conjunction with the intrinsic viscosity,[21] (η), 39 ml./ml.,[6] an axial ratio of 18.3 results for the hydrated particle. This value of the axial ratio can be used in the Perrin equation to yield a friction ratio due to asymmetry of 1.92. Allowing for the fact that a hydrated particle has a frictional resistance greater than that of an anhydrated particle produces a value, 2.01, for the over-all friction ratio. Combination of this (f/f_0) value with the sedimentation constant, 185 S, leads to 34×10^6 for the anhydrous molecular weight. This corresponds to a rod about 270 by 14 mμ.[22]

Diffusion and sedimentation data can also be used to predict the size of the anhydrous particles. The calculation of molecular weight by this method is independent of hydration and the value, 31×10^6, previously reported still obtains. This corresponds to a rod-like particle 250 by 14 mμ for the case in which 15% by volume of water on a wet basis are associated with the virus.

The size and shape of the particle can be calculated, also, by a combination of viscosity and diffusion data. Results obtained in this manner correspond to a rod-shaped particle 250 by 13 mμ and a molecular weight of 27×10^6 when 15% of volume hydration is assumed.

(21) Intrinsic viscosity is defined as the ratio, as the volume fraction approaches zero, of specific viscosity to volume fraction.

(22) This calculation was made on the assumption that hydration increases the thickness but not the length of a virus particle.

Summary

The hydrodynamic density of tobacco mosaic virus was determined by centrifugation in bovine serum albumin and sucrose solutions of various densities. In both sets of experiments, straight lines could be used to express the dependence of sedimentation rate upon density of the medium. A hydrodynamic density of 1.13 was obtained for the experiments carried out in serum albumin solutions, and a value of 1.27 was obtained for experiments carried out in sucrose solutions. It was shown that this great discrepancy can be attributed to the effect upon the buoyancy of a virus particle produced by a disturbance of the homogeneous distribution of solute molecules in the immediate neighborhood of a virus particle. This effect depends upon the radius of the solute molecule and is therefore greater for serum albumin than for sucrose. When this effect is taken into account, the data can be interpreted to indicate that tobacco mosaic virus has an intrinsic hydration of approximately 15% by volume on a wet basis. The size and shape of the virus particle were calculated by several methods on the basis of this new hydrated model. Excellent agreement was obtained when the calculations from viscosity and sedimentation data were compared with direct measurements obtained by electron microscopy and X-ray diffraction. Calculations involving the diffusion constant gave less satisfactory agreement.

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

The Separation of the Antibodies, Isoagglutinins, Prothrombin, Plasminogen and β_1-Lipoprotein into Subfractions of Human Plasma[1a,b]

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The separation of the protein and lipoprotein components of human plasma into a series of fractions by the use of a five-variable system, as well as the principles involved in this fractionation, have been described in a previous paper.[2] The procedure developed depends upon the use of organic precipitants (ethanol in this case), low temperatures (0 to $-8°$), low ionic strengths of electrolytes (below 0.16 mole per liter), and accurate control of the pH and protein concentration. In this work the albumins were concentrated in Fraction V, fibrinogen in Fraction I, most of the α-globulins in Fraction IV-1 and IV-4. Fraction II + III contained isoagglutinins, prothrombin, plasminogen, certain lipoproteins with properties ascribed to the X-protein of plasma, as well as antibodies.[3]

The aim of this study was to devise methods

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(1b) This paper is Number 73 in the series "Studies on the 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, and Number XIX in the series "Preparation and Properties of Serum and Plasma Proteins" from the same laboratory.

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(2) 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).

(3) The importance of these methods for the concentration of antibodies was pointed out in an earlier paper of this series: E. J. Cohn, J. A. Luetscher, Jr., J. L. Oncley, S. H. Armstrong, Jr., and B. D. Davis, *ibid.*, **62**, 3396 (1940).

for large scale subfractionation of as many constituents of Fraction II + III as possible into useful and stable concentrates which could then be made available for more extensive chemical and clinical work.

I. Isolation of Lipoproteins

Approximately three-quarters of the lipoproteins of plasma were concentrated in Fraction II + III. Measurements at low ionic strengths (0.005 to 0.02) indicated that both the carotenoid-bearing[4] and cholesterol-bearing proteins in this fraction had a minimum solubility at about *p*H 5.9. Isolated lipoprotein fractions, however, were usually isoionic near *p*H 5.5 (Table I).[5] The lipoprotein of Fraction II + III has the peculiar property of sedimenting in the ultracentrifuge with a rate that was very sensitive to the density of the solution. This behavior is characteristic of the so-called "X-protein" of plasma, first described by McFarlane,[6] and later by Pedersen.[7,8]

Processing of Fraction II + III, as of whole plasma, was made difficult by the presence of this lipoprotein. The total fraction could not be dried, or even frozen and thawed, without some denaturation.[10] Ammonium sulfate fractionation had yielded precipitates which either centrifuged poorly[7] or rose to the surface.[11] Separation of the lipoproteins from Fraction II + III thus became of the utmost importance.

This lipoprotein fraction, bearing the carotenoid pigment, cholesterol and most other lipids found in Fraction II + III, was extracted almost quantitatively from the residue of plasminogen, prothrombin, isoagglutinins and γ-globulins at 0.07 mole fraction (20%)[12] ethanol if the *p*H were about 7.6 and the ionic strength about 0.005. This ionic strength was readily achieved by resuspending Fraction II + III in a volume of this solvent equal to or twice that of the plasma from which it was obtained. The resulting precipitate (Fraction II + IIIW) contained considerable cholesterol if the extraction was carried out below *p*H 7, but almost none if at *p*H 7.6 or above, and could then be frozen and dried with little or no denaturation of the protein. The lipoprotein fraction separated was designated Fraction III-0. The advantages of this procedure, which yielded both a lipoprotein concentrate soluble in aqueous solutions and a lipid-poor precipitate, were so great that it became the first step in our subfractionation of Fraction II + III (globulin methods 8 and 9).

The extracted lipoproteins were precipitated at 0.09 mole fraction ethanol, *p*H 5.6 to 5.9. Because of its very low density, quantitative separation of lipoprotein from the suspension was somewhat difficult,[13] but was more complete in the presence of traces of calcium ion. The resulting lipoprotein precipitate contained a large percentage of β-globulin, and 60 to 70% of the fraction behaved as X-protein in the ultracentrifuge. Analyses revealed about 35% total lipid, about half of which was cholesterol and cholesterol esters. Materials with the properties usually ascribed to "plasmin inhibitor" and "antithrombin" were found in this fraction.[14]

Fraction III-0, like Fraction II + III, could not be dried from the frozen state without denaturation. Most of the lipoprotein was, however, precipitated as euglobulin by resuspension in a large volume of water at an ionic strength below 0.002 and at a *p*H between 5.4 and 5.9. The remaining protein (Fraction II + IIIW) could then be precipitated with ethanol, dried from the frozen state, and reconstituted to give a satisfactory solution.

II. Isolation of γ-Globulin Antibodies

Many antibody molecules fall into the γ-globulin class when studied by electrophoresis.[15] Proteins of this group have the most alkaline isoelectric points and the smallest electrical charges at neutral *p*H values of any of the major components of plasma. The γ-pseudoglobulins from horse serum[16] have an isoelectric point near *p*H 6.4. Human γ-globulin of this isoelectric point appears to be largely euglobulin,

(4) We are indebted to J. W. Mehl of the Department of Biochemistry, University of Southern California School of Medicine, for these observations, which were carried out at the Harvard Plasma Fractionation Laboratory during the summer of 1944.

