[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Preparation and Properties of Serum and Plasma Proteins. XXVIII. The β_1-Metal-combining Protein of Human Plasma[1a,b]

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Following purification from Fraction IV-7, the β_1-metal-combining protein of human plasma has been crystallized from ethanol–water mixtures of controlled *p*H, ionic strength and temperature. The physical and chemical properties of this protein have been studied. The crystallized protein was isoelectric near *p*H 5.9 and had a molecular weight of 90,000. Each molecule of protein was capable of binding two atoms of iron or copper. Iron in complex with the protein was in the ferric state.

The state in which various metals normally exist in the plasma has been of interest since the observations of Barkan[2] that there was a small amount of iron in plasma which was non-dialyzable at physiological reaction, but which appeared in the ultrafiltrate when the plasma was acidified. Similar findings have been made with respect to the behavior of the even smaller quantity of copper normally observed.[3] Later, Barkan[4] demonstrated that the iron in serum was quantitatively precipitated with the globulins by half-saturation with ammonium sulfate. Valquist[5] interpreted electrophoretic observations as indications that the iron in serum is bound to albumin, as well as to α- and β-globulins.

Schade and Caroline[6] first demonstrated that an iron-binding protein of egg white was capable of inhibiting the growth of certain bacteria which require iron for their metabolism. They next demonstrated the presence of an iron-binding protein in plasma, first by this technique and then by spectrophotometric studies.[7] The iron-binding activity was found to be concentrated in Fraction IV-4 of our earlier method of plasma fractionation.[8] Their colorimetric titration, based on the formation of a red complex on addition of iron to the protein, together with the microbiological assay of this activity, made possible the further concentration of this component of plasma into Fraction IV-7, as described in previous papers in these series.[9,10] Striking confirmation of these *in vitro* observations has been obtained in clinical studies using sterile concentrated solutions of Fraction IV-7 as a convenient source of this component for intravenous injection,[11,12] as well as by immunological studies with the crystallized protein.[13] We have called it a metal-combining globulin[14] because *in vitro* studies indicate that it is not specifically an iron-binding protein, but may also bind other metals and because it binds copper by the same groups that bind iron, by which copper is readily displaced.[10,17] That this protein is not concerned with the *in vivo* transport of copper has been suggested by clinical studies[11,12] as well as by chemical studies on the copper-containing protein of plasma. Holmberg and Laurell[16] have shown that the major part of the copper in plasma was normally associated with an α-globulin, precipitable by half-saturation with ammonium sulfate. Some of the properties of this protein, caeruloplasmin, have recently been described.[18]

II. Materials and Methods

Fraction IV-7 was the starting material for these studies. It was derived from blood collected by the American National Red Cross by methods previously described.[9]

Iron-binding capacity was measured by a modification of the method of Schade.[6,9,10]

Copper-binding capacity was measured in a similar manner, using a standard solution of copper (as the sulfate) to titrate a solution of the protein in sodium diethyl barbiturate buffer of ionic strength 0.05 at *p*H 8.4–8.6. Extinction at 435 mμ of the complex was read against the metal-free protein.

Solubility studies were made by adjusting aliquots of an aqueous solution to the desired *p*H, temperature, ionic strength and protein concentration. The resulting suspensions were allowed to equilibrate 24 hours at the desired temperature, after which the solid phase was separated by centrifugation at the same temperature. The solid phase was in the amorphous state. Protein concentration of metal-combining proteins in solution was estimated from the optical density of the solu-

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(2) G. Barkan, *Z. physiol. Chem.*, **171**, 194 (1927).

(3) O. Warburg and K. A. Krebs, *Biochem. Z.*, **187**, 255 (1927); A. Locke, E. R. Main and D. O. Rosbash, *J. Clin. Invest.*, **11**, 527 (1932); R. Boyden and V. R. Potter, *J. Biol. Chem.*, **122**, 285 (1937).

(4) G. Barkan and O. Schales, *Z. physiol. Chem.*, **248**, 96 (1937).

(5) B. Valquist, Das Serumeisen, Diss. Uppsala, Appelbergs Boktrych., 1941.

