CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION.

I. THE CHARACTERIZATION OF THE PROTEIN FRACTIONS OF HUMAN PLASMA [1,2]

By EDWIN J. COHN, JOHN L. ONCLEY, LAURENCE E. STRONG, WALTER L. HUGHES, JR., AND S. HOWARD ARMSTRONG, JR.[3]

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(*From the Department of Physical Chemistry, Harvard Medical School, Boston*)

The object of the series of investigations herewith reported has been to determine how blood plasma, fractionated into its component parts, may be used with the maximum effect in the treatment of clinical conditions. Extensive experience has demonstrated that plasma is of unquestioned value in the treatment of shock, burns, and diseases in which there has been depletion of one or another of the components of plasma. Moreover, either pooled or convalescent plasma has been injected in man because of either proven or implied effectiveness in the prophylaxis or treatment of certain infectious diseases.

The constituents of plasma are not all equally effective in the treatment of diverse conditions, however. Therefore, the utilization of the whole plasma in therapy may often prove both less effective and less economical than the use of parts thereof. In the treatment of infectious disease, for example, such antibodies as may be present in blood constitute only a small fraction of the plasma globulins. The rest of the plasma has little proven value in the prevention or modification of contagious diseases. In the control of a measles epidemic, injection of the albumin of the plasma, or of the fibrinogen or prothrombin, would appear to serve little purpose.

Conversely, in shock, injection of the human antibodies, though they exert some colloid osmotic pressure, would be far less effective and economical than the injection of an equal amount of albumin. Twice the amount of the immune serum globulins—present in but small amount—would be necessary to produce the same colloid osmotic pressure as the albumin. Albumin is responsible for nearly 80 per cent of the colloid osmotic pressure of the plasma and blood and is thus responsible to a far greater extent than other constituents of the plasma for the maintenance of blood volume.

The significant functions of the blood are by no means all performed by the plasma. The respiratory function of the blood is carried out by the hemoglobin, the carbonic anhydrase, and other proteins within the red cells; and the hematopoietic function of certain of these proteins has been repeatedly claimed. Though such studies are in progress, the present series of communications makes no contribution to the important problem of the conservation of the valuable labile cellular constituents of the blood. As in the case of the plasma, however, it is possible that technics for the concentration and preservation of these physiologically active substances would lead to further therapeutic advances.

The number of components that may be identified in the plasma proteins, either by chemical, physiological, or immunological criteria, remains far greater than the number of fractions into which it has been convenient to

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[2] This paper is Number 13 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.

[3] Welch Fellow in Internal Medicine of the National Research Council, Member, Society of Fellows, Harvard University, during the first years of these investigations.

separate the plasma proteins. Certain of the components are present in amounts so small that they cannot effectively be demonstrated in the whole blood. When they are separated from each other, concentrated, and made available in the dry state, or redissolved to yield stable solutions, their properties and functions can be investigated, and, in many cases, the products can be utilized. Among the constituents of plasma that have thus far been concentrated are the antibodies against infectious diseases, two of the components of complement, the anti-A and anti-B isohemagglutinins and Rh antibodies, hypertensinogen, thyrotropin and, of course, fibrinogen and thrombin, and the various groups of electrophoretically defined globulins and albumins.

Chemical characterizations are here reported of those products for which clinical appraisals either have been completed or are under way and which are now being produced in considerable amounts as part of the program for the plasma fractionation of Red Cross blood for the armed forces. These include (*1*) normal human serum albumin for use in shock and burns, (*2*) immune serum globulins for use in measles prevention and modification, (*3*) isohemagglutinins for use in blood grouping, (*4*) thrombin used with (*5*) fibrinogen for the formation of clots in certain surgical conditions including skin grafting and coagulum pyelolithotomy, (*6*) fibrin foams, prepared from fibrinogen and thrombin for use as hemostatics, (*7*) fibrin and fibrinogen tubes and plastics prepared to determine their most valuable surgical uses, and (*8*) fibrin films thus far used as a covering for burns and more recently as a dura substitute in neurosurgery.

The plasma fractionation program may from one point of view be compared with the early development of aniline dyes from coal tar. Until the intermediates were available, the cost of the preparation of any single product might have been considered prohibitive. After the intermediates were available, one could not afford not to develop the various products that could be derived from them.

The chemical methods that have been developed for large scale plasma fractionation, and which are now in nationwide use in the preparation of normal human serum albumin for the United States Navy, are not considered in this report.

In following the fractionation of a system as complex as blood plasma, it has become convenient to characterize protein molecules on the basis of behavior involving their size, shape, and electrical charge, rather than on the basis of specific reactive groups which may be involved in a given physiological function. The analytical methods used were chosen to be convenient, reproducible, and rapid. The value of the fractionation depends upon the correlation between the physiological properties and the components revealed by the analytical procedures. The first aim of the process was thus to separate and concentrate proteins of defined chemical properties into various fractions. These initial fractions were then tested for physiological activities. In actual practice, several analytical methods have been employed, and certain physiological reactions which could be followed simply have also been of considerable aid in developing the fractionation scheme.

This, and the papers which follow, are wholly concerned with the characterization of the products and the appraisal of their uses. The availability of these human proteins in large amounts opens diverse possibilities for the use of concentrated physiologically active components of plasma, both in surgery and in medicine.

THE PHYSICAL CHEMICAL METHODS EMPLOYED IN THE CHARACTERIZATION OF THE PLASMA PROTEINS

The aim of our plasma fractionation program has been to separate the components responsible for each function, and the aim of our physical chemical studies has been to evaluate the properties of the molecules as a means to this end. Certain properties are, moreover, of direct physiological importance. Thus, diffusion through membranes, resistance to flow through capillary vessels, and distribution of water among various body tissues are directly related to the size, shape, and electrical charge of the molecules involved. Indeed, many of the pioneer experiments in the fields of osmotic pressure, viscosity, and diffusion have been carried out by physiologists during

their studies of such phenomena. More complex behavior, particularly protein interactions such as antibody-antigen reactions, blood clotting, the agglutination of red cells by isohemagglutinins, and the activities of specific enzymes and hormones, involves more detailed molecular characteristics. Information concerning the molecular characteristics of the various plasma proteins yields (*1*) insight into the reactions with which the clinician must deal and (*2*) understanding of the properties the chemist must employ in the further development of the inclusive fractionation of plasma.

Many of the methods in use for the study of the physical chemical properties of proteins (1) involve the application of an external field of force and the study of the behavior of the various components of the system in an appropriate apparatus. The ultracentrifuge and the electrophoresis apparatus have been found helpful in studying mixtures of proteins, largely because of optical systems [4] which render it possible both to distinguish and to determine the relative amounts of the several components by their motion under the conditions imposed.

