

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

Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids<sup>1a,b,c,d</sup>BY E. J. COHN, L. E. STRONG, W. L. HUGHES, JR., D. J. MULFORD, J. N. ASHWORTH, M. MELIN AND H. L. TAYLOR<sup>1c</sup>

Natural products rarely exist in a state of maximum purity or maximum concentration. They are generally found in plant or animal tissues or fluids in the presence of other natural products, and often in small amounts, or in inactive physical states in which they are stored as biological reserves. Natural function may demand the liberation of the active component in but a small, constant concentration. The greatest value of each active component, however, whether as reagent in therapy or in chemical technology, is often in a highly purified, stable and concentrated state.

The present series of reports describes the development of a system for the separation of the protein and lipid components of a biological tissue, first into a small number of fractions in which the major components are separated, and then into a large number of subfractions into which they are further concentrated and purified. The tissue that has thus far been most thoroughly investigated is human blood. The methods that have been employed are, however, general and can be applied to give comparable inclusive fractionations of other biological systems in the interest of obtaining as many as possible of their valuable

components as nearly as possible in their natural state.

The separation of the many protein and lipid components of a biological fluid or tissue can be accomplished by control of their relative solubilities in a multi-variable system. The larger the number of components and the more nearly alike their physical chemical properties, the more variables, each under accurate control, may be needed in order to determine conditions in which sufficiently large differences in solubility obtain to permit satisfactory separations.

In practice, conditions must be determined such that the protein to be separated has (1) a high solubility when most other components of the system have low solubilities, or (2) a low solubility when most of the components of the system have high solubilities. Solubilities of 0.01 to 0.1 g. per liter generally suffice to separate fair yields of a component as a precipitate. Solubilities of 10 g. or more per liter are generally adequate if other components of the system are to be precipitated. Conditions such that the solubility can be varied from a hundred to a thousand fold should thus be known if satisfactory fractionation and purification are to be carried out. The larger the variety of conditions under which a given protein in a mixture can be maintained in either the higher or the lower solubility range, the greater is the chance of effecting sharp separations from other proteins whose solubilities are influenced by the same variables.

**Euglobulin Precipitation.**—Certain proteins can be separated in aqueous systems because of their insolubility in the isoelectric condition. Of these, those termed globulins are readily dissolved by neutral salts. "Solution of globulin by a neutral salt is due to forces exerted by its free ions. Ions with equal valencies, whether positive or negative, are equally efficient, and the efficiencies of ions of different valencies are directly proportional to the squares of the valencies."<sup>2</sup> The ionic strength thus defined for the first time in terms of this interaction influences protein solubility so profoundly that the phenomenon was noted by Denis a century ago.<sup>3</sup> The influence of the ionic strength is so great that fractions of serum globulin with a solubility of approximately 0.07 g. per liter in water at 25° "readily dissolved to a water-clear liquid in 0.1 *N* solution of sodium chloride."<sup>4</sup> The quantitative separation of pro-

(1a) This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. Since August, 1941, it has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(1b) This paper is Number 43 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross. We are indebted to the Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health, for preparing in sterile and safe form the various fractions that have been made available for clinical use; are now licensed products under the National Institute of Health and have been prepared in large amounts in industrial plants under contract with the United States Navy which, for nearly five years, has collaborated in this program.

(1c) This article has been released for publication by the Division of Publications of the Bureau of Medicine and Surgery of the United States Navy. The opinions and views set forth in this article are those of the writers and are not to be considered as reflecting the policies of the Navy Department.

(1d) Our various chemical and clinical collaborators in this and other institutions are making their results available in this and other journals. We would like to acknowledge their assistance in solution of many problems as well as that of members of the

\* Pilot Plant which has been in continuous operation for one and one half years and included, besides the authors, M. J. E. Budka, J. H. Cameron, M. Y. Clark, M. E. Odum, H. T. Gordon, P. M. Gross, M. M. Hasson, H. H. Larsen, S. G. Miller, D. A. Richert, M. H. Row, and J. H. Weare.

3) USNR.

(2) J. Mellanby, *J. Physiol.*, **33**, 338 (1905-1906); cf. p. 373.

(3) J. Scherer, *Liebigs Ann. d. Chem. und Pharm.*, **40**, 1 (1841).

(4) E. J. Cohn, *J. Gen. Physiol.*, **4**, 697 (1922); cf. p. 710.

teins of this kind has thus depended upon the removal of salt by dilution, dialysis or electrodialysis and adjustment of the pH to the isoelectric zone.

Globulins differ from each other and can be separated from each other since they have different isoelectric points, different solubilities in the absence of salt and different activity coefficients in the presence of salts, especially at very low concentrations of salts. "The precipitation of a globulin passes through a sharper maximum the freer it is from salt. Freedom from salt, at least as applied to the proteins is, however, relative . . . Moreover, three distinct sources of error may make . . . the point of maximum precipitation of a protein appear at a hydrogen ion concentration other than its isoelectric point.

"The first of these is the presence of another protein with a slightly different isoelectric point. The observed precipitation is in this case the sum of the precipitations of the two proteins. As a result the zone is usually widened, and the point of maximum precipitation shifted in the direction of the isoelectric point of the second protein."<sup>5</sup>

In order to achieve satisfactory separation between such proteins, therefore, the precipitation zone of each should be approached under conditions in which all the proteins in the system either bear a negative electric net charge, that is, are alkaline to their respective isoelectric points, or all bear a positive electric net charge and are acid to their respective isoelectric points.<sup>6</sup> The

formation of protein complexes when precipitation takes place at reactions between the isoelectric points of the proteins is, however, only one form of protein-protein interaction.

In all but the very few proteins that have been separated and purified from all other proteins, solubility is influenced by the nature and the amount of protein in the system. Four variables thus determine solubility in such systems: (1) pH, (2) salt concentration, (3) temperature and (4) the protein in the system.<sup>7</sup> If the temperature and the amount and nature of the protein in the system are maintained constant, a family of curves can be constructed describing solubility as a function of pH and salt concentration. Globulins are, however, known (1) whose solubility is increased by salt on either side of the isoelectric point, and (2) whose solubility is increased on one side, but diminished on the other.<sup>8</sup> The latter effect is often a function not only of the nature of the protein, but also of the nature of the salt. In any such system, however, curves of constant solubility can be constructed in which pH and salt concentration determine solubility at constant temperature and total protein concentration.

In many systems only a small proportion of the proteins are insoluble in water near their isoelectric points. The separation and crystallization of these has heretofore not been carried out at low salt concentrations. And yet it is precisely at the lowest salt concentrations that the interactions with electrolytes are most intense. These depend upon the number and distribution of the electrically charged groups, as well as of the other side chains, and therefore upon the same structural pattern that determines the highly specific nature of the protein molecule.

The system of fractionation here reported involves lowering the solubility of the protein so that separations may be carried out in the range of low ionic strengths at which the interactions of proteins with electrolytes differ from each other markedly, both in the isoelectric condition and when dissociated as acids or bases.

**Salting-Out.**—The separation and crystallization of water-soluble proteins has heretofore most generally been carried out in very concentrated salt solutions. The practice of reducing the solubility of proteins by salting-out, introduced in the middle of the last century by Panum, Virchow and Claude Bernard and Hofmeister, has been developed to take advantage of the influence of pH and temperature upon protein solu-

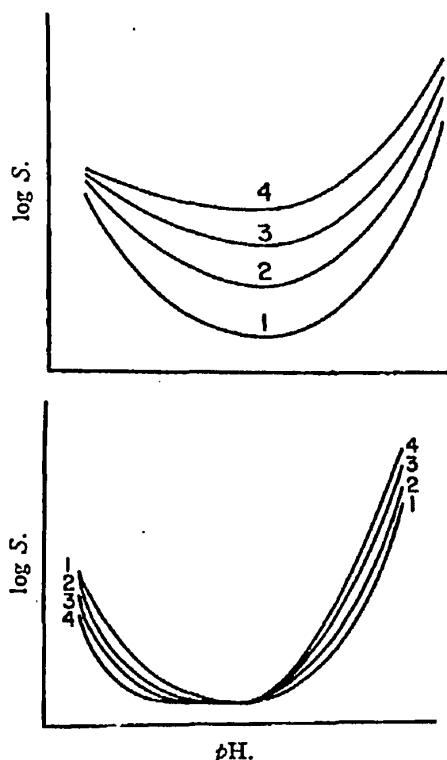


Fig. 1.—Protein solubility as a function of pH for various salt concentrations.

(5) E. J. Cohn, *J. Gen. Physiol.*, 4, 697 (1922); cf. p. 699.

(6) A. A. Green, *This Journal*, 60, 1108 (1938).

(7) For convenience the sum of the proteins in the system is treated as constituting the environmental variable. A more detailed analysis would treat each protein component as a separate variable.

(8) This "effect of minute quantities of acid on the solubility of globulin in salt solutions" was early observed by Osborn (see *This Journal*, 19, 482 (1897)) and has since been studied [W. B. Hardy *Proc. Roy. Soc. London*, B75; K. Linderström-Lang and S. Kodama, *Compt. rend. Acad. Sci. Paris*, 16, No. 1, (1925); S. P. L. Sørensen and I. M. Kolthoff, *J. Biol. Chem.*, 75, 1 (1922); and A. A. Green, *J. Biol. Chem.*, 100, 1 (1934)].

bility.<sup>9,10,11,12</sup> There are, however, limitations both practical and theoretical to the separation of proteins by salting-out. From a practical point of view a protein separated from a concentrated salt solution must next be freed of the salts precipitated with it. This is particularly necessary since the substances commonly used in salting-out are usually not desirable when the protein preparation is to be used clinically. The removal of unwanted salt has generally been accomplished in the past by dialysis, a process which exposes the protein for relatively long periods of time to conditions which are rarely bacteriostatic.

From a theoretical point of view the highly specific forces that determine the interactions between proteins and electrolytes at low ionic strengths are masked in concentrated salt solutions. The predominant forces determining solubility in concentrated salt solutions, far from being specific for proteins, are common to interaction with gases, electrolytes and non-electrolytes.<sup>11,13,14,15</sup> Salting-out for proteins, as for simpler molecules, even gases, depends largely upon the volume of water displaced. The constant defining the salting-out of proteins by sulfates or phosphates varies only by a factor of two or three to one<sup>16</sup> as contrasted with the very great variation in the interaction of different proteins with electrolytes at low salt concentrations. It was in order to take advantage of the more specific forces between proteins and electrolytes that another method of reducing protein solubility was employed.

**Alcohol Precipitation.**—The solubility of proteins may also be reduced by the addition of alcohols, acetone or other water miscible organic solvents to protein solutions.<sup>17,18,19,20</sup> Such additions at ordinary temperatures generally lead to protein denaturation. Irreversible changes in the labile protein molecules can generally be minimized, however, if the temperature is maintained sufficiently low. Thus egg albumin, a readily denatured protein, could be recrystallized after being in 25% ethanol at  $-5^{\circ}$  for a month, if the ethanol was removed before the temperature was raised.<sup>21</sup> Neutral salts increase solubility under these conditions much as they do the solubility of

globulins in water, and this observation has been extended to other proteins.<sup>22,23,24</sup>

Reducing the solubility of a protein to any desired extent by the addition of a water miscible organic liquid, at a temperature sufficiently low to prevent protein denaturation, has both theoretical and practical advantages. From a practical point of view the volatile organic liquid can be removed at low temperature. A most convenient procedure for this operation consists in lowering the temperature sufficiently below that employed in processing to freeze the wet protein precipitate, and then removing both the organic liquid and the water under reduced pressure.<sup>25,26,27</sup> The dangers of bacterial growth which beset dialysis are completely avoided, and the final product is a dry preparation of the protein and of such non-volatile substances as precipitate with it.

**Protein-Electrolyte Interactions in Alcohol-Water Mixtures.**—Theoretical advantages in carrying out plasma fractionation in a system in which solubility is reduced to a convenient level by an organic liquid are manifold. The electrolyte concentration may be maintained in the low range in which interactions with proteins depend largely upon the ionic strength and the specific electrochemical properties of the protein.<sup>23</sup> Indeed, the interactions between proteins and ions, as those between ions, are increased by the addition of water miscible organic liquids which decrease the dielectric constant of the solution. By balancing precisely the solvent action of the electrolyte with the precipitating action of the organic liquid widely different conditions can be defined, such that the solubility of the proteins under consideration remains constant. The solubility of the other proteins in certain of these

(22) R. M. Ferry, E. J. Cohn and E. S. Newman, *J. Biol. Chem.*, **114**, Proc. 34 (1936); *This Journal*, **60**, 1480 (1938).