(5) Euglobulins isoelectric in this range were reported early by W. B. Hardy (*J. Physiol.*, **33**, 251 (1905)), J. Mellanby (*ibid.*, **33**, 338 (1905)), H. Chick, (*Biochem. J.*, **8**, 261 (1914)), and E. J. Cohn (*J. Gen. Physiol.*, **4**, 697 (1922)), and more recently by A. A. Green (THIS JOURNAL, **60**, 1108 (1938)). The β-globulins as a whole were reported by A. Tiselius (*Biochem. J.*, **31**, 313, 1464 (1937)); (*Trans. Faraday Soc.*, **33**, 524 (1937)) to be isoelectric near *p*H 5.1.

(6) A. S. McFarlane, *Biochem. J.*, **29**, 407, 660, 1175, 1202, 1209 (1935).

(7) K. O. Pedersen, "Ultracentrifugal Studies on Serum and Serum Fractions," Almqvist and Wiksells, Boktryckeri AB, Upsala, Sweden, 1945; *J. Phys. and Colloid Chem.*, **51**, 156 (1947).

(8) Solutions containing 86% of X-protein in the ultracentrifuge have been obtained in some of the euglobulin lipoprotein fractions, which were found by electrophoresis to consist largely of β_1-globulin. Further purification in the air-driven preparative ultracentrifuge has given fractions still having the sechmentation behavior of X-protein and containing as much as 97% β_1-globulin.[9]

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

(10) L. Pillemer, during the time he was working at the Harvard Plasma Fractionation Laboratory, showed that high concentrations of certain agents, especially sucrose, seemed to render such products soluble to a considerable extent.

(11) G. S. Adair and M. E. Adair, *J. Physiol.*, **102**, 17P (1943).

(12) Ethanol concentrations are expressed as mole fraction, and as per cent. by volume at 25°; see Table I, reference 2.

(13) Some additional protein could be precipitated by lowering the *p*H to 4.8 and slightly increasing the ethanol concentration. More albumin and α-globulin and considerably less lipid were found in this fraction.

(14) Measurements of antithrombin activity have been made by J. T. Edsall and S. G. Miller, who also have found appreciable amounts of antithrombin in Fraction IV-1.

(15) See K. Landsteiner, "The Specificity of Serological Reactions," revised edition, Harvard University Press, Cambridge, Massachusetts, 1945, Chapter IV.

(16) E. J. Cohn, T. L. McMeekin, J. L. Oncley, J. M. Newell and W. L. Hughes, Jr., THIS JOURNAL, **62**, 3386 (1940).

human γ-pseudoglobulin being isoelectric near *p*H 7.4 (Table I).

TABLE I

ISOIONIC POINTS OF VARIOUS PROTEIN FRACTIONS[a]

Fraction	*p*H of solution of Euglobulin	*p*H of solution of Pseudoglobulin
	β-Globulin	
III-0-1,2,3:181	5.5	4.8
III-0-1,2:S362-3,4	5.5	5.4
III-0-1:183–184	5.2	4.6
III-0-3:183–184	5.6	5.7
III-0-4:S362-3,4	5.6	5.5
III-0-5:S362-3,4	5.7	5.5
III-0-5:AVL7	5.6	5.4
	γ-Globulin	
II-1:L371	6.4	7.3
II-1:LY3	6.5	7.2
II-2:L371	6.5	7.6
II-2:122	6.5	7.4
II-1,2:160A	6.3	7.1
II-1,2:171	6.8	7.6
II-3:SW1	6.4	7.4
II-3:42-W3F	6.2	7.0

[a] Determined by measuring the *p*H of the pseudoglobulin and euglobulin solutions after exhaustive dialysis, and hence representing the isoionic points of these proteins, rather than the isoelectric points. *Cf.* E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publishing Corporation, New York, N. Y., 1943, p. 446.

Solubility studies have indicated that although γ-globulin was readily precipitated by 0.09 mole fraction (25%) ethanol at neutral reaction, its solubility at *p*H 5 was considerably higher than that of albumin.[17] Sodium acetate was found to dissolve considerably more γ-globulin than sodium chloride of equal ionic strength, and both electrolytes decreased solubility with increasing ionic strength; results similar to those described for the influence of neutral salts on casein and edestin at reactions acid to their isoelectric points.[18] Great dependence of solubility upon the amount of saturating body in the system, as was earlier described for serum globulin[19,20,21] has also been observed and assumed to demonstrate the molecular heterogeneity of the γ-globulin precipitate.

The lipid-poor, readily dried, antibody-rich Fraction II + IIIW has formed an ideal starting material for the separation of γ-globulin (Fraction II) from other components (Fraction III). The γ-globulins have been extracted from these components at *p*H values of 5.8 and lower, usually 5.2. This was desirable because the γ-globulins are more soluble under these conditions than at reactions nearer neutrality, and the other components less, since they have isoelectric points in this *p*H range (5.9 to 4.8).

At any given *p*H an ethanol concentration was chosen which would permit solution of at least 3 g. of γ-globulin per liter, and in which only traces of contaminating substances were soluble.[22] Because of possible instability of various antibody molecules at acid *p*H, most efforts at isolation were carried out above *p*H 5. Reactions acid to *p*H 4.7 led to appreciably increased solubility of β-globulin, unless the ethanol concentration was greater than mole fraction 0.059 (17%). A *p*H value of about 5.2, 0.015 ionic strength, and an ethanol concentration of 0.059 mole fraction (17%) gave a very satisfactory solubility differential between the γ-globulin and the other components. A *p*H of 5.4, 0.005 ionic strength in 0.024 mole fraction ethanol (7.5%) was also satisfactory for this separation. A total Fraction II was precipitated from the supernatant by increasing the ethanol to 0.09 mole fraction and the *p*H to 7.2.

Attempts were made to separate the γ-globulins in Fraction II into subfractions. Addition of 0.05 mole/l. of sodium chloride to the γ-globulin solution at *p*H 5.2, mole fraction ethanol 0.059 (17%), and ionic strength of 0.015,[23] led to the separation of a fraction designated Fraction II-3. After Fraction II-3 had been removed, the solution was adjusted to *p*H 7.2, holding the ethanol and ionic strength constant. The precipitate formed under these conditions was called Fraction II-1. The γ-globulin remaining in solution precipitated at 0.09 mole fraction (25%) ethanol, and was called Fraction II-2.

Some physical–chemical,[9] chemical[25] and immunological[26] properties of these γ-globulin fractions have already been recorded. Clinical studies have indicated the usefulness of Fractions II-1 and II-2 in prophylaxis against measles and

(17) We are indebted to Mrs. M. H. Blanchard of this Laboratory for these studies.

(18) T. B. Osborne, THIS JOURNAL, **24**, 39 (1902); T. B. Osborn and I. F. Harris, *Am. J. Physiol.*, **14**, 151 (1905) (or *ibid.*, **13**, 436 (1905)); K. Linderstrøm-Lang and S. Kodama, *Compt. rend. trav. lab. Carlsberg*, **16**, No. 1 (1925).

(19) W. B. Hardy, *J. Physiol.*, **33**, 251 (1905).

(20) J. Mellanby, *ibid.*, **33**, 338 (1905).

(21) S. P. L. Sörensen, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 11 (1923); THIS JOURNAL, **47**, 457 (1925); "Proteins," The Fleischmann Laboratories, 1925, p. 1.

(22) The γ-globulin fractions obtained by these methods have often been found to contain substances which cause a blood pressure lowering effect upon the blood pressure of certain test animals, especially cats. A number of experiments suggested that the temperature coefficient of solubility of the depressor causing substance was of a larger magnitude than that of the γ-globulin, and made it desirable to use as low a temperature as possible for the separation. L. H. Woodruff, C. A. Janeway and W. Berenberg made the earlier assays of depressor activity. More recently O. Krayer and his associates in the Department of Pharmacology, Harvard Medical School, have investigated these effects. Some studies have also been made by E. Shorr and B. W. Zweifach of the New York Hospital.