Measurements of diffusion, viscosity, double refraction of flow, osmotic pressure, electric moment, and the light-scattering properties of certain of the proteins in plasma have been made, but these studies are of great value only when applied to systems of one protein component, or at most, of fairly simple combinations of components. They contribute greatly, however, to our knowledge of the separated proteins.

ELECTROPHORESIS

If an external electric field of force is applied to protein molecules dissolved in a suitable solvent, it is found that in acid solutions, proteins move in one direction and in alkaline solutions, in the opposite direction. The significance of this phenomenon was recognized toward the end of the last century by the late Sir William Hardy. He and subsequent workers, notable among them Pauli and Michaelis, studied this amphoteric property of a variety of proteins. It remained for Tiselius,[5] however, to design the modern electrophoresis apparatus used in these studies.

Under a given field strength, the speed and direction of the movement of a protein will be determined by the pH, concentration and nature of the salts,[6] and the viscosity of the solvent. Two or more different protein molecular species in the same solvent will usually move with different velocities. This makes it possible to use these velocities as a means of distinguishing protein molecules differing in kind; the velocity in a unit electric field is defined as the electrophoretic mobility.

In this manner, analysis of plasma has yielded at least 6 electrophoretic components (2), as illustrated below in Figure 1. The fastest moving component in neutral or alkaline pH ranges is albumin. From the relative area of the peak which moves with this velocity, albumin is found to constitute about 55 per cent of the total plasma proteins.[7] Next in order of decreasing

[4] The optical systems have been devised and developed by Svedberg, Tiselius, Lamm, Philpot, Svensson, Longsworth, and others. In perhaps their most convenient form, the so-called Toepler schlieren (shadow) is employed to project a pattern on the photographic screen which can be resolved into a series of more or less skewed probability curves. The rate of motion is calculated from the change with time of the center of mass of each area. The area under each of such curves measures the concentration of the protein moving with that speed.

[5] By the use of a rectangular shaped cell with a maximum surface and minimum change of density when heated by the current generated by the electric field (near 4° C.), Tiselius was able to subject a protein solution to electrical gradients much larger than those previously used (up to 5 or 10 volts per cm., depending upon the buffer used) which permits the observation of a series of protein boundaries, moving through the solvent or solution with characteristic electrophoretic mobilities. The mobility, u, calculated from the rate of motion of each component, is generally expressed in cm. per second when the protein is in an electric field with a gradient of one volt per cm.

[6] The salts are usually selected to buffer at the desired pH. We have used potassium phosphate buffers of pH 7.7 and 0.2 ionic strength, or sodium diethylbarbiturate buffers of pH 8.5 and 0.1 ionic strength.

[7] The electrophoretic measurements have been made by M. J. E. Budka, A. H. Sparrow, and K. C. MacDonald, under the direction of S. H. Armstrong, Jr. The percentage composition of a protein solution derived from the areas under the peaks of schlieren diagrams, under certain conditions, represents the percentage of the total refractive increment (difference between the refractive indices of the solution and the solvent) contributed by each electrophoretic component (3). To convert this to weight percentage requires knowledge of the specific refractive increments of each component.

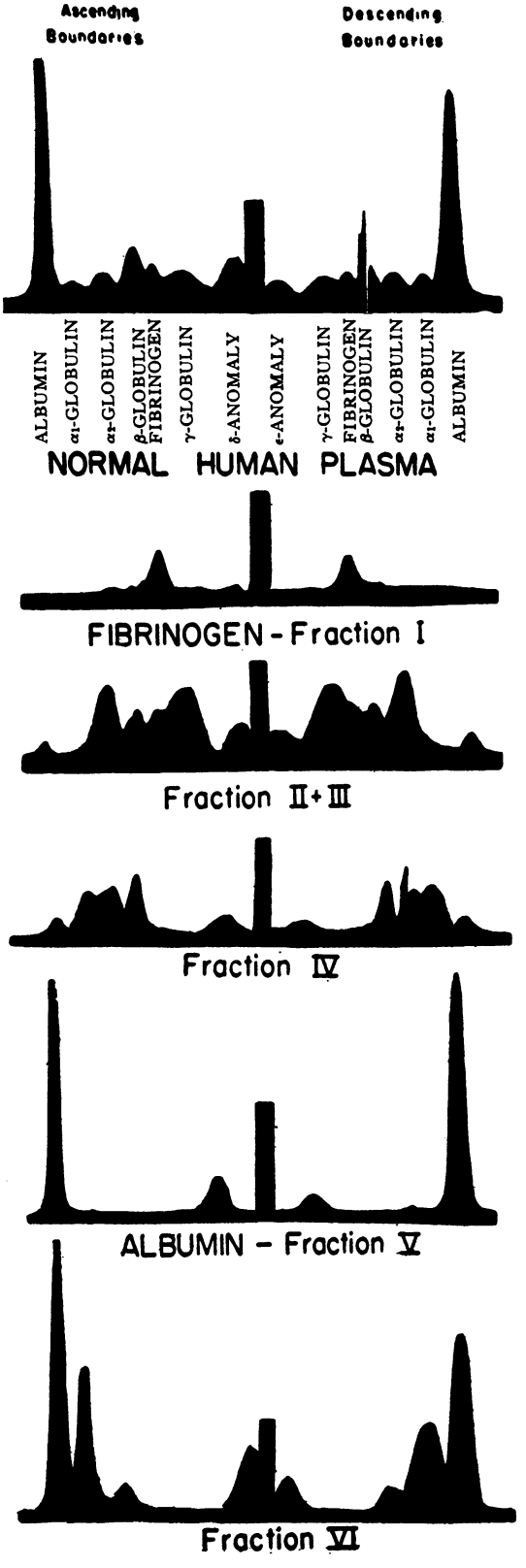

FIG. 1. ELECTROPHORETIC SCHLIEREN DIAGRAMS OF HUMAN PLASMA AND FRACTIONS I TO VI

velocity come the α-globulins, which under certain conditions divide into 2 separate components, designated as α_1 and α_2. Moving more slowly than these are the β-globulins, likewise sometimes separated into 2 components, β_1 and β_2. The total α- and β-globulins comprise about 13 and 14 per cent, respectively, of the total plasma proteins, as indicated by the areas of the separated peaks. Still slower moving is fibrinogen, which represents approximately 7 per cent of the plasma proteins. Slowest of all are the γ-globulins, estimated as representing 11 per cent of the plasma proteins.[8]

The electrophoretic components into which the Tiselius apparatus resolves the plasma proteins do not necessarily represent homogeneous molecular species, either with respect to size, shape, charge distribution, solubility characteristics, or physiological functions. The great advantage of the apparatus lies in its speed and simplicity of operation and in the reproducibility of the results.