(23) E. J. Cohn, J. A. Luetscher, Jr., J. L. Oncley, S. H. Armstrong, Jr., and B. D. Davis, *ibid.*, **62**, 3395 (1940).

(24) B. J. Cohn, *Chem. Rev.*, **28**, 395 (1941).

(25) The process of drying proteins from the frozen state yields soluble, undenatured preparations in most cases [ref. 26]. For certain lipoproteins, however, freezing appears to weaken the attachment between lipid and protein. This effect is even greater if the freezing takes place in the presence of an organic liquid. Indeed, freezing in the presence of a lipid solvent insoluble in water has been used as a method to remove lipid from the protein to which it is attached (ref. 27). The removal of organic precipitants from lipoproteins thus raises special problems.

(26) E. W. Florsdorf and S. Mudd, *J. Immunol.*, **29**, 389 (1935); see also E. W. Florsdorf, F. J. Stokes and S. Mudd, *J. Am. Med. Assn.*, **115**, 1095 (1940); R. I. N. Greaves and M. E. Adair, *J. Hyg.*, **39**, 413 (1939); J. M. Hill and D. C. Pfeiffer, *Ann. Internal Med.*, **14**, 201 (1940); M. M. Strumia, J. J. McGraw and J. Reichel, *Am. J. Clin. Path.*, **11**, 480 (1941); M. M. Strumia and J. J. McGraw, *J. Lab. Clin. Med.*, **28**, 1140 (1943).

(27) A. S. McFarlane, *Nature*, **149**, 439 (1942).

(28) The experimental evidence that the logarithm of the activity coefficient of a protein increases with the dipole moment and, as a first approximation, by slightly less than the first power of the dipole moment of the protein, and the expectations from the theories of Scatchard and Kirkwood [*Phys. Z.*, **33**, 297 (1932)] and Kirkwood [*J. Chem. Phys.*, **2**, 351 (1934)] are considered in detail by Cohn and Edsall ["Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publishing Corp., New York, N. Y., 1943, cf. pp. 609-617].

(9) H. Chick and C. J. Martin, *Biochem. J.*, **7**, 380 (1913).  
 (10) S. P. L. Sprensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 213 (1915-1917).  
 (11) E. J. Cohn, *Physiol. Rev.*, **5**, 349 (1925).  
 (12) A. A. Green, *J. Biol. Chem.*, **93**, 517 (1931).  
 (13) P. Debye and J. McAulay, *Physik. Z.*, **26**, 22 (1925); P. Debye, *Z. physik. Chem.*, **130**, 86 (1927).  
 (14) G. Scatchard, *Chem. Rev.*, **3**, 383 (1927); *Trans. Faraday Soc.*, **23**, 454 (1927).  
 (15) M. Randall and C. F. Failey, *Chem. Rev.*, **4**, 271 (1927).  
 (16) E. J. Cohn, *Ann. Rev. Biochem.*, **4**, 93 (1935); cf. p. 130.  
 (17) J. Mellanby, *Proc. Roy. Soc. (London)*, **B80**, 399 (1908).  
 (18) W. B. Hardy and S. Gardiner, *J. Physiol.*, **40**, lxviii (1910).  
 (19) L. D. Felton, *Bull. Johns Hopkins Hosp.*, **38**, 33 (1926).  
 (20) H. Wu, *Chinese J. Physiol.*, **7**, 125 (1933-1934).  
 (21) R. M. Ferry, E. J. Cohn and E. S. Newman, *J. Biol. Chem.*, **109**, Proc. 32 (1935); *This Journal*, **58**, 2370 (1936).

systems will generally be sufficiently different to permit satisfactory separations to be made.

A further advantage in carrying out protein fractionations in systems containing the desired concentration of electrolyte and organic precipitant is that five independent variables may be maintained under accurate control. In a system containing five variables, three of which are maintained constant and the other two are chosen such that one will increase and the other decrease solubility, conditions can be determined and curves constructed defining the constant solubility of any protein. For example, in the system that we have employed, the desired solubility for a given protein can be reproducibly achieved under a variety of conditions of electrolyte and ethanol concentration at the given  $pH$ , temperature and protein concentration. If the protein is a mixture, the composition of the precipitate and that of the solution will vary along a curve of constant total solubility. The smaller the protein concentration, the more accurately can the solubility curve of a mixture and the compositions of the precipitate and the solution be predicted from the solubility curves of the components.

In a system in which  $pH$ , salt and ethanol concentrations are variables, curves will not suffice to define conditions for constant solubility; a surface must be constructed. The conditions for constant solubility are then given with three variables as parameters.

Variation of the temperature has also been employed in order to effect reproducible separations in the fractionations that have been carried out. A more complex diagram would be necessary to represent graphically the conditions for constant solubility—either high solubility where the precipitation of other components was desired, or low solubility when a component was to be separated as a precipitate—in the multi-component systems that have been fractionated.

Other or more complex systems can readily be employed with other limits to these variables, or to which other substances are added at variable concentrations in order to vary still further the properties of the solution in which separations are to be effected. Were the proteins to be separated strongly basic, like histones or protamines, a more alkaline  $pH$  range would be required than has sufficed for the plasma proteins; were they more soluble in organic liquids, like the prolamines, a different range of concentration of the organic liquid would be required. Certain separations may be more effectively carried out in solutions containing either higher alcohols, acetone, dioxane or ether, or polyhydric alcohols, or mixtures of organic liquid with sugars or with dipolar ions, such as glycine, which increase the dielectric constant of the solution. Moreover, specific interactions with ions, which either have solvent or precipitating actions other than those due to their ionic strength, very often prove useful within

the framework of this system. However, in the fractionation of plasma proteins that has been carried out for the Armed Forces on a large scale with the blood of over two million donors to the American Red Cross, a five-variable system has sufficed in which the limits were as follows

Variable	Limits employed
$pH$	4.4 to 7.4
$I/2$	0.001 to 0.16
Ethanol concn., mole fraction	0 to 0.163
Ethanol concn., vol. % at 25°	0 to 40
Protein concn., g./liter	0.2 to 66
Temperature, °C.	0 to -10

These conditions have been attained, and the variables maintained constant, by the use of acetate and carbonate buffer systems to control  $pH$  and ionic strength, and by the use of ethanol as precipitant since it was readily removed by distillation. These reagents are both convenient for the processing of biological systems and safe to use in the preparation of protein and lipid products, even where large quantities are destined for intravenous use. The high values for protein concentration,  $pH$  and ionic strength are those of the original plasma. Precipitation was carried out at the lowest convenient ethanol concentration and temperature, and at the optimum  $pH$  and ionic strength for each separation. The lower protein concentrations decreased interactions and permitted sharper separations to be made, although each protein species constituted a separate component and its concentration a variable influencing the interactions of proteins with electrolytes, with the organic precipitant and with each other.

## II. Materials and Methods

**Plasma.**—The normal human plasma employed in this investigation was separated from blood collected by the Boston Metropolitan Chapter of the American Red Cross in standard bleeding bottles, containing 50 cc. of 4% U. S. P. sodium citrate solution into which approximately 500 cc. of blood was drawn. The plasma was separated from the red cells by centrifugation<sup>29</sup> at the Massachusetts Department of Public Health Antitoxin and Vaccine Laboratory, and pooled after completion of serological tests. Fractionation was begun within seventy-two hours of the time that the blood was drawn. Except for a few hours occupied in separation of the cells, the plasma was refrigerated.

Analyses of 34 large pools of citrated plasma, carried out under the direction of Dr. H. B. Vickery of the Connecticut Agricultural Experiment Station, gave an average value of 9.88 g. per liter as the total nitrogen content of citrated plasma. Of this 9.65 g. per liter was protein nitrogen.<sup>30</sup> Multiplying this value by the con-

(29) A number 3 International Centrifuge manufactured by the International Equipment Company, Boston, Massachusetts, was used. Comparable, and in some cases better yields have been obtained using either cup centrifuges or special continuous centrifuges designed by either DeLaval Separator Company, 165 Broadway, New York City (Model Number 54) or Sharples Corporation, 23rd and Westmoreland Streets, Philadelphia 40, Pennsylvania, bowls, type 18 CY-54 for the separation of plasma in one or another of the seven commercial laboratories who have had Navy contracts for plasma fractionation by these methods.

(30) Non-protein nitrogen was determined after precipitation of the protein with 10% trichloroacetic acid.

ventional nitrogen factor, 6.25, gave 60.3 g. per liter as the protein content of the average pool of citrated plasma.

**Water.**—Distilled water was prepared by a single distillation of Boston city water through a still with a "Spanish prison" type baffle.<sup>31</sup> In order to minimize bacterial contamination the hot water collected from the still was stored in borosilicate glass containers at a temperature above 50°. All apparatus was rinsed with this hot water and cooled immediately before use.

**Ethanol.**—A commercial grade of 95% ethanol has been used without further purification for the major part of this work.<sup>32</sup>

**Ethanol-Water Mixtures.**—Ethanol solutions were made up with hot water and then cooled and stored at -5° or below. The concentrations were determined by density measurements at 25° and are recorded either as volume per cent. at this temperature or as mole fraction ethanol. The composition and the criteria for the standardization of a few of the ethanol-water mixtures used repeatedly in this investigation are given in Table I. Ethanol concentrations of solutions containing non-volatile solutes were determined by sublimation *in vacuo* of the solvent from the frozen solution. The refractive index, read on a Pulfrich dipping refractometer, proved a convenient method of analyzing for ethanol in the distillate.

TABLE I

PHYSICAL CHEMICAL CONSTANTS OF ETHANOL-WATER MIXTURES USED IN PLASMA FRACTIONATION

Volume % ethanol at 25°	Mole fraction, ethanol	Density at 25°C. referred to water at 4° <sup>a</sup>	Refractive index, 25° <sup>b</sup>	Freezing point, °C. <sup>c</sup>
8	0.0200	0.9800	1.3366	-2.6
10	.0328	.9835	1.3377	-3.4
15	.0509	.9775	1.3405	-5.4
18	.0624	.9741	1.3422	-6.8
25	.0907	.9659	1.3463	-10.7
40	.1630	.9448	1.3537	-23.0
53.3	.2462	.9198	1.3583	-33.5

PHYSICAL CHEMICAL CONSTANTS OF ETHANOL-WATER PROTEIN SYSTEMS USED IN METHOD 6

Supernatant solution of fraction	Mole fraction ethanol	Temp., °C.	Estimated densities Ethanol-water (salt-free)	Protein solution <sup>d</sup>
Plasma	0	0		1.03
I	0.027	-2.5	0.990	1.01
II + III	.091	-5	.977	0.99
IV-1	.062	-5	.981	1.00
IV-4	.163	-5	.963	0.97
V	.163	-5	.963	0.97

<sup>a</sup> Data at 25° taken from Landolt-Börnstein, "Physikalisch-chemische Tabellen," J. Springer, Berlin, 1923, p. 448. Densities at other temperatures estimated by extrapolation. <sup>b</sup> Taken from Official and Tentative Methods of Analysis, 3rd ed., published by the Association of Official Agricultural Chemists, Washington, D. C., 1930. <sup>c</sup> Note <sup>a</sup> p. 1457. <sup>d</sup> The densities of plasma and of the supernatant solution from which each fraction was separated brought to room temperature have been taken as: 1.02, 1.02, 0.98, 0.98, 0.95, 0.95.

(31) Model SMQ-5 from Barnstead Still and Sterilizer Company, Inc., 2 Lancashire Terrace, Jamaica Plain, Massachusetts. In localities where tap water contains an excessive amount of organic matter more extensive treatment may be necessary to eliminate contaminants which give rise to febrile reactions following intravenous injection.

(32) In commercial production denatured ethanol formula 3A obtained from various sources has been substituted without detected change in the fractionation. When denatured alcohols are used it is important to be sure that all denaturants are readily removed by the method used to remove ethanol.

By this method a determination could readily be made in a few minutes with as little as 1 cc. of solution.