(6) A. L. Schade and L. Caroline, *Science*, **100**, 14 (1944).

(7) A. L. Schade and L. Caroline, *ibid.*, **104**, 340 (1946).

(8) 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).

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

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

(11) C. E. Rath and C. A. Finch, *ibid.*, **28**, 79 (1949).

(12) G. E. Cartwright, P. Black and M. M. Wintrobe, *ibid.*, **28**, 86 (1949).

(13) B. V. Jager, personal communication.

(14) This component has been called "siderophyllin" by A. L. Schade, R. W. Reinhart and H. Levy[15] and "transferrin" by C. G. Holmberg and C. B. Laurell.[16]

(15) A. L. Schade, R. W. Reinhart and H. Levy, *Arch. Biochem.*, **20**, 172 (1949).

(16) C. G. Holmberg and C. B. Laurell, *Acta Chim. Scand.*, **1**, 944 (1947).

(17) E. J. Cohn, *Blood: J. of Hematology*, **III**, 471 (1948).

(18) C. G. Holmberg and C. B. Laurell, Abstracts 1st Intl. Cong. Biochem., Cambridge, England, 1949, p. 367; *Acta Chim. Scand.*, **I**, 944 (1947); **2**, 550 (1948).

tion, measured against a protein-free blank at 280 mμ ($E^{1\%}_{1\,cm.}$ 11.2).

Partial specific volume and viscosity were determined as described by Oncley, Scatchard and Brown.[19]

The general procedures and equipment used in this Laboratory for the fractionation of proteins, have been described previously.[8]

The author is grateful to C. G. Gordon and P. M. Baker, working under J. L. Oncley, for the ultracentrifugal analyses, and to M. J. E. Budka for the electrophoretic analyses.

III. Purification of β_1-Metal-combining Protein From Fraction IV-7

The β_1-metal-combining protein represented 76% of Fraction IV-7,[9] albumin and α_2-globulins being the main impurities. At this stage of the fractionation the protein had been freed of most of the plasma iron by previous exposure to relatively low *p*H's. The β_1-metal-combining globulin was isoelectric near *p*H 5.8, while the albumin and α_2-globulin were both in an isoelectric condition near *p*H 5.0. Separation of the β_1-globulin in pure state from Fraction IV-7 at reactions near or alkaline to *p*H 5.8 was first attempted, since all proteins should be negatively charged, and interaction due to protein salt formation was expected to be minimal under these conditions. However, strong protein–protein interaction between the α_2- and β_1-globulins rendered these attempts unsuccessful.[9]

Attention was therefore turned to conditions acid to the isoelectric points of all proteins in the fraction, where the tendency toward interaction might also be expected to be minimal. At *p*H 4.3 to 4.5, ionic strength 0.10 and 0.0907 mole fraction (25%) ethanol[20] at −5°, most of the α_2-globulin was observed to precipitate. Even at this *p*H, however, some β_1-globulin precipitated with it. Ultracentrifugal studies suggested that although the β_1-globulin was not altered by this acid treatment, even at relatively high ionic strength, the precipitated α_2-globulin was largely denatured.

The protein remaining in solution following this treatment consisted principally of β_1-globulin and albumin. The former could be separated in nearly pure state at *p*H 6.2, ionic strength 0.24 and 0.1630 mole fraction ethanol (40% at 25°) at a temperature of −5°. The precipitate so obtained consisted to 90–95% of β_1-metal-combining protein and contained approximately 70% of the β_1-globulin from Fraction IV-7.

In order to decrease the salt concentration, which was inconveniently high for crystallization, the protein paste, separated at *p*H 6.2, was triturated with three volumes of 40% ethanol at −5°. The detailed procedure is given below.