SEDIMENTATION

When a protein solution is placed in an intense centrifugal field,[9] the molecules are observed to

[8] The almost stationary peaks, known as the delta and epsilon boundaries are nearly always observed in electrophoretic diagrams. It has been shown, however, that these peaks are not due to a protein component of plasma but to electrolyte concentration gradients (4). The discrepancy between the value for the fibrinogen content of the plasma proteins as obtained by electrophoretic analysis and by the nitrogen analysis of the washed clots is discussed in Paper XV of this series (5).

[9] During the last 18 years, Svedberg has developed the velocity ultracentrifuge for the measurement of the sedimentation rate in an intense centrifugal field (now as high as 400,000 times gravity) of molecules of the size of most proteins. This most important tool, the various improvements it has undergone, and many of the results that have been obtained with it, as well as the theory employed for the calculation of molecular weights from ultracentrifugal measurements, have been quite completely reported in a recent monograph by Svedberg and Pedersen (6). At its present stage of development, the centrifugal force developed is limited only by the strength of the alloys available for construction of the rotor. Recently, work of Beams, Bauer, Wykcoff, Pickels, and others have made the ultracentrifuge available to a somewhat larger number of

move with various rates of sedimentation. These rates, usually expressed and defined as the rates in a unit centrifugal field and called the sedimentation constants, vary almost directly with the viscosity and the difference in density between the protein and the solvent, but not in general to any large extent with pH and concentration.

The air-driven centrifuge designed by Bauer and Pickels has been used for studies of sedimentation in our laboratory,[10] while the oil-driven ultracentrifuge designed by T. Svedberg has been used by J. W. Williams at the University of Wisconsin for studies of many of these products (7).

Plasma or serum has been shown to contain a considerable number of components of different rates of sedimentation. Although undiluted plasma shows a more complex diagram, in dilute solutions, the main component is found to have a sedimentation constant near 4.6 S, characteristic of albumin. Fibrinogen, γ-globulin, and a part of the α- and β-globulins, have sedimentation constants near 7 S. In addition, there are small amounts of globulins sedimenting with constants between 7 and 18 S; and, although the results are not yet conclusive, it would appear that at least a part of the α- and β-globulins sediment, under certain conditions, with a rate close to that of albumin. Sedimentation constants of some of the constituents of plasma are listed in Table I.

Another important use of the ultracentrifuge is in studying protein denaturation. Proteins subjected to certain treatments, such as heating, become irreversibly altered from their native state, and sufficiently drastic treatment renders them insoluble in water and neutral salt solutions. Before properties of the proteins are changed greatly, it is often possible to detect preliminary subtle changes in the ultracentrifuge. Thus, solutions normally exhibiting a single protein component may show a number of components after being subjected to drastic treatment. We have accordingly established standards, making use of the ultracentrifuge, to detect changes in protein products, both during preparation and during storage.

OSMOTIC PRESSURE

Pfeffer and de Vries, while considering the forces necessary to draw water into tissues, made the pioneering studies on the passage of water through membranes which led Van't Hoff to the development of the concept of osmotic pressure. This phenomenon, extensively studied in the last century in terms of the rupture of plant and red blood cells, remains the most important factor in connection with the distribution of water, the membranes surrounding plant and animal cells being permeable to water and to many smaller molecules, but impermeable to the larger proteins which make up the greater part of the constituents of such cells. The pressure difference across such membranes at equilibrium measures the osmotic pressure, sometimes termed the colloid osmotic pressure or more recently the oncotic pressure.

Quantitative measurements of osmotic pressure at low protein concentrations can be used as a means of estimating the size of protein molecules, since the osmotic pressure depends as a first approximation upon the number of molecules or ions in a given volume of solution. The osmotic pressures of serum albumin solutions have been accurately measured and are considered in Paper VI of this series (9). A molecular weight of 69,000 has been estimated for this protein. Preliminary values for the colloid osmotic pressure of γ-globulin solutions have also been obtained. The osmotic pressure-concentration ratio for this protein varies much less with increasing concentration than is the case for albumin, and the molecular weight is in the neighborhood of 160,000 (see Table I).

In more concentrated solutions, the osmotic pressure depends to a considerable extent upon

workers by the development of a less complex air-driven machine.

The great strides made possible by this development depend in no small part upon the optical systems already mentioned which render it possible to distinguish more than one boundary and therefore components of more than one sedimentation rate. The sedimentation rates are usually expressed in terms of the rate in a unit centrifugal field, the quantity known as the sedimentation constant, s. Recorded values are usually expressed in Svedberg units (one Svedberg unit equals 10^{-13} cgs. units), corrected to a medium of the viscosity and density of pure water at 20° C., and designated $s_{20,w}$.

[10] The measurements have been made by C. G. Gordon and G. N. Thurber under the direction of J. L. Oncley.

TABLE I

Dimensions and physical constants of plasma proteins and other blood constituents

	Sedimentation constant $s_{20,w}$	Diffusion constant $D_{20,w} \times 10^4$	Viscosity coefficient F	Osmotic pressure-concentration ratio	Electrophoretic mobility pH 7.7 Γ/2 0.2	Molecular weight	Approximate dimensions (Ångstroms)		Negative net charge per molecule pH 7.4	Electric moment per molecule (Debye Units)
							Length of ellipsoid	Equatorial diameter of ellipsoid		
Sodium ion		139.[a]		6,000.[b]		23.0	1.9[c]	1.9[c]	−1	
Chloride ion						35.5	3.6[c]	3.6[c]	1	
Glucose		64.[a]	4.[a]	1,000.[a]	0	180.	9.5[d]	6.5[d]	0	
Serum albumin	4.6[e]	6.1[e]	5.8[f]	2.7[g]	5.3[h]	69,000.[i]	150.[j]	38.[j]	18[f,k]	400[m]
Serum γ-globulin	7.1[n]	3.8[n]	8.5[f]	1.2[f]	0.9[h,p]	156,000.[n]	320.[q]	36.[q]	8[k,r]	1,200[r,s]
Fibrinogen	7.[e]		70.[f]		1.9[h,p]	500,000.[t]	900.[u]	33.[u]		
Red blood cells			[v]			[w]	24,000.[x]	86,000.[x]		

