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A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma[1a,b,c]

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Introduction

Human blood is a tissue of great complexity: of formed elements, erythrocytes, lymphocytes, granulocytes and platelets; of plasma proteins; serum albumins of more than one kind, with a sulfhydryl group and without,[2] globulins of many kinds, some euglobulins, some pseudoglobulins, some lipoproteins, glycoproteins or mucoproteins; proteins concerned with blood coagulation; the metal-combining protein; the hormones which, by definition, are the chemical messengers transported in the blood, but which have but rarely been separated from the blood and characterized in this state of nature; diverse antibodies and complement components concerned with immunity; and the enzymes from which substrate proteins must be separated, if optimum conditions for their preservation are to be discovered.

"The methods would appear to be at hand.... for the study of all proteins. There is no theoretical obstacle to the isolation of all the protein constituents of any given tissue, or to their characterization as chemical substances, and to the study of their interactions as biological components. Proceeding thus, often with new techniques, but employing the classical methods of physical chemistry, we may hope in time to achieve an understanding of the morphology and physiology of biological systems in terms of the properties of their components."[3] The fractionation of human plasma carried forward during World War II made possible the preparation and characterization of many of the protein components of human plasma[4,5,6] (see Table I). Their size, shape,[7] electric moments, chemical interactions[8] and solubility characteristics in a variety of solvents were investigated. The advantages of separating proteins in ethanol–water mixtures of controlled *p*H, ionic strength, temperature and protein concentration were amply demonstrated. However, this system of fractionation was developed under the pressure of war-time demands. Research and development were rarely far in advance of commerical production, and major changes in procedure could not readily be incorporated in the system.

"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 .... 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 .... 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[9] donors to the American Red Cross, a five-variable system has sufficed....and the variables maintained constant, by the use of acetate and carbonate buffer systems to control *p*H and ionic strength, and by the use of ethanol as precipitant .... 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

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(2) W. L. Hughes, Jr., *Cold Spring Harbor Symposia Quant. Biol.*, in press (1949).

(3) E. J. Cohn, *Bull. N. Y. Acad. of Med.*, **15**, 639 (1939).

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

(5) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., *ibid.*, **71**, 541 (1949).

(6) D. M. Surgenor, L. E. Strong, H. L. Taylor, R. S. Gordon, Jr., and D. M. Gibson, *ibid.*, **71**, 1223 (1949).

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

(8) E. J. Cohn, *Experientia*, **3**, 125 (1947).

(9) At the time the present paper went to press this number had increased to 3,200,000.

TABLE I

PROPERTIES AND INTERACTIONS OF PROTEIN COMPONENTS OF HUMAN PLASMA[a]

Protein components	Estimated % plasma proteins	Sedimentation constant $S_{20,w}$	Approximate isoelectric point	Specific chemical interactions
Major Protein Components				
Serum albumins	52	4.6	4.9	Fatty acids, dyes, bile salts, drugs and mercury
Mercaptalbumin	(34[e])			
α_2-Glycoproteins	1.2	9	4.9	Carbohydrates and barium
α_2-Mucoproteins	0.5	9[f]	4.9	
Fibrinogen	4	9	<5.3[g]	Thrombin
Cold insoluble globulin	0.15		<5.3	
α_1-Lipoproteins	3	5	5.2	Steroids and carotenoids
β_1-Lipoproteins	5	7	5.4	
β_1-Lipid-poor euglobulins	2 1	7 20	5.5	
β_1-Metal combining protein	3	5.0[h]	5.8	Iron and copper
β_2-Globulins	3	7	6.3	
γ-Globulins	11	7 10	6.3 7.3	Antigens
Total	86			
Other Protein Components				
Prothrombin	0.1			Calcium and thromboplastin
Plasminogen				Streptokinase
Hypertensinogen				Renin
Iodoproteins				
Isoagglutinins	(0.03)		6.3	Incompatible red cells
Complement components C'1, C'2	0.4			Antigen–antibody complex
Amylase				Starch
Choline esterase	0.005[d]		4.5	Choline esters
Alkaline phosphatase				Phosphate esters
Peptidase				L-Leucylglycylglycine
β-Glucuronidase				β-Glucuronides
Caeruloplasmin			4.4[c]	Copper
α_1 Small acid protein[b]	0.5	2.9	3.0	
α_1-Bilirubin globulin	0.05		4.7	Bilirubin
α_2-Protein[b]	0.1	2.9		Barium
β_1-Protein[b]	0.05	5		

[a] This table is based largely on data from refs. (7) and (8). Revisions and new data are from the sources noted below. [b] E. J. Cohn and K. Schmid, in preparation. [c] C. G. Holmberg and C. B. Laurell, *Abstracts 1st Int. Cong. Biochem., Camb., Eng.*, No. 291-B; *Acta Chem. Scand.*, 1, 944 (1947); *Nature*, **161**, 236 (1948). [d] D. M. Surgenor and D. Ellis, in preparation. [e] Ref. (2). [f] Ref. (6). [g] P. R. Morrison, J. T. Edsall and S. G. Miller, THIS JOURNAL, **70**, 3103 (1948). [h] Ref. (3).

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."[4]

The knowledge gained in the earlier system of plasma fractionation made possible a reconsideration of each separation. Instead of the sodium salts of buffers, advantage has been taken of the far more specific nature of the interactions of different proteins and protein enzymes with bivalent cations. The lower solubility of the protein salts formed made possible the lowering of the ethanol concentration to mole fraction ethanol 0.066 as a maximum. The highest concentration of ethanol in any reagent added has been reduced to 0.09 mole fraction ethanol (25% of 95% ethanol at 25°). The *p*H range in the earlier system was from 4.4 to 7.4. In the present system the *p*H is never lower than 5.5 and the precipitation of all but traces of the plasma proteins may be effected at constant *p*H, 5.8, at constant ethanol concentration, mole fraction 0.036, at constant temperature, −5°, in a matter of hours after collection of the blood. The new system, in which all components are brought to the relatively inert solid state, as rapidly as possible, and maintained insoluble at −5°, until separated from each other and from the enzymes for which they are the substrate, is described in this report. Many investigations upon which it depends were carried out by a large number of able collaborators, from November, 1946, to November, 1949, are referred to in this communication, and will be reported in detail in subsequent communications.[10]

Fractional Extraction.—Separation of a pure component, or group of components, by fractional extraction of a precipitate has the advantage that the material which remains insoluble is protected from the various changes, chemical and enzymatic, which occur rapidly in solution. The greater stability of proteins in the solid state has long been recognized. The present system of fractionation has been devised so that many separations, following the initial fractional precipitation, proceed by fractional extraction. In this way each component remains continuously in the solid state during operations introduced before it is dissolved. This procedure contrasts

(10) The continuous advice of J. T. Edsall, especially regarding the components concerned with blood coagulation; of J. L. Oncley especially upon the γ-globulins and β-lipoproteins and upon the estimation of the physical properties of the proteins; and of W. L. Hughes, Jr., upon their chemical modification and crystallization, has been greatly appreciated. We are indebted to C. G. Gordon for ultracentrifugal analyses, to M. J. E. Budka for electrophoretic analyses, to M. M. Hasson, C. T. Qua, E. H. Russ, R. M. Irland, E. Schultz and R. A. Aronson for chemical analyses and to L. E. Strong, D. J. Mulford, L. H. Larsen, W. J. Small and R. Kelty for aid in large scale preparations.

