# THE PROTEINS

**CHEMISTRY, BIOLOGICAL ACTIVITY, AND METHODS**

VOLUME II, PART B

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# The Proteins

## CHEMISTRY, BIOLOGICAL ACTIVITY, AND METHODS

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# CONTENTS

## CONTENTS OF VOLUME II, PART A

# CONTENTS OF VOLUME I (TWO PARTS)

## PART A



## PART B

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CHAPTER 21

# Interstitial Proteins: The Proteins of Blood Plasma and Lymph[1,2]

BY WALTER L. HUGHES



(1) This chapter holds no claims to completeness having been developed around my particular interests. However, it is hoped that the gain in perception and in critical appraisal so achieved will offset the otherwise flagrant omissions. Fortunately, some proteins which might naturally fall in this chapter have been covered in other chapters and so will be mentioned here only in passing. These include hemocyanin (Chap. 14), and fibrin (Chap. 20). It is unfortunate, from my point of view, that omission of fibrin could not justify complete omission of the clotting process since this appears hopelessly complex at the present time. Instead, a brief description has been included centering around fibrinogen and prothrombin with references to more extended treatments. The discussion of immunity has been restricted largely to the non-immune properties of antibodies, a more complete discussion being found in Chap. 22.

(2) While taking full responsibility for the views here expressed, I would like to express my appreciation to the many friends with whom I have spent hours of stimulating discussion, and whose ideas must naturally appear throughout this chapter. My particular thanks go to David Gitlin, Henry Isliker, John Pappenheimer and the editors.

## I. Origin and General Properties of Plasma Proteins

### 1. Limits of Free Diffusion

The all-important purpose of a circulatory system is obviously the convection of metabolites. Simple calculation will show that diffusion processes cannot supply sufficient metabolite at distances greater than a few millimeters. Thus the diffusion distance for $O_2$ at normal atmospheric tension at the rate of consumption of typical mammalian tissue has been calculated as 0.2 mm.[3,4]

(3) O. Warburg, *Biochem. Z.* **142,** 317 (1923).
(4) R. W. Gerard, *Am. J. Physiol.* **82,** 381 (1927).

## 2. Description of Blood, Blood Cells and Plasma

The convecting system evolved in the higher animals developed first through a hemocoele (as in crustacea)—a blood reservoir from which the fluid is pumped through a continuously branching system to the tissues from whence it drains back to the blood cavity, and eventually to a closed vascular system in the higher mollusks (octopus) and the vertebrates.[5,6] Throughout this evolution blood has consisted of a variety of "formed elements" (cells and smaller particles such as the blood platelets) and a large variety of molecules ranging from salts through sugars, amino acids, and other metabolites to the large molecules, the plasma proteins.

The withdrawal of blood, for the purpose of obtaining plasma proteins, requires special precautions if rapid changes following withdrawal are to be avoided. Blood is a self-sealing fluid, and the clotting process accomplishing this is activated by a chemical or physical stimulation of certain components of blood or of the injured tissues. In the case of vertebrate blood such changes may be delayed by preventing contact of the blood with injured cells or with "wettable" surfaces (by insertion of a tubular needle directly into the vascular system and by the use of vessels, tubing, etc., coated with paraffin or other nonwetting material). At the present time this may be most conveniently accomplished by the use of tubing, vessels, and even needles made of plastic[7] or by "siliconing" glassware[8] and coating needles and other metal parts with silicone oil.

Alternatively, blood coagulation may be prevented by stopping one of the chain of events leading to the formation of a clot. This has proven a successful method when study of the blood-clotting process itself was not intended. For this purpose workers have added citrate,[9,10] oxalate, or the sodium salt of ethylenediaminetetraacetic acid[11] as complexing agents for calcium. These interrupt the clotting process by preventing the conversion of prothrombin to thrombin (a process involving calcium ions). Clotting may also be prevented by drawing the blood through a column of cation-exchange resin on the sodium cycle so as to exchange sodium for the blood calcium.[12] Heparin may also be used. This can act at a later stage in the clotting process to prevent the action of thrombin on fibrinogen.[13]

(5) C. L. Prosser, Comparative Animal Physiology, Chap. 15, W. B. Saunders, Phila., 1950.
(6) M. Florkin, Biochemical Evolution, Academic Press, New York, 1949.
(7) C. Walter, *Surg. Forum, Proc. 36th Congr. Am. Coll. Surgeons. 1950* (*Pub. 1951*), p. 483.
(8) E. G. Rochow, An Introduction to the Chemistry of the Silicones, Wiley & Sons, New York, 1951.
(9) G. A. Pekelharing, *Beitr. wissensch. Med.* **1,** 433 (1891).
(10) R. Lewisohn, *Med. Record* **87,** 141 (1915).
(11) G. Schwarzenbach, *Helv. Chim. Acta* **30,** 1798 (1947); F. Proescher, *Proc. Soc. Exptl. Biol. Med.* **76,** 619 (1951).
(12) A. Steinberg, *Proc. Soc. Exptl. Biol. Med.* **56,** 124 (1944).
(13) However, *in vivo*, it appears to prevent prothrombin conversion. D. S. Riggs, *New Engl. J. Med.* **242,** 179, 216 (1950); M. Burstein, *Compt. rend. soc. biol.* **144,** 750, 1338 (1952).

If "defibrinated" blood is desired, the blood may be drawn without any precautions and stirred (whipped) while clotting takes place. This prevents gross occlusion of the cells by the clot which contracts to small shreds.[14]

Plasma, the noncellular portion of blood, may be obtained by simple settling (used with horse blood) or by centrifugation of the cellular elements.

The various types of cells in human blood possess quite different densities permitting their separation in a density-gradient column.[15-17] On a larger scale this may be accomplished by differential centrifugation. This process has been made more efficient by the design of special centrifuges[18] and by use of aggregating reagents, such as dextran,[19] for the erythrocytes. The blood platelets have been separated by differential centrifugation and by adsorption on ion-exchange resins.[20]

Blood plasma so obtained from fasting animals is a straw-colored fluid, which visually appears stable, if sterile. However, continuous changes take place on storage, not only among the clotting components, as mentioned above, but also among the lipoproteins, complement, etc.[21] Some changes may be due to enzymes present and some to the natural instability of the protein components. Certainly, these changes may be minimized by storage in the cold, although at 0°C. considerable precipitation of fibrinogen or of cold-insoluble globulin may occur. For this reason the storage temperature of blood has frequently been specified as 4°C. Blood plasma may be stored frozen or dried (by "lyophilization" —sublimation of water under vacuum after freezing the plasma) with marked improvement in stability for many components. However, some of the lipoproteins are badly damaged by this technic (see page 705).

The constancy in composition of blood plasma led to Claude Bernard's concept of the *milieu interieur* which bathed all the cells and buffered them from an adverse external environment. The constancy to which Bernard referred was that of the circulating electrolytes (Table I), which is, in fact, unusually invariant for a "biological constant." Proteins (including lipoproteins) constitute the bulk of the remaining plasma components and these show much greater variation, which can be measured by physicochemical methods against this constant ionic background.

(14) This process of some historical interest was described by Hewson, who first isolated fibrin: W. Hewson, An Experimental Inquiry into the Properties of the Blood, T. Codell, London, 1771.
(15) B. L. Vallee, W. L. Hughes, Jr., and J. G. Gibson, 2d, *Blood* **1**, 82 (1947).
(16) J. W. Ferrebee and Q. M. Geiman, *J. Infectious Diseases* **78**, 173 (1946).
(17) D. W. Fawcett and B. L. Vallee, *J. Lab. Clin. Med.* **39**, 354 (1952).
(18) E. J. Cohn, personal communication.
(19) E. S. Buckley. Jr., and J. G. Gibson, 2d, *Proc. Univ. Lab. Phys. Chem. Related Med. and Public Health* **1**, 45 (1950).
(20) J. L. Tullis, personal communication.
(21) L. E. Krejci, L. Sweeney, and E. B. Sanigar, *J. Biol. Chem.* **158**, 693 (1945).