a. From International Critical Tables, McGraw Hill, New York, 1929.
b. Calculated from molecular weight and osmotic coefficient.
c. These are the diameters of the sodium and chloride ion, as given by Pauling, L., "The Nature of the Chemical Bond," 2nd Edition, Cornell University Press, Ithaca, New York, 1940.
d. Calculated from dimensions of glucose models. See J. Phys. Chem., 1941, **45**, 776.
e. Unpublished measurements of Oncley, J. L.
f. Unpublished measurements of Scatchard, G., and Brown, A., and Batchelder, A. C.
g. Scatchard, G., Brown, A., and Batchelder, A. C., Paper VI of this series (9).
h. Unpublished measurements of Armstrong, S. H., Jr.
i. Calculated from sedimentation and diffusion constants, and from osmotic pressure measurements.
j. Computed for an axial ratio of 4 and a hydration of 0.2 gram of water per gram of protein (8).
k. Unpublished electromotive force measurements of Cohn, E. J., Strong, L. E., and Blanchard, M. H.
m. Unpublished measurements of Oncley, J. L., and Gross, P., Jr.
n. The sedimentation and diffusion constants recorded here are those quoted by Svedberg and Pedersen (6) for total, electrophoretically isolated, human γ-globulin. There are indications that this sedimentation constant and the molecular weight, given as 176,000, are somewhat larger than those for our γ-globulin Fraction II, which represents only a part of the total γ-globulin of plasma, and probably more of the pseudoglobulin than of the euglobulin. Complete ultracentrifuge and diffusion studies have not yet been carried out on this subfraction, but a value of 156,000 for the molecular weight of such a preparation has been obtained from osmotic pressure measurements of Scatchard, G., Brown, A., and Batchelder, A. C. This value may well vary with the euglobulin/pseudoglobulin ratio of the γ-globulin fraction, lower values being characteristic of pseudoglobulin.
p. Luetscher, J. A., Jr., Electrophoretic analysis of the proteins of plasma and serous effusions. J. Clin. Invest., 1941, **20**, 99.
q. Computed for an axial ratio of 8 and a hydration of 0.5 gram of water per gram of protein. Oncley, J. L., The investigation of proteins by dielectric measurements. Chem. Rev., 1942, **30**, 433.
r. Measurements on horse-globulin were used to estimate this value.
s. Oncley, J. L., Electric moments and relaxation times of protein molecules. J. Phys. Chem., 1940, **44**, 1103.
t. Estimated from measurements of sedimentation constant, viscosity, and double refraction of flow, the latter by J. T. Edsall and I. H. Scheinberg.
u. Computed for an axial ratio of 30.
v. Estimated from shape to be about 4.
w. A value of about 6,000,000,000,000 is obtained for the weight of Avagadro's number of red blood cells, which shows them to be about a billion times the weight of a serum albumin molecule.
x. These values are the maximum thickness and the equatorial diameter of the red blood cell in plasma as recorded by Ponder, E., The Mammalian Red Cell and the Properties of Haemolytic Systems. Gebrüder Borntraeger, Berlin, 1934.

properties of the protein other than size. Molecular properties of most interest in this case are the number and the distribution of the electric charges the protein bears. Measurements of both osmotic pressure and distribution of chloride ion across the membrane as the magnitude of the charge varies with change in pH and salt concentration reflect forces of physical chemical and physiological significance, also considered in Paper VI of this series (9).

DIFFUSION

Molecules of a solution originally confined to a given space will uniformly disperse in the course of time throughout a larger volume. Measurements of the rate of transfer of molecules across a given area under specific conditions [11] yield a diffusion constant, D, characteristic of the molecules. Protein molecules have small, and simpler molecules like sodium chloride and glucose have large values for this constant, as shown in Table I. The slow diffusion of proteins through certain animal membranes was one of the early observations which led to their classification as colloids by Thomas Graham and others. Although diffusion can be used for an approximate estimate of the size of the molecules, we have used the equations of Svedberg which include both sedimentation and diffusion constants for the calculation of molecular weights. These calculations also yield the deviation in hydrodynamical behavior of the actual molecule from a spherical particle of the same weight, and this makes possible an estimate of the shape and hydration of the molecules. The relations used were either in the form of the original equations, tables, nomograms, or graphs (6, 11, 12).

VISCOSITY

Resistance to the flow of fluids through capillaries is caused almost entirely by the viscosity of the fluid involved. Indeed, it was the physiologist Jean Poiseuille who, in order to learn more about the flow of blood, made the first precise measurements of viscosity and discovered the laws of flow through capillary tubes. The viscosity of a protein solution is influenced far more by the shape of the molecules than by their size. The simple theory demands that the viscosity of solutions of spherical, incompressible, uncharged molecules be the same, regardless of their size, provided they occupy the same volume fraction. Solutions of fibrinogen and the globulins are more viscous than those of albumins, not because the molecules are larger, but because they are more asymmetrical. Thus, the viscosity of blood is of the same order as twice concentrated plasma, a 25 per cent albumin, a 15 per cent γ-globulin, or a 2 per cent fibrinogen solution. The importance of this observation in the preparation of blood substitutes which will offer a minimum of resistance to flow is immediately apparent.

In dilute solutions, the increase of relative viscosity is proportional to the volume concentration of dissolved substance, the constant of proportionality being the viscosity coefficient, F. Measurements of F for certain plasma proteins are recorded in Table I and have led to estimates of the shape and solvation of the molecules [12] (11, 12).

DOUBLE REFRACTION OF FLOW

When a solution containing molecules of long rod-like shape is caused to move in such a manner that velocity gradients are set up, orientation of the elongated molecules will occur as logs are lined up due to the variations of velocity in different parts of a fast flowing stream. A beam of polarized light passed through such a solution will usually behave in the manner observed for birefringent crystals such as Iceland spar. This double refraction of flow, or streaming birefringence, can be quantitatively evaluated by proper measurements. It is particularly pronounced in solutions containing fibrinogen, indicating that fibrinogen molecules are many times longer than they are broad. Studies in our laboratory [13] have indicated a length in the neighborhood of 900 Ångstroms for fibrinogen, a length 6 times that of the albumins, the most symmetrical of the plasma proteins.

CHARGE DISTRIBUTION AND NET CHARGE

Considerations of size and shape alone do not yield complete characterization of proteins from

[11] Diffusion measurements have been made in this laboratory by J. L. Oncley. We have used the diffusion cell designed by Lamm (10).

[12] In the case of viscosity, we have a choice of several calculations for the relationship between viscosity coefficient and asymmetry. The equations of Simha (13) have given results in better agreement with other methods when protein solutions are involved. Values computed from this equation are tabulated by Mehl, Oncley, and Simha (14). For a recent review of viscosity measurements of substances of high molecular weight see Lauffer (15).

[13] We are indebted to J. T. Edsall and I. H. Scheinberg for these preliminary values made with an apparatus which will be described elsewhere and which reaches velocity gradients as high as 30,000 cm. per second per cm. (8).

the standpoint of chemical behavior nor of physiological function. Thus, a hydrocarbon chain of the same size and shape as albumin will stand in obvious contrast to the latter in low solubility in water or dilute salt solutions, in base-binding capacity, and in effective colloid osmotic pressure, as well as in nutritive function.

A plasma protein molecule may be considered as an array of acidic and basic groups of various spatial relations and strengths. These groups, when ionized, are the principal sites of electrical charge. The degree of ionization varies according to the chemical environment of the molecule and the position of the group in the molecule.

At blood pH, plasma proteins bear a negative charge, being present largely as sodium salts. This negative charge is due to the dissociation of free carboxyl groups of the dicarboxylic amino acids.[14] At strongly acid reactions, the proteins carry a positive charge, for under these conditions the carboxyl groups are not ionized and the positive charge is entirely due to ionization of the free groups of the basic amino acids.