**Acetate Buffers.**—The control and adjustment of the acidity of the solution was effected by the use of acetate buffer solutions of known pH and ionic strength.<sup>33</sup> Preparation of such solutions is facilitated by the use of the Cartesian nomogram<sup>34</sup> or the more recently developed D'Ocagne nomogram.<sup>35</sup>

Reagent grade acetic acid and sodium acetate were used without further purification for the preparation of buffers. Stock solutions of 10 M acetic acid and 4 M sodium acetate have proven convenient in the preparation of the buffers used in adjusting pH to a more acid reaction. The use of buffer solutions rather than free acid, which was never employed in these investigations, protected the proteins in the system from acidities greater than those of the buffer solution. The addition of all reagents, either by diffusion through membranes, or from capillaries with constant stirring, was designed to prevent the development of local excesses in order to minimize the denaturation of proteins from acids or alcohols.

**pH.**—The pH in these systems was controlled, wherever possible, by the use of buffers to yield the desired ionic strengths. The values of pH recorded do not refer to those in the ethanol-water mixtures at the low temperatures at which the proteins were fractionated. The pH was always determined by dilution of these systems to concentrations sufficiently low in ethanol that the pH value did not appear to change significantly with further dilution. In systems of low ionic strength, neutral salt solution was used as diluent. The measurements of pH were made with a glass electrode at a temperature near 25°.

**Temperature.**—All temperatures during processing were maintained at 0° or lower. The plasma, as soon as it was pooled, was brought to 0° before the addition of an ethanol-water mixture. During the addition in all but method 1 the temperature was maintained close to the freezing point by carrying out the process in a -5° room. In order to increase the rate at which the temperature could be decreased, cooling coils were introduced into, or surrounding, the glass-lined or stainless steel kettles in which processing took place.<sup>36</sup>

The processing room at the Harvard Pilot Plant was maintained at -5°. The deviations of the air temperature within the room rarely exceeded ±1°. The temperature within the processing tanks was maintained to within ±0.5°.

In certain steps the temperature was reduced below -5°. This was accomplished by the use of accessory refrigeration equipment to cool the processing tanks, centrifuge or other equipment.

Centrifugation was the principal means used for separating the precipitates at each step in the process. Where the volumes were more than a few liters, a continuous centrifuge was used.<sup>38</sup>

(33) E. J. Cohn, F. F. Heyroth and M. F. Menkin, *THIS JOURNAL*, 50, 696 (1928).

(34) A. A. Green, *ibid.*, 56, 2331 (1933).

(35) W. C. Boyd, *ibid.*, 67, 1035 (1945).

(36) In large-scale commercial processing it was found convenient to operate with closed, insulated, brine-jacketed kettles, which could then be located outside the cold processing room but connected with it by stainless steel, insulated pipes. Connections to centrifuges and filters within the cold room were as short as possible. All equipment and connections used in processing were either glass-lined or of stainless steel, and installed so as to be readily cleaned and sterilized. Rubber gaskets and rubber connecting tubes were eliminated wherever possible.

(37) The temperature control was effected by means of a Duplex air operated control and recorder manufactured by the Foxboro Co., Foxboro, Massachusetts.

(38) Sharples supercentrifuge Model 16, manufactured by The Sharples Corporation, Philadelphia, Pennsylvania. All parts coming in contact with the material to be centrifuged were stainless steel. When using the high speed centrifuge it was necessary to dissipate the heat produced by air friction. This was accomplished by refrigerating the air space between the rotor and the frame. Even momen-

Filtration was, in some cases, used as a method for recovering precipitates, but it was used principally as a means of removing traces of suspended material not removed by centrifugation or gravity sedimentation. By careful clarification of solutions small amounts of material were removed which, if present during a subsequent precipitation, would contribute impurities to the precipitate. In most cases it was found useful to add calcined diatomaceous earth as a diluent for the filter cake and thus an aid to rapid filtration.<sup>39,40</sup>

In order to ensure adequate removal of suspended matter by filtration nephelometric measurements<sup>41</sup> were often used as a means of control. In the separation of albumin a correlation was established between the clarity of the supernatant from which the albumin was to be precipitated and the thermal stability of the albumin.

Drying from the frozen state was used in many cases as a means of removing ethanol employed in the processing, as well as a means of reducing the proteins to a dry, stable state for storage or resolution in an appropriate solvent. Most of the drying at the Harvard Pilot Plant was carried out on Folsom type driers. Where more rapid drying, or drying many small final containers, especially to very low moisture content, was desired, one of the more recently developed biological desiccating units was employed. The precipitates to be dried were suspended in water, and where ethanol was present it was usually diluted to less than 15%. The suspensions were frozen in a Dry Ice-ethanol-bath and the volatile material removed *in vacuo*. For preparations which are to be immediately redissolved the drying process need go no further than is necessary to remove the ethanol. In order to preserve many labile components in the dry state, however, very low moisture contents must be achieved rapidly and the final product stored *in vacuo*.

Sterility was obtained in liquid products by filtration through asbestos-paper pulp pads. This operation was carried out immediately following solution of the dry product and sterility achieved as rapidly as possible, since the conditions in the reconstituted aqueous solutions, in contrast to those in ethanol-water mixtures at low temperatures, are not bacteriostatic.<sup>42</sup>

tary failure of the refrigeration can lead to a rise in temperature sufficient to denature proteins in the presence of ethanol.

(39) Several grades of calcined diatomaceous earth filter aids manufactured by the Johns Manville Corporation, 22 East 40th Street, New York 16, N. Y., have been used. The filtration equipment used at the Harvard Pilot Plant consisted mainly of a 14" stainless steel horizontal plate filter manufactured by the Sparkler Manufacturing Company, Mundelein, Illinois. Buchner funnels were used for small-scale filtrations, and a few experiments were carried out with a continuous rotary filter manufactured by the Oliver United Filters, Inc., 2900 Glascock Street, Oakland, California. In most cases asbestos paper pulp pads were used of the types manufactured by F. R. Hormann & Co., Inc., 127 Boerum Place, Brooklyn, New York and Republic Filters, Inc., 204 21st Avenue, Patterson 3, New Jersey.

(40) Filter aids were usually washed before use with several volumes of 2% sodium chloride, followed by several volumes of an ethanol-water solution of the same ethanol and salt concentration as the system to be filtered. In certain systems, where the buffer concentration was low, it was found necessary to wash the filter aids and filters with sufficient buffered solution to bring the pH of the filter close to that of the material to be filtered.

(41) Most of the nephelometric measurements were made in a Zeiss-Pulfrich photometer with nephelometer attachment, in which the scattered beam makes an angle of 45° with the unscattered emergent beam. More recently, a photoelectric tyndallometer designed by Hans Mueller and C. Rado has been used. This latter instrument was developed under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts Institute of Technology, primarily for use in the study and acceptance of normal human serum albumin (see G. Scatchard, S. T. Gibson, L. M. Woodruff, A. C. Batchelder and A. Brown, *J. Clin. Invest.*, 23, 445 (1944)).

(42) Pads manufactured for this purpose by the F. R. Hormann & Company and Republic Filters have been used successfully. Small

Nitrogen and Dry Weight Analyses.—Nitrogen was determined by micro-Kjeldahl. To permit estimation of non-protein nitrogen (NPN), protein was precipitated by 10% trichloroacetic acid. Dry weights were estimated after heating for twenty-four to forty-eight hours in an air oven at 110°. These analyses have largely been carried out by M. M. Hasson and M. H. Smith.

Lipid Analysis.—Total cholesterol was determined by the method described by Bloor, Pelkan and Allen.<sup>43</sup> No attempt was made to carry out detailed analyses for the various lipids in plasma.<sup>44</sup> For the purpose of controlling separation and standardizing products, measurements were occasionally made of lipid phosphorus, and routinely of total cholesterol, by P. Gross.

Electrophoretic analysis of plasma and the various fractions has been used as a convenient means of characterizing certain of the separations. Its application to the problems of plasma fractionation has been previously discussed.<sup>45,46</sup> The measurements reported for methods 1 and 2 were carried out in a phosphate buffer at pH 7.7 and an ionic strength of 0.2. A more complete resolution of the  $\alpha$ -globulins and albumin was obtained in the analysis of the fractionation by methods 5 and 6 by substituting a sodium diethyl barbiturate buffer at pH 8.6<sup>40</sup> and an ionic strength of 0.1. The electrophoretic measurements have been made by M. J. E. Budka, A. H. Sparrow, K. C. MacDonald and M. Clark under the direction of S. H. Armstrong, Jr.

Ultracentrifuge analysis of the various fractions was employed as an aid in determining conditions for their separation, as a method for characterizing the separated proteins, and as a means of determining whether denaturation had occurred.<sup>45</sup> An air-driven Pickels type ultracentrifuge equipped with Schlieren Toepler optical system was used. All ultracentrifuge measurements were made by C. G. Gordon and G. N. Thurber under the direction of J. L. Oncley.

### III. System of Plasma Fractionation

The system of plasma fractionation described here has been repeatedly modified and is subject to further modification. The possible conditions for the separation of the components of the system are many, and one method has replaced another in our effort, which still continues, to determine the optimum conditions for the separation of each component from all the others.

The experimental conditions that obtained in four of the six methods that have thus far been employed in the fractionation of normal human plasma are given in Table II. The number of major fractions was purposely maintained as laboratory filters manufactured by F. R. Hormann & Company and Model 200 IE plate and frame press manufactured by Republic Filters have been used to hold the filter sheets for sterilizing filtrations.

(43) W. R. Bloor, K. F. Pelkan and D. M. Allen, *J. Biol. Chem.*, 52, 191 (1922).

(44) More detailed studies of the lipids in Fraction IV have been carried out by H. E. Carter, A. Chanutin, and E. Chargaff (personal communications). See also doctor's thesis of G. Phillips carried out under H. E. Carter, University of Illinois.

(45) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H. Armstrong, Jr., *J. Clin. Invest.*, 23, 417 (1944).

(46) A re-appraisal of the electrophoretic analyses of the plasma proteins based on the consideration of the technical problems involved in the interpretation of Schlieren diagrams (refs. 47 and 48) and the refractive index increments of the plasma proteins (ref. 49) are considered in two subsequent papers in this series.

(47) S. H. Armstrong, Jr., M. J. E. Budka and K. M. Morrison, in preparation.

(48) H. Svensson, *Arkiv. Kemi, Mineral. Geol.*, 17A, 14, 1943.

(49) S. H. Armstrong, Jr., M. J. E. Budka, P. Gross and M. Hasson, in preparation.

(50) L. G. Longworth, *Chem. Rev.*, 30, 323 (1942).



TABLE II  
CONDITIONS FOR THE SEPARATION OF HUMAN PLASMA PROTEINS INTO FRACTIONS BY VARIOUS METHODS

Fraction	Method	pH	$\Gamma/2$	Temp., °C. <sup>a</sup>	Mole fraction ethanol	Protein in system g./liter	Protein in fraction N X 6.25 g./liter of plasma <sup>b</sup>
Plasma		7.4	0.16			60.3	
I	1	Uncontrolled	.08	0	0.027	30.1	4.2
I	2	Uncontrolled	.13	0	.033	29.0	4.1
I	5	Uncontrolled	.14	-3	.027	51.1	4.3
I	6	7.2	.14	-3	.027	51.1	3.8
II	1	Uncontrolled	.04	-5	.051	28.0	6.8
III	1	Uncontrolled	.02	-5	.091	24.6	7.0
II + III	2	Uncontrolled	.08	-5	.091	29.0	13.5
II + III	5	6.8	.09	-5	.091	29.7	16.3
II + III	6	6.8	.09	-5°	.091	30.0	15.5
IV	1	5.5	.05	-5	.163	10.6	13.4
IV	2	5.8	.09	-5	.163	10.6	14.1
IV	5	5.8	.09	-5	.163	9.9	9.7
IV-1	6	5.2	.09	-5	.062	15.8	4.3
IV-4	6	5.8	.09	-5	.163	10.1	5.8
V	1	4.8	.06	-5	.163	7.2	26.7
V	2	4.8	.11	-5	.163	6.9	27.9
V	5	4.8	.11	-5	.163	6.7	29.6
V	6	4.8	.11	-5	.163	7.5	29.9
VI	1	4.8	.06	-5	.163	0.7	2.8
VI	2	4.8	.11	-5	.163	0.2	0.6
VI	5	4.8	.11	-5	.163	0.2	0.6
VI	6	4.8	.11	-5	.163	0.2	0.9

<sup>a</sup> The temperature control in method 1 was accurate to  $\pm 2^\circ$ , in subsequent methods to  $\pm 0.5^\circ$ . <sup>b</sup> The size of fraction was reproducible to about 0.5 g. per liter plasma. <sup>c</sup> Certain preparations have been carried out at  $-10^\circ$  in the interest of diminishing depressor nature of lipoprotein fractions.

small as possible so as to facilitate and render economical large-scale processing. Further division of these major fractions into subfractions and the final separation, wherever possible, of chemical components will be the subject of succeeding papers.