Thus density measurements can be used to estimate total protein, although they will err if the lipide content of the plasma varies. Actually the density method largely ignores the lipides since these have densities close to that of water. Therefore the measurements give estimates for protein agreeing well with nitrogen analysis if one assumes all of the proteins to contain 16% N.[22]

TABLE I
IONIC COMPOSITION OF BLOOD PLASMA

| Cations, meq./l. | | Anions, meq./l. | |
|---|---|---|---|
| $Na^+$ | 142 | $Cl^-$ | 103 |
| $K^+$ | 5 | $HCO_3^-$ | 27 |
| $Ca^{++}$ | 5 | $HPO_4^=$ | 2 |
| $Mg^{++}$ | 3 | $SO_4^=$ | 1 |
| | | Organic acids | 6 |
| | | Protein | 16 |
| | 155 | | 155 |

Alternatively, refractive-index measurements may be used. Since the refractive-index increment of lipide is relatively close to that of protein, this method agrees well with the estimation of protein as total non-dialyzable solids.[22a]

### 3. REQUIREMENTS FOR CIRCULATORY FUNCTION

The proteins of mammalian plasma show related physicochemical properties, all being negatively charged at physiological pH and having molecular weights varying from 40,000 to 150,000 (a few larger—see Table VI). This relative homogeneity would appear related to function in the blood stream. These molecular sizes are similar to the "pore" sizes of the reticulo-endothelium (these pores being apparently designed so that relatively small variation in membrane structure may permit wide variation in the amount of protein passed). Only traces of protein pass through the membranes of the general capillary bed (including the renal glomerulus) and larger amounts through the membranes of the liver capillaries.[23] Certainly, limited diffusibility would appear desirable

(22) D. D. Van Slyke, A. Hiller, R. A. Phillips, P. B. Hamilton, V. P. Dole, R. M. Archibald and H. A. Eder, *J. Biol. Chem.* **183,** 331 (1950).

(22a) S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison, and M. Hasson, *J. Am. Chem. Soc.* **69,** 1747 (1947).

(23) E. M. Landis, *Physiol. Revs.* **14,** 404 (1934).

for the maintenance of a high concentration of these substances in the blood. However, perhaps the more interesting question is why the membranes permit any passage at all. Is this an imperfection of nature, a compromise in the interests of high permeability of certain small metabolites, or is such protein permeability necessary as an aid to their function? In the case of some proteins, such as the hormones, the latter interpretation would seem correct. Also, in the case of proteins whose function is transport of metabolites or protection against infection, permeability might seem desirable. It will be interesting in the following pages to look for further functional correlation of the relative permeability for different plasma proteins.

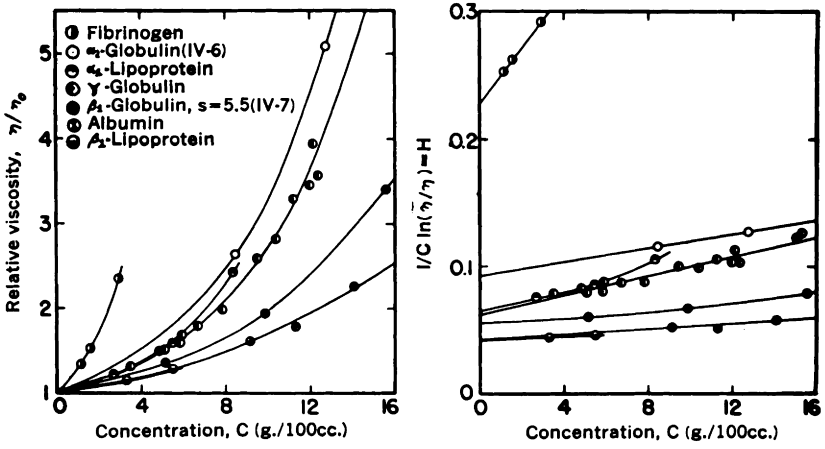



FIG. 1. Viscosity of various plasma proteins at 37°C. (From Oncley *et al.*)[61]

In the interests of osmotic efficiency (see sec. II) all plasma proteins at physiological pH are negatively charged and also they are all closely related in size (Table VI). These factors may be further related to hydrodynamic efficiency. Certainly, protein–protein interaction, which is accentuated when the proteins bear opposite electric charges, would decrease osmotic pressures and increase blood viscosity. This relative weakness of interaction between plasma proteins has been a boon to the investigator interested in the isolation of individual components.

The plasma proteins, excepting fibrinogen, also show similar and relatively small intrinsic viscosities (see Fig. 1). This, too would be a desirable feature in the circulation since it would decrease the work of the heart. Fibrinogen is, of course, necessarily asymmetric (and hence viscous) since it is a precursor to the network of the blood clot.

## 4. Distribution in Plasma, Extravascular Fluid, and Cells

### *a. Reservoirs of Plasma Proteins*

While the proteins of plasma include a number of readily identifiable components of dimensions such as to favor their confinement to the vascular system, absolute retention is not achieved. Thus it has been shown by tracer technics that approximately 50 per cent of small (relative to plasma content) infusions of serum albumin disappear from the circulation within the first day.[24–26] A considerable portion of this "lost" protein may be explained as being contained in the intercellular spaces due to leakage from the capillaries.[26a] The volume of such spaces is generally considered to be about ⅕ of the total volume of the mammalian organism[27] and hence is approximately 3 × the plasma volume. The protein content of this extravascular fluid must vary widely, the average value lying somewhere between zero and 2–4 per cent—the protein content of lymph.[28] Thus, there would appear sufficient latitude to explain the protein loss following transfusion.

However, lymph represents a concentrated (with respect to protein) extravascular fluid, and the very small permeability of capillaries to plasma proteins coupled with practically complete equivalence between plasma colloid osmotic pressure and average capillary hydrostatic pressure (above tissue pressure)[29] suggests that the extravascular interstitial fluid must contain far less, on the average, than the 1 per cent plasma protein which the above argument would demand. Drinker has pointed out that a quiescent limb does not normally become edematous despite lack of lymph flow.[28] This again suggests that leakage of protein must be extremely small. These arguments apply to the extravascular fluid of skeletal muscle and the appendages. In

(24) A. M. Seligman, *J. Clin. Invest.* **23,** 720 (1944); K. Sterling, *ibid.* **30,** 1228 (1951).

(25) M. P. Deichmiller, F. J. Dixon, and P. H. Maurer [(*Federation Proc.* **12,** 386 (1953)] measured half-lives of several homologous serum albumins ($I^{131}$ tagged): Man 15 days, dog 8 days, rabbit 6 days, and mouse 1.2 days.

(26) This effect was probably first demonstrated in humans by Janeway and Heyl, who followed by Heidelberger's quantitative precipitin technic the disappearance of bovine serum albumin from the blood stream and found 50 per cent disappearance during the first day followed by a much slower rate thereafter.

(26*a*) The dynamic equilibrium between vascular and extra vascular protein has been demonstrated with homologous antibody by D. Gitlin and C. A. Janeway [*Science* **118,** 301 (1953)].

(27) J. L. Gamble, Jr., and J. S. Robertson, *Am. J. Physiol.* **171,** 659 (1952); M. Gaudino, I. L. Schwartz, and M. F. Levitt, *Proc. Soc. Exptl. Biol. Med.* **68,** 507 (1948).

(28) C. K. Drinker and J. M. Yoffey, Lymphatics, Lymph, and Lymphoid Tissue, Harvard Univ. Press, Cambridge, 1941.

(29) E. M. Landis, *Am. J. Physiol.* **82,** 217 (1927); **83,** 528 (1928).

keeping with them, perfusion of the isolated hind limb[30] shows no loss of plasma protein to extravascular space.[31]

Therefore, the large protein losses described above would not appear to be generally distributed, and some extravascular "reservoir" of plasma proteins approaching blood plasma concentrations must exist in some of the internal organs (e.g., the liver) where capillary permeability is known to be very large. (High permeability for the capillaries of the portal system would seem necessary because of the low hydrostatic pressure—see sec. II.)

### *b. Evidence for Plasma Proteins within Cells*

Another possible reservoir of plasma proteins lies within cells. Recent immunochemical studies have demonstrated the rapid appearance of heterologous proteins within cells and particularly within cell nuclei following their injection. Homologous plasma proteins within cells and cell nuclei have also been observed.

An "immunohistochemical method" developed by Coons for this purpose consists in staining alcohol-fixed tissue slices with an antibody to which has been coupled a fluorescent dye. After suitable washing procedures to remove unspecifically adsorbed antibody, the location of the protein against which the antibody is directed may be observed microscopically under ultraviolet light.[32] In this way Coons has been able to observe the rapid penetration (i.e., within 10 minutes) of proteins such as ovalbumin and serum albumin within a variety of cells following their intravenous injection into mice.[33] Haurowitz has also demonstrated the penetration following injection of foreign proteins labeled with radioactive iodine into liver cells of the rabbit. In this way he found radioactivity in the fractions designated: microsomes, the mitochondria, and the nuclei. However, the activity could be separated from the nucleoprotein itself.[34]

Such penetration within cells might be assumed to be the first stage in the immune response of the animal similar to the previously observed

(30) J. R. Pappenheimer, E. M. Renkin, and L. M. Borrero, *Am. J. Physiol.* **167,** 13 (1951).