Each kilogram of Fraction IV-7 paste was dissolved in 9 liters of water, about one-third of which was in the form of fine ice crystals. The solution was adjusted to *p*H 4.45, Γ/2 0.1, 0.091 mole fraction (25%) ethanol and −5° at a protein concentration of 1%. This was accomplished by adding, per kilo of starting paste, sufficient *p*H 4.0 sodium acetate buffer (0.8 molar) to adjust the *p*H to 4.45, and sufficient *p*H 4.45 sodium acetate buffer to bring the total sodium acetate in the two buffers to 2.5 moles. The combined buffers were added in sufficient water so that the volume, after addition, was 13.9 liters. Addition was carried out, with stirring, through capillary jets. To this solution at *p*H 4.45 was then added 11.1 liters of 53.3% ethanol, the temperature being maintained at the freezing point until a temperature of −5° was attained, and at −5° thereafter. The resulting suspension was stirred for two hours.[21]

Fraction IV-7-1 was removed by centrifugation at −5° at a rate of 30 liters per hour.

The solution after centrifugation was adjusted to *p*H 6.2, 0.163 mole fraction (40%) ethanol, Γ/2 0.24 at −5°. Sodium bicarbonate, suspended in a small volume of water was added to adjust the *p*H.[22] This demanded efficient stirring, as copious amounts of carbon dioxide were evolved. The *p*H was read after 1–2 hours equilibration. Sufficient 95% ethanol was then added through capillary jets, at −5°, to raise the ethanol to mole fraction 0.16.

Fraction IV-7-2 was separated by centrifugation at −5° at a rate of 30 liters per hour. It contained 90–95% β_1-metal-combining protein. The paste obtained from the centrifuge was suspended in three volumes of ethanol of mole fraction 0.167 (40%) ethanol at −5°, stirred for at least 2 hours, and again centrifuged in order to lower its salt content. The final washed paste was then dissolved in two volumes of water containing ice, and dried from the frozen state.

IV. Crystallization of β_1-Metal-combining Protein

Crystallization of the metal-combining globulin from Fraction IV-7-2 was carried out by a procedure similar to those used in the crystallization of human serum albumin.[23,24] The range of conditions for crystallization acid and alkaline to the isoelectric point, are given in Table I.

TABLE I

CONDITIONS FOR THE CRYSTALLIZATION OF β_1-METAL-COMBINING PROTEIN

*p*H	5.2–5.5	6.1–6.5
Γ/2	0.01–0.02 (chloride)	0.01–0.05 (chloride or acetate)
Ethanol concn., %	10–14	14–20
Protein concn., %	8–10	8–12
Temp., °C.	0–−5	0–−5

Unlike the albumins, however, the β_1-globulin crystallized with considerable difficulty. Spontaneous crystallization at *p*H 6.2 occurred only with preparations of at least 95% purity. Less pure fractions generally yielded crystals after seeding. Only the highly purified protein crystallized at *p*H 5.2–5.5. The impurities in Fraction IV-7-2 tended to precipitate amorphously during the crystallization. After two or three recrystallizations it was possible to obtain β_1-globulin which was homogeneous by electrophoresis and in the ultracentrifuge. The purified metal-combining globulin was crystal-

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

(20) Ethanol concentrations are given both as mole fraction and as volume per cent at 25°.

(21) If dry Fraction IV-7 were used as the starting material, these same conditions were obtained when each 250 g. of dry protein was dissolved in 10 liters of water at 0°. The same amount of buffer was required, the volume was adjusted to 13.3 and 11.7 liters of 53.3% ethanol was added.

(22) We have found it advantageous to keep to a minimum the time that the reaction of the solution was acid to *p*H 5. It has usually been possible to carry out the precipitation of Fraction IV-7-1 and add at least part of the bicarbonate to the supernatant solution in one day.

(23) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(24) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

lized by the following procedure, which is taken from a typical experiment.