(23) The precipitation of γ-globulin under these conditions was predicted from solubility studies,[17] and by work simultaneously carried out at the University of Wisconsin.[24] Fraction II-3 was not extracted from Fraction II + III by the earlier methods, which had been carried out in the presence of the lipoprotein now concentrated in Fraction III-0 and at higher ionic strengths.

(24) H. F. Deutsch, L. J. Gosting, R. A. Alberty and J. W. Williams, *J. Biol. Chem.*, **164**, 109 (1946).

(25) J. W. Williams, M. L. Petermann, G. C. Colovos, M. B. Goodloe, J. L. Oncley and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 433 (1944).

(26) J. F. Enders, *ibid.*, **23**, 510 (1944).

epidemic jaundice.[27] Fraction II-3 has been shown effective in measles prophylaxis,[28] but studies in prophylaxis against epidemic jaundice have not yet been completed. Although differences among these γ-globulin fractions were observed in the above-mentioned studies, further fractionation of purified γ-globulin must be undertaken to explore more fully the possibility of separating the γ-globulin antibodies from one another.

III. Separation of Isoagglutinins, Prothrombin and Plasminogen

Solubility studies of anti-A, anti-B and anti-Rh isoagglutinins indicated a minimum solubility near 6.3, and a strong solvent action of neutral salts in water or in ethanol–water mixtures at that *p*H, and at reactions acid to the isoelectric point[29,30] Under most conditions employed, the solubility of the fractions containing the isoagglutinins was considerably lower than that of other γ-globulins. Quantitative measurements indicate, however, that less than 1% of even the most highly purified fractions were agglutinating antibody.[31]

Highly purified human prothrombin has thus far not been prepared. However, as Mellanby's important observations suggested, it appears to be a euglobulin with a minimum solubility near *p*H 4.8, dissolved by most electrolytes in aqueous solutions when the ionic strength was 0.08 mole per liter or greater.[32,33] Most investigators have reported inactivation of prothrombin at reactions acid to *p*H 4.8 and alkaline to *p*H 10. If it is assumed that human prothrombin has the same activity as the bovine prothrombin highly purified by Seegers and reported to be 1500 u./mg,[33] then fractions with activities of less than 30 u./mg., routinely prepared in the course of this work, contained less than 2% prothrombin.[34] Although there was no need to purify this component further, when it is prepared to yield human thrombin for clinical purposes[35] the fractions containing prothrombin which have been obtained must be considered only as starting material for further chemical investigations.

Plasma, or a fraction of plasma, has been rendered proteolytic by the use of certain reagents[36]; the activated enzyme termed plasmin (fibrinolysin), and the inactive enzyme (or precursor), plasminogen.[37] Under most conditions investigated, plasminogen precipitated with prothrombin. The observation that plasminogen was adsorbed by fibrin made possible its separation from prothrombin.

The separation of the isoagglutinins from prothrombin and plasminogen was made possible by utilizing the different isoelectric points of these materials. At *p*H 5.4 there were large differences in solubility, and the solubilities of the two components to be separated can be adjusted to suitable values by the variation of the ionic strength of an aqueous solution. Since it has been observed that prothrombin was considerably less soluble in sodium acetate buffers[38] than in sodium chloride solution with *p*H adjusted to the same value, and since a specific effect of the acetate ion upon the solubility of the isoagglutinins was not noted, it was found advantageous to use acetate buffers for this step. This separation was effected by completely dissolving Fraction III in a small volume of acetate buffer of about 0.2 ionic strength, and then precipitating the prothrombin and plasminogen (Fraction III-2,3) together with most of the remaining fibrinogen and a part of the other proteins present by diluting this solution

(27) C. W. Ordman, C. G. Jennings, Jr., and C. A. Janeway, *J. Clin. Invest.*, **23**, 541 (1944); C. A. Janeway, *Bull. N. Y. Acad. Med.*, **21**, 202 (1945); *J. Am. Med. Assoc.*, **126**, 674 (1944); M. Greenberg, S. Frant and D. D. Rutstein, *ibid.*, **126**, 944 (1944); J. Stokes, Jr., and J. R. Neefe, *ibid.*, **127**, 144 (1945).

(28) C. A. Janeway, personal communication.

(29) The presence of anti-A and anti-B isoagglutinins in Fraction II + III was noted by W. C. Boyd, and reported in some detail by J. F. Enders, *J. Clin. Invest.*, **23**, 510 (1944), (*cf.* table on p. 515, compiled by W. C. Boyd), and by L. Pillemer, J. L. Oncley, M. Melin, J. Elliott and M. C. Hutchinson, *J. Clin. Invest.*, **23**, 550 (1944).

(30) Studies of the anti-Rh isoagglutinins have been carried out in collaboration with L. K. Diamond, Department of Pediatrics, Children's Hospital, Boston, Mass.

(31) E. A. Kabat, private communication. See also E. A. Kabat and A. E. Bezer, *J. Exptl. Med.*, **82**, 207 (1945).

(32) For a discussion of the earlier literature see J. T. Edsall, R. M. Ferry and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 557 (1944).

(33) A recent survey of the properties of prothrombin has been made by W. H. Seegers, E. C. Loomis and J. M. Vandenbelt, *Proc. Soc. Exptl. Biol. and Med.*, **56**, 70 (1944); and *Arch. Biochem.*, **6**, 85 (1945).

(34) The assay of thrombin and prothrombin was briefly described by Edsall, Ferry and Armstrong.[32] More recently, it has been defined in terms of a standard thrombin reference preparation, whose activity is fixed by definition. This new thrombin unit is identical within the limits of experimental error with the thrombin unit described by Seegers,[33] and has been officially adopted by the National Institute of Health (defined in detail in Minimum Requirements for Dried Thrombin, National Institute of Health, August 28, 1945). See the reports on the determination of prothrombin in "Blood Clotting and Allied Problems," Josiah Macy, Jr., Foundation New York, 1948.

(35) E. A. Bering, Jr., *J. Clin. Invest.*, **23**, 586 (1944); O. T. Bailey and F. D. Ingraham, *ibid.*, **23**, 591 (1944); F. D. Ingraham and O. T. Bailey, *J. Neurosurg.*, **1**, 23 (1944); F. D. Ingraham, O. T. Bailey and F. E. Nulsen, *ibid.*, **1**, 171 (1944); O. T. Bailey, F. D. Ingraham, O. Swenson, J. J. Lowrey and E. A. Bering, Jr., *Surgery*, **18**, 347 (1945).

(36) In Fraction III-3, the separation of which is described below, D. A. Richert found a slow spontaneous activation of plasminogen to plasmin, even in sterile solution, without the addition of chloroform, streptokinase, or any other activator. The final concentration of plasmin obtained in this way was about one-third of the maximum obtainable on complete activation with streptokinase. Chloroform treatment of Fraction III-3 did not give any increase in the rate or in the final amount of activation, as compared with the control sample without added activator. This is perhaps explainable by the fact that the lipids present in Fraction II + III were previously separated in Fraction III-0. The function of the chloroform in whole plasma or crude plasma fractions may well be due to its extraction of fat soluble inhibitors which normally prevent the spontaneous conversion of plasminogen to plasmin. For further discussion of the proteolytic enzyme system of plasma, see J. T. Edsall, "Advances in Protein Chemistry," **3**, 449–51 (1947).

(37) In order to describe the protease system the revised terminology suggested by L. R. Christensen and C. M. MacLeod, *J. Gen. Physiol.*, **28**, 559 (1945), has been followed.