(31) J. R. Pappenheimer, personal communication. Pappenheimer measures osmotic-pressure changes and therefore might not detect changes in extravascular protein which was not osmotically active. The presence of such "bound" plasma protein is suggested by the occurrence of plasma proteins together with interstitial connective tissue throughout the body in histological sections stained immuno-chemically.[37] If this is not an artifact in the preparation of the histological specimen, further modification of the picture of capillary permeability may be required.

(32) A. H. Coons and M. H. Kaplan, *J. Exptl. Med.* **91,** 1 (1950).

(33) A. H. Coons, E. H. Leduc, and M. H. Kaplan, *J. Exptl. Med.* **93,** 173 (1951).

(34) C. F. Crampton and F. Haurowitz, *Science* **111,** 300 (1950); F. Haurowitz and C. F. Crampton, *J. Immunol.* **68,** 73 (1951).

phagocytosis of particulate antigens.[35] However, the distribution observed by Coons was much more widespread than the cells usually implicated in this mechanism.[36] Using Coons's technic, Gitlin *et al.* have studied the distribution of homologous plasma proteins within cells[37] and have found a similar distribution of serum albumin, $\gamma$-globulin, and $\beta$-lipoprotein in human tissues. Furthermore, that this represents a true penetration for $\gamma$-globulin was indicated by injecting this into a child who genetically was unable to synthesize this protein. The child's tissues, which before injection did not stain for $\gamma$-globulin, showed a normal staining pattern after injection.

It is not known whether plasma proteins can reversibly enter and again leave the cell, although heterologous proteins have been shown to disappear from cells at about the same time as disappearance from the circulation should occur.[33] A quantitative approach to these experiments has not been possible, so that the magnitude of the effects is unknown. However, it was noted that only traces of plasma proteins could be detected within muscle cells.[37] Therefore, any large cellular depots must again reside within the body cavity.

The significance of intracellular plasma proteins may lie in their close relation to protein metabolism. Schoenheimer and Rittenberg *et al.* have demonstrated the dynamic equilibrium of plasma proteins[38] with tissue proteins, and Whipple and Madden have shown their importance as amino acid reservoirs in the fasting animal.[39] However, it is not clear whether the plasma proteins are transported intact to the point of their incorporation or are first broken down to the constituent amino acids (see Tarver, Chap. 26). A choice cannot be made from known physical parameters, for while the concentration of most combined amino acids (as proteins) is 10 to 100 times as great as the free concentration in plasma, the capillary permeability to the free amino acid is probably 100-fold that of the protein;[40] and while evidence for cellular uptake of proteins is slowly accumulating, the evidence for uptake of amino acids is much more striking.[41]

(35) A. Bowin and A. Delaunay, Phagocytose et Infections, Herman & Co., Paris, 1947.
(36) For the effect of altered physicochemical properties on protein distribution within cells (phagocytosis?) compare: D. Gitlin, *Proc. Soc. Exptl. Biol. Med.* **74,** 138 (1950).
(37) D. Gitlin, B. H. Landing, and A. Whipple, *J. Exptl. Med.* **97,** 163 (1953).
(38) R. Schoenheimer, S. Ratner, D. Rittenberg, and M. Heidelberger, *J. Biol. Chem.* **144,** 545 (1942).
(39) G. H. Whipple and S. C. Madden, *Medicine* **23,** 215 (1944).
(40) J. R. Pappenheimer, *Physiol. Revs.* **33,** 387 (1953).
(41) H. N. Christensen, T. R. Riggs, and N. E. Ray, *J. Biol. Chem.* **194,** 41 (1952).

### c. *Evidence for Cellular Proteins in Plasma*

The presence of stray proteins in plasma as leakage from tissues might be expected either from dying cells or as a result of a slight permeability of the cell membranes. Substances whose presence in plasma might be so explained are the hydrolytic enzymes such as amylase, phosphatases, and esterases. The presence of catalase, occasionally considered to be a plasma enzyme, has been traced to hemolysis during blood collection, since its concentration is proportional to that of hemoglobin in the plasma.[42] The presence of peptidases in serum has been similarly ascribed to erythrocyte destruction.[42a] Recently, ovalbumin has been detected in chicken plasma, highest in laying hens. However, the concentration never exceeded 0.1 per cent of the total protein.[43]

In pathological conditions, changes in the plasma proteins are frequently observed which are proving increasingly useful in diagnosis. These changes can either involve an altered level of a normal plasma constituent (e.g., decreased albumin concentration in nephrosis) or the appearance of one or more new components, normally not detectable in plasma. An interesting example of the latter case is McCarty's crystalline "C reactive component"[44] (a protein occurring during the acute phase of certain infections). Further discussion of pathological changes is considered outside the scope of this chapter, and the reader is referred to the excellent review by Gutman.[44a]

## 5. Metabolism of Plasma Proteins

Metabolism of the plasma proteins is considered at greater length in Chap. 26 (Tarver), but it is interesting to point out in passing that the major component of plasma, serum albumin, appears to act as a protein reserve in the fasting animal. The turnover rate for human serum albumin is nevertheless quite slow (7 per cent/day),[25] indicating the relative stability of this protein toward catabolism or exchange phenomena. Nevertheless, the stability *in vitro* of albumin under physiological conditions is so great[45,46] that disappearance cannot be a function of "wearing out" in the circulation. Some sites of formation of certain

(42) G. E. Perlmann and F. Lipmann, *Arch. Biochem.* **7**, 159 (1945).
(42*a*) E. L. Smith, G. E. Cartwright, F. H. Tyler, and M. M. Wintrobe, *J. Biol. Chem.* **185**, 59 (1950).
(43) M. E. Marshall and H. F. Deutsch, *J. Biol. Chem.* **189**, 1 (1951).
(44) M. McCarty, *J. Exptl. Med.* **85**, 491 (1947).
(44*a*) A. B. Gutman, *Advances in Protein Chem.* **4**, 156 (1948).
(45) G. Scatchard, S. T. Gibson, L. M. Woodruff, A. C. Batchelder, and A. Brown, *J. Clin. Invest.* **23**, 445 (1944).
(46) P. D. Boyer, G. A. Ballou, and J. M. Luck, *J. Biol. Chem.* **162**, 199 (1946).

plasma proteins have been found; serum albumin is formed in liver slices,[47] immune globulin in plasma cells.[48,48a]

## II. Maintenance of Vascular Volume

### 1. Physiology of Peripheral Circulation

In the simpler circulatory systems, as exemplified by the crustacea, blood upon leaving the heart passes through smaller and smaller vessels in a continuously ramifying network, the "vascular tree," until it is eventually discharged into intercellular spaces from which it seeps back to the body coelom and thence by veins to the heart. The osmotic relationships which must exist are thus obscured by active transport phenomena across cell surfaces.

In the closed vascular system of the vertebrate, on the other hand, the laws of hydrodynamics and diffusion suffice to explain the phenomena. To prevent their collapse, pressure within the blood vessels must be greater than in the surrounding tissue spaces. Since there is only slight net fluid flow through the permeable capillary walls, the hydrostatic pressure must be balanced by a chemical potential (osmotic pressure).[49] In fact, the *mean* hydrostatic pressure drop across the capillary wall equals, within experimental error, the colloid osmotic pressure.[50,51] The ionic and small-molecule composition of lymph is identical with that of plasma, when allowance is made for Donnan equilibria across the capillary membrane.[52] Consequently, active secretion by capillaries is unimpor-

(47) T. Peters and C. B. Anfinsen, *J. Biol. Chem.* **186,** 805 (1950).

(48) A. Fagraeus, Antibody Production in Relation to the Development of Plasma Cells, Esselts Aktiebolag, Stockholm, 1948.

(48*a*) L. L. Miller, W. F. Bale, and C. G. Bly [*J. Exptl. Med.* **99,** 125, 133 (1954)] have shown the incorporation of $C_{14}$ ($\epsilon$-lysine) in all of the plasma proteins, except $\gamma$-globulin, by perfused liver and in $\gamma$-globulins by the perfused eviscerated carcass. The perfused carcass also incorporated small amounts of activity into the $\alpha$ and $\beta$ globulins. However, this may have represented merely binding of metabolic products of lysine by the lipoproteins, rather than protein synthesis.

(49) E. H. Starling, *J. Physiol.* **19,** 312 (1896).

(50) A. Krogh, E. M. Landis, and A. H. Turner, *J. Clin. Invest.* **11,** 63 (1932).

(51) An interesting difference arises in the octopus, which in spite of a high hydrostatic pressure[28] shows a low colloid osmotic pressure.[51a] It would be interesting to know how the octopus has resolved this difficulty, whether by a large pressure drop between the heart and the permeable capillary, or by a much larger amount of lymph flow, or still otherwise.