Between blood pH, where the molecule is an anion, and an acid pH, where it is a cation, there is an intermediate reaction, the isoelectric point, at which the molecule behaves as though uncharged in an electric field. Nonetheless, the molecule under these circumstances contains large and equal numbers of positively and negatively charged groups.

The symmetry of distribution of groups capable of carrying a charge is of great importance for the behavior of a large molecule, for even though the net charge be zero, if a great many groups bearing charges of similar sign are closely crowded together at one portion of the molecular surface, they will give rise to an appreciable electrical field of force which can act both on other large charged molecules and on smaller electrolytes of the environment. If, however, positively and negatively charged groups are evenly distributed, the proximity of positive to negative charges will tend to cancel their electrostatic fields, and thus interaction with other charged molecules will be small. The distribution of these groups, and thus the electrical symmetry of the molecule, is expressed in terms of an overall electric moment.[15]

Of the plasma proteins thus far studied, the γ-globulins have the most asymmetric charge distribution and thus have strong interactions with other proteins and electrolytes. By contrast, the albumins have a very symmetric electrical structure. Although they possess nearly 100 negatively and 100 positively charged groups per molecule in the isoelectric condition, these are spaced so as to produce a small electric moment (Table I) and albumins interact weakly with other proteins and electrolytes.

The net charge of a protein molecule determines to a great extent the rate of its migration in an electrical field. It cannot be quantitatively evaluated from this rate however for, in electrophoretic measurements, there enter many factors not at present [16] susceptible of analysis. However, electrometric titration curves of proteins, yielding data on acid- and base-binding capacities, measure this net charge and the number of positively and negatively charged groups on the molecule at reactions over the isoelectric and physiological ranges of pH.

The amphoteric behavior of proteins as revealed in electrometric titration curves is a factor in many physiological reactions; it is by reason of the base-binding capacity that the proteins account for 16 of the 155 m.eq. of anions per liter of normal plasma. Although the most important mechanism for maintenance of blood pH is the carbonic acid-bicarbonate buffer

[14] At pH more alkaline than blood, the negative charge is further increased by dissociation of the phenolic hydroxyl and sulfhydryl groups. For a discussion of the amino acid composition of the plasma proteins, see Brand (16).

[15] The electric moment of a molecule with two equal and opposite charges, e, separated by a distance, d, is $\mu = de$. The electric moment of a more complex set of charges can be computed by calculating such an electric moment for each pair of charges and then obtaining a vector sum of these moments. Electric moments are usually expressed in Debye units, equal to 10^{-18} cgs. units.

[16] In measurement of electrophoretic mobility, the electrostatic forces between the charged molecule and the ions of the surrounding electrolyte give rise to the formation of an atmosphere of ions around the molecule. This ionic atmosphere alters the strength of the electrical field in the immediate vicinity of the protein molecule and increases the force resisting the motion. For a theoretical discussion see chapter by Hans Mueller in Cohn and Edsall (1).

system, a secondary buffering is provided by the plasma proteins.

The rôle of this secondary system may be quantitatively illustrated as follows:[17] If, in the plasma electrolyte distribution, the proteins are replaced by their equivalent amount of chloride and the carbonic acid concentration of the system maintained constant, the addition of about 16 m.eq. of fixed acid will result in a drop of pH from the physiological mean of 7.4 to 7.0. The presence of the plasma proteins in normal concentration permits the addition of 3 more m.eq. of fixed acid to attain the same drop. Approximately 75 per cent of the additional buffering power is contributed by the albumins which, gram for gram, have about twice that of the globulins in this pH range.

The high buffering power of the albumins is reflected in the fact that they possess a net charge of 18 at blood pH, the highest of the plasma proteins (Table I). The significance of high net charge (taken in conjunction with low molecular weight and high molecular symmetry from the standpoint of both shape and charge distribution) in fitting albumin for its physiological rôle in the osmotic activity of plasma has previously been discussed (17 to 20) and is considered in earlier studies and in subsequent papers of this series.

THE CHARACTERIZATION OF THE PLASMA FRACTIONS

Knowledge of the physical properties of the various plasma proteins has been an invaluable aid in so dividing plasma that proteins responsible for a particular function have been concentrated and can be used appropriately in the therapy of specific conditions. Our goal has been not the preparation of a limited number of products to the exclusion of others, but the development of an inclusive method by which all components of plasma may be preserved and made available in useful forms. In developing our system of plasma fractionation, two criteria have been followed as guides to the success of separations: (*1*) each fraction should be as homogeneous as possible, and (*2*) the more labile components should be preserved.

The criterion that each fraction shall approximate homogeneity leads to a series of fractions, each possessing a different specific function or functions connected with chemically discrete protein molecules. The criterion that the more labile components are preserved throughout the fractionation not only demands that the methods be completely inclusive but also gives some assurance that subtle changes have not occurred in the more rugged components. Since many biological reactions are sensitive to small chemical changes that are difficult to detect by *in vitro* methods, it is of immense importance to know whether the isolated proteins have been irreversibly altered or maintained in their native state. In human therapy, it is always necessary to prove that any proteins foreign to the human body do not initiate undesirable reactions. If proteins from a human source, such as normal human blood plasma, are irreversibly altered during or subsequent to their separation, they must be considered to be foreign to the human body and only used with great caution.

Undoubtedly, dynamic equilibria exist within whole blood so that destruction and synthesis of its components go on continually. However, a purified protein solution for therapeutic use should possess stability of both its physical and chemical properties. It has been found in many cases that the proteins isolated from plasma are stable under conditions that seriously alter plasma. This fact may depend to a considerable extent on the presence of enzymes and reactive groups within plasma which are capable of acting upon protein molecules, otherwise inherently stable. In some cases, it has been found that careful separation of lipoid material from a product by precise fractionation enhances thermal stability greatly.

Clearly, a subdivision of all of the plasma into 6 fractions is not adequate in order to achieve the high concentration and separation of all of the useful components of plasma. Subfractionations of all our initial fractions have, therefore, been developed, and in the case of Fractions I, II + III, and IV it has been possible further to separate and concentrate components possessing specific physiological

[17] The calculation is arbitrary in the sense that it ignores the rôle of the phosphates and is inapplicable to whole blood in that the effect of hemoglobin is not considered.