The advantages of an initial acid precipitation at low concentrations of ethanol were first suggested by H. L. Taylor during World War II. The presence of Fraction II in this precipitate was first demonstrated in collaboration with D. Mittelman and the dissociation of the γ-globulin complex by glycine in collaboration with D. Mittelman, E. Uroma and F. W. Kahnt. The use of bivalent cations as protein precipitants, also considered during World War II, was extended to mercury in collaboration with W. L. Hughes, Jr., to iron and copper in collaboration with D. M. Surgenor and B. A. Koechlin, to barium and zinc in collaboration with J. M. Gillespie. The use of zinc hydroxide rather than magnesium hydroxide in the precipitation of small proteins and peptides at *p*H 7.5 was first introduced in collaboration with K. Schmid.

with the complete resolution which preceded each fractional precipitation in the earlier methods.[4,5,6]

Fractional extraction can be carried out more rapidly than fractional precipitation since a shorter time of equilibration has generally been found adequate. Since the ethanol concentration need not be lowered initially, to permit complete solution of a precipitate, less concentrated ethanol may be added to achieve final conditions. Equally important, the use of less concentrated ethanol allows a very high rate of mixing of the suspended precipitate with the precooled reagent, both because temporary exposure to higher concentrations of ethanol is avoided, and because very little heat of mixing need be dissipated.

Protein-Metal Interactions.—The earlier procedures for the separation of the plasma proteins in ethanol–water mixtures at low temperatures made use of electrolytes in far lower concentrations than are employed in the older "salting out" methods.[4] In the initial precipitation of the proteins from a tissue extract or body fluid, the electrolyte concentration is necessarily higher than the very low range in which interactions with proteins depend largely upon the specific electrochemical properties of ion and protein.[4] Following precipitation of the proteins, fractional extraction may be carried out at very low ionic strengths. Moreover, under these conditions, very specific interactions may be achieved. The possibility of employing such interactions with ions, having either solvent or precipitating actions other than those due to their ionic strength, was clearly recognized but was purposely avoided at the time.

A large number of salts of human serum albumin and of certain other proteins have recently been crystallized[11,12] from ethanol–water mixtures at low temperature. The various cations and anions in these salts have been present in very low, indeed in stoichiometric concentrations; for example, one mercury to two molecules of serum albumin,[11] two iron atoms to one of the metal-combining protein.[13] The difference in solubility of these protein salts from that of the uncombined protein, depends upon the separation of new saturating bodies. The number of new solid phases that can be formed greatly increases the phase complexity of the system and thus increases the degrees of freedom which render possible precise, quantitative separations from such multicomponent systems as body fluids and tissue extracts.

The solubility minima of the metal salts of proteins are somewhat alkaline to the isoelectric points of the protein. This phenomenon is similar to that exhibited by calcium caseinates. The saturating protein salts employed in the present system are water soluble, for the concentrations of interacting ions employed are much lower than would be required to bring about precipitation from aqueous solution. Advantage of the specificity of these interactions for protein separations thus depends upon insolubility in systems containing a non-aqueous, generally an organic, solvent to diminish protein solubility.

The knowledge gained from studies on the specific interactions of metal ions with purified plasma proteins is the basis of the present system of plasma fractionation. Low concentrations of zinc and barium acetates have been employed most advantageously. The resulting interactions have not only been used selectively to effect separations of protein components, in low concentrations of ethanol, but have been used routinely to effect, quantitatively, reprecipitation of the soluble components separated by fractional extraction. The low solubility of these metal protein salts in ethanol–water mixtures at low temperatures has made possible quantitative recovery of proteins from dilute solutions. These specific metal–protein interactions have often favored the separation of undenatured proteins by reducing the extremes of ethanol concentration and of *p*H required in the various separations and reprecipitations.

Protein–Protein Interactions.—Formation of salt-like complexes between proteins of opposite net charge is usual in systems containing mixtures of proteins. The effect of such protein–protein interactions in reducing the solubilities of both components in a binary mixture over a broad range of *p*H between the isoelectric points of the two proteins has long been recognized.[14] Separations of interacting proteins have been effected most satisfactorily under conditions of *p*H in which both proteins bear the same sign of net charge, that is, either acid or alkaline to the isoelectric points of both proteins.[15] Since the isoelectric points of the albumins of plasma are in the neighborhood of *p*H 5 and of certain of the γ-globulins alkaline to the *p*H of blood, a wide *p*H range would be necessary to achieve these conditions.[16]

During the preliminary studies leading to the development of the present system of fractionation, it was recognized that for certain separations it would be preferable to take advantage of protein–protein interactions rather than to avoid

(11) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947).

(12) J. Lewin, in preparation.

(13) D. M. Surgenor, B. A. Koechlin and L. E. Strong, *J. Clin. Invest.*, **28**, 73 (1949); B. A. Koechlin, in preparation.

(14) E. J. Cohn, *J. Gen. Physiol.*, **4**, 697 (1922).

(15) A. A. Green, THIS JOURNAL, **60**, 1108 (1938).