(51*a*) Aortal pressure = 25–80 mm. Hg.[28] Colloid osmotic pressure = 2.7 mm. P. Meyer, *Compt. rend. soc. biol.* **120,** 305 (1935).

(52) E. Muntwyler, R. C. Mellors, F. R. Mautz, and G. H. Mangun, *J. Biol. Chem.* **134,** 389 (1940).

tant or non-existent. (The possibility of special secretory mechanisms for certain trace components cannot, of course, be excluded.)

Since there is a continuous pressure drop along the length of the capillary, at the arterial end the hydrostatic pressure exceeds osmotic pressure and fluid flows out through the capillary wall, while at the venous end fluid is drawn into the capillary from the interstitial space. Fluid not reabsorbed is collected by the lymphatic system and emptied into a vein. This lymph contains the plasma proteins which have leaked out, now concentrated by water reabsorption. Nevertheless, filtration appears to be relatively unimportant as a physiological process; the rate of flow even at the extreme arterial end is slow enough that for most substances diffusion is the important consideration.[40] That is, filtration (motion of solute molecules with the stream) is unimportant compared to Brownian movement. (This is, of course, fortunate at the venous end of the capillary where the metabolite must diffuse against the current.) Another corollary of this is the fact that capillaries must be geometrically so distributed as to permit adequate diffusion to every cell, as indicated in sec. I. (Krogh has shown them to be of the order of 0.02 mm. apart.)[53]

Thus far we have dealt with capillary pressure drops across the capillary wall. Absolute capillary pressure must of course exceed atmospheric pressure by this drop (colloid osmotic pressure) plus tissue pressure. Tissue pressure may be considered as the elastic resistance to expansion of the tissue. Consequently, if the colloid osmotic pressure decreases (hypoproteinemia) while the hydrostatic capillary pressure remains constant, the tissue must swell (edema). Conversely, in therapy the injection of plasma protein raises the colloid osmotic pressure, and fluid flows back into the blood stream, from whence it is excreted through the kidney, relieving the edema. (The mechanism of the diuresis produced by elevating the colloid osmotic pressure of plasma is not so obvious, since by decreasing the chemical potential of water, glomerular filtration should be decreased.)

### 2. Capillary Porosities and Renal Function

Membranes may be considered as molecular sieves permitting the separation of molecules on the basis of size. This has been well demonstrated for artificial membranes[54] although completely uniform pore size has not been attained.

Viewed as a sieve, the vascular membrane would appear to have different pore sizes in different organs, varying from the smallest pores

(53) A. Krogh, *J. Physiol* **52**, 409 (1919).

(54) W. J. Elford and J. D. Ferry, *Brit. J. Exptl. Path.* **16**, 1 (1935).

in the kidney glomeruli to the largest in the liver capillaries.[23,55] Extensive data are available on the renal glomerulus, based on kidney function studies and on the beautiful experiments of Richards *et al.* on isolated glomeruli. It is generally agreed that proteins larger than albumin do not pass the glomerular membrane. However, among molecules of sizes similar to albumin some discrepancies appear.

Although Richards was unable to detect any protein in the ultrafiltrate from the glomerulus,[56] albuminuria is a common occurrence in kidney disease. Although this might be attributed to an altered glomerular permeability, over-all kidney function studies are complicated by the reabsorption of molecules from the glomerular filtrate by the renal tubules. In kidney disease, when glomerular abnormalities are not present, albuminuria could be attributed to a lack of tubular reabsorption. Therefore altered glomerular permeability appears an unnecessary hypothesis.

We might ask whether the sensitivity of Richards' test for protein was adequate. Clearance studies on hemoglobin excretion indicate that once the threshold is passed (about 100 mg. per cent—a measure of tubular reabsorption capacity), the urinary concentration bears a direct relation to other small molecules which are not reabsorbed. From these data, the concentration of hemoglobin in the glomerular filtrate has been calculated to be 3 per cent of that in plasma.[57]

Albumin has the same molecular weight as hemoglobin, although hemoglobin may dissociate into half molecules. However, albumin is definitely more asymmetric, with an axial ratio of 4:1 vs. 1:1 for hemoglobin, and it is not certain what dimension of the molecule should be used as a measure of size when discussing membrane permeability. Since Brownian motion precludes the possibility of orientation in the stream flowing through the pore, it would seem likely that effective size might prove more a function of the length of the molecule than of its volume, and if the path through the pore were tortuous, a rigid rod would be still further impeded.

Therefore, the concentration of albumin in the glomerular filtrate might be estimated at $<60$ mg. per cent (i.e., $<2$ per cent of the plasma level). (Dock measured 20 mg. per cent in perfusates of chilled rabbit kidneys.[57a]) This concentration might well be below the sensitivity of Richards' test.[56]

The permeability of the renal glomerulus to molecules as large as

(55) A recent electronmicrograph of the kidney appears to confirm the physiological evidence. B. V. Hall, *Federation Proc.* **12,** 467 (1953).

(56) P. A. Bott and A. N. Richards, *J. Biol. Chem.* **141,** 291 (1941).

(57) C. L. Yuile, *Physiol. Revs.* **22,** 19 (1942).

(57a) W. Dock, *New England J. Med.* **227,** 633 (1942).

albumin may merely point to the importance of a renal mechanism for the excretion of large metabolic fragments of proteins and other structural elements of cells. Owing to their extremely low concentration, little is known about the proteinaceous components of normal urine.[58]

### 3. Osmotic Properties of the Plasma Proteins

The osmotic efficiencies of several plasma proteins have been studied by Scatchard *et al.*[59–61] (Fig. 2). Serum albumin is responsible for ¾ of the colloid osmotic pressure of human plasma, although it represents approximately only ½ of the plasma proteins. This high efficiency is the result of its smaller size (65,000 vs. 90,000 for the average molecular weight of plasma proteins).

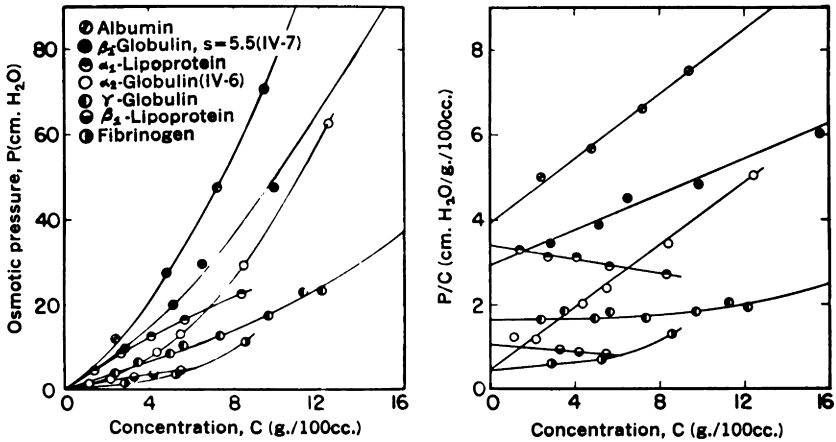



Fig. 2. Osmotic pressure of various plasma proteins at 37°C. (From Oncley *et al.*)[61]

Under physiological conditions approximately ⅓ of the colloid osmotic pressure is contributed by the Donnan effect.[59] A proper evaluation of this effect cannot be obtained from titration curves because of ion-binding. It is most readily derived from a complete study of the ion distribution in osmotic measurements where it can be obtained directly from the distribution of free (not bound) ions. Thus Scatchard *et al.* have obtained it from chloride-activity measurements on both sides

(58) A very large mucoprotein (mol. wt. = $7 \times 10^6$) which obviously cannot pass the glomerular membrane is present in amounts of 0.02 g./l. of normal human urine. I. Tamm and F. L. Horsfall, Jr., *J. Exptl. Med.* **95**, 171 (1952).

(59) G. Scatchard, A. C. Batchelder, and A. Brown, *J. Clin. Invest.* **23**, 458 (1944).

(60) G. Scatchard, A. C. Batchelder, and A. Brown, *J. Am. Chem. Soc.* **68**, 2320 (1946).

