ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

Some Physical Properties of Hen's Egg Conalbumin[1]

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Conalbumin prepared from hen's eggs was purified by repeated crystallization as the iron complex, and rendered electrophoretically homogeneous by electrophoresis–convection. Molecular weights of the iron-complexed and iron free proteins were determined by light scattering, osmotic pressure, and sedimentation–diffusion methods. A value of 85,000 ± 2000 was obtained. Sedimentation constants, diffusion constants, partial specific volumes, refractive index increments and frictional ratios are reported for each form of the protein.

Recent work on the conalbumin of the hen's egg has indicated it to be a source of crystallizable protein comparable in availability and homogeneity with ovalbumin or serum albumin. These observations suggest that conalbumin may find a similar wide degree of usefulness in protein investigations. Although considerable work has been done on its preparation,[3-7] metal binding properties,[8-12] and electrophoretic behavior,[6,7] data on many of the physical properties of this protein are limited and, in some cases, conflicting.

Conalbumin and an iron–conalbumin complex were prepared in crystalline form by Warner and Weber,[7] who studied the electrophoretic behavior and determined the isoelectric points of both forms. From measurements of its combining capacity with iron and other metals, these authors calculated a molecular weight for conalbumin of 76,600. Earlier determinations by Bain and Deutsch[6] on a highly purified, but non-crystalline preparation of conalbumin (which they then believed to be a "flavin protein"), gave values for the molecular weight and isoelectric point which differed from those determined by Warner and Weber. Bain and Deutsch employed sedimentation and diffusion data and an assumed value for the partial specific volume to calculate a molecular weight of 87,000. Longsworth, Cannan and MacInnes[4] were the first to estimate the molecular weight of conalbumin. Based on a Stokes' law calculation of their sedimentation data, these authors suggested a value of 70,000.

In the present study, the molecular weights of both iron and iron-free conalbumin have been determined by three independent methods: light scattering, sedimentation–diffusion and osmotic pressure. In addition, values of the following constants for both the iron and the iron-free forms of the protein were determined: sedimentation constant, diffusion constant, frictional ratio, partial specific volume and refractive index increment. Some values obtained for the electrophoretic mobility are also reported.

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(5) G. Alderton, W. H. Ward and H. L. Fevold, *Arch. Biochem.*, **11**, 9 (1946).

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Experimental

Crystalline iron–conalbumin was prepared by the method of Warner and Weber.[7] Metal-free conalbumin was prepared from the crystalline iron–conalbumin according to the instructions of these authors, but despite repeated efforts it was not itself obtained in crystalline form.

An impurity, which was not removed by repeated recrystallization of the iron complex, was encountered in four separate preparations. The impurity exhibited the mobility of ovalbumin but had a higher particle weight, which suggested that it was a denatured form of that protein. Preparative ultracentrifugation and passage through an ultrafine sintered glass filter removed some, but not all, of this impurity. Electrophoresis–convection[13] was used to remove the last traces of this contaminant from both the iron and iron-free forms of the protein.

Electrophoresis–convection of iron–conalbumin was carried out in an acetate buffer of *p*H 6.5 and ionic strength 0.1. The impurity was concentrated in the bottom compartment of the electroconvection apparatus.[14] Iron-free conalbumin was freed of the impurity by electrophoresis–convection at *p*H 5.0 in an acetate buffer of 0.1 ionic strength. Under these conditions the conalbumin was concentrated in the bottom compartment of the apparatus. After 24 hours, when no further concentration was effected as determined by absorbance measurements at 280 mμ, the electrophoretic pattern of the bottom fraction showed that a small trace of the impurity remained. Consequently, the top fraction was removed and replaced with more buffer solution, the contents of the cell were mixed, and electrophoresis–convection was carried out for another 24 hours. This process was repeated a second time so that a triple fractionation was accomplished. The electrophoretically detectable impurity was entirely removed by this method.