(16) The previous system of plasma fractionation relied principally on differences in solubility of the sodium or acetate salts of the various protein components. Whenever possible, protein-protein interactions were avoided, and low solubilities were obtained by the increase in ethanol concentration rather than by permitting the formation of insoluble complexes between proteins. To mimimize interactions, Fractions I and II + III were separated alkaline to the isoelectric points of nearly all the protein components of plasma. However, the separation of Fraction II + III at *p*H 6.8, in Methods 5 and 6, introduced protein-protein interactions.[17] Protein-protein interactions were also recognized in the separation of Fractions IV-1 and IV-4 at more acid reactions.[4,6]

(17) J. L. Oncley and F. R. N. Gurd, in preparation.

them. Reduction of the solubility of the interacting proteins, of the ionic strength, and of the ethanol concentration were advantageous. Moreover, the *p*H range over which the highly insoluble complexes form is generally broader than the range of minimum solubility of either component, and allows considerable latitude for the choice of a *p*H for their separation from other proteins. Extreme *p*H values have thus been avoided. Although the ability to form interaction complexes is not restricted to particular proteins, or groups of proteins, the strength of the interactions between proteins, as between proteins and other ions, differs widely.[17,18] Accordingly, selective separations may be made in which one protein competes for another to the exclusion of a third which would otherwise have been involved in the interaction. In the present system the initial precipitation of the γ-globulins in combination with β-lipoproteins is effected at *p*H 5.8, since at more acid reactions γ-globulins also combine with the albumins, which cannot therefore be quantitatively extracted.

Formation of protein–protein complexes, in a system of fractionation, requires subsequent release of the interacting components. Quantitative decomposition of the protein–protein complexes has generally been carried out in a *p*H range beyond the zone between the isoelectric points of the interacting proteins.[15] Just as the strength of the interaction between oppositely charged protein ions is increased in the alcohol–water mixtures,[4] decomposition of the complex is favored by increasing the ionic strength or the dielectric constant of the medium. Increase in dielectric constant has been conveniently employed by the addition to the ethanol–water mixtures of dipolar ions such as glycine or β-alanine.[19]

Materials and Methods

The normal human plasma employed in this investigation was obtained from blood collected in one of the following ways: (1) mixture of the blood with a solution (A. C. D.) containing citric acid, sodium citrate and glucose[21]; (2) passage of the blood through an ion exchange resin[22]; (3) mixture of the blood with a solution containing fibrinogen and sodium citrate[23]; (4) passage of the blood through an ion exchange resin into solutions of human fibrinogen and glucose.

The formed elements of the blood were separated by centrifugation in methods (1) and (2), and by sedimentation of erythrocytes and centrifugation of lymphocytes, granulocytes and platelets in methods (3) and (4). Plasma proteins should be precipitated as soon as possible and always in less than six hours after collection.

Reagent solutions were freshly prepared from 95% ethanol, water and standard solutions containing C. P. buffers or other solutes, according to specifications, at 25°, and precooled before use.

Sodium Acetate Buffers.—Reagent grade acetic acid and sodium acetate were used without further purification. Stock solutions of 10 *M* acetic acid and 4 *M* sodium acetate have proved convenient in the preparation of the buffers. The use of buffer solutions rather than free acid protects proteins in the system from exposure, even momentarily, to acidities greater than those of the buffer solution. In general a special buffer of the highest *p*H consistent with ionic strength requirements has been prepared for each operation. The composition of any particular buffer has been defined by the acetic acid required to adjust the *p*H, determined by titration of aliquots (then discarded), and the sodium acetate required to adjust the ionic strength to the desired level. The sodium acetate has been added as a buffer of the desired *p*H.

For the adjustment of the *p*H of A. C. D. plasma, in the first separation, it was necessary to use a very acid buffer in order to maintain the ionic strength sufficiently low to prevent dissociation of the γ-globulin–β_1-lipoprotein complex. The buffer used had a molar ratio of acetic acid to sodium acetate of 5:1, and when diluted to 0.01 ionic strength had a *p*H of 4.0. The development of calcium-free plasma may permit use of a less acid buffer while maintaining the same or lower ionic strength.

Zinc acetate buffer contained 54.8 g. of zinc acetate dihydrate and 34 ml. of *M* acetic acid per liter. The *p*H of this buffer was 5.80 ± 0.05 at 25°.

Sodium glycinate buffer contained 75 g. of glycine, and 20 g. of sodium hydroxide per liter. The *p*H of this buffer was about 9.5.

Ammonia buffer contained 3.1 g. of ammonium chloride, 57 ml. of concentrated ammonia and 200 ml. of 95% ethanol per liter. This buffer, like the others, was precooled to −5° and used in a closed system to avoid loss of ammonia to the atmosphere. The *p*H was about 10.4.

Glycine was introduced in the appropriate ethanol–water–buffer mixtures.

Removal of the bivalent cations, calcium, zinc, barium and iron was effected either by the addition of solutions of complex-forming reagents such as sodium citrate or ethylenediamine tetraacetate buffers[26] or by removal with ion exchange resins. Proteins were freed of bivalent cations before being dried from the frozen state.

Analytical Methods

Nitrogen, Biuret and Dry Weight Analyses.—Nitrogen was determined by micro-Kjeldahl. Non-protein nitrogen (NPN) was determined after precipitation of the proteins by 10% trichloroacetic acid. Biuret determinations were carried out by a modification of the method of Mehl[27] in which each series of readings was standardized against a standard preparation of normal human serum albumin. Determinations were made by diluting each aliquot to 10 ml. with 0.15 *M* sodium chloride before addition of 1 ml.

(18) E. J. Cohn and D. Mittelman, unpublished results.

(19) ". . . Precisely as the interaction between ions and one dipolar ion species may be shown to be diminished by the concentration of a second dipolar ion, so also increase in ionic strength may be shown to diminish the interaction between two species of dipolar ions. There is thus a multifunctional relation between the physical chemical behavior of the ionic and the dipolar ionic components of biological systems as a function of temperature, ionic strength and dielectric constant, as well as of their specific properties."[20]

(20) E. J. Cohn, *J. Gen. Physiol.*, **21**, 651 (1938).

(21) Approximately 450 ml. of blood was drawn into 75 ml. of a solution containing per liter 26.7 g. of trisodium citrate (5.5 H_2O), 8.0 g. of citric acid, and 22.0 g. of dextrose.

(22) We are indebted to Drs. C. Walter, J. G. Gibson, 2nd, E. S. Buckley, Jr., and C. P. Emerson for supplying plasma collected in this way as part of the continuing investigation being carried out for the American National Red Cross on the preservation of the formed elements of the blood.

(23) Approximately 450 ml. of blood was drawn into 100 ml. of a solution containing 2.5 g. of human fibrinogen and 2.0 g. of sodium citrate at a *p*H of 6.3.[24]

(24) E. S. Buckley, Jr., M. J. Powell and J. G. Gibson, 2nd, in preparation. See also ref. (25).