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

of the membrane, or by measurements of sodium chloride distribution, assuming that sodium is not bound.[60]

The effect of albumin administration on plasma volume has been studied by Heyl, Gibson, and Janeway who report that 1 g. of albumin increases plasma volume by 17 ml.[62] in striking agreement with the predicted value of 18 ml. (at 25 mm. Hg pressure) calculated from osmotic-pressure measurements.[59]

## 4. Serum Albumins

### *a. Definitions*

The mammalian serum albumins, which as indicated above provide most of the colloid osmotic pressure of plasma, have been variously defined as our knowledge has increased, each new redefinition having been generally in the direction of excluding some related protein found to differ from the bulk of the serum albumins in some particular. Thus the earliest classifications on the basis of solubility defined serum albumins as those proteins soluble in 0.5 saturated ammonium sulfate or soluble upon dialysis against distilled water saturated with carbon dioxide.[63,64] That such albumins contained appreciable quantities of quite different components was realized at that time by chemical studies of the composition of subfractions obtained from them by crystallization[65] or other means. Electrophoretic analysis, as developed by Tiselius,[66] further resolved the components and made possible a new definition of human serum albumin as the major component in electrophoretic analysis at pH 7.7 or 8.6 and having mobilities of $-5.2$ and $-6.0$ Tiselius units in phosphate $\Gamma/2$ 0.2, pH 7.7 and veronal $\Gamma/2$ 0.1, pH 8.6, respectively.[67] (The veronal buffer would appear to be more critical as it better resolves any $\alpha_1$-globulin present.)[67] Bovine albumin and probably those of other species can be similarly defined.

Analysis for carbohydrate appears to be a simple chemical test for albumin purity, since from such electrophoretically homogeneous (i.e., 98 per cent or better) human or bovine albumin the majority of the protein may be crystallized free of carbohydrate, and the carbohydrate

(62) J. T. Heyl, J. G. Gibson 2d, and C. A. Janeway, *J. Clin. Invest.* **22,** 763 (1943).
(63) P. Panum, *Virchow's Arch. pathol. Anat. Physiol.* **4,** 419 (1852); Kauder, *Arch. exptl. Path. Pharmakol.* **26**; P. S. Denis, Memoir sur le Sang, Paris, 1859.
(64) For further historical data see E. J. Cohn, *Physiol. Revs.* **5,** 349 (1925).
(65) A. Gürber, *Sitzber. physik. med. Ges., 1894, Würzburg,* p. 143 (1895).
(66) A. Tiselius, *Trans. Faraday Soc.* **33,** 524 (1937).
(67) S. H. Armstrong, Jr., M. J. E. Budka, and K. C. Morrison, *J. Am. Chem. Soc.* **69,** 416 (1947).

present before crystallization can reasonably be allocated to the small amounts of $\alpha$-globulins present by assigning them a carbohydrate content of 10 per cent or less.[68,69]

### *b. Heterogeneity*

However, albumins so defined still do not represent a homogeneous protein; nor do they become so upon repeated recrystallization from ethanol–water mixtures.[68] Heterogeneity may be detected by electrophoretic analysis at more critical pH values. Thus the most highly purified albumins have continued to show two or more components at pH 4.0 as first observed by Luetscher.[70] They also show reversible boundary spreading at pH 4.6.[71] The latter test is more informative. The pH 4.0 measurements, although quite reproducible, are more difficult to interpret, since they are carried out in a region where the stability of albumin is questionable and, furthermore, the patterns obtained are particularly sensitive to the ionic environment.

Serum albumins, repeatedly recrystallized, were also found to be heterogeneous when tested for constancy of solubility with excess saturating body in buffered ethanol–water systems.[72] As an exception, McMeekin reported a fraction of horse serum albumin crystallized as the sulfate at pH 4.0, which showed constant solubility when recrystallized from ammonium sulfate and then equilibrated with the same solvent.[73] Unfortunately, he did not extend his studies over a wide enough range of saturating body to see how rigorously this criterion was obeyed. Kendall[74] has also reported a crystalline complex of human serum albumin with fatty acids. This showed remarkably constant solubility. However, his material must have been heterogeneous with regard to fatty acid content (i.e., mixed stearic, oleic, linoleic, etc.) and the albumin itself was presumably heterogeneous as regards its sulfhydryl content, as will be discussed below. Therefore, it seems probable that Kendall was studying a solid solution in equilibrium with a solution of similar composition. Northrop *et al.* have observed a similar phenomenon with pepsin.[75]

(68) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *J. Am. Chem. Soc.* **69,** 1753 (1947).

(69) By definition are thus excluded previously described "carbohydrate-containing albumins." (These will be discussed under glycoproteins, sec. VIII.)

(70) J. A. Luetscher, *J. Am. Chem. Soc.* **61,** 2888 (1939).

(71) R. L. Baldwin, P. M. Laughton, and R. A. Alberty, *J. Phys. Chem.* **55,** 111 (1951).

(72) W. L. Hughes, unpublished observations.

(73) T. L. McMeekin, *J. Am. Chem. Soc.* **61,** 2884 (1939).

(74) F. E. Kendall, *J. Biol. Chem.* **138,** 97 (1941).

(75) R. M. Herriot, V. Desreux, and J. H. Northrop, *J. Gen. Physiol.* **24,** 213 (1940).

Simple chemical evidence of heterogeneity lies in the sulfhydryl content of bovine and human serum albumins. The author has always found considerably less than one thiol group per molecule of human or bovine albumin unless the albumin had been fractionated with the aid of mercury.[76] However, material so fractionated has still appeared heterogeneous by solubility criteria and by electrophoretic analysis at acid pH.

A small degree of heterogeneity is also found upon ultracentrifugal analysis in 0.1 ionic strength buffers. This is usually evinced as a slight asymmetry of the schlieren diagram. However, in "bad" preparations a distinct shoulder may be observed moving ahead of the main peak with a sedimentation constant attributable to an albumin dimer. The amount of this component is a function of the age and past history of the albumin sample. Storage in the "dry" state at room temperature appears to be particularly deleterious. However, relative to the other measurements of heterogeneity, ultracentrifugal evidence would appear of minor importance, since the amount of heterogeneity is usually less than 10 per cent and in a good preparation is probably less than 5 per cent.

As to the ultimate basis of albumin heterogeneity, several postulates might be kept in mind. First, it might be assumed that the biosynthetic mechanism is not precise enough for exact duplication of molecules of the complexity of serum albumins. However, such a postulate appears unlikely from what is known already of biosynthetic specificity. A second postulate, of consequences similar to the first, would be that the albumin molecule may undergo slight alterations during its life in the circulation. Thus a population of young and old molecules would be heterogeneous. In terms of structure this may be exemplified by the fractional sulfhydryl content of serum albumin. The sulfhydryl is known to be a very labile group subject to oxidative or coupling reactions under physiological conditions. However, to extend such reasoning to other groupings is difficult at the present state of our knowledge. Oxidative alterations in the phenolic group of the tyrosyl residues of this protein have not been reported. Hydrolytic changes which might appear as changes in net charge (hydrolysis of amides) do not occur in large degree, as judged by electrophoretic homogeneity. The small amount of reversible boundary spreading observed by Alberty at the isoelectric point could be explained, he feels, by a unit variation in the net charge, and yet there are 86 hydrolyzable amide linkages.[71]

The heterogeneity observed electrophoretically and by solubility studies may be due to tightly bound charged impurities. Serum albumins bind many anions with extreme avidity although not in simple stoichiometric amounts (see sec. 4*g*. and Chap. 8). Thus purified albumins

(76) W. L. Hughes, Jr., *J. Am. Chem. Soc.* **69**, 1836, (1947).

usually contain small amounts of the higher fatty acids in amounts of a fraction of a mole of each per mole of protein.[68] Whether such heterogeneity can explain solubility behavior is uncertain. The author has found but slight evidence for fractionation in terms of fatty acid content under the conditions of his solubility tests (ethanol–water systems). On the other hand, Kendall reports considerable fractionation of fatty acid content during crystallization from ammonium sulfate.[74]

A final postulate regarding albumin heterogeneity may be that there is a variation among individual animals of the same species. This would make the albumins, now available in large amounts obtained from pooled plasmas, more heterogeneous than those described by the early workers, which were frequently obtained from a single animal. Immunological evidence for individual variations in proteins or protein conjugates is available, being the explanation of blood types. Such variation is presumably also the cause of failure of tissue grafts from one individual to another.

In spite of the evidence for heterogeneity cited above, the properties of serum albumin studied by most workers can be described in terms of a single protein component. Therefore in the following paragraphs serum albumin will be treated as if it were homogeneous unless specifically stated to the contrary.

### *c. Crystallization*

Serum albumins are generally purified by crystallization procedures, although a remarkably pure product has been obtained by ethanol fractionation.[77] Salting-out procedures for crystallizing horse[65,73,78,79] and human[74,80] serum albumins have been described, bovine serum albumin thus far not having yielded to such methods.[81] The salts used have included ammonium sulfate, magnesium sulfate, sodium sulfate, and sodium dihydrogen phosphate. Of these, ammonium sulfate is by far the most convenient because of its greater solubility, magnesium sulfate and sodium sulfate being too insoluble to precipitate albumin in the cold. Sodium dihydrogen phosphate provides a useful buffering

(77) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin, and H. L. Taylor, *J. Am. Chem. Soc.* **68,** 459 (1946).

(78) S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg* **18,** No. 5, 1 (1930).