(38) These observations were made by L. Pillemer, D. I. Mulford, J. T. Edsall, S. G. Miller and D. A. Richert.

with water to an ionic strength of about 0.08.[39]

The recovery of isoagglutinin in the supernatant solution from Fraction III-2,3 was accomplished by raising the *p*H to 6.3 (minimum solubility of the isoagglutinins), and adding a small amount of ethanol. The recovery of the isoagglutinins was quite satisfactory, and the precipitate so obtained (Fraction III-1) was dried from the frozen state without deterioration.

Prothrombin and plasminogen were separated by dissolving Fraction III-2,3 in about 0.1 ionic strength sodium chloride and sufficient buffer to bring the *p*H to about 6.9. Under proper conditions a fibrin clot[40] formed and was removed by centrifugation. This clot lysed in a few hours at room temperature, or after standing overnight in the cold room, and contained a large part of the plasminogen.[41]

The supernatant solution from the clot contained most of the prothrombin, which could be reprecipitated by lowering the *p*H and adding a small amount of ethanol, or could be dried from the frozen state. Prothrombin was not stable indefinitely, but could be readily converted to thrombin by suitable agents.[32,42]

IV. Procedures for the Subfractionation of Fraction II + III[43]

Studies of the subfractionation of Fraction II + III involving isoelectric precipitation at various ionic strengths, but without the use of ethanol, yielded fairly active prothrombin fractions, but failed to yield concentrated antibody-containing fractions which were free of lipid. Various methods involving precipitation with ethanol, with careful control of the *p*H, ionic strength and temperature, were then introduced. Only the last methods have led to the separation of all protein components in conditions approximating the native state. For this reason it has not seemed desirable to describe in detail earlier attempts at complete fractionation. Since they may be useful for specific purposes, however, they have been briefly outlined.[44] Although developed for the fractionation of normal human plasma, they have also been applied with some success to certain types of convalescent human plasma and to the plasma of certain animals. Modifications of these methods may lead to more satisfactory separations of the comparable proteins of different species, because of their somewhat different concentrations and solubilities.

Method 1 for preparing the total Fraction II + III for immunological studies was introduced in 1942. The precipitated Fraction II + III, removed and stored at a temperature of −5°, contained approximately 35% protein by weight, 0.062 mole fraction (18%) ethanol, and 0.06 ionic strength of salts (largely sodium chloride with some citrate and bicarbonate). This material was suspended in about an equal volume of 0.1 *M* sodium chloride solution previously chilled to 0°, and dialyzed against 0.1 *M* sodium chloride at 0° for two or three days with frequent changes of dialysate to remove the ethanol present in the precipitated material. During dialysis a clot of fibrin usually formed and was removed by filtration and centrifugation. The precipitate was then washed with cold 0.5 *M* sodium chloride solution, recentrifuged, and the supernatant solution was added to the dialyzed material. The resulting protein solution, containing only traces of ethanol, was adjusted to 0.15 *M* sodium chloride, approximately 15% of protein by dry weight. It could be sterilized by filtration through Seitz type filter pads. Thrombin was largely removed during clotting, and prothrombin was removed by the filtration.

Method 2 made available γ-globulin and thrombin. Fraction II + III was suspended in cold sodium chloride solution, allowed to clot, and clarified. Prothrombin (Fraction III-2) was removed by adjusting to *p*H 4.7–5.3 and an ionic strength of about 0.08. The remaining α- and β-globulin (Fraction III-1) was removed at 0° by adjusting the *p*H to 5.7, the ionic strength to 0.015 or 0.03 and the ethanol concentration to about 0.051 mole fraction (15%). The volume used was about twice that of the original plasma. The γ-globulin (Fraction II) was then precipitated by increasing the ethanol concentration to mole fraction 0.091 (25%), which precipitated Fraction II-2. By then adjusting the *p*H to 7.0, Fraction II-1 was precipitated.

Method 3 involved suspension of Fraction II + III paste, clotting of fibrinogen, and removal of thrombin by methods similar to those used in Method 2. Fraction III-1 was then removed at more acid *p*H (5.2 to 4.5), lower volume (approximately equal to the volume of original plasma), and ethanol concentrations from 0.04 to 0.07 mole fraction (12 to 20%) (Table II). The total γ-globulin was usually precipitated in one fraction, but in some cases Fractions II-1 and II-2 were removed separately.

Method 4 involved suspension of Fraction II + III in a volume of water twice that of the original plasma at *p*H 5.9, 0.005 ionic strength, 0°. The resulting euglobulin precipitate represented about half the total protein, and contained considerable γ-globulin. The supernatant solution, precipitated by bringing the ethanol concentration to 0.1 mole fraction (30%), contained large amounts of γ-globulin and lipoprotein.

Method 5 was developed to make isoagglutinins available in good yield. Suspension, clotting of fibrinogen, and removal of prothrombin were accomplished as in Methods 2 and 3, except that Fraction III-2 was precipitated at a slightly lower ionic strength (0.06). Fraction III-1, containing β-globulin, lipoprotein and isoagglutinin, was precipitated in a volume 1.8 times that of the plasma represented, at *p*H 6.3, 0.015 ionic strength, 0.026 mole fraction (8%) ethanol, 0°. The ethanol was removed by suspending the precipitate in about 20 volumes of water at 0°, *p*H 6.3, and ionic strength less than 0.001. The resulting lipoprotein-rich precipitate could not be dried without denaturation, but the paste could be suspended in 0.15 *M* sodium chloride and adjusted to *p*H 7.2. The γ-globulin was precipitated as in Method 3.

(39) These conditions are very nearly those used in the original Mellanby procedure.[20] A similar procedure, using somewhat lower ionic strengths, was used in the isolation of the C'1 component of complement from guinea pig serum, L. Pillemer, E. E. Ecker, J. L. Oncley and E. J. Cohn, *J. Exptl. Med.*, **74**, 297 (1941).

(40) If sufficient thrombin had been formed by converting agents in the plasma, spontaneous clotting occurred. If insufficient thrombin were present, a small amount was added at this point.

(41) The products of the lysis of the fibrin clot have been studied by W. H. Seegers, M. L. Nieft and J. M. Vandenbelt, *Arch. Biochem.*, **7**, 15 (1945), who found two main components called α- and β-fibrin. The α-fibrin, *pI* = 5.5, has an electrophoretic behavior similar to fibrinogen; the β-fibrin, *pI* = 4.2, is somewhat more like α-globulin or albumin.

(42) See L. E. Strong, Encyclopedia of Chemical Technology, Vol. II, p. 571–572. The Interscience Encyclopedia, Inc., New York, N. Y., 1948.

(43) The materials and method used for these subfractionations are completely described in ref. 2.

(44) Detailed procedures for carrying out most of these methods have been published in the Bulletin of the Blood Substitutes Committee, Committee on Medical Research, Office of Scientific Research and Development. They will be made available upon request.

TABLE II

CONDITIONS FOR THE SEPARATION OF FRACTION III-1 FROM γ-GLOBULIN

Method	pH	Ionic strength, moles/l.	Ethanol concn. Mole fraction	Ethanol concn. Vol. %(25°)	Temperature, °C.	Volume compared to plasma
2	5.7	0.015–0.03	0.051	15	0	2.0
3A	5.1	.040	.048	14	−3	1.0
3B[a]	5.2	.059	.040	12	−3	1.0
3C	5.2	.036	.051	15	−5	0.9
3D	5.2	.036	.059	17	−3	.9
3E	5.2	.036	.059	17	−5	.9
3F	5.2	.036	.059	17	−6	.9
3G	4.8	.036	.070	20	−7	.9
3H[a]	4.5	.036	.070	20	−7	.9
5	6.3	.015	.026	8	0	1.8
8	5.4	.005	.024	7.5	−2	0.7
9	5.2	.015	.059	17	−6	0.7

[a] The γ-globulin fraction obtained from these conditions was found to contain considerable β-globulin as impurity.