The attempt was made to have as much as possible of the fibrinogen in Fraction I, of the  $\gamma$ -globulins in Fraction II, of the lipid-bearing  $\beta$ -globulins in Fraction III, of the  $\alpha$ -globulins in Fraction IV, and of the albumins in Fraction V. In method 1, Fraction II and Fraction III were removed separately by increasing the ethanol concentration at constant temperature and without adjustment of the pH, first to mole fraction 0.051, and then to mole fraction 0.091 ethanol. This attempt at fractionation by simple ethanol precipitation proved unsatisfactory since both  $\gamma$ -globulin and prothrombin were distributed between the two fractions. In methods 2 to 6, therefore, Fractions II and III have been precipitated together and the various important and useful components in the mixture separated from each other by subfractionation.

On the other hand, Fraction IV, separated in methods 1 to 5 as one precipitate, was removed in method 6 in two steps. Fraction IV prepared by methods 1 to 5 contained denatured protein. Method 6 was therefore devised in which a frac-

tion, IV-1, rich in lipid and in  $\alpha$ -globulins and readily denatured by high ethanol concentrations, was first removed at a lower ethanol concentration and a more acid reaction than was employed for the complete precipitation of the globulins in Fraction IV. This change in procedure, which again brought the number of primary precipitates to five, thus made available  $\alpha$ - and  $\beta$ -globulins as soluble fractions available for chemical and clinical studies.

The high albumin yield in Fraction V was first obtained with method 5, which was specifically developed for that purpose, since the clinical use of this fraction had been established before the value of the  $\gamma$ -globulins for prophylaxis in measles and infectious jaundice, of the isohemagglutinins in blood-grouping, or of the various products of fibrinogen and thrombin as hemostatic agents, films and plastics<sup>51</sup> was demonstrated. The present uses of these diverse products of plasma fractionation are again leading to revision of the earlier steps in the process so as to increase the yield of fibrinogen in Fraction I and of  $\gamma$ -globulin in Fraction II. These developments will be considered in subsequent papers in this series.

In method 1 reagents were introduced through cellophane membranes.<sup>23</sup> The experimental con-

(51) See *J. Clin. Invest.*, **23**, 417-608 (1944).

TABLE III

DISTRIBUTION OF PLASMA PROTEINS INTO FRACTIONS BY VARIOUS METHODS ESTIMATED BY ELECTROPHORETIC ANALYSIS AND A NITROGEN FACTOR OF 6.25 FOR ALL PROTEINS

Fraction	Method	Albumin	$\alpha$ -Globulin	Cholesterol	$\beta$ -Globulin	$\gamma$ -Globulin	Fibrinogen <sup>b</sup>
Plasma		33.2	8.4	1.6	7.8	6.6	4.3
I	1	0.3	0	0	0.2	0.7	3.0
I	2	1.0	0.3	...	.4	.2	2.4
I	5	0.2	.2	0.02	.8	.5	2.6
I	6	0.3	.3	.01	.6	.3	2.3
II <sup>a</sup>	1	1.5	0	.2	1.3	3.7	0.3
III	1	1.5	.1	.3	2.6	2.6	0.2
II + III	2	0.6	.9	...	5.9	4.7	1.4
II + III	5	.7	1.8	1.1	6.2	6.0	1.6
II + III	6	.6	0.9	1.3	6.7	5.7	1.6
IV	1	5.6	4.2	0.7	3.0	0.4	0.2
IV	2	4.9	4.9	...	3.7	.6	0
IV	5	1.0	5.4	0.4	3.1	.2	0
IV-1	6	...	3.9	.4	0.4	.04	0
IV-4	6	0.9	2.7	.04	2.2	0	0
V	1	26.0	0.3	0	0.4	0	0
V	2	27.0	.6	...	0.3	0	0
V	5	29.0	.6	<.01	0	0	0
V	6	28.4	1.2	<.01	0.3	0	0
VI	1	2.2	0.1	0.02	0	0	0
VI	2	0.5	.1	...	0	0	0
VI	5	.3	.3	...	0	0	0
VI	6	.7	.2	...	0	0	0
Totals	1	37.1	4.7	1.2	7.5	7.4	3.7
	2	33.5	6.7	...	10.3	5.5	3.8
	5	31.2	8.3	1.5	10.1	6.7	4.2
	6	30.9	9.4	1.7	10.2	6.0	3.9

<sup>a</sup> This early Fraction II should not be confused with Fraction II prepared in later methods by subfractionation of II + III. <sup>b</sup> Estimated by electrophoresis. The amount of clottable protein is always lower and presumably more reliable.

ditions obtaining for each separation are reported in Table I. The pH and ethanol concentrations at which separations were made are graphically represented in Fig. 2. The method of adding reagents by diffusion through a membrane was introduced so that the equilibrium conditions for separations would be attained without risk of local excesses, either of ethanol or of acid, which might lead to protein denaturation. Where fractionation is carried out on a small scale advantages inhere in this procedure which will sometimes outweigh the manipulative advantages of the subsequent methods, which were specifically designed for large-scale processing.

**Method 2.**—It was found possible to avoid the manipulative difficulties of carrying out dialysis on a large scale by substituting direct addition of reagents through capillaries at a slow and controlled rate. The ethanol was always added in an ethanol-water mixture as dilute as possible and the acid as a buffer mixture as high in pH as possible. The volumes in which the reagents were added were as large and the temperature as low as convenient. The systems to which the reagents were added were constantly stirred so as to avoid local excesses of the added reagents, but without entrainment of air and the resulting foaming of

the solution. The rates of addition through capillary jets varied from 50 cc. to 500 cc. per minute at different stages in the process. Throughout the additions, temperature was carefully controlled so that the heat evolved as a result of mixing ethanol and water did not lead to a rise in temperature,<sup>52</sup> and so that the desired temperature was attained at the completion of the addition.

The conditions of pH and ethanol concentration at which separations were made were much as in method 1. The ionic strengths were, of course, higher, since salt was not constantly being lost through the dialyzing membranes<sup>53</sup> at the same time that the reagents were being added. The greatest difference in ionic strength occurred

(52) Whenever possible ethanol was added as an ethanol-water mixture. Since the final volume in the process was 4 times plasma volume (see Table I) an 0.246 mole fraction ethanol-water mixture proved convenient for many operations. Adding 0.246 mole fraction ethanol at  $-5^{\circ}$  to 0.051 mole fraction ethanol at  $-5^{\circ}$  can easily lead to a mixture with a temperature of  $0^{\circ}$  or higher if provision is not made for adequate absorption of the heat evolved. Such a temperature rise is quite sufficient to injure even the more stable plasma proteins in the presence of ethanol.

(53) The removal of electrolytes and of most other small molecules is conveniently accomplished in the later methods by reprecipitation or by titration of a fraction under conditions such that the protein is insoluble.



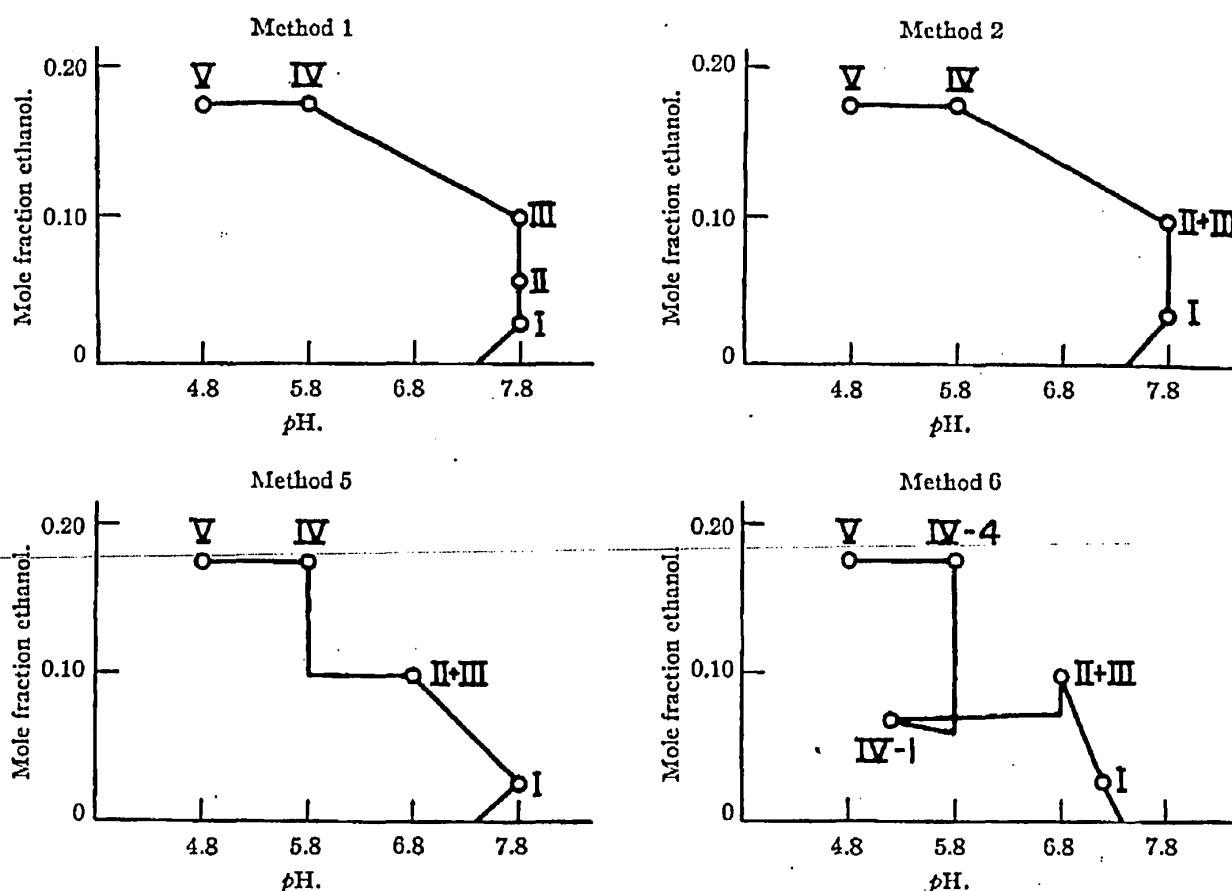


Fig. 2.—Ethanol concentration and pH for separation of plasma fractions by various methods.

at the separation of Fraction III, which was as low as 0.02 in method 1, and close to 0.09 for Fraction II + III in subsequent methods. Increased solubility, especially of globulins, due to increased ionic strength can generally be balanced either by change in pH, increase in ethanol concentration or decrease in temperature.<sup>54</sup>

Since the smaller molecules, especially the carbonates and citrates removed by dialysis in method 1, were still present in the supernatant of Fraction II + III in subsequent methods 2, 5 and 6, the acid required to neutralize the dialyzable buffers was large in comparison with that required to neutralize the proteins. Any variation in the proportion of citrate, carbonate and protein in the collected blood imposed a variation in the buffer required, so that each fraction might be removed under conditions closely similar to those that have been found satisfactory to effect the desired separations.

The albumin was present in the filtrate from Precipitate IV and was separated by isoelectric precipitation in Fraction V. A small amount of the albumin was found in the mother liquors (Fraction VI). There was no difference between

the procedures for recovering Fraction V or Fraction VI in method 1 and methods 2, 5 or 6.

The albumin in Fraction V was readily further purified of salts and other molecules, including thermally unstable impurities, either by crystallization<sup>55</sup> or by reprecipitation. In methods 1 and 2 Fraction V was largely dissolved in ethanol of mole fraction 0.051 at 0° at a concentration of about 3% protein. A small amount of material, containing some albumin and a large part of the  $\alpha$ -globulins, remained insoluble. The dissolved albumin was then reprecipitated by raising the ethanol concentration to mole fraction 0.163.