(25) "The Preservation of the Formed Elements and of the Proteins of the Blood." American National Red Cross (1949).

(26) Supplied by Alrose Chemical Company, Providence, Rhode Island, under the name Sequestrene AA.

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

of the biuret reagent. When barium ions were present, the substitution of a reagent prepared with copper chloride in place of copper sulfate was recommended. Dry weights were estimated after heating to constant weight for twenty-four to forty-eight hours under vacuum at 75°.

Lipid Analyses.—Total cholesterol was determined by the method of Bloor, Pelkan and Allen.[28] Lipid phosphorus was determined on Bloor extracts by the method of Fiske and SubbaRow[29] following the ashing procedure of Gortner.[30]

Hexose determinations were carried out on protein fractions following the modification by Friedmann of the orcinol procedure of Sørensen and Haugaard.[31]

The β_1-metal combining protein was estimated by the method of Surgenor, Koechlin and Strong.[13]

Optical densities were measured in the Beckman quartz spectrophotometer. Extinction coefficients at 280 mμ, to be reported in subsequent communications, were used in estimating protein concentrations.

Clottable protein was determined by the method of Morrison.[32]

Determinations of *p*H were carried out in the glass electrode at 25° after dilution with 0.02 *M* sodium chloride.

Protein Separation Methods

The physical chemical methods employed in developing these procedures—ultracentrifugal, diffusion, osmotic, viscometric, double refraction of flow, and especially electrophoretic methods—but not employed in carrying them out, have been considered in previous communications.[4,7,33]

Method 10 of plasma fractionation has been designed to be equally applicable on any scale from a few milliliters to thousands of liters of plasma. Regardless of the quantities used, or the mechanical procedures employed, separation of precipitates from solutions is effected at the same conditions of *p*H, ionic strength, and temperature, and the same concentrations of solutes and ethanol. Separations may often be effected either by fractional precipitation or by fractional extraction. Depending on the scale and the objectives of the operations, considerable latitude has been found permissible in the choice of dilutions, of method and rate of addition of reagents, of mechanical equipment, and often of order of separation of the protein components. Provided the phase conditions remain the same, the same result may be achieved in the preparation of large amounts of stable blood derivatives and in the analysis of a small amount of blood in the clinical laboratory.

Choice of Volumes for the Separations.—Introduction of bivalent cations such as zinc and barium for the quantitative recovery of plasma proteins from dilute solutions has allowed great freedom in the choice of the volumes of reagents employed. In general, the largest convenient volumes for a given quantity of plasma protein have been used in order to minimize occlusion of soluble components in the insoluble fractions, and to obtain sufficiently low ionic strengths for the sharp separations. To maintain low ionic strengths it has also been necessary to avoid, as far as possible, wide changes in *p*H in successive operations.

Since the precipitated fractions separated in the various ways generally contain 75–90% by weight of the solutions from which they have been removed, the recovery of occluded soluble proteins required special attention. The use of the highest possible dilutions, of washing on filters, resuspension and centrifugation of precipitates, or reprecipitation may all be used successfully. Each alternative will be best suited to a given scale of operations, and will be discussed in subsequent communications dealing with each of the detailed techniques. By these means it is possible to effect quantitative separations to any desired degree of accuracy.

Separation in the refrigerated angle centrifuge,[34] in plastic cups, was the method of choice in developing quantitative procedures, since surfaces are minimal and there is no loss, or denaturation, caused by filters or filter aids, or due to air interfaces as in the Sharples centrifuge. From 5 to 25 ml. of plasma proved convenient, yielding sufficient quantities of the various fractions for all analytical determinations, both chemical and electrophoretic. Reagents were added dropwise, from automatic pipets, directly into centrifuge tubes partially immersed in a bath at −5° and arranged in a battery of mechanical stirrers. As many as six or more samples were carried through operations simultaneously.

Separation through sintered glass filters partially immersed in a cold bath have been employed in carrying out these procedures with 2–10 ml. and will be reported in a subsequent communication by Lever and others.[35]

Separations by continuous centrifugation on a large scale in the refrigerated Sharples supercentrifuge have been carried out as previously described[4] and will be reported in a subsequent communication in collaboration with L. E. Strong and others.[36]

Filtration of large amounts of plasma, such as that from one or more donors, using filter aids to decrease processing time has also been studied. This procedure renders possible substitution of continuous operation for the usual batch system. The advantages include rapid equilibration, reproducible conditions, the use of counterflow techniques, fine temperature control, convenient use of exchange resins, and the possibility of operation in a closed system from which air can be excluded. The period of delay before a large pool of plasma is collected is eliminated, and the various protein components are rapidly brought to conditions where they are protected from enzymatic and autoxidative processes.

Quantitative Separation of Protein Components by Fractional Precipitation

Method 10.—The present system has evolved gradually, and will continue to evolve. The development of each operation has involved first an exploration to discover the range of conditions which would separate major components and then a series of adjustments to avoid distribution of the minor components into more than one fraction.[37]

The plasma was first separated into the smallest possible number of fractions, after which each fraction was subjected to further subfractionation to yield highly purified components. In contrast to the earlier procedures, in which the serum albumins were separated in the last fraction,[4] and the γ-globulins after laborious sub-

(28) W. R. Bloor, K. F. Pelkan and D. M. Allen, *J. Biol Chem.*, **52**, 191 (1922). Color development was measured either with a Coleman Universal Spectrophotometer or with a Klett-Summerson Colorimeter.

(29) C. H. Fiske and Y. SubbaRow, *ibid.*, **66**, 375 (1925).

(30) W. A. Gortner, *ibid.*, **159**, 97 (1945).

(31) R. Friedmann, *Biochem. J.*, **44**, 117 (1949).

(32) M. Sørensen and G. Haugaard, *Compt. rend. trav. lab. Carlsberg*, **19**, No. 12 (1933). P. R. Morrison, THIS JOURNAL, **69**, 2723 (1947).

(33) E. J. Cohn, T. L. McMeekin, J. L. Oncley, J. M. Newell and W. L. Hughes, Jr., THIS JOURNAL, **62**, 3386 (1940); E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 417 (1944).

(34) Manufactured by International Equipment Company, Boston, Massachusetts.

(35) W. F. Lever, F. R. N. Gurd, R. K. Brown, B. A. Barnes, E. Schultz and R. A. Aronson, in preparation.