Method 6 was used to obtain a satisfactory isoagglutinin fraction when other fractions were not desired. Fraction II + III was suspended and taken to *p*H 6.3, 0.01 ionic strength, 0.024 mole fraction (7.5%) ethanol, 0°, and a volume about 0.6 times that of the original plasma. Isoagglutinins were precipitated along with lipoprotein. The resuspended precipitate was adjusted to *p*H 7.2 in water at 0°, 0.005 ionic strength, and a volume 0.7 times that of plasma. Much of the lipoprotein and γ-globulin were soluble under these conditions, but the isoagglutinins remained in the precipitate. This precipitate was dissolved in 0.1 *M* sodium chloride, and thrombin was added to remove fibrinogen as a fibrin clot.

Method 7 recovered only the isoagglutinin fraction. Fraction II + III was suspended at 0° or 25° in a volume of water eight times that of the plasma, *p*H 6.3, and an ionic strength of 0.005. The water was saturated with toluene. The isoagglutinin fraction settled in about twenty-four hours. Resuspension under the same conditions was sometimes desirable. Fibrinogen and most of the γ-globulin and lipoprotein were removed by this procedure.

Method 8 was similar to method 9, except that Fraction III was removed at *p*H 5.4, 0.005 ionic strength, 0.024 mole fraction (7.5%) ethanol, −2°, and a volume 0.7 times the volume of plasma represented.

Method 9 evolved from the combination of method 8 with the modification of method 3 to a lower ionic strength developed at the University of Wisconsin[24] for the separation of Fraction III from γ-globulin. This method is given in detail in the remainder of this section.

Precipitate II + III from standard preparations of normal serum albumin (human) is used as the starting material for this preparation.[45] Each kilogram of this precipitate is suspended in 2 kg. of water containing ice (about one-quarter of the water should be frozen, in the form of very fine ice crystals). When the suspension is fairly uniform, add 3 kg. of water (cooled to 0°) to which 112 cc. of 0.5 *M* disodium phosphate (*p*H 9.2) has been added.[46] The suspension should be stirred slowly and kept at a temperature of 0° until all lumps are dissolved and a nearly complete solution is obtained. After the suspension is complete, add it to 20 kg. of water (cooled to 0°), and stir slowly at 0° for thirty to sixty minutes. The *p*H of this suspension[47] should be 7.6 ± 0.2. Then bring to 20% ethanol by adding 15 liters of 53.3% ethanol keeping the temperature as low as possible until −5° is reached. This suspension should stand at −5° with a slow stirring for several hours before centrifuging. Centrifuge at a rate of about 30 liters per hour.

Precipitate III-O is precipitated from the supernatant from precipitate II + IIIW by bringing the *p*H to about 5.7, with *p*H 4 calcium acetate buffer,[48] and the ethanol concentration to 25%. This suspension should be allowed to stand for about forty-eight hours at −5° before centrifugation, and then centrifuged at a rate of about 20 liters per hour. The supernatant solution still contains appreciable protein, a large part of which is precipitated by adjusting the *p*H to 4.8, and the ethanol concentration to 35%.

Precipitate II + IIIW is resuspended in acetate buffer[49] and taken to *p*H 5.2, 0.015 ionic strength and 17% ethanol. This can be done by suspending each kilogram of precipitate II + IIIW in 2 kilograms of water and ice, as before, and then adding 2 kg. of cold water to which 0.35 mole of sodium acetate has been added. When this suspension is complete, add sufficient *p*H 4 acetate buffer,[51] diluted with one liter of cold water per kilogram of precipitate II + IIIW, to lower the *p*H of the suspension to 5.0 ± 0.1 and stir for several hours. Then add 13.5 liters of cold water and then 8.66 liters of 53.3% ethanol per kilogram of precipitate II + IIIW, raising the ethanol concentration to 17%. The temperature should be lowered during the addition of the ethanol, keeping the suspension near the freezing point until it is cooled to −6°. The precipitate is re-

(45) This method can also be applied to precipitate II + III or I + II + III from special lots of plasma or serum, such as convalescent serum or plasma, or to various similar fractions obtained by these methods. Occasionally, where materials have been stored in the frozen or dry state, the original suspension will yield a considerable quantity of insoluble material consisting of denatured protein and lipid. In these cases, material can be centrifuged and clarified with filtration through Seitz-type pads in a solution of 1 or 2 per cent. protein and 0.15 *M* sodium chloride at *p*H about 7. The clarified solution can then be taken to −5°, *p*H 6.8, 25% ethanol, ionic strength 0.1 just as plasma would be treated (but omitting the removal of Fraction I), and the precipitate corresponding to the usual Fraction II + III can then be used in this preparation.

(46) A sodium glycinate buffer solution was also used for this *p*H adjustment. See ref. 53.

(47) Because of the low ionic strength of this suspension, the *p*H value obtained depends considerably upon the method of reading in the glass electrode. We have used the value obtained by dilution of the suspension just before the alcohol addition with an equal volume of 0.02 *M* sodium chloride solution. Dilution with an equal volume of 0.15 *M* sodium chloride solution will give a *p*H nearer 7.2

(48) The *p*H 4 calcium acetate buffer is that used in the fractionation of plasma except that the sodium acetate is replaced by calcium acetate. It consists of 4.0 *M* acetic acid and 0.4 *M* calcium acetate, and is diluted before use.

(49) When we are dealing with plasma collected so that it contains a high titer of the various anti-Rh isoagglutinins[50] it is often advantageous to process the Fraction II + IIIW by a modification of Method 7. The procedure which has been used follows:

Precipitate II + IIIW is resuspended in water and taken to *p*H 6.3, 0.005 ionic strength, using acetate buffer. This can be done by suspending each kilogram of precipitate II + IIIW in two liters of cold water and ice. When this suspension is complete, pour it with good stirring into 90 liters of 0.005 ionic strength *p*H 6.3 acetate buffer per liter of starting II + IIIW paste. This resulting suspension may be held at either 0° or 25°. It is usually carried out at 0°, but where there is a large amount of fibrinogen present in the precipitate II + IIIW the 25° extraction procedure is advantageous. When the extraction is carried out at room temperature, the buffer is saturated with toluene as a bacteriostatic agent (approximately 0.5 cc. per liter). The dilute suspension is then stirred for about an hour, and then allowed to settle overnight. The precipitate is suspended in a small amount of *p*H 6.3 buffer of 0.005 ionic strength (sodium acetate), frozen and dried in vacuum.

This method can also be applied to precipitate III as obtained from method 9 for the subfractionation of Fraction II + III.

The use of this material as a reagent for blood typing has been discussed in an earlier paper.[50]

(50) J. L. Oncley, M. Melin, J. W. Cameron, D. A. Richert and L. K. Diamond, *Ann. N. Y. Acad. Sci.*, **47**, 899 (1946).

(51) The *p*H 4.0 sodium acetate buffer is that used in the fractionation of plasma.[3] It consists of 4.0 *M* acetic acid and 0.8 *M* sodium acetate.

moved by centrifugation at a rate of about 30 liters per hour and at a temperature of $-6.0 \pm 0.5°$. The effluent from this centrifugation should then be clarified by filtration[52] at a temperature of $-6.0 \pm 0.5°$.

