode boundary eventually becomes complex, a phenomenon which may be related to the dissociation that occurs in dilute solutions (Caspary & Kekwick, 1954).

For a series of preparations uniform with respect to the properties just discussed, the clottable N/total N ratio has varied between 97·0 and 98·7 %, and from this standpoint it is still not possible to conclude what value 'pure' human fibrinogen should attain. Evidence has been provided by Bailey *et al.* (1951), that the primary action of thrombin on fibrinogen is to split off a peptide or peptides, the residue of the main molecule then polymerizing to form the characteristic clot. For bovine fibrinogen Lorand (1952) suggests that the peptide or peptides account for about 3·5 % of the fibrinogen nitrogen, or in other words the clottable N/total N ratio of pure bovine fibrinogen should be close to 96·5 %. A species variation in this value would not be unexpected, and in fact the data presented here suggest that the clottable N/total N ratio for human fibrinogen is probably higher than the value for bovine fibrinogen deduced from Lorand's data.

For these preparations of human fibrinogen it can be stated that, from the point of view of the clotting test, at least 97 % of the molecules are identical, and the physicochemical and other data obtained are consistent with this interpretation.

SUMMARY

1. Experiments are described which led to a method for the separation and purification of human fibrinogen using systems containing diethyl ether.

2. The purified fibrinogen obtained, which is uncontaminated with other components of the clotting mechanism, has a clottable N/total N ratio in excess of 97 %, and is substantially free from plasminogen and plasmin.

3. The purified fibrinogen sediments as a single component in the ultracentrifuge. After prolonged electrophoresis at pH 8·0 the anode limb boundary remains a single peak but the cathode limb boundary becomes complex.

4. The characteristics of some plasma protein fractions separated during the course of the purification of fibrinogen are briefly described.

This work was carried out on material used for the production of human plasma fractions for clinical purposes by the Blood Products Research Unit (Medical Research

Council and Lister Institute). Blood was provided to the Blood Products Research Unit by the South London Blood Transfusion Depot.

Fig. 1 is reproduced from the *Medical Research Council Special Report*, no. 286, by permission of the Controller, H.M. Stationery Office.

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The Fermentation of L-Threonine, L-Serine, L-Cysteine and Acrylic Acid by a Gram-negative Coccus

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A Gram-negative coccus isolated from the rumen of the sheep (Elsden, Gilchrist, Lewis & Volcani, 1951) ferments a variety of substances with the production of hydrogen, carbon dioxide and volatile fatty acids (Elsden & Lewis, 1953). This organism, referred to as LC, is of considerable interest, since it produces volatile fatty acids (VFA's) higher than butyrate. Its metabolism has been shown to resemble, in many respects, that of *Clostridium kluyveri* (Stadtman & Barker, 1949*a*). During the fermentation of glucose the main products are fatty acids containing an even number of carbon atoms, whereas during growth on lactate considerable amounts of propionate and valerate are formed and little or no hexanoate. The production of fatty acids containing an even number of carbon atoms probably occurs via a condensation of 2-carbon fragments (Elsden & Lewis, 1953).

Lewis, Nisman & Elsden (1952) reported that LC fermented L-serine, L-threonine, L-cysteine and acrylate, and in the present paper we give the results of a more detailed investigation of the anaerobic metabolism of these four compounds by this organism.

During recent years the microbial degradation of serine, threonine and cysteine has been actively studied. Thus, Woods & Clifton (1937) showed that *Cl. tetanomorphum* ferments DL-serine and L-cysteine with the formation of hydrogen, carbon dioxide, ammonia and VFA's. Gale & Stephenson (1938) showed that washed suspensions of *Escherichia coli* under anaerobic conditions rapidly produced ammonia from DL-serine; the progress curve of ammonia formation indicated that one isomer was preferentially attacked. Chargaff & Sprinson (1943), also using *Esch. coli*, showed that the deamination of serine and threonine was accom-