Method 3 differed from methods 2, 5 and 6 in that the albumin in the supernatant of Fraction IV was not concentrated by isoelectric precipitation, but by vacuum distillation. Although the ethanol and water could be conveniently removed by this process, which could be carried out at sufficiently low temperatures to prevent denaturation of the protein, the non-volatile components were concentrated with the albumin, and the concentrate was therefore very rich in salt. This step, which thus yielded Fraction V + VI as a concentrated solution, was followed by dialysis. Method 3 had advantages for specific purposes. It required neither a centrifuge for

(54) In later methods the pH at which Fraction II + III was removed was adjusted to 0.8. In the reprecipitation of the  $\gamma$ - and certain of the  $\beta$ -globulins of this fraction, when the pH was again brought to a more alkaline reaction in order to remove lipid, the ionic strength had again been reduced (see subsequent papers in this series).

(55) E. J. Cohn and W. L. Hughes, Jr., subsequent paper in this series.

the separation of the albumin nor drying equipment for the removal of the alcohol. On the other hand, where human albumin was to be separated for intravenous use its removal and purification by isoelectric precipitation at low temperature was preferred.

Method 4 differed from method 2 in that the albumin in Fraction V was finally crystallized under somewhat different physicochemical conditions of pH, temperature, ionic strength and ethanol concentration than sufficed for amorphous reprecipitation.<sup>55</sup> However, normal human serum albumin could be prepared in far larger amounts and with higher yields if further purification by crystallization was not superimposed upon the process. The standards of purity determined upon for the albumin delivered to the Navy in large amounts were chosen so as to assure freedom from untoward reactions with maximum efficiency in large-scale production.

Method 5 was a further development of method 2. New physicochemical conditions defining optimum points for separation had been determined, as well as optimum conditions for approaching these points, with the result that the  $\gamma$ -globulins were more completely concentrated in Fraction II + III and the albumin in Fraction V. This was accomplished largely by more careful consideration of the charged condition of the proteins and therefore of the protein-protein interactions interfering with their separation. The ethanol concentration, the ionic strength, and the temperature at which fractionation occurred were tentatively maintained as before (these variables being reconsidered in method 6), since our investigations demonstrated that substantial advances could be made by better definition of pH and of the order in which the adjustment in pH and the increase in ethanol concentration were effected.

The changes in the separation of Fraction I were to lower the temperature to approach  $-3^\circ$  during the addition of the ethanol-water mixture added to precipitate the fibrinogen, and the ethanol was decreased from mole fraction 0.033 to 0.027, so as to minimize loss of albumin or  $\gamma$ -globulin into this fraction.

Increasing the ethanol concentration to mole fraction 0.091 and lowering the temperature to  $-5^\circ$  did not suffice in method 2 to precipitate all the  $\gamma$ -globulins or all the isoagglutinins<sup>56</sup> in Fraction II + III. For during processing carbon dioxide was lost and the alkalinity increased, often to a pH as high as 7.8. By careful control of the pH it was found possible to obtain more nearly quantitative precipitation of the  $\gamma$ -globulins without further increase in ethanol concentration.

The pH of the supernatant of Fraction I was reduced in method 5 by the addition of an acetate buffer so that Fraction II + III was precipitated

near pH 6.8. This adjustment was made by the addition to the ethanol solution used as a precipitant of a sodium acetate-acetic acid buffer with a molar ratio of salt to acid of 1.77, and served to protect labile components of plasma from alkaline reactions. The yield of prothrombin was also increased in method 5, and components of complement were concentrated in it which had been destroyed in the earlier methods.<sup>58</sup>

Loss of albumin into Fraction IV was minimized in method 5 by changing the order of the addition of the reagents. This change is illustrated in Fig. 2. The pH of the supernatant of Fraction II + III was adjusted in method 5 before the ethanol concentration in the system was increased. As a result, protein complexes formed at pH values between 6.8 and 5.8 and insoluble in 0.091, rather than in 0.163 mole fraction ethanol, were more likely to be those precipitated in Fraction IV. Albumin represented over 30% of Fraction IV as precipitated in method 2. The amount expected from occlusion should not have exceeded 2%. Since albumin is relatively soluble in ethanol of mole fraction 0.163 at pH 5.8 and  $\Gamma/2 = 0.09$ , the amount retained in this fraction was presumed to be precipitated in combination with proteins of more nearly neutral isoelectric points. Washing of the precipitate with ethanol of mole fraction 0.163, 0.1 molar in sodium chloride did not remove albumin. Albumin was, however, much reduced when the washing was carried out at pH 6.2. The results of the experiments leading to method 5 are given in Table IV.

TABLE IV  
CONDITIONS FOR SEPARATION AND EXTRACTION OF ALBUMIN FROM FRACTION IV

Method	Preparation number	Fraction IV washed at pH	Electrophoretic analysis % fraction IV			
			Albumin	$\alpha$	$\beta$	$\gamma$
2	82-84	*	28	40	30	2
5	87	*	16	52	29	3
5	91-95	5.8	17	52	29	2
5	96-98	6.2	11	55	32	2
<hr/>						
			% Fraction V			
2	82-84	*	99	1	0	0
5	91-93	5.8	98	2	0	0
5	96-98	6.2	98	2	0	0

\* Fraction IV not washed.

These changes in procedure resulted in precipitating less albumin in Fraction IV without, however, significantly decreasing the purity of the albumin in Fraction V. Fraction V was precipitated under the same conditions in methods 2 and 5. Conditions for the resolution of Fraction V were, however, modified. The ethanol concentration was reduced to 0.033 mole fraction ethanol and the temperature to  $-2^\circ$  in the interests of minimizing protein denaturation. As in method 2 a small precipitate was removed and the albumin was reprecipitated at  $-5^\circ$  and 0.163 mole fraction ethanol.

(55) We are indebted to Lt. L. Pillemer for his collaboration in a preliminary study of the isoagglutinins and of the components of complement in human plasma.

Method 6 was developed in the interest of making available not only fibrinogen and thrombin,  $\gamma$ -globulin and albumin, but also as many as possible of the diverse components of plasma, as nearly as possible in their native state. The  $pH$  of the plasma was adjusted before the precipitation of Fraction I so as to avoid reactions alkaline to that of blood. Increasing the ethanol concentration beyond mole fraction 0.027 or decreasing the  $pH$  increases the size of Fraction I, largely, however, by the precipitation of other proteins in addition to fibrinogen. Optimum conditions for the quantitative precipitation of fibrinogen and the antilemophilic globulin, without the inclusion of prothrombin, the fibrinolytic enzyme, or  $\gamma$ -globulin in Fraction I, are being further explored and will be reported subsequently.

Although  $\gamma$ -globulin was almost completely precipitated by 0.091 mole fraction ethanol at  $pH$  6.8 and at  $-5^\circ$ , the temperature of this separation has been further reduced, in certain experiments which are continuing, to  $-10^\circ$ . This was attempted in order to concentrate in Fraction II + III depressor substances which previously were distributed between Fractions II + III and IV, rendering their subfractions unsafe for intravenous use. At least certain of these depressor substances appeared to have a very high heat of solution in the range of ethanol concentrations from mole fraction 0.0624 to 0.091 and could therefore be partially separated from Fraction II + III by reprecipitation at a slightly higher  $pH$  and temperature,  $-5^\circ$ . The ionic strength at which reprecipitation took place could be reduced to any desired extent by varying the volume of the system in which the  $\gamma$ -globulins were insoluble and many of the other components of Fraction II + III extracted. The further purification of the  $\gamma$ -globulins, the isoagglutinins, prothrombin, and the fibrinolytic enzyme by subfractionation of Fraction II + III will be reported in subsequent papers in this series.<sup>57</sup>

The supernatant solution from Fraction II + III had the same composition in method 6 as in method 5. The greatest change in this method, however, had to do with the precipitation of the lipid rich  $\alpha$ -globulins in Fraction IV at a more acid reaction where they were insoluble at a lower ethanol concentration. This fraction of lipoproteins was denatured at higher ethanol concentrations, such as the ethanol of mole fraction 0.163 employed in the precipitation of Fraction IV in previous methods. Separated at 0.062 mole fraction ethanol and a  $pH$  near 5.2, these lipoproteins could be redissolved to yield water-clear solutions. These  $\alpha$ -globulin lipoproteins differ in many respects from the  $\beta$ -globulin lipoproteins precipitated in Fraction II + III.

The precipitate separated near 0.062 mole fraction ethanol and at  $pH$  5.2 and  $-5^\circ$  was called

Fraction IV-1. It can be readily separated into at least three subfractions, and their properties will be described in a later communication in this series. One of these fractions contains most of the lipid of the plasma that is combined with  $\alpha$ -globulin, has a low solubility in water near  $pH$  5.2, is soluble at  $pH$  5.8 and 4.8, and is readily precipitated at the latter reaction by even low concentrations of neutral salt.<sup>58</sup> Another component contains the blue-green pigment previously described in one or another study of plasma proteins,<sup>6,59</sup> and appears to be isoelectric at an even more acid reaction.

Essentially all of the protein remaining in solution after the precipitation of Fraction II + III could be precipitated by maintaining the ethanol concentration at mole fraction 0.091 and the temperature at  $-5^\circ$  by increasing the acidity to close to  $pH$  5. Since it has often been convenient to precipitate Fractions I and II + III together, it is thus readily possible to divide the plasma proteins into such a fraction and Fractions IV + V precipitated together under these conditions. The first fraction would then contain most of the proteins of neutral isoelectric point, such as the isoagglutinins and the  $\gamma$ -globulins and most of the proteins of low solubility such as fibrinogen and the lipid rich  $\beta$ -globulins and prothrombin, the latter being isoelectric near  $pH$  5.2. Were the first precipitation carried out in ethanol at acid reaction under conditions such that interactions were minimum, most of the proteins in Fractions I + III + IV + V would be precipitated together.

In the present process, water insoluble proteins of closely the same isoelectric point, such as prothrombin (III-2), the lipid rich  $\beta$ -euglobulin (III-0), and the lipid rich  $\alpha$ -globulins (IV-1) were readily separated. Moreover, the ethanol concentration necessary for the precipitation of the latter fraction was close to the limit at which it was denatured at this  $pH$  and temperature.

The precipitation at  $pH$  4.7 and mole fraction ethanol 0.091 at  $-5^\circ$  of all the proteins remaining in solution after the precipitation of Fraction IV-1 would have definite advantages, once satisfactory procedures were developed for the separation by subfractionation of the lipid poor  $\alpha$ - and  $\beta$ -globulins (IV-4)<sup>59</sup> and the albumins (V). The molecular dimensions and osmotic behavior of the globulins in Fraction IV-4 resemble those of the albumins in Fraction V rather than those of the other globulins of the plasma.<sup>60</sup> Fraction IV-4 precipitated in combination with Fraction V under these conditions contained nearly all of the hypertensinogen<sup>61</sup> of plasma, whereas precipitation

(58) J. A. Luetscher, *J. Clin. Invest.*, **19**, 313 (1940).

(59) This fraction was designated IV-3,4 in previous communications.

(60) G. Scatchard, J. L. Oncley and A. Brown, unpublished work.

(61) We are indebted to Dr. Lewis Dexter in the Department of Medicine of the Harvard Medical School for the assay of hypertensinogen.

(57) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Cross, subsequent paper in this series.

of Fraction IV-4 by 0.163 mole fraction ethanol at pH 5.8 and  $-5^{\circ}$  led to destruction of hyper-tensinogen and presumably of other labile components of the fraction without, however, destroying gross molecular structure. Fraction IV-4 contains the esterase of plasma,<sup>62</sup> and an iron binding globulin fraction.<sup>63</sup>

The stability of the globulin fraction (IV-4) is far lower than that of the albumin fraction (V). Moreover, the reagents that have rendered it possible to increase the stability of albumin<sup>64</sup> to the point where it can be pasteurized in the final container<sup>65</sup> do not interact with the globulins in the same way. In order to take advantage of the greater stability of the albumin, the procedure followed in method 6 has been to precipitate IV-4 under the same conditions as were employed in the precipitation of Fraction IV in methods 2 and 5.<sup>66</sup> Fraction IV-4, prepared in this way, was completely soluble after having been dried from the frozen state, and has been prepared routinely free from depressor substances and safe for intravenous injection. Method 6 has thus yielded two water-soluble plasma fractions not previously available: IV-1, rich in  $\alpha$ -globulins and lipid in a form that can be dried from the frozen state, and IV-4, rich in relatively soluble  $\alpha$ - and  $\beta$ -globulins of low lipid content.