Each kilogram of precipitate III is suspended in 2 kg. of water and ice, and then diluted with 2 kg. of 0.5 ionic strength *p*H 5.4 acetate buffer cooled to 0°. The suspension is stirred until uniform, and then diluted with 7.5 kg. of 0° water. This suspension should be at *p*H 5.4, ionic strength 0.08, and 0 to +1°, and should be stirred for several hours. The precipitate is removed by centrifugation at a relatively slow rate of speed and at 0 to +1°.

Precipitate III-2,3 should be suspended as soon as possible in 1 liter of 0° water per kilogram of precipitate and the *p*H adjusted to 6.8 to 7.0 with sodium glycinate buffer.[53] Then add 1 liter of 0° water containing enough sodium chloride to give a total ionic strength of 0.1. After one to two hours of stirring, test the solution for preformed thrombin and remove a sample to see if the fibrinogen has clotted. To do this, centrifuge a small sample and then add thrombin to the supernatant solution to see whether more fibrin is formed. If all the fibrinogen has clotted, do not add additional thrombin. If there is fibrinogen still left in solution, then add enough thrombin to give a total amount (including that already present) of 10,000 units per kilogram of Fraction III-2,3. Stir this suspension for an additional hour and centrifuge at 0° for about an hour, or at a very slow rate if continuous centrifugation is required. The supernatant containing Fraction III-2 can either be dried directly if prothrombin is desired, or immediately converted to thrombin before drying. To accomplish the conversion of prothrombin to thrombin, calcium chloride and thromboplastin are added, according to the directions given elsewhere,[42] and the temperature is raised to 20–25°. This solution is then clarified, sterilized and dried.

Precipitate III-3 should be suspended[54] in sodium chloride and bicarbonate solution to give an ionic strength of 0.15 and a *p*H of 7.1 ± 0.1, and allowed to stand at 0° until all of the fibrin has been lysed. After clarification and sterilization, this material can be dried from the frozen state.

Each kilogram of the supernatant from precipitate III-2 is adjusted to *p*H 6.3 by the addition of sodium bicarbonate (approximately 20 cc. of 1 *M* sodium bicarbonate should be required), and then the ethanol is adjusted to 15% by the addition of an equal volume of 30% ethanol, keeping the temperature at or near the freezing point until lowered to −5°, where it should be maintained. The precipitate can then be removed by centrifugation at −5°, and at a speed of about 80 liters per hour.

Precipitate III-1 can be dried from the frozen state, and reconstituted to about 5% protein in isotonic saline at *p*H 7 for a blood grouping solution if the pool were group specific and of sufficiently high titer.[50,55]

Each kilogram of filtrate from precipitate III is taken to about 0.06 ionic strength by the addition of 50 millimoles of sodium chloride, holding the *p*H at 5.2, the ethanol concentration at 17%, and the temperature at −6°.

Precipitate II-3 can be removed by centrifugation of the above suspension at a rate of about 50 liters per hour and at a temperature of −5°. It may be dried from the frozen state[56] preferably without the addition of sodium chloride or glycine.

Each kilogram of filtrate from precipitate II-3 is taken to *p*H 7.4 ± 0.2 by addition of about 15 millimoles of sodium bicarbonate, and to 20–25% ethanol by the addition of 40–114 cc. of 95% ethanol. The temperature is maintained at −5°.

Precipitate II-1,2[57] can be removed by centrifugation of the above suspension at a rate of about 50 liters per hour, and at a temperature of −5°. It may be dried from the frozen state preferably without the addition of sodium chloride or glycine.

Precipitate II.—A total Fraction II, rather than the Fractions II-1,2 and II-3, can be prepared from the filtered effluent from precipitate III by increasing the *p*H to 7.4 ± 0.2 with sodium bicarbonate solution, and the ethanol concentration to 25% with 95% ethanol. The temperature should be maintained at −5°. The precipitate may be removed by centrifugation at the same rate as recommended for Fractions II-1,2 and II-3.

V. Distribution of Proteins in the II + III Subfractions

Analyses of the various steps in the fractionation procedure have been carried out by the same methods already reported in a previous study of this series (2). The results obtained during the fractionation of a large lot of Fraction II + III prepared by method 6 for the fractionation of citrated plasma (2) are recorded in Table III. Nitrogen determinations and volume measurements were made on all suspensions and supernatant solutions obtained during subfractionation, and of all resuspended precipitates. Values for the protein in the precipitates are calculated from the analyses of the resuspended precipitates, and the protein lost in centrifugation or filtration, and the protein remaining in the supernatant solutions at the end of the process were calculated from the analyses of the suspensions or supernatant solutions. These results are tabulated as the percentage of total protein nitrogen in the fraction referred to the protein nitrogen in the Fraction II + III used for subfractionation. To convert these values to the amount of protein by nitrogen (N × 6.25) which would have been obtained from a liter of plasma, we have used the value of 15.1 g. of Fraction II + III per liter of plasma previously reported.[58] The nitrogen factors estimated from nitrogen and dry weight determinations of each resuspended precipitate are also recorded in Table IV. Using these nitrogen factors, instead of 6.25 as used in Table III, we obtain the values recorded in the last column of Table

(52) We have used Seitz S-1 pads and 0.5% 505 filter aid and sometimes K-5 pads and 1% Hyflow filter aid for this filtration. The filter pad should be washed with 0.30 *M* sodium chloride and then with 0.01 *M* acetic acid, and finally with 0.05 ionic strength *p*H 5.2 acetate buffer in 17% ethanol. The *p*H of the final washings should be checked to see that it is 5.2 after filtration (this can be easily done with a spot-plate and suitable indicator solution). This washing procedure is essential because the low ionic strength of the solution and changes in *p*H at this point can substantially lower the recovery of γ-globulin.

(53) Sodium glycinate buffer is prepared by half-neutralizing a glycine solution with sodium hydroxide. The solution we have used contains 1 mole of glycine and 0.5 mole sodium hydroxide per liter. It has a *p*H of about 9.5 and has the advantage of causing less of a drift in *p*H upon aeration than is observed when sodium bicarbonate is used to increase the alkalinity.

(54) The suspension of this material is conveniently carried out using a Waring blendor to break up the fibrin clot.

(55) M. Melin, *J. Clin. Invest.*, **24**, 662 (1945).

(56) We have found it helpful to suspend precipitate II-3 and II-1,2 in 1.5 volumes of water–ice mixture (1.3 of water frozen). If this mixture is kept cold during the suspension, most of the globulin remains undissolved, and does not "lump up" upon freezing.

(57) Fraction II-1,2 represents the more soluble part of the γ-globulin and is taken off at conditions used for preparation of Fraction II by earlier methods. Such material has been used clinically in both measles and epidemic jaundice. This fraction should be dissolved to make solutions contalning 16.5 g. of γ-globulin per 100 cc. and sterilized by Seitz filtration.

(58) See Ref. 2, Table VII.