(79) L. F. Hewitt, *Biochem. J.* **30,** 2229 (1936).

(80) M. E. Adair and G. L. Taylor, *Nature* **135,** 307 (1935).

(81) The author has observed crystallization in bovine albumin solutions concentrated by vacuum distillation of the supernatant after precipitation of the globulins by Cohn's method 6 (precipitates I through IV-4). In this case the albumin was salted-out by simultaneous concentration of the acetate buffer added in fractionation.

action. Crystallization at low ionic strengths with the aid of organic precipitants has been used for human and bovine albumins.[68,74] Ethanol has been the reagent of choice, but methanol would appear equally useful, and at least upon occasion acetone may be used.[82] When sufficiently purified, usually by previous crystallization, bovine and human serum albumin (or at least a fraction of them) may be crystallized from water.[68] Horse albumin has also been crystallized as its sulfate at pH 4 from water.[73]

In general, serum albumins crystallize most readily at a pH slightly alkaline to their isoelectric points (0.2–0.5 pH units) from concentrated solutions containing 10 per cent or more of protein. A variety of small molecules which are tightly bound to albumin facilitates crystallization from ethanol–water systems. These include the higher alcohols from pentanol to decanol, toluene, chloroform, and sodium oleate. Fatty acid salts also appear to be involved in Kendall's method for the crystallization of human albumin from ammonium sulfate.[74] Lewin has further shown that a variety of ions and small molecules may be bound to serum albumin without hindering the ability of the albumins to crystallize.[82] The mechanism of action of these agents is unknown. However, it is suggestive that sodium oleate and decanol have been found to decrease markedly the dielectric increment of serum albumin.[83] This indicates a change in the charge distribution, which must be one of the orienting forces for crystallization.

Both human and bovine serum albumins crystallize as dimer units when linked with a mercury atom.[84] The amount of mercuric salt added must be carefully adjusted to equivalence with the sulfhydryl content if optimal yields are to be obtained. The crystals form from aqueous ethanol solutions under conditions comparable to those for crystallizing the monomer, excepting that the dimer solubility is appreciably lower, so that they can be readily recrystallized from water. In this way the mercaptalbumin may be readily separated from the thiol-free protein, two or three recrystallizations usually sufficing to give the pure dimer as judged by ultracentrifugal analysis (Fig. 5) or content of mercury.

### *d. Physicochemical Properties*

The physicochemical properties of serum albumin have been frequently mentioned in Vol. I of this text and will be only summarized here. In general, they can be explained in terms of an elongated ellipsoid of

(82) J. Lewin, *J. Am. Chem. Soc.* **73**, 3906–11 (1951).

(83) H. Dintzis, Doctor's Dissertation, Harvard University, Cambridge, Mass., 1952.

(84) Details of these crystallization procedures are in preparation (Hughes and Dintzis).

revolution with a molecular weight (anhydrous) of 65,000, a length of 150 A., and a diameter of 38 A. (axial ratio of 4:1). Assuming an ellipsoid of revolution, these dimensions best coordinate the various physicochemical data (i.e., unit cell dimensions of the crystal, sedimentation and diffusion constants, viscosity, and length from double-refraction of flow). However, the assumption of an ellipsoid of revolution may be a poor approximation to the true shape, and even small deviations from this (e.g., a cylinder or a molecule with holes in it) will require quite different dimensions, as Schulman has pointed out for fibrinogen.[90]

Even the molecular weight, which is capable of direct measurement, is a matter of some uncertainty. It will be noticed that the value of 65,000 is appreciably smaller than the previously accepted value of 69,000.[68] This new value is based primarily on the recent x-ray crystallographic data of Low,[85] who calculated 65,200 and 65,600 for two types of human serum albumin crystals and was confirmed in the ultracentrifuge by Creeth[86] and by Koenig and Perrings,[87] who calculated molecular weights of 65,400 and 64,500, respectively.

Regarding the earlier data leading to a mean value of 69,000, methods giving an average molecular weight (such as osmotic pressure and light scattering) will all overestimate the true molecular weight if heavier components, as discussed above, are present. Previous sedimentation constants have also varied owing to technical difficulties, of which the most serious has been correct measurement of temperature.[88] Recently Waugh has discovered a temperature change in the rotor upon acceleration, due to expansion of the metal.[89]

Various physicochemical evidence in the past has indicated a molecular weight of serum albumin much smaller than 65,000.[91,92] However, further investigation has always revealed either faulty experimental technic or incorrect interpretation of the data. Now that such methods are bolstered by analytical composition indicating that human albumin contains only one thiol and one tryptophanyl per 65,000, and bovine one thiol and one polypeptide chain (one free $\alpha$-amino and one free $\alpha$-carboxyl group),[93,94] the evidence would appear fairly conclusive.

(85) B. W. Low, *J. Am. Chem. Soc.* **74,** 4830 (1952).
(86) J. M. Creeth, *Biochem. J.* **51,** 10 (1952).
(87) V. L. Koenigs and J. D. Perring, *Arch. Biochem. and Biophys.* **41,** 367 (1952).
(88) S. Shulman, *Arch. Biochem. and Biophys.* **44,** 230 (1953).
(89) D. F. Waugh and D. F. Yphantis, *Rev. Sci. Instruments* **23,** 609 (1952).
(90) See Ref. 310.
(91) T. Svedberg, *Nature* **139,** 1051 (1937).
(92) G. Weber, *Biochem. J.* **51,** 155 (1952); *Discussions Faraday Soc.* **13,** 33 (1953).
(93) H. Van Vunakis and E. Brand, Abst. 119th meeting Am. Chem. Soc., p. 28c (1951).
(94) P. Desnuelle, M. Rovery, and C. Fabre, *Compt. rend.* **233,** 987 (1951).

Thus it would seem better to use albumin to test the physicochemical methodology, rather than vice versa.

Isoelectric serum albumin contains approximately one hundred pairs of positive and negative charges. While the word pair implies a very special charge distribution, its choice is suggested by the apparent high symmetry in charge distribution, resulting in a net charge asymmetry which can be represented by a dipole vector of a positive and a negative charge separated by 150 Å (700 Debye units) and inclined at an angle of 30° with the long axis of the molecule. The variation in magnitude of this dielectric increment from preparation to preparation has now been explained in terms of the fatty acid content of the albumin. Removal of fatty acid (by passage through anion-exchange columns on the acetate cycle) increased the dielectric increment, and subsequent addition of sodium oleate reduced it to the original value. The most marked effects were noticed with the first mole of fatty acid added per mole of protein. More surprisingly, perhaps, decanol had a similar effect.[83]

The solubility of serum albumin is higher than that of the other major plasma components in practically all solvent systems, so that separations are usually based on precipitating the other components while leaving the albumin in solution. Albumin is generally considered to be water-soluble, this being one of the early definitions of the albumin fraction. However, fractions of serum albumins can be crystallized from distilled water and then prove to be relatively insoluble in this solvent.[68,73] This is particularly true of the mercury albumin dimer.

Albumin solubility may be conveniently investigated in alcohol–water systems where the alcohol concentration, ionic strength, pH, temperature, and specific ion effects may be independently varied. Since constant solubility with variation of saturating body cannot be achieved, solubility may only be expressed in relative terms as a function of changing the parameters. In this way the effect of several variables on the solubility can be studied and reproducible values obtained for any given albumin preparation. The effects of ionic strength and ethanol concentration are thus illustrated in Fig. 3 over a limited pH range.[95] Albumin possesses a marked solubility minimum at the isoelectric point, the solubility increasing manyfold as the charge on the protein increases. Albumin solubility may increase with rising temperature as in alcohol–water systems, be largely independent of temperature as in the presence of polyelectrolytes,[96] or decrease with rising temperature as in the presence of zinc ions.[97]

(95) D. Mittelman, *in* E. J. Cohn, Blood Cells and Plasma Proteins, Academic Press, New York, 1953, p. 24.

(96) H. Morawetz and W. L. Hughes, *J. Phys. Chem.* **56**, 64 (1952).

(97) A. Weber, personal communication.