The albumin in the supernatant of Fraction IV-4 was separated as Fraction V by the same procedure that was employed in methods 2 and 5. The preparation of albumin with as small a ratio of sodium to protein as convenient became of interest, however, in connection with certain clinical applications.<sup>65</sup> This was accomplished by changing the conditions for the reprecipitation of Fraction V to a pH near 5.3 instead of acid to 4.7 as in earlier methods, in order to reduce the acetic acid precipitated with the albumin.

The procedures describing the detailed fractionation with each method were made available, as they were developed, in directives to the laboratories with Navy contracts for the fractionation of plasma to yield blood derivatives for the Armed Forces. Publication of all these descriptions in this Journal would appear unwarranted, since so many of the earlier procedures in use in 1941 have been superseded. Method 6 is, however, given below in the form in which it and the earlier methods were described to the manufacturers.

(62) We are indebted to Dr. Ralph W. Brauer in the Department of Pharmacology of the Harvard Medical School for the assay of esterase.

(63) Personal communication from A. L. Schade, in press in *Science*.

(64) C. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lum, *J. Clin. Invest.*, 23, 454 (1944).

(65) G. Scatchard, L. E. Strong, W. L. Hughes, Jr., J. N. Ashworth and A. H. Sparrow, *ibid.*, 24, 671 (1945).

(66) A somewhat greater yield of albumin in Fraction V may be obtained if Fraction IV-4 is precipitated at pH 6.0 to 6.2 (see Table IV). This does not, however, always result in an albumin preparation of high purity and satisfactory thermal stability.

**Method 6: Directions for the Preparation of Normal Human Serum Albumin.**—The plasma<sup>67</sup> is stirred gently but thoroughly and cooled as quickly as possible to  $0^{\circ}$  without permitting the formation of ice.

The stirring is continued while sufficient sodium acetate-acetic acid buffer in a 53.3 volume per cent. (at  $25^{\circ}$ —see Table I) ethanol-water mixture is added through capillary jets to bring the pH to  $7.2 \pm 0.2$  and the final ethanol concentration of the system to 8%. The addition rate is 80–100 cc. per jet per minute and the over-all time for the addition should be about one and one-half hours. During the addition, the temperature is allowed to fall so that the system is maintained close to its freezing point and so that the final temperature is between  $-2.5$  and  $-3^{\circ}$ . This first step requires 0.177 liter (measured at  $-5^{\circ}$ ) of 53.3% ethanol for each liter of plasma (measured at  $0^{\circ}$ ) and about 1 cc. of 0.8 molar sodium acetate buffered at pH 4.0<sup>68</sup> with acetic acid for each liter of plasma should suffice for the pH adjustment.

Precipitate I consists principally of fibrinogen. The precipitate is removed by centrifugation at a temperature between  $-2$  and  $-3^{\circ}$ . If the undried fraction is stored at  $-5^{\circ}$  for periods longer than a few months loss of fibrinogen occurs. Storage at lower temperatures slows but does not completely stop this loss. The fibrinogen may be preserved by dissolving in four volumes of sodium citrate buffer at pH 6.1 and ionic strength 0.3. After clarification and sterilization<sup>69</sup> by filtration the solution is dried from the frozen state and for best stability the dried fraction is stored *in vacuo*.

The supernatant is next brought to 25% ethanol and a pH of about 6.9 by the addition of cold 53.3% ethanol containing a sodium acetate-acetic acid buffer. Capillary jets are used as before, the rate of addition is about 100 cc. per jet per minute, and the over-all time is about five hours. During this addition the temperature is held at the freezing point until  $-5^{\circ}$  is reached and then maintained at  $-5^{\circ}$  throughout the remainder of the addition. This step requires for each liter of supernatant I an ethanol buffer mixture made as follows: 601 cc. of 53.3% ethanol at  $-5^{\circ}$ , 0.88 cc. of 10 M acetic acid at  $25^{\circ}$ , 0.44 cc. of 4 M sodium acetate at  $25^{\circ}$  and 2.30 cc. of 95% ethanol. The buffer used in this step has a mole ratio of sodium acetate to acetic acid of 0.2 and if, before the 53.3% ethanol is added, it is diluted eighty-fold with water, it should have a pH of  $4.00 \pm 0.02$  at  $25^{\circ}$ . No attempt has been made to vary the composition of the buffer so as to adjust the system to an exact pH and, indeed, the system varies by several tenths of a pH unit as a result of changes in carbon dioxide concentration. The buffer added in the two additions contributes 14 milliequivalents of acetic acid for each liter of plasma.

Precipitate II + III is removed by centrifugation at  $-5^{\circ}$  and by electrophoretic measurements it consists principally of  $\beta$ - and  $\gamma$ -globulins. It contains nearly all of the immune globulins and the isoagglutinins. Nearly all the prothrombin is precipitated in this fraction. It also contains large amounts of cholesterol and other

(67) While these procedures were developed with relatively large volumes of plasma, the same methods have been successfully applied to plasma volumes as small as 100 cc. They are being further developed as an analytic method for characterizing very small amounts of plasma.

(68) This buffer has a mole ratio of sodium acetate to acetic acid of 0.2 and is conveniently made up by taking 200 cc. of 4 M sodium acetate, 400 cc. of 10 M acetic acid and water to make 1 liter. This buffer diluted with water eighty times should have a pH of  $4.00 \pm 0.02$  when measured in a glass electrode at  $25^{\circ}$ .

(69) When this crude fraction is used as a source of antihemophilic globulin (ref. 70) the fraction should be dissolved and sterilized as soon as possible after fractionation, and with a minimum amount of filtration necessary to insure sterility. The loss during filtration of this component of plasma has rendered it necessary to prove its presence by *in vitro* studies of its effect in lowering the clotting time of hemophilic blood.

(70) G. R. Minot, C. S. Davidson, J. H. Lewis, H. J. Tagnon and F. H. L. Taylor, *J. Clin. Invest.*, 24, 704 (1945).

lipid substances. The undried fraction has been stored at  $-5^{\circ}$  for at least a year without measurable loss of antibody activity, and there is no evidence of deterioration at longer periods. Substantial loss of isoagglutinin activity, however, may occur in six months time, and prothrombin often shows a measurable loss after two or three months. Storage at temperatures low enough to free the precipitate or drying from the frozen state result in alterations of the lipid containing proteins. To ensure the stability of the various components of this fraction, they should be separated from each other and stored by procedures suitable to their particular properties.

The supernatant from Precipitate II + III is brought to a pH of 5.2, and 18% ethanol by the addition of water and a sodium acetate-acetic acid buffer. This addition is carried out in two steps.

1. The first step consists of the addition of 311 cc. of water at  $0^{\circ}$  for each liter of supernatant II + III at the rate of 400 cc. per jet per minute, holding the temperature constant at  $-5^{\circ}$ .

2. The second step is the addition to each liter of supernatant II + III of 78 cc. of water at  $0^{\circ}$  containing enough of sodium acetate-acetic acid buffer to lower the pH to  $5.2 \pm 0.2$ . The mole ratio of sodium acetate to acetic acid in the buffer is 0.2.<sup>63</sup> The amount of buffer necessary is determined by preliminary titration. The rate of addition is 100 cc. per jet per minute. The temperature is held at  $-5^{\circ}$ .

After the addition is complete this system is stirred for one hour and allowed to stand six to eight hours at  $-5^{\circ}$  in order to complete the formation of the precipitate.<sup>71</sup>

Precipitate IV-1 is removed by centrifugation at a temperature of  $-5^{\circ}$ . By electrophoretic measurements it consists primarily of  $\alpha$ -globulins and a considerable quantity of lipid material.<sup>72</sup> This lipid rich  $\alpha$ -globulin is readily denatured by standing at low pH in the presence of high alcohol concentration, even at low temperatures. If the fraction is to be preserved in soluble form, it should be dissolved in about four volumes of water at a pH near 6 and then frozen and dried immediately from the frozen state, or else separated into its various components by further fractionation.

The supernatant is next brought to a pH of  $5.80 \pm 0.05$ , an ionic strength of 0.09 M, and an ethanol concentration of 40% at  $-5^{\circ}$ . The buffer<sup>73</sup> is added first over a period of about half an hour or more and the temperature is held at  $-5^{\circ}$  throughout. Following this addition the system is brought to 40% ethanol by the addition of 456 cc. of cold 95% ethanol for each liter of supernatant IV-1, while the temperature is maintained constant at  $-5^{\circ}$ .

Precipitate IV-4 is removed by centrifugation at  $-5^{\circ}$ . By electrophoretic measurements it consists principally of  $\alpha$ - and  $\beta$ -globulins with some albumin. The esterase activity in plasma is largely concentrated in this fraction, as well as some of the hypertensinogen. The precipitate can be dissolved in cold water and dried from the frozen state.

The supernatant from Precipitate IV-4, after centrifugation, contains a small amount of suspended material and is therefore clarified by filtration.<sup>74</sup> It is essential to the sta-

bility of the final albumin that the filtrate be highly clarified so that it possesses at most only a slight Tyndall effect.

After clarification is complete, all filtrates and washings<sup>75</sup> are combined and the pH is lowered to 4.8 by the addition of a sodium acetate-acetic acid buffer while the temperature is held at  $-5^{\circ}$  and the ethanol concentration at 40%. The addition takes about two hours, and when completed the system is allowed to stand for at least three hours without stirring. During the addition the temperature is held between  $-5$  and  $-6^{\circ}$ . For this addition, the buffer is made up by taking for each liter of clarified supernatant<sup>68</sup> 5.0 cc. of 10 N acetic acid, 2.5 cc. of 4 M sodium acetate, 10.5 cc. of 95% ethanol and enough water to make 25 cc.

Precipitate V contains the bulk of the albumin present in human plasma. It generally contains less than 3% of  $\alpha$ -globulin as measured by electrophoresis at pH 8.0 in a barbiturate buffer of ionic strength 0.1. By the same measurement it should contain less than 0.5% of  $\beta$ -globulin. The precipitated protein is removed by centrifugation or filtration at a temperature between  $-5$  and  $-6^{\circ}$ . The supernatant should be almost completely clear and should not contain more than 1% of the plasma protein as albumin.

Purification of Precipitate V.—For certain clinical applications it is advantageous to reduce the electrolyte content of the albumin to a minimum. Impurities with a stability inferior to that of albumin which are present at this point must also be removed. If this is not done, the final 25% solution will not remain clear when heated at  $50^{\circ}$  and visible particles will form.

Precipitate V, as obtained from the centrifuge, is in the form of a paste, each liter of which contains about 250 g. of protein, 0.015 mole of sodium acetate, 0.075 mole of acetic acid and 0.035 mole of salt contributed by the plasma, of which about 85% is sodium chloride.<sup>76</sup>

Purification is carried out by the removal of substances insoluble in 10% ethanol, 0.01 M salt, a temperature between  $-2$  and  $-3^{\circ}$ , a protein concentration of 3%, and a pH between 4.5 and 4.7. Precipitate V is dissolved in six volumes of water at  $0^{\circ}$  and to the solution is added one volume of 53.3% ethanol over a period of about two hours. During this addition the temperature is reduced to between  $-2$  and  $-3^{\circ}$ . The resulting turbid solution is stirred gently but thoroughly for about two hours, and then clarified by filtration.<sup>77,78</sup>

Albumin is precipitated from the filtrate by raising the ethanol concentration to 40%, lowering the temperature to  $-5^{\circ}$ , and, in order to minimize the absorption of acetic acid by the precipitate, raising the pH to 5.2. This is done by adding sufficient sodium bicarbonate to bring the pH to between 5.0 and 5.2, followed by the addition of 545 cc. of cold 95% ethanol to each liter of filtrate over a period of about two hours at a temperature of  $-5^{\circ}$ .

Albumin is removed by centrifugation or filtration at  $-5$  to  $-6^{\circ}$  and dried from the frozen state at as low a temperature as is practical. By electrophoretic analysis

(71) This fraction precipitates rather slowly and should stand for a period if Fraction IV-4 is not to contain some of the easily denatured material precipitated at this point.