TABLE III

DISTRIBUTION OF PROTEINS IN SUBFRACTIONS OF FRACTION II + III

Experiment	Per cent. of total protein nitrogen in fraction 1	2	3	4	Mean	G. N × 6.25 per liter of plasma
II + III	(100)	(100)	(100)	(100)	(100)	(15.1)
II + IIIW	66.0	65.0	62.5	61.5	63.7	9.6
Cent. loss	0.7	1.0	0.9	1.5	1.0	
III-0	24.4	26.2	22.7	24.3	24.4	3.7
Cent. loss	0.6	0.8	1.8	0.7	1.0	
Supernatant	8.3	7.0	12.1	12.0	9.9	
III	.... 26.8	....	.... 24.2	....	25.5	3.9
Cent. loss	.... 1.2	....	.... 2.0	....	1.6	
Filt. loss	.... 2.8	....	.... 2.0	....	2.4	
II-3	.... 20.9	....	.... 16.9	....	18.9	2.9
Cent. loss	.... 0.2	....	.... 0.2	....	0.2	
II-1,2	.... 13.2	....	.... 15.5	....	14.4	2.2
Cent. loss	.... 0.1	....	.... 0.1	....	0.1	
Supernatant	.... 0.4	....	.... 0.9	....	0.6	
III-1	.... 11.9	....	.... 11.2	....	11.6	1.8
Cent. loss	.... 0.6	....	.... 0.5	....	0.5	
Supernatant	.... 1.3	....	.... 0.7	....	1.0	
III-2	.... 8.8	....	.... 7.2	....	8.0	1.2
Cent. loss	.... 0.9	....	.... 0.7	....	0.8	
III-3	.... 2.7	....	.... 2.6	....	2.7	0.4
Cent. loss	.... 0.6	....	.... 1.3	....	0.9	

V(b) for the grams of protein per liter of plasma recovered in fractions.

We have recorded the electrophoretic analyses of the various II + III sub-fractions in Table V(a). In some cases the separations between these components is incomplete, especially in the case of the β_1-globulin and the β_2-globulin + fibrinogen components. The protein distributions in Tables V(b) and V(c) have been corrected for the nitrogen factor, but not for the refractive index increment of the components. Recent papers in this series[59] have indicated that these refractive index corrections would be small, and they can properly be neglected at this time. The unusually high mobilities assigned to the β_1 component in the lipid-rich fractions has also been discussed.[59]

TABLE IV

ESTIMATES OF NITROGEN AND CHOLESTEROL IN SUBFRACTIONS OF FRACTION II + III OF HUMAN PLASMA

Fraction	Grams protein/gram nitrogen (1)	(2)	(3)	(4)	Mean	Wt. per cent. Nitrogen	Cholesterol
II + III	7.9	7.8	7.8	7.8	7.8	12.8	6.0
II + IIIW	—6.8—		6.7	6.7	6.7	14.9	1.8
III-0	10.5	10.4	9.9	9.8	10.1	9.9	12.
II-1,2	—6.2[a]—				6.2[b]	16.0	< 0.06
II-3	—6.2[a]—				6.2[b]	16.0	< 0.06
III	—7.1—		—7.0—		7.0	14.3	3.5
III-1	—7.3—		—7.2—		7.3	13.8	3.7
III-2	—6.8—		—6.6—		6.7	13.0	3.0
III-3	—6.5—		—6.7—		6.6	15.2	1.2

[a] After filtration of combined 16.5% γ-globulin solution. [b] Also value of E. Brand, B. Kassell and L. J. Saidel, *J. Clin. Invest.*, **23**, 437 (1944).

(59) S. H. Armstrong, Jr., M. J. E. Budka and K. C. Morrison, THIS JOURNAL, **69**, 416 (1947); S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. Hasson, *ibid.*, **69**, 1747 (1947).

TABLE V

DISTRIBUTION OF PLASMA PROTEINS INTO II + III SUBFRACTIONS BY METHOD 9

Fraction	A	α	β_1	$\beta_2 + \beta$	γ	Total
	(a) Per cent. of fraction					
II + III	4	6	35	18	37	100
III-0	6	5	68	16	5	100
II + IIIW	2	8	15	19	56	100
II-1,2	0	0	0	2	98	100
II-3	0	0	0	4	96	100
III	3	15	33	34	15	100
III-1	2	18	15	45	14	100
III-2	1	20	65	12	2	100
III-3	3	20[a]	39	37[b]	2	100
(b) Grams per liter of plasma recovered in fractions						
II + III	0.7	1.1	6.5	3.4	6.9	18.6
III-0	0.3	0.3	4.1	1.0	0.3	6.0
II + IIIW	0.2	0.8	1.6	1.9	5.8	10.3
II-1,2	0	0	0	0	2.2	2.2
II-3	0	0	0	0.1	2.8	2.9
III	0.1	0.7	1.5	1.5	0.6	4.4
III-1	0	0.4	0.3	1.0	0.3	2.1
III-2	0	0.3	0.8	0.2	0	1.3
III-3	0	0.1	0.1	0.2	0	0.4
(c) Grams per liter of plasma estimated in plasma						
II + III	0.8	1.1	6.6	3.5	7.0	19.0
II	0	0	0	0.1	5.4	5.5
III-1	0.1	0.5	0.4	1.2	0.4	2.6
III-2	0	0.4	1.0	0.2	0.1	1.7
III-3	0	0.2	0.3	0.3	0	0.8
III-0	0.5	0.5	5.7	1.3	0.4	8.4
Total	0.6	1.6	7.4	3.1	6.3	19.0

Including β-fibrin.[61] [b] Including α-fibrin.[61]

In order to estimate the composition of plasma from these data, we have also calculated the amounts of each of these subfractions present in a liter of plasma (Table V(c)), using the data in Table III to estimate the losses. Although the lost material probably does not have the same electrophoretic composition as the isolated subfractions, we have calculated the distribution of proteins using this assumption, and the total found in the corrected subfractions is near to that observed for the Fraction II + III.

The distribution of the lipoprotein components has been followed by cholesterol determinations in most of these fractions, as recorded in Table IV. These values, when combined with the yield of the fractions from Table V(c), indicate that about 85% of the cholesterol of II + III, or about two-thirds of the cholesterol of plasma, is concentrated into Fraction III-0. The purified γ-globulin fractions (II-1,2 and II-3) contain less than one-fourth molecule of cholesterol per molecule of γ-globulin.

TABLE VI

DISTRIBUTION OF CERTAIN ANTIBODIES INTO II + III SUBFRACTIONS BY METHOD 9

Fraction	Weight of fraction	Typhoid agglutinin O	Typhoid agglutinin H	Influenza A Hirst test	Influenza A mouse Prot.	Diphtheria Antitoxin	Streptococcal Antitoxin
(a) Preparation 184 (Ratio to IIG141)							
II-1,2		1.1	1.5	0.6	0.7	0.8	1.0
II-3		4.6	1.3	1.2	.9	.6	0.9
III-1		40	0.9	0.7	.1	.3	...
III-2,3		20	0.7	.2	.1	.1	...
III-0		...	...	.2	.1	.1	...
Plasma		4.6	0.3	.3	.2	.1	...
(b) Preparation S362 (Ratio to IIG141)							
II-1,2		1.0	1.0	1.0	1.0	1.2	1.0
II-3		3.8	1.4	1.0	0.7	1.3	0.8
III-1		41	3.8	0.7	.5	0.5	...
III-2		10	0.6	0.3	.1	.1	...
III-0		0.7	0.2	0.2	.1	.1	...
II + III		10	1.5	1.3	.3	.7	...
(c) Preparation 184 (per cent. of plasma)							
II-1,2	3.8	1	18	7	15	20	
II-3	4.6	5	18	19	22	25	
III-1	4.0	36	12	9	3	8	
III-2,3	3.8	17	8	2	2	1	
III-0	12.7	...	...	6	6	10	
Total	28.9	59	56	43	48	64	
(d) Preparation S362 (per cent. of II + III)							
II-1,2	13	1	9	10	39	21	
II-3	16	6	15	13	34	29	
III-1	14	59	35	7	23	10	
III-2	9	9	4	2	2	1	
III-3	4	...	..	..	...	..	
III-0	44	2	2	8	10	7	
Total	100	77	65	40	108	68	

VII. Distribution of Antibodies and Physiologically Active Components

Studies of the antibody content of the various fractions have been reported by Enders.[26] More extensive studies are now available.[60] Nearly all antibodies which have been studied are found concentrated into Fractions II-1,2, II-3 or III-1, and Fractions I, III-0, III-2, III-3, IV-1, IV-4 and V are found to contain only traces of antibody. Table VI gives the results obtained on two preparations which were carefully studied. The results are first compared with a reference solution containing the same concentration of protein Table VI (a) and (b), and then with the plasma (or Fraction II + III for preparation S362) from which it was fractionated. The totals recorded in Table VI (c) and (d) represent the amount of antibody accounted for in the various fractions. Deviations from 100% represent destruction of activity, loss into other fractions, and the collected errors of these tests. The latter cause probably is the most significant. Errors in antibody assay are most serious when the activity is low, such as is found in plasma, and Fractions III-0, III-2 and III-3.