One hundred grams of dry Fraction IV-7-2 was dissolved in 500 ml. of water at 0°. The *p*H was adjusted to 6.3 by the addition of the required amount of *p*H 4 sodium acetate buffer. The solution was then clarified by passage through a D-5 filter pad. It was then diluted to 700 ml. with water. The amount of 53.3% ethanol necessary to decrease the solubility of the protein just to the point of amorphous precipitation at 0° was next determined with an aliquot of this solution. The required amount of 53.3% ethanol was added through a capillary jet with adequate stirring. The temperature was maintained at 0°. The solution remained clear at the end, but if cooled rapidly to −3°, a copious precipitate formed due to the decreased solubility of the protein at the lower temperature. The solution, contained in a glass flask or bottle, was seeded and placed in a closed dewar flask, which contained an ethanol–water mixture at 0° and the dewar flask allowed to stand in the −5° cold room. The temperature was slowly decreased in this way over a period of 2–3 days to that of the cold room. Crystals that formed were centrifuged at −5°. Further crystals were obtained from the mother liquors by readjustment of the *p*H to 6.3 and addition of approximately 50 ml. of 53.3% ethanol. The resulting suspension was allowed to cool once more from 0 to −5°. A 50–60% yield was obtained in the first two crops of crystals. The crystal mass was redissolved in water and ice, clarified by filtration if necessary, and dried from the frozen state.

Recrystallization was carried out in a similar manner. One hundred grams of dried crystallized protein was redissolved in 500 ml. of water containing 0.02 mole of *p*H 6.2 sodium acetate buffer. The solution was diluted to 660 ml., and approximately 270 ml. of 53.3% ethanol was added through a capillary jet while keeping the temperature of the solution at 0 to −2°. After cooling to −5° as before, over a period of two to three days, the solution was quickly warmed to 0° and approximately 70 ml. of ethanol was added. Vigorous stirring was required to prevent local precipitation of amorphous protein. After standing at −5° another two or three days, the crystals were centrifuged at −5°, redissolved and dried from the frozen state. A yield of 80–90% was obtained on the second crystallization. Further recrystallizations, if necessary, were made by this same procedure. The crystals so obtained were elongated six-sided prisms (Fig. 1).

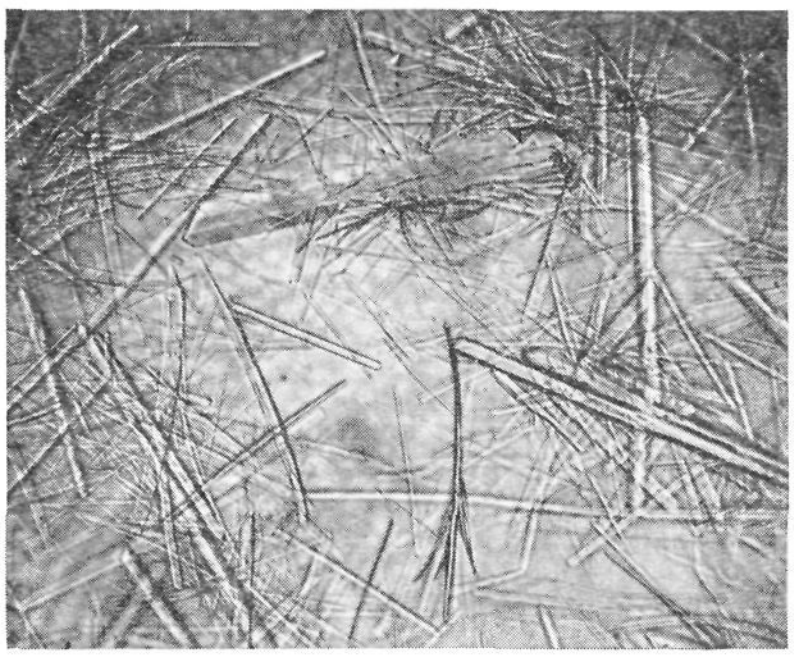

Fig. 1.—Crystals of β_1-metal-combining protein obtained at *p*H 6.2; magnification 250 ×.

Physical and Chemical Properties.—The four times recrystallized protein was found to be homogeneous both by electrophoresis and in the ultracentrifuge. The properties of the pure protein are presented in Table II.