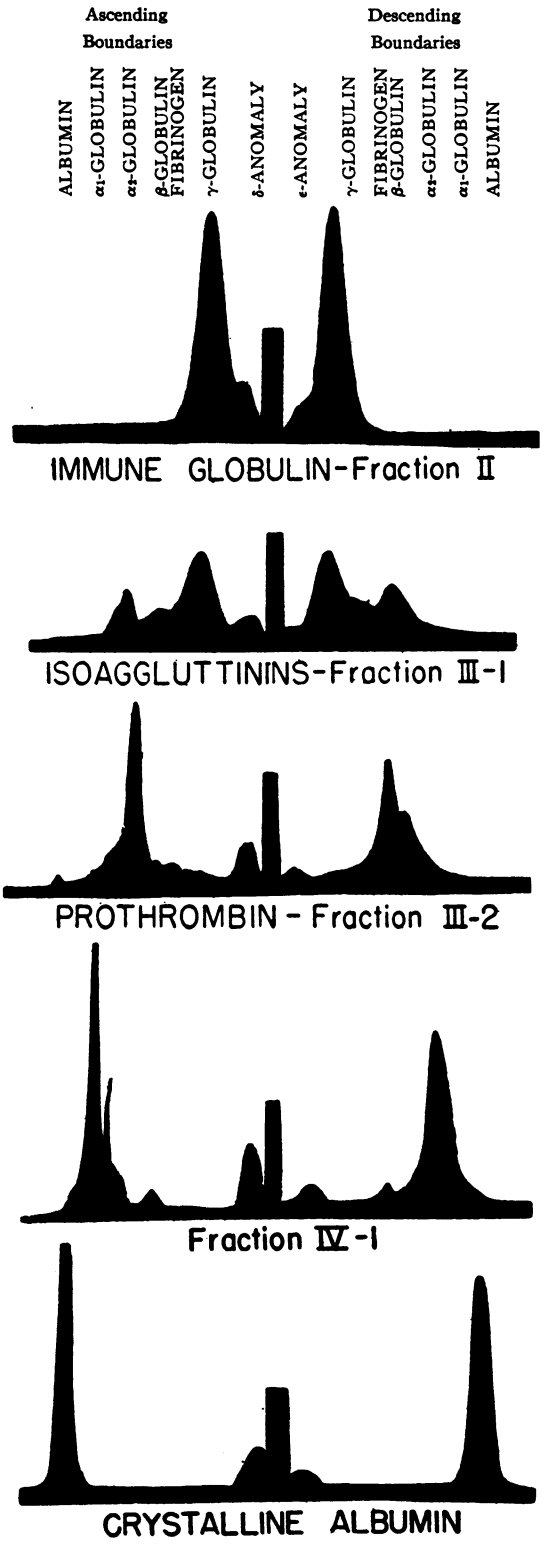

FIG. 2. ELECTROPHORETIC SCHLIEREN DIAGRAMS OF CERTAIN SUBFRACTIONS OF HUMAN PLASMA

activities (Figure 2). Moreover, it should be stressed that the smaller the fraction in which a component of therapeutic value can be concentrated, the more potent the activity of the concentrate, especially as compared to the plasma as a whole. The value of attaining the greatest possible concentration of each component, as well as its separation from other components, has emerged in the partial successes that have thus far been achieved.

Thus, in the case of antibodies, the higher their concentration in a fraction over that in plasma, the smaller the dose that may be necessary and, more important still, the more antibodies that can be injected in any practical dose. Thus, it is always possible that enough antibodies to give protection, or especially for treatment, could not conveniently be injected if the maximum concentration over the plasma that was achieved was 3- to 10-fold, whereas a 30- to 300-fold concentration of these specific antibodies might render them of clinical value. Moreover, certain active principles are present in such small amounts that they cannot be detected in plasma. The possibility that these human proteins have value for therapeutic use is opened when they are concentrated, and the useful limits of concentration cannot yet be envisioned.

The present state of development may be illustrated by the electrophoretic schlieren diagrams of the fractions in Figure 1. These diagrams should be compared with the diagram for whole plasma, whose analysis has been previously described. The diagrams, taken after electrophoresis has progressed to a comparable point in each case, are placed above each other so that proteins of like mobility in the plasma and in the fractions thereof should lie above each other in vertical lines. The peaks which have moved farthest from the central boundary are those of albumin. Albumin is largely concentrated in Fraction V, whereas the amounts in Fractions I, II + III, and IV are so small as to constitute a minor part of the total diagram. Likewise, Fraction II + III contains the larger part of the γ-globulin, which moves slowly. The quantitative information which can be obtained from these diagrams is given in Table II. Fraction I contains approximately 60 per cent of the fibrinogen in plasma; Fraction II + III contains

about 90 per cent of the γ-globulins; Fraction IV contains about 65 per cent of the α-globulins; and Fraction V contains about 85 per cent of the albumins.

The fractions upon which are based the clinical studies reported can also be characterized in terms of ultracentrifugal studies (7). Thus, the component of molecular weight comparable to the albumins is found in Fraction V. There is, however, a larger amount of material in the other fractions sedimenting with the velocity characteristic of albumin than can be accounted for on the basis of the amounts of albumin in these fractions, as estimated from electrophoretic analysis. Presumably, therefore, there is globulin in these fractions of smaller molecular weight than is characteristic of the bulk of the globulin, as has previously been claimed for some of the globulin in serum.

Conversely, the high molecular weight globulins have been largely concentrated in Fraction III–1, although a smaller amount has also been detected in other fractions. None is present in Fraction V.

PRODUCTS OF PLASMA FRACTIONATION

Proteins of interrelated functions do not always possess comparable chemical properties and are thus not always found in the same fraction. The products of plasma fractionation must also be considered, therefore, from the point of view of physiological function and clinical use. The largest categories are (*1*) the albumins, (*2*) the immune globulins, (*3*) the isohemagglutinins, (*4*) the hormones, enzymes, and related substances, and (*5*) the proteins concerned with the clotting of the blood.

The clotting of the blood depends upon a complex mechanism and upon a number of plasma proteins. Of these, fibrinogen, separated in Fraction I, may be considered the most important since its molecular properties determine to a large extent the mechanical properties of the clot (21). The conversion of a fibrinogen solution into the fibrin clot is brought about by the interaction of fibrinogen and thrombin. Prothrombin, rather than thrombin, is found in circulating plasma. Both are separated in Fraction III–2. The properties of these proteins, as well as of the other components that presumably play a rôle in clotting, such as the fibrinolytic enzyme and the hemophilic factor (both in Fraction II + III) are considered in Paper XV of this series (5).