Albumin shows a tendency to interact with other proteins so that its solubility in natural systems may be quite different from that in the isolated state. Thus it is frequently carried down in globulin fractions under conditions where it should be soluble. In protein fractions of liver

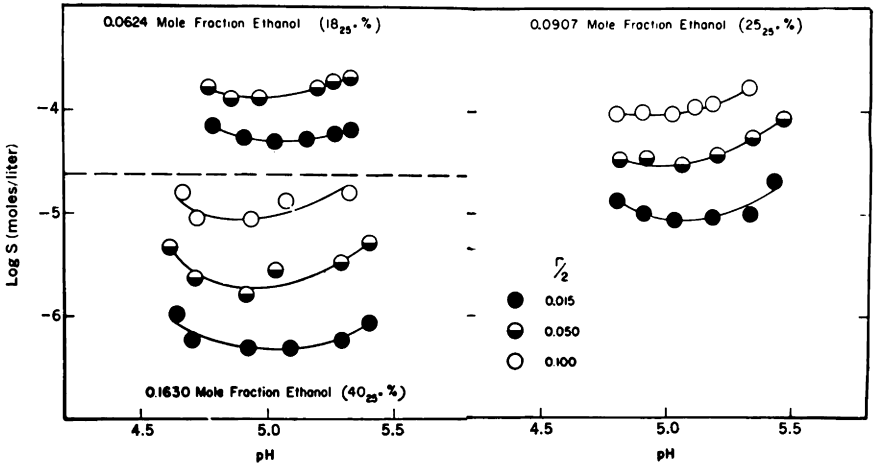



FIG. 3. Solubility of crystallized human serum albumin in ethanol–water mixtures at −5°C.[95]

cells, combination was so firm that only immunochemical methods could detect the albumin present.[98] Fortunately, albumin is present in plasma in much larger amounts than any other component, so that interaction usually does not greatly diminish yields.

### *e. Stability*

Any discussion of protein solubility leads naturally into a discussion of protein stability, since the effect of time on solubility measurements as well as the extremes in variation of pH, temperature, and solvent composition which can be tolerated must be known. Serum albumin is one of the most stable of the plasma proteins. In the cold it appears stable from pH 10 to pH 4. It may be stable at even greater acidities; however, it shows progressive degrees of aggregation in the ultracentrifuge.[99] This may be the fibril formation described by Waugh.[100]

At elevated temperatures serum albumin in concentrated aqueous

(98) E. J. Cohn, Enzymes and Enzyme Systems, Harvard Univ. Press, Cambridge, Mass., 1951.
(99) H. A. Saroff, personal communication.
(100) D. F. Waugh, *J. Am. Chem. Soc.* **68,** 247 (1946).

solution shows a stability maximum between pH 6.5 and 7.0.[45] The stability may be further increased by the addition of many anions.[45,46,101] Sodium caprylate, 0.04 *M*, has proven particularly useful in this regard, making it possible to heat ("pasteurize") albumin for several hours at 60°C.[101] Under such conditions the stability of albumin relative to many other components of plasma is further increased, rendering possible the purification of albumins by selective denaturation of some of the globulins.[68] Under these conditions the rate of denaturation approximately doubles for each 2° rise in temperature,[101] appearing kinetically similar to the heat denaturation of other proteins.

In concentrated urea solutions serum albumin immediately undergoes changes associated with protein denaturation: increase in optical rotation and increase in viscosity[102–104] (see also Chap. 9). These are followed by further changes in the viscosity, finally resulting in a gel. The first rapid changes appear to be quite reversible. The secondary changes have now been interpreted as a disulfide interchange reaction resulting in the formation of intermolecular disulfide bridges and thus a three-dimensional network. Experimental evidence indicates that sulfhydryl groups are essential for the secondary changes.[105] The mechanism may be formulated thus:

$$\mathrm{R{-}SH} + \begin{matrix}\mathrm{S}\\ \diagup\\ \diagdown\\ \mathrm{S}\end{matrix}\!\!>\mathrm{R_1} \rightarrow \mathrm{R{-}S{-}S{-}R_1{-}SH}$$

Where R and $R_1$ represent two protein molecules. The new sulfhydryl group formed may then react again and a chain reaction is started. Serum albumin dissolved in glacial acetic acid also gels over the course of some days, indicating that a similar mechanism may be involved.

Perhaps of greater interest is the amount of reversibility achieved in the first rapid reaction in urea. Albumin recovered from this treatment has been shown to have approximately the same solubility, electrophoretic mobility, viscosity, anion-combining ability, and immunological properties as the starting material.[104,106] Apparently the structure of serum albumin is largely determined by the peptide backbone and the

(101) G. Scatchard, L. E. Strong, W. L. Hughes, Jr., J. N. Ashworth, and A. H. Sparrow, *J. Clin. Invest.* **24,** 671 (1945).
(102) R. B. Simpson and W. Kauzmann, *J. Am. Chem. Soc.* **75,** 5139 (1953).
(103) W. Kauzmann, *in* McElroy and Glass, The Mechanism of Enzyme Action, Johns Hopkins Univ. Press, Baltimore, 1954.
(104) H. Neurath, G. R. Cooper, and J. O. Erickson, *J. Biol. Chem.* **142,** 249 (1942).
(105) C. Huggins, D. F. Tapley, and E. V. Jensen, *Nature* **167,** 592 (1951).
(106) J. O. Erickson and H. Neurath, *J. Exptl. Med.* **78,** 1 (1943).

disulfide cross-links, and, provided these links are unaltered, considerable amounts of configurational changes may occur reversibly. Further examples of this will appear in the following discussion.

### *f. Composition*

Serum albumin appears to be composed exclusively of amino acid residues. By definition (see sec. II-4*a*) no carbohydrate (giving tests by methods involving furfuraldehyde formation) is present,[68] and nothing resembling a prosthetic group has ever been reported. The absorption spectrum can be interpreted in terms of the aromatic amino acid content.[107–109] The elementary composition has been reported by Brand.[110] Complete amino acid analyses[110–113] by several workers are summarized in Table II. Recoveries of up to 98 per cent of the protein give further evidence for the presence of only amino acids in this protein.

Particular uncertainty dwells upon the tryptophan determination, since all hydrolytic methods appear to give considerable destruction of this amino acid.[114] However, color reactions on the intact protein confirm the fact that bovine serum albumin contains more tryptophan than human. Tryptophan analyses were regularly carried out by the early workers during the fractionation of horse serum albumin, obtaining values which indicated one or two groupings per albumin molecule. However, inconsistencies concerning the effect of protein fractionation on the tryptophan content appear in their work.[115] Certainly a reliable method of tryptophan analysis is badly needed, and would provide an additional criterion for protein homogeneity.

The sulfur distribution in serum albumin should also be further investigated. Brand found in bovine serum albumin 42 sulfurs per 69,000 g. of protein (this becomes 40 per 65,000 g.) and *even* numbers of cysteine and methionine residues[110] (Table II). However, there is only

(107) D. Gitlin, *J. Immunol.* **62,** 437 (1949).
(108) Frequently, an absorption maximum at 405 mμ appears due to contamination by hemin.
(109) There is a very small amount of absorption in the range 340–400 mμ which cannot reasonably be assigned to aromatic amino acids or Tyndall scattering.
(110) E. Brand, *Ann. N. Y. Acad. Sci.* **47,** 187 (1946).
(111) E. Brand, B. Kassel, and L. J. Saidel, *J. Clin. Invest.* **23,** 437 (1944).
(112) S. Moore and W. H. Stein, *J. Biol. Chem.* **178,** 53 (1949).
(113) D. Shemin, *J. Biol. Chem.* **159,** 439 (1945).
(114) J. R. Spies and D. C. Chambers, *Ind. Eng. Chem., Anal. Ed.* **21,** 1249 (1949).
(115) Thus Hewitt[79] found that the tryptophan content of horse albumin decreased to 0.3 per cent upon isolation of the carbohydrate-free fraction. Whereas McMeekin[73] found a tryptophan content of 0.5 per cent for the carbohydrate-free fraction, which was not altered by further fractionation.

TABLE II
COMPOSITION OF SERUM ALBUMINS[a]
(Residues per 65,000 grams)

| | Bovine | Human | Comparative analyses | |
|---|---|---|---|---|
| | | | Bovine | Equine |
| Alanine | 46 | — | | |
| Glycine | 17 | 14 | (16) | |
| Valine | 33 | 43 | (36) | |
| Leucine | 61 | 54 | (68) | (50) |
| Isoleucine | 13 | 8 | (14) | |
| Proline | 27 | 29 | (32) | |
| Phenylalanine | 26 | 31 | (24) | |
| Tyrosine | 18 | 17 | (20) | (17) |
| Tryptophan | 2 | 1 | | (1–2) |
| Serine | 26 | 20 | | |
| Threonine | 32 | 25 | | |
| Cysteine | 1 | 1 | | |
| Cystine/2 | 34 | 34 | 34 | 34 |
| Methionine | 4 | 6 | 4 | 0 |
| Arginine | 22 | 23 | (23) | (21) |
| Histidine | 17 | 15 | (16) | (18) |
| Lysine | 55 | 55 | 55 | |
| Aspartic acid | 50 | 48 | 50 | |
| Glutamic acid | 75 | 75 | 75 | |
| Amide N | (36) | (41) | | |
| Total S | 40 | 41 | 40 | 37 |

Nitrogen content of bovine serum albumin: measured = 16.07
calculated from above composition = 16.2[b]

[a] Data are largely from Tristram's Chap. 3 but corrected for a molecular weight of 65,000. However, cysteine data have been corrected in the light of my own evidence (see text), and the probably more precise estimates of Shemin[113] by isotope dilution for glycine, lysine, aspartic and glutamic acids have been substituted for Stein and Moore's and Brand's values. The values in columns 3 and 4 were included for direct comparison with column 2 having been carried out for a given amino acid by a single laboratory.[111–113]

[b] i.e. number of nitrogen atoms × 14 ÷ 65,000 × 100.