The minimum molecular weight of the crystallized protein, estimated from its iron-binding capacity, was 45,000. Oncley, Scatchard and Brown[19] reported a molecular weight of 90,000 for Fraction IV-7, on the basis of osmotic pressure and ultracentrifugal measurements. Preliminary measurements on the crystallized protein have given values for the sedimentation constant, specific volume and intrinsic viscosity quite similar to those already reported for the less pure material in Fraction IV-7.[19] Since 75% of this fraction represented the β_1-metal-combining globulin, the molecular weight of the crystallized protein has been taken as 90,000, or twice the minimum molecular weight, determined from its iron-binding capacity. A molecular weight of about 90,000 has also been confirmed by light scattering measurements.[25] Of the major

TABLE II

SOME PROPERTIES OF THE CRYSTALLIZED β_1-METAL-COMBINING PROTEIN OF HUMAN PLASMA

Nitrogen content (g. N/g. protein)	0.147
Carbohydrate content (g. hexose/g. protein)	0.018
Extinction coefficient ($E^{1\%}_{1\ cm.}$, 280 mμ)	11.2
Optical rotation α^{25}_{5461}	−57±2°
Iron-binding capacity, *p*H 7.4 (γ/mg. protein)	1.25
Copper-binding capacity, *p*H 8.6 (γ/mg. protein)	1.42
Molecular weight[a]	90,000
*p*H of minimum solubility	5.9

[a] Estimated from iron-binding capacity.

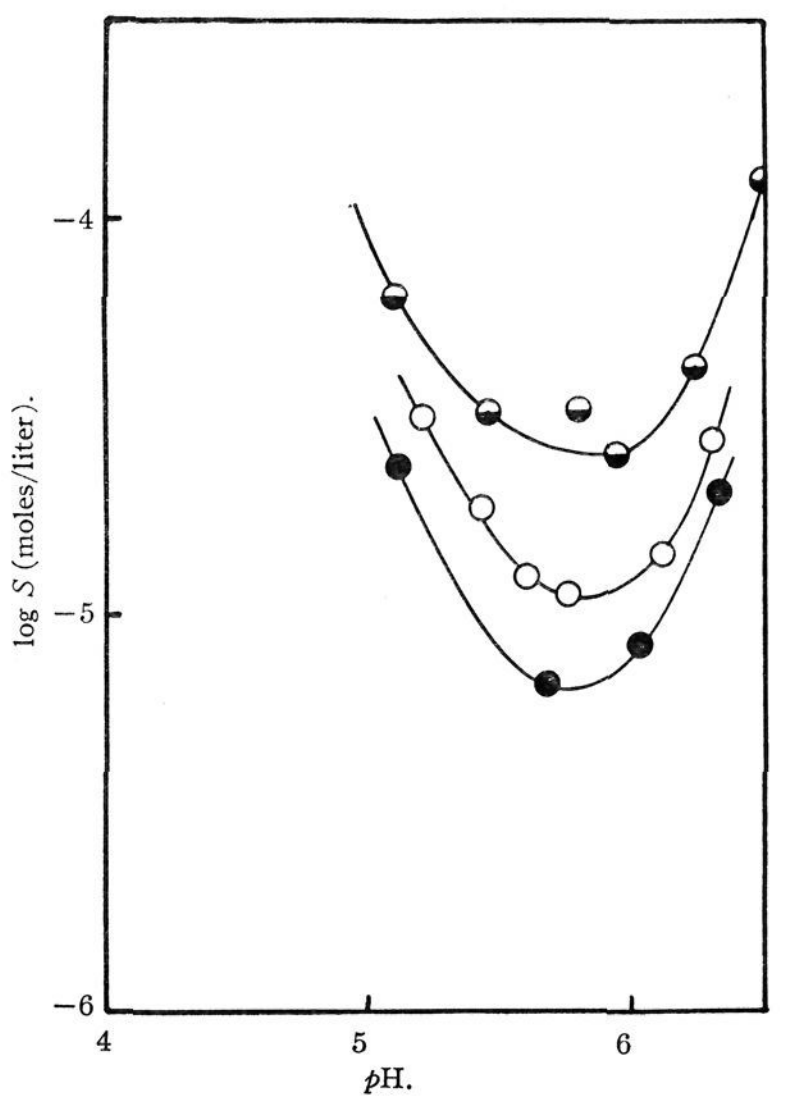

Fig. 2.—Solubility of β_1-metal-combining protein in 0.070 mole fraction (20%) ethanol at −5°: ◒ ionic strength 0.10; ○, ionic strength 0.04; ● ionic strength 0.01.