The immune globulins are also found in Fraction II + III. These antibodies are largely concentrated in Fraction II, which consists in recent preparations of over 98 per cent γ-globulins as judged by electrophoretic analysis. The principal impurities consist of small amounts of β-globulin and albumin, of cholesterol and phospholipid. Like albumin, Fraction II can be dried and dispensed in the dry state. It has proved convenient, however, to make the immune globulins available in a concentrated solution. The solution is nearly colorless. In measles prophylaxis, only small amounts of this concentrated solution of antibodies are generally used. Immunological studies upon the anti-

TABLE II

Distribution of proteins of plasma[a]

	Grams protein per liter plasma	Grams protein per liter of plasma in fractions					Protein in fractions
		I	II+III	IV	V	VI	
Protein	60.3[b]	4.3	16.3	9.7	29.6	0.6	60.5
Albumin	33.2	0.2	0.7	1.0	29.0	0.3	31.2
α-globulin	8.4	0.2	1.8	5.4	0.6	0.3	8.4
β-globulin	7.8	0.8	6.2	3.1			10.1
γ-globulin	6.6	0.5	6.0	0.2			6.7
Fibrinogen	4.3	2.6	1.6[c]				4.2
Totals	60.3	4.3	16.3	9.7	29.6	0.6	60.6

a. The distribution of components in plasma and in each fraction is based on electrophoretic analysis which gives the fraction of the total refractive increment contributed by each component. The size of each fraction is based on nitrogen analysis, assuming a nitrogen factor of 6.25. It has been assumed that the refractive increment per gram of nitrogen is the same for all components. Work is in progress to determine the relations among nitrogen content, refractive increment, and dry weights for the various proteins.

b. For the total nitrogen of citrated plasma, an average value of 9.88 grams per liter has been taken. Of this, 9.65 grams per liter is protein nitrogen. Accordingly, the protein content of the plasma, which is obtained by multiplying by the conventional factor 6.25, is 60.3 grams per liter.

c. The electrophoretic analysis of Fraction II + III has been corrected to include 10 per cent of fibrinogen, precipitated as fibrin previous to analysis.

TABLE III

Products of human plasma fractionation

Components of plasma	Concentrated in plasma fraction	Percentage of plasma protein in fraction	Distribution of electrophoretic components in main fractions					Concentration ratio of principal electrophoretic component to that in plasma	Concentration of active function times that in plasma	Concentration of activity in final product times that in plasma
			Albumin	α	β	γ	Fibrinogen			
Albumin (Crystallized)	V	48	100	0	0	0	0	1.8	1.3[a]	5.4[b]
Albumin (Standard)			98.5	1.5	0	0	0[c]			
Hypertensinogen	IV	10	15	55	28	2	0	3.9	8[d]	
Complement C'2									8	
Complement C'1	III-2	3	0	10	75	15	0	5.8	15	
Thrombin									15[e]	20[f]
Isohemagglutinin	III-1	8	0	4	35	61	0	5.5	12	18[g]
Immune globulins	II	10	1	0	1	98	0[h]	8.8	8[j]	25[j]
Fibrinogen (Standard)	I	6	5	4	19	11	61	8.7		
Fibrinogen (Purified)			0	0	9	0	91	13		

a. Determined from osmotic pressure measurements. See Paper VI of this series (9).
b. Final product contains 250 grams of protein per liter.
c. Electrophoretic analyses are reported in Paper II (7).
d. Hypertensinogen activity estimated in cat units per gram.
e. Estimated in prothrombin units.
f. Calculated as ratio of thrombin units per mgm. of protein in thrombin preparation to prothrombin units per mgm. of protein in the original plasma pool.
g. Estimated as an approximately 9 per cent protein solution.
h. Electrophoretic analysis of Fraction II, prepared by more recent methods and shown by clinical trial to contain measles antibody. The preparations made earlier contained about 85 per cent γ-globulin, 10 per cent β-globulin, less than 1 per cent α-globulin, and 5 per cent albumin. Values for individual preparations are given in Paper II (7).
j. Average of estimates of activity of several different antibodies as reported in Paper X of this series (22).

bodies of plasma are reported in Paper X of this series (22), and clinical studies on their use in the prevention and modification of measles in Papers XI and XII of this series (23, 24).

The isohemagglutinins are also separated quantitatively in Fraction II + III and are further concentrated in Fraction III-1. When group specific plasma is used as the starting material for the fractionation, the resulting concentrate of the isohemagglutinins makes an acceptible blood grouping material. Both anti-A and anti-B isohemagglutinins have been concentrated at the Harvard pilot plant in this way at the suggestion of and in collaboration with Col. Callender and Lt. Col. Kendrick at the Army Medical School (Papers XIII and XIV (25, 26). The anti-Rh globulin has also been concentrated in this fraction and reduced to the dry state from low titered Rh positive blood collected by Louis Diamond of the Children's Hospital, Boston. It is hoped that the development of these typing materials as a part of the plasma fractionation process will contribute to the program of making available whole blood and resuspended red cells.

Complement is made up of a series of components which together participate in certain immunological phenomena. They are less stable than the antibodies and present in but small amounts. Though destroyed in our earlier attempts at plasma fractionation, it has recently been possible, in collaboration with Lt. Louis Pillemer, to obtain in almost quantitative yield, both the midpiece of complement, C'1, and the end piece, C'2. As was to have been anticipated from earlier work with complement of animal origin (27, 28), these components are concentrated in quite different fractions, C'1 in

Fraction III–2, and C'2 in Fraction IV–2. Their use in physiology and medicine has not yet been established.

Enzymes and hormones serve many different functions, possess very different properties, and are found in different fractions. Thus, a fibrinolytic enzyme is largely concentrated in Fraction III–2, as is the globulin which promotes the clotting of hemophilic blood. Alkaline phosphatase is concentrated in Fraction IV. Whereas the concentration of enzymes from animal and vegetable sources has yielded products of great value and this may be expected also of the enzymes of human plasma, our efforts thus far have been concentrated on freeing the albumins and immune globulins from enzymes in the interest of high thermal stability.

The hormones found in the blood stream will presumably be present in varying amount from time to time and have their origin in many cases in the various glands of the body. It has not been possible to prove thus far that the hormone, concentrated from a tissue extract, is identical to that concentrated in a fraction of the plasma, but it has been possible, in collaboration with F. L. Hisaw, to detect, in plasma fractions, the presence of luteinizing, follicle stimulating, and thyrotropic hormones. The 2 gonadotropic hormones have been found in only certain plasma pools, the luteinizing in Fraction II + III, the follicle stimulating hormone in Fraction VI. The thyrotropic hormone appears to be a more constant component of our fractions where it is concentrated in Fraction IV–3, 4.

The protein-bound iodine of plasma, for which assay has been made by W. T. Salter, has also been largely found in Fraction IV, some also in Fraction V. The investigation of these proteins is continuing.

Hypertensinogen has been concentrated in Fraction IV–2, 3. Special problems, however, have been encountered in obtaining a satisfactory stable hypertensinogen concentrate. We are indebted to Lewis Dexter for assay for this blood pressure regulating substance.

The lipo-proteins are largely concentrated in Fractions III and IV and appear to be associated with α- and β-globulins. Indeed Fraction I, rich in fibrinogen, Fraction II in the immune globulins, and Fraction V in albumin have been prepared with such low content of lipid material as to suggest that its presence represents impurities in these fractions.