*one* cysteine in serum albumin,[116] hence, *if* the total sulfur content is correct, there must be an *odd* number of methionine residues or an additional unknown type of sulfur linkage must exist.

Serum albumin may contain a single polypeptide chain since only one terminal $\alpha$-amino group (aspartyl) exists in human, equine, porcine, and bovine serum albumin.[93]

(116) W. L. Hughes, *Cold Spring Harbor Symposia Quant. Biol.* **14,** 79 (1950).

*g. Reactivities*

The reactivities of serum albumin, as of proteins in general, are so numerous that discussion will be limited to those particularly significant as regards to structure and those more or less peculiar to serum albumin. A more general discussion will be found in Chap. 10.

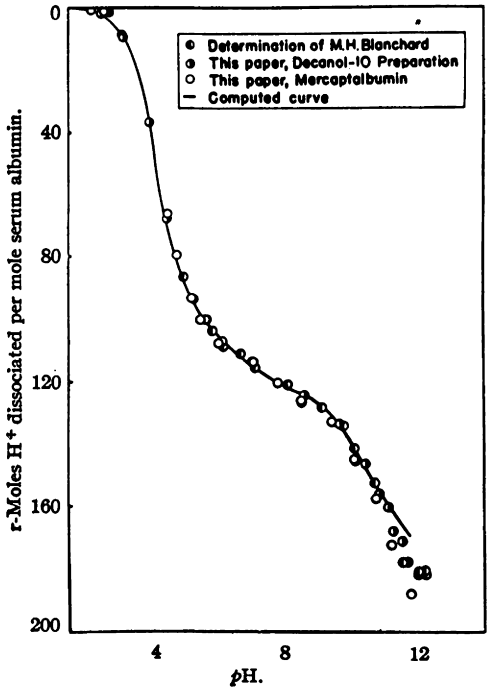



FIG. 4. Titration curve of human serum albumin. (From Tanford.)[117]

*Hydrogen-ion equilibria* will be considered first since they involve practically all of the reactive groupings of serum albumin and must be considered as competitive reactions in most of the other interactions to be discussed later (for further details see Chap. 6, sec. II and Chap. 8, sec. VI-1). The titration curve has been recently redetermined by Tanford[117] (Fig. 4), substantially in agreement with earlier data.

The points in Fig. 4 are experimental; the curve is theoretical, calculated from the amino acid composition with the assignment of appropriate intrinsic p$K$'s (Table VII, Chap. 8, page 791) to each type of residue and allowing for the effect of protein charge on the equilibrium (excepting in the acid range) after the manner of Scatchard.[118]

(117) C. Tanford, *J. Am. Chem. Soc.* **72**, 441 (1950).
(118) G. Scatchard, *Ann. N. Y. Acad. Sci.* **51**, 660 (1949).

The intrinsic p$K$ of the tyrosyl was estimated by a spectrophotometric titration. The intrinsic p$K$'s of the carboxyl, imidazole, and amino groups were then estimated from the experimental data (Fig. 4) by curve-fitting with the help of measurements of the effect of temperature on the equilibria. The heats of ionization of carboxyl, imidazole, and amino groups are sufficiently different (Table VI, Chap. 6 and Table VII, Chap. 8) to allow the estimation of the contribution of each to any portion of the titration curve.

It will be noted that the calculated intrinsic constants correspond fairly closely to those for model compounds (Table VI, Chap. 6). However, the differences may have some significance. Thus the carboxyl groups definitely seem to be more acidic, and this weakened proton affinity appears to be a generally weakened cation affinity, being reflected in zinc binding and calcium binding,[119] which is much weaker per carboxyl grouping for isoelectric albumin than for simple carboxylate compounds. It is interesting to speculate whether this weak cation affinity is a corollary of the strong anion affinity of this protein (see below).

Another interesting feature of the titration curve is the very steep portion in the acid range, corresponding to titration of carboxyl groups as though no charge effects were present. Part of this effect has been explained by anion binding;[117] however, to attribute it completely to anion binding requires the assumption of the unreasonably large value of 89 chlorides bound at pH 2. Therefore it would seem more reasonable to explain it partly by structural changes in the molecule—an uncoiling which would decrease the electrical potential. The decreased sedimentation constant observed in acid solutions[120] could be similarly interpreted in terms of increased asymmetry. Such uncoiling must be quite reversible since there is no hysteresis in the titration curve upon neutralizing an albumin sample which has been brought to any acidity down to pH 2.0.[117]

*The thiol grouping* would seem a logical starting point when discussing the specific reactivities of serum albumin: first, because it is the most reactive grouping (in the sense of organic chemistry); secondly, because its nature and location permit a dimerization reaction between mercaptalbumin molecules which can be followed by the usual physicochemical techniques (Figs. 5–7); and thirdly, because its singular presence makes unnecessary the statistical considerations which complicate studies of the other groupings.

The reaction of this grouping with mercuric ions to form crystallizable dimers, as described above, permitted separation of the mercaptalbumin from the remaining albumin. However, excepting the thiol group (usually present on ⅔ of the albumin molecules), no other differences

(119) F. R. N. Gurd and W. L. Hughes, unpublished observations.

(120) H. Gutfreund, *Discussions Faraday Soc.* **13** (1952).

have been observed in these two fractions, suggesting that the remainder may have lost their sulfhydryl by oxidative or coupling reactions. This view is strengthened by the discovery that a thiol group may be liberated in this fraction by thioglycolate under conditions where the mercaptalbumin fraction does not form additional thiols.[121]

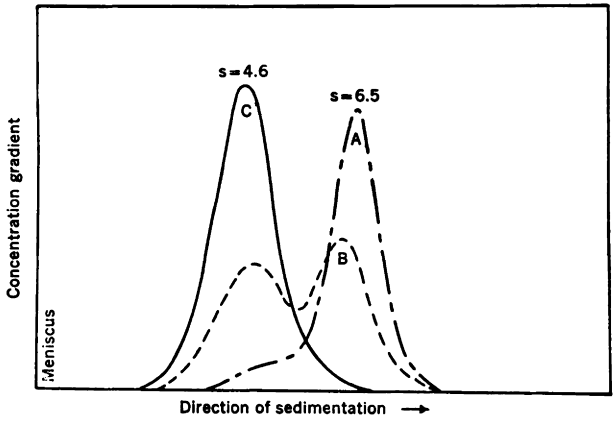
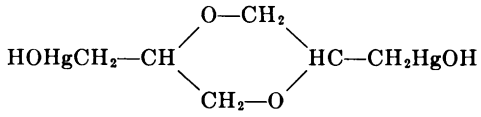



FIG. 5. Sedimentation diagrams of crystallized mercury serum albumin at pH 7. A fresh solution of the crystal sediments at the rate of an albumin dimer ($s$ = 6.5 — curve A). After standing one week, some dissociation has taken place (curve B). Addition of excess mercuric chloride causes immediate dissociation and curve C is obtained with a sedimentation constant ($s$ = 4.6) typical of serum albumin.[116]

While no other metal ions have been found to dimerize albumin like mercury, a similar reaction occurs with a "bivalent" mercurial:




whereupon reaction proceeds manyfold faster.[122] Both dimers may be dissociated by reagents competing with albumin for the mercury (thiols, $CN^-$, and $I^-$ being particularly effective) and by reagents competing with the mercury for the albumin ($Ag^+$, $Hg^{++}$). The dissociating effect of excess mercuric salts (Fig. 6) is explained by the following reaction scheme if it is assumed that the first reaction proceeds much further to completion than the second:

$$\begin{array}{l} \mathrm{AlbSH + HgCl_2 \rightleftharpoons AlbS{-}HgCl + H^+ + Cl^-} \\ \qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad \mathrm{AlbSH} \\ \qquad\qquad\qquad\qquad\quad \Downarrow\Uparrow \\ \qquad\qquad\qquad\qquad\quad \mathrm{(AlbS)_2Hg + H^+ + Cl^-} \end{array}$$

(121) M. Hunter, personal communication.

(122) R. Straessle, *J. Am. Chem. Soc.* **73**, 504 (1951).