(25) F. R. Morrison and R. Lontie, personal communication.

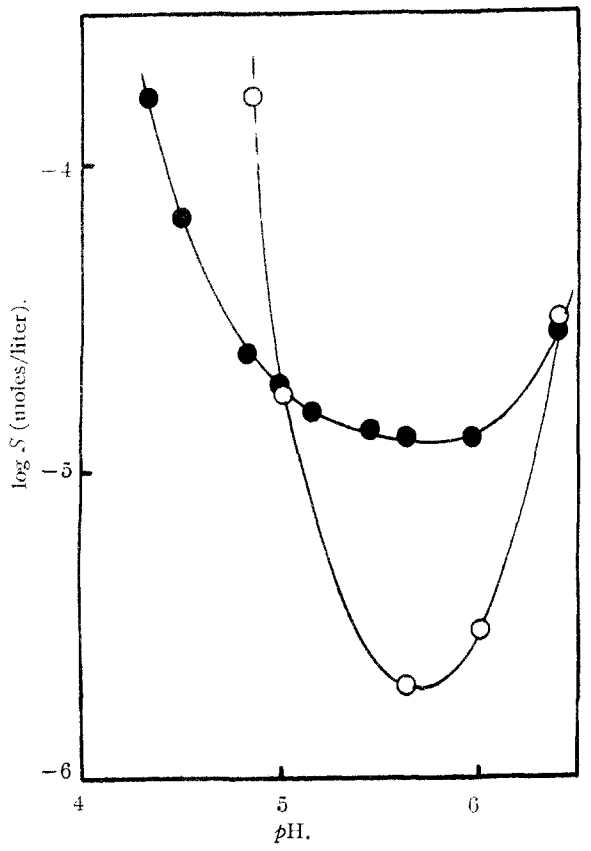

Fig. 3.—Solubility of β_1-metal-combining protein in 0.091 mole fraction (25%) ethanol at −5°, ● ionic strength 0.10; O, ionic strength 0.02.

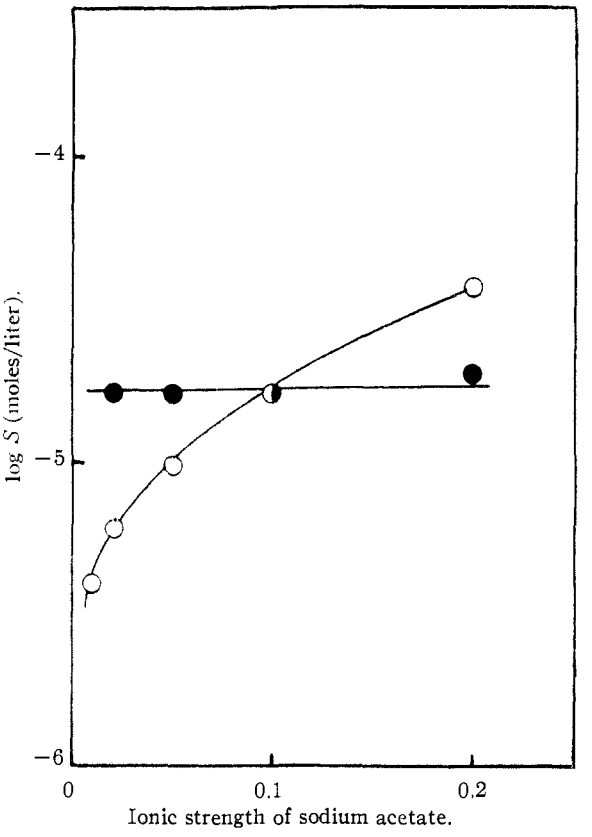

Fig. 4.—Solubility of β_1-metal-combining protein in 0.091 mole fraction (25%) ethanol at −5° as a function of ionic strength of sodium acetate: O, *p*H 5.7; ●, *p*H 5.1.

components of plasma, the β_1-metal-combining globulin is thus the closest to albumin in size and shape, as well as other properties. Among the differences that have been noted is the extremely high dielectric increment[26] of this fraction. Another difference is the complete absence of any free sulfhydryl group[27] such as that of albumin upon which its dimer formation depends.