Although detailed investigations are in progress [18] of the nature of the lipid material which has been concentrated largely in Fractions III–1, IV–1, and IV–4, distribution has thus far been followed by the determination of cholesterol, carotene,[19] and phospholipid. Approximately two-thirds of the total plasma cholesterol separates in Fraction II + III where it appears to be associated with the β-globulins with which it is further concentrated in Fraction III–1. Over half of the carotene and phospholipids are also concentrated in Fraction II + III.

The albumin of plasma represents by far its largest component and is concentrated therefore in its largest fraction, V. Whereas there is evidence of the existence of more than one albumin, this fraction appears homogeneous in the ultracentrifuge and in the electrophoretic apparatus at neutral or slightly alkaline reactions.[20] The standard of purity specified in the Navy contracts for the production of this blood substitute permits 2 per cent of globulin. Electrophoretic analyses, reported in Paper II of this series (7), reveal the constancy of the product under the conditions of industrial production.

Human serum albumin has been still further purified by crystallization, and study of the crystallized albumin that we have prepared has demonstrated that the very high thermal stability and low viscosity of our standard preparations are indeed ascribable to the albumin, instability largely to globulin and lipid impurities. Comparison of thermal stabilities of crystallized and of standard normal human serum albumin is considered in Paper IV of this series

[18] Large amounts of Fraction IV-1 have been made available to A. Chanutin and H. E. Carter for these investigations which will subsequently be reported elsewhere. The distribution of these by-products of plasma fractionation for chemical studies has been directed by H. B. Vickery.

[19] We are indebted to N. Talbot for carrying out assays for carotene and cholesterol in our earlier fractions, assays which are now being continued and expanded by P. Gross.

[20] Conditions under which electrophoretic separations among the albumins have been effected are not considered at this time, though the studies of Luetscher (29) have been extended by S. H. Armstrong, Jr., and M. J. E. Budka.

(30), the electrolytes which appear to contribute the most favorable environment for the concentrated albumin solutions in Papers IV (30) and V (31) of this series. Normal human serum albumin can be prepared in far larger amounts and with higher yields if further purification by crystallization is not superimposed upon the process. The standards of purity determined upon for the albumin now being delivered to the Navy in large amounts were chosen so as to assure freedom from untoward reactions with maximum efficiency in large scale production.

Serum albumin was developed in order to attain a blood substitute which could be distributed in solution ready for immediate emergency use. It has been made available as a 25 per cent solution to render the package as compact [21] as possible for transport. Osmotically 4 times as concentrated as plasma, 25 per cent albumin is no more viscous than whole blood. Both the advantages and the contraindications to the use of this hypertonic solution are considered in other papers, both from the point of view of theory and of practice.

The properties and uses of albumin are considered in Papers II to IX (7, 9, 16, 30 to 34); those of the immune globulins in Papers X to XII (22 to 24); those of isohemagglutinins in Papers XIII and XIV (25, 26); while the properties and uses of the various products that have been prepared from the clotting proteins are considered in Papers XV to XXII (5, 21, 35 to 40) of this series.

The development and interrelations of these different products has been our special concern. The physical chemical characterization of the protein products which are given in this paper are supplemented by their amino acid analyses in Paper III of this series (16). Wide differences in the nature of the specific chemical groups upon which the fine structure and specificity of behavior of these proteins presumably depend are revealed in this preliminary report. Analytical knowledge of this kind was sought in the interest not only of chemical control but also of determining the significance of the various fractions from the point of view of nutrition, especially of plasma and blood regeneration, considered thus far only in a single preliminary communication (Paper XXIII of this series) (41).

As each of these products is developed at the Harvard Plasma Fractionation Laboratory, sufficient amounts are prepared in our pilot plant [22] to permit investigation of conditions for its distribution in a sterile [23] and stable state and of the reproducibility of the preparative process and of its clinical appraisal under diverse conditions.

All the products of plasma fractionation that have thus far been recommended to the armed forces have been reduced to the dry state in their preparation. In the dry state, stability may be expected to be maximal, whereas stability in solution will vary from product to product and depend also upon the properties of the solvent. The final form for a given product has thus been decided following consideration both of its maximal stability and of medical and military requirements.

SUMMARY

Plasma has been subjected to a large scale fractionation process which yields the different proteins of which it is composed, separated and concentrated. Each concentrate can then be used in therapy in relation to its specialized natural properties and functions.

The physical dimensions and properties of the plasma proteins differ widely. All that have been studied, however, have equatorial diameters close to 36 Å, a dimension which prevents their rapid loss from the blood stream.

The plasma proteins, however, vary in length from 150 Å, characteristic of serum albumin, to 900 Å, characteristic of fibrinogen. It follows that solutions of the more symmetrical albumin

[21] The package adopted by the armed forces for the standard 25 per cent albumin solution was developed by Capt. Lloyd R. Newhouser, Medical Corps, United States Navy, and Lt. Col. Douglas Kendrick, Medical Corps, United States Army.

[22] The continuous operation of our pilot plant for two and a half years has made possible the preparation of each product developed and its standardization for clinical trial. The staff of the pilot plant have included: L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, M. Melin, J. N. Ashworth, J. H. Cameron, H. T. Gordon, H. L. Taylor, D. A. Richert, R. W. Kelty, L. H. Larsen.

[23] We are indebted to the Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health, for its collaboration in preparing for distribution in sterile and safe form both normal human serum albumin and the immune serum globulins which are now licensed products under the National Institute of Health.

molecules are far less viscous than are those of other plasma proteins.

The molecular weights of the albumins being less than half as great as those of most globulins, their osmotic activity is more than twice as great. Albumins have large numbers of charged groups, symmetrically arranged, and a large net charge which also results in increased osmotic activity.

The immune globulins have a smaller net charge, but far greater electric moments, rendering them more reactive with other proteins and electrolytes.

The size and shape of the molecules and the nature and structural interrelations of their free groups determine the physico-chemical characteristics and presumably the physiological functions of the plasma proteins. The plasma proteins differ in their solubility and in their stability in solutions of different pH, temperature, dielectric constant, and concentration of dipolar ions, electrolytes or molecules rich in hydroxyl or other groups. The methods of separation that have been developed depend upon these differences in physico-chemical properties.

The products that have thus far been made available as stable, dry white powders in sufficiently large amounts for clinical appraisal include: (*1*) ***fibrinogen***, upon which the physical properties of the blood clot largely depend, which can be made into plastics, or in connection with (*2*) ***thrombin*** into (*3*) ***fibrin films***, used as membranes and (*4*) into ***fibrin foams***, used as hemostatic agents in neurosurgery; (*5*) the ***immune globulins*** proven of value in measles prophylaxis; (*6*) the ***isohemagglutinins*** in blood typing; and (*7*) ***albumin***, which represents less than 60 per cent of the plasma protein but is responsible for approximately 80 per cent of the osmotic activity of the blood and is used in the treatment of shock.

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