(36) Pilot plant operations, begun in the spring of 1949, using these new chemical procedures and the same equipment as in earlier methods of plasma fractionation, were interrupted and are being resumed using the new methods of blood collection and of formed element separations. See also refs. (24) and (25).

(37) Details of the alternative systems that have been studied, in the search for optimum conditions for each separation, will be described in the communications to follow.

fractionation of Fraction II + III,[5] these important components, representing 63% of the plasma proteins, have now been quantitatively separated; each in two simple operations. In the initial step, the albumins, together with certain α-globulins and the β_1-metal combining protein were separated from the other components of the plasma by taking advantage of the solubility of their sodium salts, at *p*H 5.8, in 0.066 mole fraction ethanol, at $-5°$. In order to effect quantitative separations of all of the components, it has been found necessary to wash the precipitate with a small amount of solvent of the same composition. In this way quantitative yields were achieved of the components extracted, as well as maximum purity of components, insoluble under these conditions, and extracted subsequently.

The γ-globulins, β_1-lipoproteins and many of the components concerned with blood coagulation were concentrated in the initial precipitate. The γ-globulins, when purified, were moderately soluble under the conditions of the precipitation, which depended on their interaction with β_1-lipoproteins. It has been found possible first to extract the β_1-lipoproteins from this precipitate, by decomposing the protein–protein interaction under conditions such that they were soluble, or first to extract the γ-globulins under conditions such that the β_1-lipoproteins were insoluble and the antibodies were soluble. Following extraction of β_1-lipoproteins, or of γ-globulins and β_1-lipoproteins, the residue could be brought to the dry state. Frequently, it has been convenient to process a number of pools of plasma to this point, awaiting the accumulation of large amounts of the residual fractions before completing the separation of the remaining components. Other convenient operations for the separation of components will be reported subsequently. The conditions which have been tentatively adopted for the separation of the various major components of A.C.D. plasma by fractional precipitation are given in Table II.

The serum albumins, α_1-lipoproteins, α_2-globulins and the β_1-metal combining protein remaining in solution[38] following the first precipitation from plasma were precipitated almost quantitatively, without change in *p*H, or temperature, or ethanol, by the addition of zinc to the solution. The first precipitate contained 37, the second 62% of the proteins of plasma.

The proteins remaining in solution,[39] comprising 1% of the plasma proteins, consisted in part of small amounts of the various proteins previously precipitated as zinc salts and presumably present in proportion to their solubilities in the system. In part, however, this fraction consisted of more soluble, smaller, nitrogenous components.

TABLE II

FRACTIONAL PRECIPITATION FROM PLASMA OF PROTEIN AND OTHER NITROGENOUS COMPONENTS IN AN ETHANOL–WATER MIXTURE OF MOLE FRACTION 0.066 AT $-5°$

Metal	Na	Zn	Zn
Concn. of metal m./l.	0.04	0.02	0.02
*p*H	5.8	5.8	7.5
% Plasma protein	37	62[a]	[a]
Designation of fraction	I+II+III	IV+V	VI
	Components concentrated in precipitate		
	γ-Globulins	Serum albumins	Traces of IV+V
	β_1-Lipoprotein	β_1-Metal combining protein	α_1-Small acid protein
	β_1-Lipid-poor euglobulins	α_2-Glycoproteins	α_2-Protein
	Caeruloplasmin	α_2-Mucoproteins	β_1-Protein
	Isoagglutinins	α_1-Lipoproteins	Other small proteins and peptides
	Plasminogen	Iodoproteins	Uric acid
	Cold insoluble globulin	Choline esterase	
	Fibrinogen	Phosphatase	
	Prothrombin		

[a] For the quantitative separation of the plasma proteins, the time for the precipitation of the zinc salts should be longer than has been found practical in routine analyses. The value for the amount of protein precipitated at *p*H 5.8 has, therefore, frequently been found to be lower, and for that precipitated at *p*H 7.5 to be higher than given here. Experiments are in progress at slightly higher ethanol concentrations and lower temperatures, which achieve more nearly quantitative precipitation of components concentrated in Fraction IV + V at *p*H 5.8 and correspondingly lower the amount of protein precipitated at *p*H 7.5. Not more than a few tenths of 1% of the protein nitrogen remained soluble at *p*H 7.5. This precipitated with lead at *p*H 9.5.

Adjustment of the *p*H from 5.8 to 7.5, without change in temperature, in the concentration of ethanol or of zinc in the system, effected separation of 80% of this nitrogenous material from solution.[40] The *p*H was adjusted by the addition of an ammonium hydroxide–ammonium chloride buffer, in 0.066 mole fraction ethanol at $-5°$, and resulted in the formation of zinc hydroxide. The nitrogenous components which separated with the zinc hydroxide were resuspended, always at $-5°$, in an ethanol–water mixture of 0.066 mole fraction ethanol, and returned to the initial *p*H, 5.8. Their concentration had been increased approximately thirty-five-fold. The electrolyte concentrations, as well as concentrations of urea and sugars, differed considerably from those obtaining in the original solutions, all free soluble components having been greatly reduced, but the concentration of zinc precipitating with the protein increased from 0.02 to nearly 0.5 molar.

Electrophoretic and chemical analyses of the proteins insoluble under these conditions indicated

(38) The amount of α_2-protein remaining in solution was smaller in plasma collected over an exchange resin. Moreover the optimum concentration of zinc necessary to effect this precipitation was lower for calcium-free plasma than for A.C.D. plasma.

(39) The protein concentration in this solution was so low that no precipitate was observed, until after concentration, on addition of trichloroacetic acid.

(40) The optical density, $E_{1\,cm.}$ at 280 mμ of the solution, was decreased by this operation from 0.49 to 0.10.

the presence of albumins, α_1-, α_2- and β_1-proteins. These proteins appeared to be identical to those first precipitated as zinc salts at *p*H 5.8. Should further investigation prove this to be the case, they may be combined with the first zinc salts separated, thereby increasing the yield in which each is separated and isolated from plasma.

The nitrogenous components which remained in solution in the presence of zinc at *p*H 5.8, included a protein of high electrophoretic mobility; another with the mobility of an α_1-protein, which was rich in sugar, had an acid isoelectric point, near *p*H 3, and, as the other non-dialyzable components in this fraction, was free of lipid. In addition, α_2- and β_1-proteins were noted. The molecular sizes of all of these components were small, their sedimentation constants ranging from about 2 to 5. These components have been further separated by the addition of 0.02 mole of barium acetate at *p*H 6.1. Under these conditions only the α_1-lipid free glycoprotein remained in solution, with a purity of approximately 95%. The nature of these components of human plasma, which have not been previously characterized, will be reported in a later communication.