Solubility Studies.—The β_1-metal-combining globulin was extremely soluble in water. Solutions containing 25% protein have been routinely prepared. The solubility of the crystallized globulin has been studies in ethanol–water mixtures at −5° under controlled conditions of *p*H and ionic strength. The solid phase in the experiments was amorphous protein. The results are presented graphically in Figs. 2–6. The minimum solubility in sodium acetate buffers was found near *p*H 5.8–5.9. Solubility in acetate buffers was not markedly affected by moderate changes in *p*H. However, a tenfold increase in ionic strength, from 0.01 to 0.10, produced nearly a tenfold change in solubility at *p*H 5.8 in 0.070 mole fraction (20%) ethanol at −5° (Fig. 2). At 0.0907 mole fraction (25%) ethanol (Fig. 3), the effect of ionic strength on the solubility at *p*H 5.8 was somewhat greater. The solubility is represented as a function of ionic strength near the isoelectric point and reactions slightly acid to it in Fig. 4. Salting-in is most pronounced near the isoelectric point and is negligible at *p*H 5.1. Corresponding data for the solubility of the protein in sodium chloride, instead of sodium acetate, are represented graphically in Fig. 5. The over-all shape of the curves is the same as those for acetate (Fig. 4); however the chloride ion exerted but one-tenth the salting-in effect of the acetate ion at *p*H 5.

Specific ion effects are further illustrated in Fig. 6, where the ionic strength was maintained at 0.1, while the ratio of sodium chloride to total ionic strength was varied from 0 to 0.5. As a result, solubility diminished by a factor of 10.[28]

Interaction with Metals.—The ability of this protein to interact with cations such as iron and copper in a characteristic fashion has been described and discussed previously.[9] Using the crystallized protein it was possible to extend our earlier findings. Maximum and firm combination with two atoms of iron per mole of protein occurs at reactions alkaline to *p*H 6.5. At more acid reactions the affinity of the protein for iron decreases gradually.

Magnetometric measurements, carried out by Dr. L. Michaelis, had demonstrated the trivalent state of the iron in the red complex.[9,29] Consistent with this evidence are our own observations that instantaneous formation of the red complex is obtained only when ferric iron is added to the protein.

(26) J. L. Oncley and N. R. S. Hollies, personal communication.

(27) W. L. Hughes, Jr., personal communication.

(28) A variable not controlled in this series of experiments was the concentration of acetic acid. This necessarily varied in the same ratio as the sodium acetate was varied, but the acetic acid concentration was of the order of 4 times the acetate ion concentration. It is unlikely, however, that this was responsible for the effects noted.

(29) L. Michaelis, personal communication.

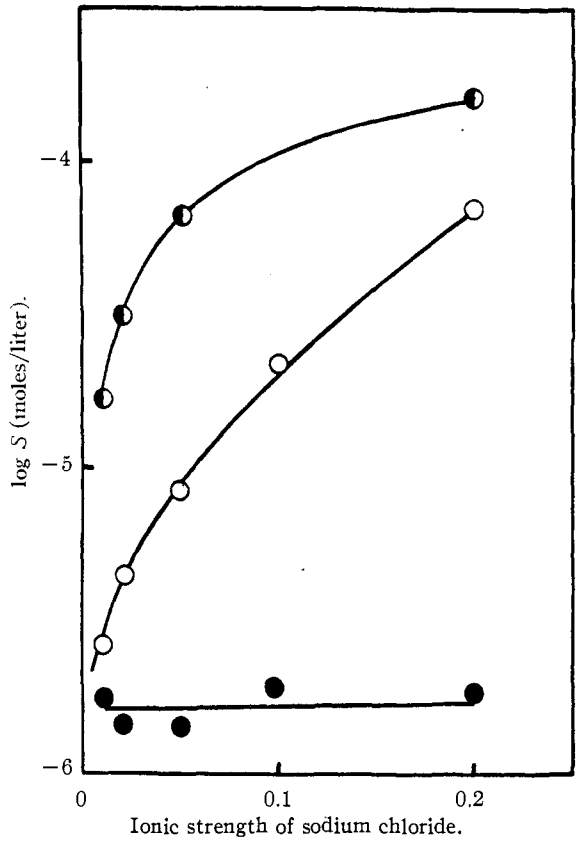

Fig. 5.—Solubility of β_1-metal-combining protein in 0.091 mole fraction (25%) ethanol at −5° as a function of ionic strength of sodium chloride: ◐, *p*H 6.3; ○, *p*H 5.7; ●, *p*H 5.1.