(72) Although rich in lipoproteins, this fraction can be dissolved in water at a pH near 6.0 and dried from the frozen state to give a material that is readily soluble in water. However, if the fraction is held for any length of time at low temperature, either as a paste or as a frozen solution, a part of the protein becomes insoluble.

(73) At the Harvard Pilot Plant we have used for each liter of supernatant IV-1 a mixture made up of 1.14 g. of sodium bicarbonate, 7.90 cc. of 4 M sodium acetate and enough water to make 77 cc. of solution. If a preliminary titration indicates that this mixture is not adequate to give the proper pH, it should be adjusted by altering the ratio of bicarbonate ion to acetate ion concentration while the total sodium concentration is held constant.

(74) This has been done at the Harvard Pilot Plant by suspending 0.5% of washed standard super cel in the supernatant and filtering at  $-5^{\circ}$  through Republic S-1 sheets pre-coated with standard super-cel and washed.

(75) In order to minimize loss of albumin in the filter pads and filter cel washing of the filter cake is desirable. At the Harvard Pilot Plant we have used, for each liter of starting plasma, 250 cc. of cold 40% ethanol containing 0.1 mole of sodium chloride per liter.

(76) If precipitate V is dried and restored to a 25% protein solution at a pH close to 6.8, the solution will contain approximately 0.12 mole of sodium acetate per liter.

(77) The practice at the Harvard Pilot Plant has consisted of suspending 0.25% of standard super-cel in the solution and filtering through a Republic K-5 pad at  $-2^{\circ}$ . The albumin occluded by the filter cake was washed out with a small volume of 10% ethanol at  $-2^{\circ}$ .

(78) When salvaging albumin from certain contaminated plasmas, or in other cases where the level of unstable impurities may be high, still further purification may be desirable. If this is the case, the ethanol concentration should be raised from 10 to 15% and the temperature lowered to  $-5^{\circ}$ . The precipitate is then removed, the solution clarified, and the albumin precipitated in the regular way. While this procedure will result in loss of albumin, a substantial purification is effected. It may be possible to recover some of the loss by recycling the precipitate through a second rework.

at pH 8.6, the albumin generally contains less than 3% of globulin impurities. When made up to a 25% solution in 0.04 *M* acetyl tryptophan solution with the pH adjusted to 6.8 with sodium bicarbonate and sterilized by filtration, heating at 50° for several months should not result in the formation of particles nor in materially increased cloudiness. This test is most sensitive to the presence of unstable impurities and therefore reflects the quality of the separations. At a temperature of 57° the albumin solution should not change in clarity markedly until after one hundred hours of heating. This test is most sensitive to the stability of the albumin itself, and therefore reflects the care with which the albumin was handled throughout the processing. Heating for ten hours at 60° should not result in a visible change in the solution. The sodium content of the 25% albumin solution prepared by this procedure should be less than 0.33 g. of sodium per 100 cc.

#### IV. The Distribution of Proteins in the Separated Fractions and in Plasma

The system of fractionation in terms of five or more variables, all under precise control, that has been described, could be applied to any body fluid or tissue extract and could be followed by any characterization of the components whose separation was desired. In the case of plasma, it has been our plan to separate into different fractions protein, lipoprotein and lipid components having different physiological functions, and therefore therapeutic value in different conditions. The clinical evaluation of the separated fractions, even their study by immunological or physiological techniques, is so time-consuming, however, that simpler chemical procedures to aid in effecting separations have been employed. It seemed reasonable to assume that sorting the proteins in terms of their size and shape, lipid and carbohydrate content, amphoteric and dielectric properties, would yield fractions homogenous as well in their physiological properties.

To simplify analytical procedures further, the primary fractionations were routinely followed by determinations of nitrogen and cholesterol and of electrophoretic mobility. The separated fractions were then made available for study and the procedure revised on the basis of other chemical, ultracentrifugal, immunological and physiological results.

During the period when the process was being developed, it proved convenient to estimate yields (1) by determining the difference in the nitrogen content of the supernatant solutions from which successive fractions had been precipitated and (2) by using the conventional factor of 6.25 to convert nitrogen to apparent weight. The errors introduced were negligible when the nitrogen factor was near 6.25, when the fraction separated was large, and the nitrogen content of its supernatant small. In the case of the albumin rich Fraction V, which represented approximately one-half the total plasma protein and which was lipid poor, no substantial error was introduced by the analysis of the supernatants rather than by direct analysis of the separated precipitates. Results obtained by these two analytical methods are compared in Table V.

TABLE V  
YIELD OF FRACTION V PREPARED BY DIFFERENT METHODS  
Total N  $\times$  6.25

Method for precipitation of Fraction IV	Prepn. no.	Protein in fraction V Difference <sup>b</sup> G./liter of plasma	Direct analysis of plasma	Discrepancy, cols. 3-4
2	82	27.9	26.8	1.1
	83	26.8	27.0	-0.2
	84	28.3	28.3	0
	91	28.9	28.4	0.5
5 <sup>a</sup>	92	30.1	29.5	0.6
	93	29.4	29.4	0
	96	31.1	30.9	0.2
5	97	30.1	30.1	0
	98	29.2	29.5	-0.3
6	182	29.6	30.1	-0.5
	183	29.0	(27.5)	1.5
	184	30.3	30.4	-0.1
	185	29.9	30.5	-0.6

<sup>a</sup> Fraction IV washed at pH 5.8.

<sup>b</sup> All results for Method 6 were calculated from weight measurements and involved no density correction. For other preparations, densities measured at the temperature of fractionation were used in calculating results of analyses made at room temperature (see Table I). All measurements listed by direct analyses were made by weights.

The smaller the precipitate removed in the process of fractionation and the higher the nitrogen content of the supernatant before and after its separation, the larger the error introduced in estimating the yield by difference. The yields were smaller because of protein lost in the spray of the centrifuge, adhering to tanks and centrifuge bowls, as well as to Filter-Cel where filtration steps were introduced for clarification. In order to estimate not only the magnitude of such losses, but the extent to which recovery was possible, the tanks and centrifuge bowls after use were rinsed with a detergent solution (Duponol C) and these rinsings analyzed for nitrogen. The loss of spray from the centrifuge was not recoverable, but was estimated from the loss in weight during centrifugation. It was found in one preparation investigated that about half the observed loss was in spray from the centrifuge, and the remainder was in protein adhering to the equipment. The results are reported in Table VI. Among the results obtained by difference, a high value for fraction IV-4 is compensated by a low value for V. Whereas the sampling and analytical errors lead to unimportant discrepancies for Fraction V, whether determined by difference or direct analysis, the losses in the case of small fractions like I and IV-1 may be as large as 10 or 20%. The loss in the case of Fraction IV-4 is even larger, because of the very careful filtration step carried out on the supernatant from Fraction IV-4 which was necessary to yield a supernatant of great clarity and an albumin preparation of great thermal stability. Nephelometric analysis of



March, 1946

## SEPARATION INTO FRACTIONS OF PROTEIN AND LIPOPROTEIN COMPONENTS

473

TABLE VI

DISTRIBUTION OF PLASMA PROTEINS INTO FRACTIONS  
COMPARISON OF ANALYTICAL PROCEDURESPREPARATION 185 BY METHOD 6. TOTAL N  $\times$  6.25

Fraction	Difference, g./liter of plasma	Protein in Fractions Direct analysis	Protein lost in processing <sup>a</sup> Column 2 - 3	Re- covered	Esti- mated <sup>b</sup>
I	3.8	3.0	0.8	0.7	0.8
II + III	15.5	15.5	0.0	0.3	0.3
IV-1	4.3	3.8	0.5	0.4	0.5
IV-4	5.8	4.2	1.6	1.0 <sup>c</sup>	1.0
V	29.9	30.5	-0.6	0.1	0.1
VI	0.9 <sup>d</sup>	0.9 <sup>d</sup>	...	...	...
Total	60.2	57.9	2.3	2.5	53.3

<sup>a</sup> Studies indicate that about half of the loss is from the fraction and the other half is loss of protein from the supernatant solution during separation of the precipitate.

<sup>b</sup> Estimated losses are derived by taking for each fraction the higher of the two figures in columns 4 and 5. <sup>c</sup> Filtration loss included as 0.5 g. per liter of plasma determined by extraction of filter cake with 0.02 *M* sodium acetate in water followed by nitrogen analysis. By analysis of the supernatant before and after filtration the loss in the filter was estimated to be 1.5 g. per liter of plasma. <sup>d</sup> The analysis of Fraction VI was corrected for non-protein nitrogen which comprised about two-thirds of the total nitrogen.

this filtrate proved a useful indication of the extent to which light-scattering material had been removed.<sup>41</sup> The protein lost in the Filter-Cel which could be extracted with 0.15 *M* sodium acetate had an electrophoretic pattern close to that of Fraction IV-4, except for an elevation of the albumin by a factor of two. In no case was it possible to extract all the protein from the Filter-Cel and the insoluble material probably contained lipids and lipoproteins readily denatured by ethanol.

The actual yields of the various fractions are, of course, most accurately given by direct analysis of the separated precipitates. The results obtained with this procedure for four consecutive preparations carried out in the Pilot Plant by method 6 are given in Table VII.<sup>79</sup> The volume of plasma fractionated in each preparation was about 44 liters. The reproducibility of the process is indicated as well as the occasional deviation in yield or in analysis (see Fraction V of preparation 183) which inevitably creeps in when much systematic work is done.

The nitrogen factors of the fractions separated in this series of preparations were also estimated by simultaneous determination of dry weight and nitrogen and are recorded in Table VII. That determined for Fraction V is remarkably close to the conventional value assumed in previous calculations and reported for crystallized human serum albumin.<sup>80</sup> The nitrogen factor of Fraction I is slightly lower, reflecting a high nitrogen con-

(79) In this series the pH at which Fraction IV was precipitated was 6.2 in order to minimize the occlusion of albumin (see Table IV). If the pH is not readjusted to 5.8 before the filtration, however, though the yield may be higher, stability is often impaired by the inclusion of impurities in Fraction V.

(80) For references, see Table VIII.

TABLE VII

DISTRIBUTION OF PROTEINS AMONG FRACTIONS. COMPARISON OF CONSECUTIVE PREPARATIONS BY METHOD 6

Fraction	Preparation number				Average
	182	183 g./liter of plasma	184	185	
I	3.13	3.17	2.91	2.99	3.05
II + III	15.1	14.9	15.1	15.4	15.1
IV-1	3.61	3.44	3.15	3.82	3.51
IV-4	3.80	3.62	3.73	4.18	3.83
V	30.1	(27.5)	30.4	30.5	30.3 <sup>b</sup>
VI	1.5	2.0	1.2	0.9	1.0
Total	57.2	54.0	56.5	57.8	56.8
Plasma <sup>c</sup>	60.8	60.0	59.0	60.2	60.2

—Grams protein/gram nitrogen<sup>d</sup>—

I	6.11	6.11	5.95	6.1
II + III	7.66	7.76	7.57	7.7
IV-1	8.48	8.42	8.49	8.4
IV-4	7.13	7.02	6.97	7.1
V	6.23	6.39	6.28	6.3
Plasma	6.82 <sup>e</sup>	6.94 <sup>e</sup>	6.79 <sup>e</sup>	6.8

<sup>a</sup> Grams calculated from nitrogen multiplied by 6.25. <sup>b</sup> Value for 183 omitted from average. 29.0 was obtained by difference. <sup>c</sup> The plasma values have been corrected for the non-protein nitrogen present. The average NPN times 6.25 was 1.4 and nearly all of this nitrogen appeared in Fraction VI, which has also been corrected to exclude this component from the table. <sup>d</sup> For 182, 183, and 184 the observed dry weight values were corrected for amount of non-protein solid calculated to be present because of occlusion during precipitation. For 185 the ratio was determined after prolonged dialysis against a buffer of known solid content and free of nitrogen. <sup>e</sup> Nitrogen factors calculated by taking weighted averages of factors for each fraction using size of corresponding fraction as weighting factor and excluding Fraction VI.

tent of 16.4% for the fraction as compared with 16.9% for the fibrinogen or fibrin separated from it. Although the nitrogen content of Fraction II is reported to be 16.0% and the nitrogen factor therefore 6.25, the value for Fraction II + III, as well as for Fraction IV, is very high, reflecting the low nitrogen but high lipid and carbohydrate contents of certain of their components. Thus, the cholesterol content of Fraction II + III is 6%, the  $\gamma$ -globulin separated from it in Fraction II is less than 0.06%, but that of the "X-protein"<sup>81</sup> in Fraction III-0 is over 20%. Comparably, the cholesterol content of Fraction IV-1 is 7%, but that of the lipid rich protein separated from it (Fraction IV-1,1) 16%. The carbohydrate content of subfractions of II + III and of IV is over 2%. The analyses of these fractions and of components separated from them that are thus far available are, for convenience, collected in Table VIII.