Quantitative Separation of Protein Components by Fractional Extraction

Release of the γ-globulins from protein complex was achieved by the addition of glycine, at very low ionic strength, at *p*H 5.5. This complex dissociated to some extent at *p*H 5.8 upon increasing either the ionic strength or the dielectric constant. To render the extraction quantitative, however, the *p*H was brought nearer the isoelectric point of the β_1-lipoproteins, and the concentration of ethanol was lowered to 0.0509 mole fraction. On washing the residue with a small additional amount of solvent the yield of γ-globulins was essentially quantitative.

β_1-Lipoproteins and a complex mixture of lipid-poor α- and β-globulins were next extracted in a solvent of the same mole fraction ethanol, temperature, and concentration of glycine at very low ionic strength and *p*H 6.8. This extraction was comparable to that employed for the separation of Fraction III-0 in Method 9[5] at *p*H 7.5. The use of glycine made possible extraction of this fraction at the more nearly neutral *p*H. The extracted β_1-lipoproteins may be quantitatively reprecipitated by the addition of zinc.

The specificity of the interactions that obtained in these systems permitted considerable freedom in the order in which the protein components could be separated. For example, the β_1-lipoproteins could be extracted before the γ-globulins by reversing the order of the operations. This, as well as various other modifications in the procedure, will be treated in detail in subsequent communications.

The residue remaining after the extraction of the β_1-lipoproteins could be dried from the frozen state and accumulated, if desired. It contained fibrinogen, cold insoluble globulin, antihemophilic globulin, isoagglutinins, plasminogen, and prothrombin, all of which had remained in the solid state throughout previous extractions.

Prothrombin and the isoagglutinins were readily extracted and separated from fibrinogen, plasminogen, and the cold insoluble globulin, by virtue of the greater change in their solubilities in a solvent of the same mole fraction ethanol, at the same temperature, upon addition of sodium citrate and adjustment of the *p*H to a slightly more alkaline reaction, 7.2. Prothrombin, prepared in this way may be reprecipitated at *p*H 5.6 following dilution to reduce the concentration of citrate ions. An alternative method involving the quantitative removal of prothrombin from calcium-free plasma by adsorption on barium sulfate,[41] before fractionation in an ethanol–water system at low temperature, has certain other advantages that are being considered.

The residue from this extraction consisted of many of the largest or the most insoluble components of plasma, which alternately could be separated, as previously, as Fraction I, as the first step in plasma fractionation. As a result of the greater precautions taken to protect the proteins in this, as contrasted to our earlier procedures, conversion of prothrombin to thrombin, or of plasminogen to plasmin had not occurred.

Extraction of serum albumins from the zinc salts precipitated at *p*H 5.8 was accomplished by taking advantage of the insolubility of the zinc salts of the α_1-lipoproteins, of the α_2-globulins and of the β_1-metal combining protein and of the barium salts of the α_2-globulin at *p*H 5.5 in 0.0509 mole fraction ethanol. A small amount of zinc may be added in the extracting solvent, to control the dissociation of the zinc–protein salts formed in the previous separation. The albumins, rendered more soluble by both the sodium and barium ions, were extracted, and were quantitatively reprecipitated as the zinc salts at *p*H 5.8 in 0.091 mole fraction ethanol.

The chief impurities in the albumins were small amounts of α_1 and β_1 proteins. In contrast to earlier procedures, in which the β_1-metal combining protein was obtained as the iron-free protein,[6] as a result of the more acid conditions of fractionation, the protein in the present system had never been acid to *p*H 5.8, and had not, therefore, discharged all of its iron. In the method here described, the iron-free β_1-metal combining globulin was largely in the precipitate. In normal plasma this protein is approximately one-third saturated with iron.[43,44] The solubility of that

(41) Suggested and being further investigated in collaboration with B. Alexander.[42]

(42) B. Alexander, A. De Vries and R. Goldstein, *N. E. J. Med.*, **240**, 403 (1949).

(43) C. E. Rath and C. A. Finch, *J. Clin. Invest.*, **28**, 79 (1949).

(44) G. E. Cartwright and M. M. Wintrobe, *ibid.*, **28**, 86 (1949).

part of the β_1-metal combining protein saturated with iron is much greater than that of the apoprotein at reactions alkaline to its isoelectric point but not at *p*H 5.5.[45]

In alternative procedures, which will be described in subsequent communications, the β_1-metal combining protein may be separated into a single fraction by removal of the iron from its complex with the protein prior to precipitation, thus yielding the apoprotein, which separates quantitatively with the α-proteins; or by saturation of the protein with iron to form the iron complex of the β_1-metal combining protein anion, which may be extracted quantitatively from the α-globulins with the serum albumins.

TABLE III

FRACTIONAL EXTRACTION OF PLASMA COMPONENTS PRECIPITATED IN AN ETHANOL–WATER MIXTURE OF MOLE FRACTION 0.066 AT −5°

Conditions for the Further Separation of the Plasma Components			
	Ppt. I+II+III	Ppt. IV+V	Ppt. VI
Metal	Na	Zn + Ba + Na	Zn
Concn. of metal, m./l.	0.005	[a] 0.01 + 0.02	0.5
*p*H	5.5	5.5	5.9
Ethanol, mole/fract.	0.051	0.051	0.066
Glycine, m./l.	0.6		
% plasma protein	11	53	0.6
Designation of fraction	Extr. II	Extr. V	Extr. VI
	Components in fraction		
	γ-Globulins	Serum albumins	α_1-Small acid protein α_2-Protein β_1-Protein Small proteins and peptides

[a] This separation depends on the presence of a small amount of zinc. The zinc content of the wet paste has been found adequate.

TABLE IV

FRACTIONAL EXTRACTION OF PLASMA COMPONENTS PRECIPITATED IN AN ETHANOL–WATER MIXTURE OF MOLE FRACTION 0.066 AT −5°

Conditions for the Further Separation of the Plasma Components			
	Ppt. I+III	Ppt. IV	Ext. VI
Metal	Na	Zn + Na	Ba
Concn. of metal, m./l.	0.005	0.0005 + 0.05	0.02
*p*H	6.8	6.2	6.1
Ethanol, mole fract.	0.051	0.051	0.066
Glycine, m./l.	0.6		
% Plasma protein	17	6	0.1
Designation of fraction	Ext. III-0	Ext. IV-6+7	Ppt. VI-1
	Components in fraction		
	β_1-Lipoprotein β_1-Lipid-poor euglobulins Caeruloplasmin	β_1-Metal combining protein α_2-Glycoprotein α_2-Mucoprotein Choline esterase	α_2-Protein β_1-Protein Small proteins and peptides

(45) The influence of iron on the isoelectric point of this protein is being investigated in collaboration with R. Zahn.