Table VII summarizes a much larger number of tests made on Fractions II-1,2, II-3 and III-1. The antibody content of the fraction, compared

TABLE VII

AVERAGE ANTIBODY CONTENT OF FRACTIONS II-1,2, II-3 AND III-1

Comparison with equal weights of plasma protein[a]

	II-1,2	II-3	III-1
Isoagglutinins	(0.2)	(0.4)	16
Typhoid O agglutinin	0.4	1.2	16
Typhoid H agglutinin	8	8	4
Influenza A, Hirst test	4	4	3
Influenza A, mouse protection	9	12	4
Influenza A, complement fixation	7	(7)	..
Mumps, complement fixation	8	(8)	..
Diphtheria antitoxin	10	7	4
Streptococcal antitoxin	9	9	..

[a] Antibody concentration of solutions of Fractions II-1,2 or II-3 containing 165 mg. protein/ml., compared with plasma, will be 2.5 times these values. Values for Fraction II-1,2 compared with plasma from Enders[6] and additional data obtained more recently (about 30 preparations). Values for Fraction II-3 were obtained largely by comparison of Fraction II-3 with II-1,2 (about 13 preparations). Values for Fraction III-1 compared with plasma or Fraction II-1,2 and II-3 obtained in about 4 preparations.

(60) We wish to thank Professor John F. Enders and his group in the Department of Bacteriology and Immunology at the Harvard Medical School—Miss Julia C. Sullivan, Miss Bettie Griffith, and Miss Jeanne E. Beauchamp—for making most of these studies. Studies of anti-A, anti-B, anti-Rh$_0$, anti-Rh', and anti-Rh" isoagglutinins were made by Dr. L. K. Diamond, Children's Hospital, Boston, Dr. W. C. Boyd, Boston University School of Medicine, Boston, and some of us (M. Melin, J. W. Cameron, and D. A. Richert). The distribution of the following antibodies has been studied: (a) On most preparations: agglutinins against typhoid O antigen; agglutinins against typhoid H antigen; antibody causing inhibition of the hemagglutinin of influenza A virus, strain PR8; mouse protection (neutralizing) antibody against influenza A virus, strain PR8; diphtheria antitoxin (iuterdermal neutralization test on rabbits). (b) On many preparations: complement-fixing antibody against the virus of mumps; complement-fixing antibody against influenza A virus, strain PR8; (c) on selected preparations: agglutinins against H pertussis, phase 1, strain 484, Philadelphia; streptococcal antitoxin (interdermal neutralization test on rabbits); antibody against streptococcal hemolysin; isoagglutinins against cells of human blood groups A^x and B; isoagglutinins against cells of human blood groups Rh$_0$, Rh' and Rh".

with the content of the same weight of plasma protein has been recorded.

TABLE VIII

ASSAY FOR PROTHROMBIN AND PLASMINOGEN ACTIVITY

Values are in units/cc. plasma

Run	Plasma	(II + III)	II + III Susp.	II + IIIW	III	III-2	III-3
			Prothombin				
171	82	63	75	70	46	..	..
173	100	54	91	60	..	..	..
175	...	95	89	50	41	..	..
176	58	83	86	60	37	..	..
177	72	61	38	17	..	..	..
178–179	88	58	18	190	35	..	..
180–181	56	68	32	55	37	34	..
182–183–184	68	73	24	18	27	..	..
185–186	63	78	53	31	21	..	..
S362-1,2	...	..	62	73	81	78	..
			Plasminogen				
S-362-1,2	...	..	32	27	30	(21)	14

Typical assays for prothrombin and plasminogen activity are recorded for the various fractions in Table VIII.[61] Assays for both of these substances are somewhat uncertain, probably due largely to the presence of inhibitors which make it difficult to completely convert prothrombin to thrombin and plasminogen to plasmin without decomposition. Little or no prothrombin and plasminogen is found in any fraction other than III-2 and III-3.

(61) These studies have been made by Miss S. G. Miller and one of us (D. A. Richert), under the supervision of Professor J. T. Edsall. The methods used are recorded elsewhere.[59,60]

Summary

1. The properties of various protein components of Fraction II + III of normal human plasma are reviewed.

2. Methods are outlined for the separation of Fraction II + III into a series of subfractions by low-temperature ethanol precipitation, in which careful control of the *p*H, temperature, and concentration of ethanol, salt and protein have been achieved. The subfractions so obtained have led to satisfactory separation and concentration of the following components: γ-globulin antibodies in Fraction II, isoagglutinins in Fraction III-1, prothrombin in Fraction III-2, plasminogen in Fraction III-3 and β_1-lipoprotein in Fraction III-0.

3. Studies of the distribution of components into these subfractions are tabulated. They have involved measurements of protein nitrogen, dry weight of protein, cholesterol, prothrombin activity, plasminogen activity and various antibody activities.

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Filter Paper Chromatography

BY HENRY B. BULL, J. WILFRID HAHN AND VICTOR H. BAPTIST

Consden, Gordon and Martin[1] reported a very ingenious method for the separation of amino acids by filter paper chromatography in which phenol or some other appropriate organic solvent sweeps the applied amino acids along the filter paper, capillarity causing the organic solvent to move. The positions of the amino acids are located by spraying the filter paper with a solution of ninhydrin and heating the filter paper strips. The amino acids develop colors, the intensity and tint of which depend upon a number of factors. A review of filter paper chromatography has recently appeared.[2]

The present paper reports an attempt to make filter paper chromatography of amino acids quantitative. The percentage light intermission along the chromatogram is measured and this transmission has been plotted on semi-log graph paper against the distance along the filter paper strips, and the areas of the segments above the plotted curve measured with a planimeter. It has been found that the areas so determined are over a limited concentration range a simple function of the concentration of the amino acids. The various factors which influence the reliability of this method are reported.

(1) Consden, Gordon and Martin, *Biochem. J.*, **38**, 224 (1944).

(2) Consden, *Nature*, **162**, 359 (1948).

Experimental

Carl Schleicher and Schuell quantitative filter paper number 507 was cut against the machine direction into strips 7.20 mm. wide and 60 cm. long. One end of the filter paper strip was placed in the bottom of an oblong butter dish and a thick glass strip placed on top of the paper to hold it in position. It was found convenient to use 5 such strips hanging at about 2 cm. intervals over each side of the butter dish. The lower ends of the filter paper strips were attached to small metal clamps affixed to a metal rod. 0.0135 cc. of the amino acid solution adjusted to a *p*H 5 to 7 was placed on a marked spot on the filter paper strip with a Blodgett pipet about 4 cm. from the edge of the butter dish. The filter strips were in a horizontal position during this operation. The amino acid solutions were air dried on the filter paper and the butter dish, with the filter paper strips hanging vertically, was placed on a stage in a tall glass jar. The jar had a layer of 80% aqueous solution of Merck reagent grade of phenol on the bottom. Eighty per cent. aqueous solution of phenol was poured into the butter dish to cover the ends of the filter paper strips. A second glass jar was inverted over this jar and the joint between the jars sealed with vaseline.