The electrophoretic analyses that have proved so useful in enabling us to determine the optimum conditions for the separation of these fractions, recorded in Table III, are subject to correction for refractive index increments and nitrogen factors.<sup>46</sup> Recalculated in terms of the nitrogen

(81) A. S. McFarlane, *Biochem. J.*, **29**, 407 (1935). K. O. Pedersen, "Ultracentrifugal Studies on Serum and Serum Fractions," Almqvist and Wiksells Boktryckeri AB, Upsala, 1945.



TABLE VIII

ESTIMATES OF NITROGEN, LIPID AND CARBOHYDRATE COMPONENTS OF PLASMA. FRACTIONS PREPARED BY METHOD 6 AND PURIFIED COMPONENTS OF HUMAN PLASMA

	Per cent. nitrogen	Per cent. cholesterol	Per cent. carbohydrate <sup>a</sup>
Fraction I	16.4	0.26	1.2
Fibrinogen	16.9 <sup>a</sup>	<.1	1.2
Fraction II + III	13.0	6.0	1.3
$\gamma$ -globulin	16.03 <sup>a</sup>	<.06	1.1
$\beta$ -globulin	14.84 <sup>ab</sup>		
X-globulin	8.0 <sup>c</sup>	20	(2.0)
Fraction IV-1	11.9	7.0	2.2
Fraction IV-1,1	10	16.0	
Fraction IV-4	14.0	1.7	2.0
Fraction V	15.0	<.04	0.10
Albumin	15.95 <sup>a</sup>		
Total protein	14.0 <sup>d</sup>	2.3	(1.16)

<sup>a</sup> Measurements made by E. Brand, B. Kassell and L. J. Saidel, *J. Clin. Invest.*, 23, 437 (1944). <sup>b</sup> Fraction III-2 representing about 10% of II + III and containing 62%  $\beta$ -globulin. <sup>c</sup> Measurements of an euglobulin separated from Fraction III-0 and containing over 80% of X-globulin. <sup>d</sup> S. H. Armstrong, Jr., M. J. E. Budka and K. M. Morrison, in preparation. <sup>e</sup> Estimated by the orcinol reaction, referred to galactose. M. Sørensen and G. Haugaard, *Compt. rend. trav. lab. Carlsberg*, 19, No. 12, (1933).

TABLE IX

DISTRIBUTION OF PLASMA PROTEINS INTO FRACTIONS BY METHOD 6

Fraction	Al- bumin	$\alpha$	$\beta$ Per cent. of fraction	$\gamma$	Fibrino- gen	Total
Plasma	55	14	16	11	4 <sup>a</sup>	100
I	7	8	15	9	61	100
II + III	4	6	48	37	5 <sup>a</sup>	100
IV-1	..	89 <sup>b</sup>	10	1	0	100
IV-4	16	46	38	0	0	100
V	95	4	1	0	0	100
VI	76	23	1	0	0	100

Grams per liter of plasma recovered in fractions

Plasma	36.3	9.2	10.6	7.2	2.5 <sup>a</sup>	65.8
I	0.2	0.2	0.5	0.3	1.8	3.0
II + III	0.7	1.1	8.9	6.9	1.0	18.6
IV-1	..	4.2 <sup>b</sup>	0.5	<.1	0	4.7
IV-4	0.7	2.0	1.7	0	0	4.4
V	29.0	1.2	0.3	0	0	30.5
VI	0.8	0.2	<.1	0	0	1.0
Total	31.4	8.0	11.9	7.2	2.8	62.2

Grams per liter of plasma estimated in plasma

I	0.2	0.3	0.5	0.3	2.1	3.4
II + III	0.8	1.1	9.1	7.0	1.0 <sup>a</sup>	19.0
IV-1	0	4.6 <sup>b</sup>	0.5	0.1	0	5.1
IV-4	0.9	2.7	2.2	0	0	5.8
V	20.9	1.3	0.3	0	0	31.5
VI	0.8	0.2	<.1	0	0	1.0
Total	32.6	10.1	12.6	7.4	3.1	65.8

<sup>a</sup> Electrophoretic components of plasma with mobilities close to fibrinogen are resolved with difficulty. The value of 4% is taken from Edsall, Ferry and Armstrong, *J.*

*Clin. Invest.*, 23, 557 (1944). The remainder of the electrophoretic component amounting to 3% is included with the  $\beta$ -globulins. A similar situation occurs with Fraction II + III. Experiments suggested a clottable protein content of about 5% so the remaining 7% was included with the  $\beta$ -globulin. <sup>b</sup> Satisfactory resolution of the  $\alpha_1$ -globulin from albumin in Fraction IV-1 has not been accomplished and since probably less than 25% of the fraction is albumin, any components with mobilities similar to that of albumin have been included with the  $\alpha$ -globulins. Further work on this point is in progress.

factors in Table VII, though not for refractive index increments, the yields of the fractions separated by method 6 are given in Table IX.

If we are to employ our knowledge to yield a clearer insight into the protein composition of plasma, correction should also be made for the losses incurred during the fractionation process. This has tentatively been done on the basis of the results in Table VI, and these estimates are also recorded in Table IX. Although the process remains subject to improvement, the separation of the components of plasma into fractions that has been achieved concentrates most of the fibrinogen in Fraction I, of the  $\gamma$ -globulin in Fraction II + III, of the  $\alpha$ -globulins in Fraction IV, and of the albumin in Fraction V.

These gross fractions are the starting materials for the subfractionations reported in the subsequent papers in this series<sup>32</sup> to yield as many as possible of the separated protein and lipoprotein components of normal, convalescent, hyperimmunized, pathological, human or animal plasma, as pure as possible, for study and utilization in connection with their specific properties.

### Summary

A system is described for the separation and purification of the protein and lipoprotein components of biological tissues and fluids and is applied to the fractionation of human plasma.

Simultaneous control of all the variables of a multi-component system permits the choice of the optimum conditions for the separation of a component from a mixture. The separations reported have thus far been achieved by the precise control of five variables: (1) salt, (2) protein, (3) water miscible organic precipitant, (4) pH, and (5) temperature.

Concentration of salt is maintained as low as possible in order (1) to take advantage of the diversity of protein-electrolyte interactions, and (2) to avoid the necessity of dialysis.

Concentration of protein is maintained (1) as low as necessary to minimize protein-protein interactions, but (2) as high as possible in order to render practical large-scale processing.

Concentration of the organic precipitant—ethanol in these studies—and the temperature are maintained as low as possible, in order to minimize protein denaturation. The ethanol-water mix-

(32) The fractionation by these methods of other tissues including bacterial toxins and toxoids and the components of erythrocytes and other enzyme and hormone rich systems, in this and other laboratories, will also subsequently be reported.

tures employed in plasma fractionation have not exceeded mole fraction 0.163 and rarely 0.091, and the temperature has been maintained between 0° and the freezing point of the solution.

The balance between (1) the precipitating action of ethanol and (2) the interaction with salt permits attainment of a variety of conditions under which the protein to be separated may be brought to any desired solubility. This balance is different at constant ethanol and salt concentration for each pH and temperature, and for each protein component.

The pH has been controlled by buffering the ethanol-water mixtures with acetates or other buffers of known ionic strength, and adjusted so as to take advantage of the differences in the iso-

electric points and the directions of the interactions with salts of the protein components to be separated.

Crystallization of a separated component has also been effected in an ethanol-water mixture of low ionic strength and low temperature.

Removal of salts and other organic molecules by trituration, reprecipitation, or recrystallization in an ethanol-water mixture at low temperature replaces dialysis of protein solutions in this system.

Removal of the organic precipitant without raising the temperature has generally been accomplished by drying the separated proteins from the frozen state yielding stable, salt-poor, dried protein products of any desired degree of purity.

BOSTON, MASSACHUSETTS RECEIVED DECEMBER 12, 1945

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY AND PHYSICS OF THE PENNSYLVANIA STATE COLLEGE]

## Organo-silicon Compounds. I. Synthesis and Properties of *n*-Alkyltrimethyl- and *n*-Alkyltriethyl-silanes

BY F. C. WHITMORE, L. H. SOMMER, P. A. DI GIORGIO, W. A. STRONG, R. E. VAN STRIEN, D. L. BAILEY, H. K. HALL, E. W. PIETRUSZA AND G. T. KERR

In 1863 Friedel and Crafts<sup>1</sup> synthesized tetraethylsilane, the first tetraalkylsilane, by heating diethyl zinc with silicon tetrachloride in a sealed tube; tetramethylsilane was synthesized by the same method.<sup>2</sup> Twenty years later Polis<sup>3</sup> utilized the reaction of sodium with a mixture of aryl halide and silicon tetrachloride for the synthesis of arylsilicon compounds, a method applied later by Kipping<sup>4</sup> to the synthesis of alkylsilicon compounds. In 1904 Kipping<sup>5</sup> reported that Grignard reagents could be used in the synthesis of organo-silicon compounds. Chemists were quick to adopt this method, since it was far superior to those previously used, and the synthesis of organo-silicon compounds went forward at a rapid pace. In 1916 Bygden<sup>6</sup> reported the preparation and physical properties of sixteen tetraalkylsilanes. Among these were five *n*-alkyltrimethylsilanes and three *n*-alkyltriethylsilanes.

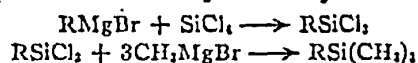
As a starting point for our program of research on organo-silicon compounds, we chose to extend the series of *n*-alkyltrimethylsilanes and *n*-alkyltriethylsilanes into a higher molecular weight range; a study of the physical properties of such compounds seemed of considerable interest. It should be noted that only six carbon analogs of the nineteen tetraalkylsilanes herein reported are known. The difficulties encountered in the syn-

thesis of neo hydrocarbons are well known<sup>7</sup> whereas the synthesis of tetraalkylsilanes, which contain a neo silicon atom (silicon attached to four carbons), is a relatively simple procedure and may be achieved in high yield, due to the ease with which silicon-halogen bonds couple with primary Grignard reagents. The higher *n*-alkyltrimethyl- and *n*-alkyltriethyl-silanes may possibly supply indications regarding the physical properties of neo hydrocarbons which have yet to be prepared.

We have synthesized and here reported seven new *n*-alkyltrimethylsilanes and six new *n*-alkyltriethylsilanes, and, in addition, we have prepared again three known *n*-alkyltrimethylsilanes and three known *n*-alkyltriethylsilanes to insure a comparable purity for determination of physical properties including viscosities. Incidental to the synthesis of new *n*-alkyltrimethylsilanes, we have synthesized five new *n*-alkyltrichlorosilanes.

### Preparation of the Tetraalkylsilanes

In the preparation of *n*-alkyltrimethylsilanes two methods were used. Method I involved preparation of an *n*-alkyltrichlorosilane from a Grignard reagent and silicon tetrachloride in yields of about 50%. The *n*-alkyltrichlorosilane, after purification by fractional distillation, was added to an ether solution of methylmagnesium bromide to give the *n*-alkyltrimethylsilane in about 70% yield. This was the method used by Bygden<sup>6</sup> for the preparation of *n*-alkyltrimethylsilanes



(7) Cf. Whitmore and Fleming, THIS JOURNAL, 55, 4161 (1933).

(1) Friedel and Crafts, *Ann.*, 127, 31 (1863).

(2) Friedel and Crafts, *ibid.*, 136, 203 (1865).

(3) Polis, *Ber.*, 18, 1540 (1885).

(4) Kipping and Lloyd, *J. Chem. Soc.*, 79, 449 (1901).

(5) Kipping, *Proc. Chem. Soc.*, 20, 15 (1904).

(6) See Krause and Grosse "Die Chemie der metall-organischen Verbindungen," Gebrüder Borntraeger, Berlin, 1937, p. 263.