The conditions for the various separations made from the major precipitates (Table II) are defined in Tables III to V. Other separations, devised because of an especial interest in specific components of the plasma, will be described subsequently from this Laboratory. However, an almost infinite number of modifications of these general procedures using specific anions, as well as cations, other organic solvents and dipolar ions, is possible in order to effect the separation, in a state of nature, of the components of this and other tissues. The choice of method in the purification of any protein component should not be based solely on convenience, however, but should follow comparative studies upon the physical and chemical properties and, when possible, upon the immunological and physiological behavior and the clinical response to the isolated component so as to determine whether it has been separated in its state in nature.

TABLE V

FRACTIONAL EXTRACTION OF PLASMA COMPONENTS PRECIPITATED IN AN ETHANOL–WATER MIXTURE OF MOLE FRACTION 0.066 AT −5°

Conditions for the Further Separation of the Plasma Components			
	Ppt. I+III-1, 2, 3	Ext. IV-6+7	Ext. VI-2
Metal	Na		
Concn. of metal, m./l.	0.3		
*p*H	7.2		
Ethanol, mole fract.	0.051		
Sodium citrate	0.1		
% Plasma protein in fraction	3	4	0.5
Designation of fraction	Ext. III-1+2	Ppt. IV-6[a]	Ppt. VI-2
	Components in fraction		
	Isoagglutinins Prothrombin	α_2-Glycoproteins	α_1-Small acid protein
	Components in residual precipitates		
% Plasma protein in fraction	6	3	0.2
Designation of fraction	I+III-3	IV-1	
	Components in fraction		
	Plasminogen Cold insoluble globulin Fibrinogen	α_1-Lipoprotein	Trace of albumins and other proteins

[a] Alternative conditions for separation of the β_1-metal combining protein and the α_2-glycoproteins are considered in the text.

Method 10. Directions for the Quantitative Separation of Plasma Protein Components.—The procedure, as carried out with 25 ml. of A.C.D. plasma, follows. The few changes in conditions for the fractionation of calcium-free plasma will be reported subsequently. Separations are effected in the refrigerated angle centrifuge. The plastic centrifuge tubes in which the fractionation is begun are referred to as master tubes. All reagents are freshly prepared at 25° and precooled to −5° before use.

First, 25 ml. of plasma is placed in the master tube in

the −5° bath. After the plasma has cooled to 0°, but before any appreciable freezing has taken place, 100 ml. of a reagent containing 250 ml. of 95% ethanol and 2.5 ml. of 0.8 Γ/2 *p*H 4.0 sodium acetate buffer per l. is added at the rate of 20 ml. per minute with mechanical stirring. Continue stirring for fifteen minutes. The *p*H which may be estimated with 1 ml. of suspension diluted with 4 ml. of 0.02 *M* sodium chloride, should be 5.8–5.9. Centrifuge for thirty minutes at −5° and 4000 r. p. m.

Measure the volume of Solution IV + V + VI and transfer to a new plastic centrifuge tube. Withdraw exactly 1 ml. into a flask for biuret determination, and another aliquot of 3 ml. for cholesterol analysis.

To the remaining Solution IV + V + VI are added with stirring 10 ml. of a reagent containing 200 ml. of 95% ethanol and 54.8 g. of zinc acetate dihydrate per l., freshly prepared each day. Let stand at least fifteen minutes before centrifuging for thirty minutes. The nitrogenous material remaining in Solution VI, comprising about 1% of the plasma proteins, may be precipitated if desired using the conditions described in Table II.

The serum albumins may be extracted from Precipitate IV + V by stirring for one hour with 175 ml. of a reagent containing 160 ml. of 95% ethanol, 2.6 g. of barium acetate, 20 ml. of *M* sodium acetate and 7.3 ml. of *M* acetic acid per l. The *p*H should read 5.5–5.6. Centrifuge for thirty minutes, and take 2 ml. of the measured volume of Solution V for biuret determination of the serum albumins.

Precipitate IV remaining after the extraction of the serum albumins is stirred for one hour with 25 ml. of a reagent containing 160 ml. of 95% ethanol, 50 ml. of 1 *M* sodium acetate and 0.1 g. of zinc acetate dihydrate per l. The *p*H of the suspension should be 6.1–6.2. Centrifuge for thirty minutes.

Precipitate IV-1 may be resuspended in 0.15 *M* sodium chloride, after which it is brought into solution by the minimum amount of 0.5 *M* sodium citrate and made up to volume for lipid analysis. It should not be frozen or dried.

Solution IV-6 + 7 contains α_2-glycoproteins and β_1-metal combining protein. Alternate methods for their separation are considered above, and will later be reported in detail.

Precipitate I + II + III in the master tube is stirred manually into a smooth paste free from visible lumps; and then stirred mechanically with 50 ml. of a reagent containing 150 ml. of 95% ethanol, 2.0 ml. of *M* sodium acetate, 1.4 ml. of *M* acetic acid, and 45 g. of glycine per l. The *p*H of the suspension should read 5.50 ± 0.05. Centrifuge for thirty minutes, and take 2 ml. of the measured volume of Extract II for biuret estimation of the γ-globulins.

Precipitate I + III in the master tube is stirred to a smooth paste and extracted for one hour with 100 ml. of a reagent consisting of 160 ml. of 95% ethanol, 45 g. of glycine, 2.5 ml. of sodium glycinate buffer (described above), 3.2 ml. of 0.5 *M* disodium phosphate and 2.4 ml. of 0.5 *M* monosodium phosphate, brought to 1 l. with water. The *p*H of the suspension should read 6.8–6.9. Centrifuge for forty-five minutes, and take 3 ml. of the measured volume of Extract III-0 for biuret determination; 2 ml. may be taken for cholesterol estimation. Extract III-0 should not be frozen or dried.

Precipitate I + III-1,2,3 in the master tube is stirred to a smooth paste and extracted for one hour with 25 ml. of a reagent containing 160 ml. of 95% ethanol, 1.2 ml. of 1 *M* citric acid and 120 ml. of *M* trisodium citrate per l. The *p*H of the suspension should read 7.1–7.2. Centrifuge for thirty minutes, and take 5 ml. of the measured volume of Extract III-1,2 for biuret determination. This fraction contains the prothrombin and part of the isoagglutinins. A more satisfactory separation so as to avoid this distribution will be reported subsequently.