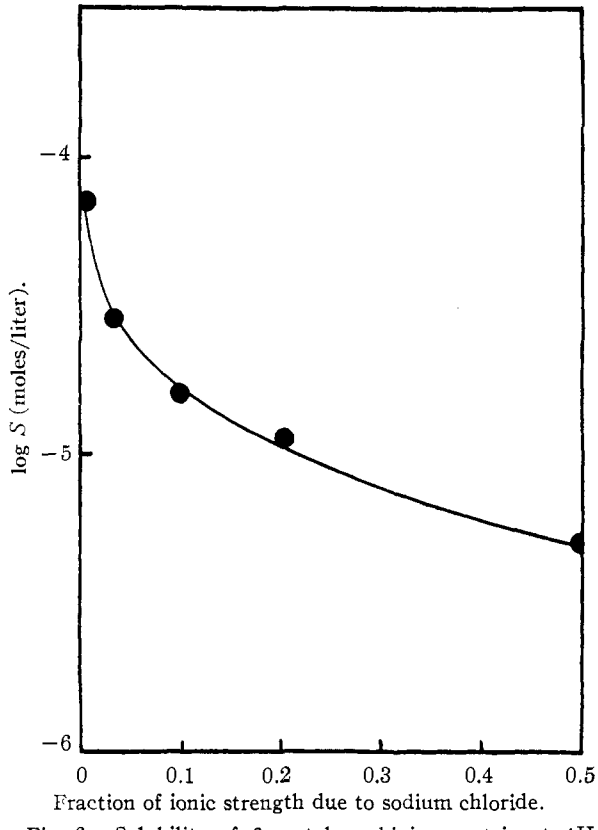

Fig. 6.—Solubility of β_1-metal-combining protein at *p*H 4.5 in 0.091 mole fraction (25%) ethanol at −5° as a function of chloride/total anion ratio; total ionic strength (acetate plus chloride) = 0.10.

When ferrous iron is added oxygen is required for the formation of the red complex and the rate of color development depends on the rate of autoxidation to ferric iron. This rate varies directly with the *p*H and is enhanced by certain catalytic factors present in plasma. In addition, Dr. L. Michaelis showed that hydrosulfite reduced the ferric iron and discharged the red color in the complex at reactions where dissociation of the complex would not normally have occurred.[29]

Copper combines most firmly with the protein in a narrow region near *p*H 8.5. With decreasing *p*H the affinity for copper decreases and the copper can be displaced by iron, the degree of displacement being a function of the *p*H as well as of the total concentrations of copper and iron. This can be demonstrated by measuring the absorption spectra of the respective solutions. Moreover zinc, if added in relatively high concentrations, partly displaces copper. However, iron bound to the protein cannot be displaced by any cation at any *p*H alkaline to *p*H 6.5. At reactions alkaline to *p*H 8.6 the binding capacity for both ions is rapidly lost.

It was observed that the color intensity following addition of copper to the impure protein in Fraction IV-7 was considerably lower than that obtained, under the same conditions, with crystallized β_1-metal-combining protein. This suggested that some contaminant present in Fraction IV-7 was competing with the β_1-metal-combining protein for copper.

The iron–protein complex is over ten times more soluble than the iron-free protein at *p*H 7, $\Gamma/2$ = 0.1 in 40% ethanol at −5°. No increase in solubility was observed, however, in the case of the copper complex nor in the presence of any other divalent cation tested, including zinc, cobalt, lead, manganese and magnesium. This observation constitutes additional evidence for the unique and specific character of the iron–protein complex.

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