Precipitate I + III-3, in the master tube, containing fibrinogen, antihemophilic globulin, cold insoluble globulin and plasminogen may be redissolved to 10 ml. in 0.02 *M* trisodium citrate, with a 1-ml. aliquot taken for biuret determination, or may be used for other purposes such as the determination of clottable protein.

Summary

1. Many proteins and protein enzymes form dissociable salts or complexes with each other, with smaller dipolar ions, with complex organic molecules and ions, and with such heavy metal ions as zinc, manganese, barium, calcium, mercury, iron and copper.

2. Heavy metal salts or complexes of proteins generally differ in solubility from each other and from sodium or potassium salts of equal protein valence. The former are often, but not always, less soluble. Both the sodium and zinc salts of the iron complex of the metal-combining protein anion are far more soluble than the sodium and zinc salts of the iron-free protein anion.

3. The solubilities in water of the albumins and pseudoglobulins of human plasma are so great that precipitates form only when the proteins are combined with many equivalents of heavy metal per mole; often in a denatured state. Even small amounts of organic solvents, such as alcohols, acetone and ether, lower the solubilities of proteins, and especially of proteins with even a small number of heavy metal equivalents. Thus 37% of all plasma proteins are precipitated from water, buffered with sodium acetate near *p*H 6, containing as little as 0.066 mole fraction ethanol, at −5°. Addition of 0.02 *M* of zinc ions results in the precipitation of an additional 62% of plasma proteins, including albumins and certain of the pseudoglobulins. Use of zinc ions has thus halved the amount of ethanol required in this system, as compared with the previous system in which only sodium buffers were used.

4. Only traces of the plasma proteins remain soluble in the ethanol–water mixture of mole fraction 0.066, containing 0.02 *M* of zinc at *p*H 6 and −5°. Of this soluble residue, 80% separates with the zinc if the *p*H is changed to 7.5. This fraction contains very soluble proteins, some of small size, some not previously characterized.

5. The additon of as little as 0.02 *M* of zinc acetate to a system containing 0.066 mole fraction ethanol (one volume 95% ethanol diluted to 5 volumes at 25°) at −5°, within the *p*H range 5.5 to 7.5, thus renders possible and convenient the recovery all but traces of the nitrogenous components of human plasma.

6. The separation of different plasma fractions from each other is also readily and often quantitatively effected in systems containing less than 0.066 mole fraction ethanol, within this *p*H range, by taking advantage of differences in the solubilities of the different metal–protein salts and complexes.

7. The albumins are quantitatively separated from the γ-globulins; the α_1-lipoproteins from the

β_1-lipoproteins and the β_1-metal combining protein from the β_1-lipoproteins by this first precipitation of the sodium proteinates in 0.066 mole fraction ethanol at *p*H 5.8 at $-5°$. The γ-globulins may then be quantitatively separated from the precipitated sodium proteinates, and the serum albumins from the precipitated zinc proteinates, by fractional extraction in 0.051 mole fraction ethanol at *p*H 5.5 at $-5°$.

8. The sodium salts of most of these fractions are soluble in the ethanol–water systems from which the heavy metal–protein salts separate, and may often be crystallized. The heavy metal salts formed with most of these proteins are soluble in water. Removal of the metal from the protein by addition to the ethanol–water mixture of citrate, ethylenediamine tetraacetate, or a comparable reagent, redissolves the protein as the sodium salt. Passage of the water soluble metal salts through an appropriate exchange resin may also be employed to remove the metal ions.

9. Many protein–protein complexes that are insoluble under conditions such that one of the proteins alone is soluble—such as those between γ-globulins and β-lipoproteins—are decomposed by the addition of glycine to the system.

10. A new system, based upon protein–protein and protein–metal interactions, is proposed for the quantitative separation of the protein components of plasma or other tissues, for analytical or preparative procedures on any scale.

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

Raman Spectra of Amino Acids and Related Compounds. VII. Glycylglycine, Cysteine, Cystine and Other Amino Acids[1]

BY JOHN T. EDSALL,* JOHN W. OTVOS AND ALEXANDER RICH

This paper reports the continuation of earlier studies[2,3] on amino acids and related compounds. The spectrum of glycylglycine, the simplest of the peptides, shows certain features not observed in the amino acids. The presence in cysteine of the sulfhydryl group, with its characteristic frequencies sets it apart from the other amino acids. The study of cystine has also revealed the presence of the characteristic vibrational frequency of the S–S linkage. Spectra for the basic amino acids, lysine and histidine, and for some β-, δ- and ϵ-amino acids are also reported.

Experimental

The experimental technique has already been fully described.[2,3,4,5] All studies were made with the Hilger E-439 glass spectrograph. For the isolation of the mercury *e* line (22938 cm.$^{-1}$) a filter composed of an alcoholic solution of *p*-nitrotoluene and rhodamine 5 GDN extra was employed[6]; and for the isolation of the mercury *k* line (24705 cm.$^{-1}$) a combination of a dilute solution of sodium nitrite with Corning red purple ultra glass. The observed spectra are recorded in Table I, together with details concerning the materials and the preparation of the solutions studied. All the compounds reported, except cystine dihydrochloride, were studied using both the mercury *k* and *e* lines as the exciting radiation. Cystine dihydrochloride could be studied only with the *e* line, since the solution showed appreciable fluorescence when *k* line excitation was employed.

Discussion of Results

Cysteine and Cystine.—The most distinctive feature of the spectrum of cysteine is the stretching frequency of the S–H group at 2572 cm.$^{-1}$. This frequency for the cysteine cation in water is identical, within two or three cm.$^{-1}$, with the S–H frequency in liquid aliphatic mercaptans. Kohlrausch[7] gives for a series of 10 aliphatic mercaptans values of the S–H frequencies ranging from 2567 to 2573, with an average of 2571. If there were any hydrogen bonding between the sulfhydryl group and the surrounding water molecules, the corresponding frequency in cysteine should be decreased below its value in the mercaptans and the line should also be broadened. On the contrary, the frequency is unchanged and the line is sharp, indicating practically complete absence of hydrogen bonding.

The intense line at 684 cm.$^{-1}$ must represent the stretching frequency associated with the C–S bond, corresponding to the lines near 705 in

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