A yellow precipitate observed by Warner and Weber on dialysis of the newly formed iron complex against 0.002 *M* ammonium hydroxide was not encountered when crystalline lysozyme was removed from the egg white[15] prior to the isolation of ovalbumin and conalbumin. In our other preparations this precipitate was obtained. Feeney[16] has found that this precipitate exhibits lysozyme activity.

Electrophoresis.—Electrophoresis determinations were made using a Tiselius–Klett electrophoresis apparatus of Longsworth design, at 4° in a cell equipped with an electrode vessel described by Alberty.[17] Electrophoresis patterns were recorded photographically using the schlieren-

(13) S. Raymond, *Proc. Soc. Exptl. Med.*, **81**, 278 (1952).

(14) EC-25 Electroconvection apparatus manufactured by E-C Apparatus Co., 23 Haven Ave., New York 23, N. Y.

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scanning technique and mobilities were determined from the descending pattern in the conventional manner.

Electrophoresis was used to follow the preparation of the protein and to ascertain its purity. Mobility determinations were made at several pH levels.

Light Scattering.—Stock solutions of conalbumin were prepared in a buffer solution 0.05 M in sodium acetate and 0.2 M in sodium chloride and of pH 5.8. Varying aliquots of these stock solutions were pressure-filtered through an ultrafine sintered glass filter into the clean, dust-free tubes used for the light scattering cells. Sufficient buffer solution was then pressure-filtered into each of the tubes to bring the final volume to approximately 20 ml. The concentration of protein in each tube was determined, after the light scattering determination, by drying 5-ml. aliquots of the solution at 70° in a hot air oven and then in a vacuum oven for four hours at 70°. The protein concentrations were calculated from the weights of these dried residues after subtracting the dry weights of the buffer salts present.

Turbidity measurements, at wave lengths of 546 and 436 mμ, were made at 90° to the incident beam in a light scattering instrument designed and built in this Laboratory.[18] The absolute turbidity, τ, due to the protein was determined for each solution by comparing its observed scattering, less that due to the solvent, with that of a benzene or polystyrene standard of known absolute turbidity, and applying a correction factor to allow for the differences in refractive index between the solution and the standard. The specific refractive index increment of the protein, required in the calculation of Hc/τ,[19] was determined with a Phoenix differential refractometer.

Turbidity measurements made at angles of 45 and 135° to the incident beam indicated that no dissymmetry corrections were necessary. The "depolarization factor," required in the calculation of the Cabannes factor,[20] was determined for the solutions by inserting a Polaroid plate between the light scattering cell and the photomultiplier tube oriented in such a way as to screen out first the horizontal and then the vertical component of the scattered light.

The high degree of light absorption by iron–conalbumin solutions and the consequent large corrections which were required, rendered light scattering results with this form of the protein unreliable. Consequently light scattering results reported are restricted to those made with the iron-free conalbumin.

Osmotic Pressure.—Osmotic pressure measurements were made using a Bull-type[21] osmometer suspended in a large water-bath which was regulated to 20 ± 0.01°. Levels of the menisci were determined with a cathetometer. Densities of the solutions were measured with a Chainomatic Westphal balance calibrated with water at 20°. Concentrations of the protein solutions were determined by drying aliquots in the manner described above for the solutions used in the light scattering experiments.

All determinations were carried out with the protein in a buffer solution of pH 6.8 and ionic strength of 0.25 (0.2 M in sodium chloride and 0.0275 M in total phosphate). Care was taken to eliminate iron from all reagents and equipment used in the experiments with iron-free conalbumin. Osmotic pressures were determined at a series of concentrations for each form of the protein.

Sedimentation.—Data relative to the sedimentation velocity of conalbumin and iron–conalbumin were obtained with a model E Spinco ultracentrifuge. Sedimentation determinations were made at 59,780 r.p.m. at several concentrations for each form of the protein. A 0.05 M acetate buffer made 0.2 M in sodium chloride (total ionic strength 0.25) was used as the iron–conalbumin solvent. In all but one determination, which was carried out at pH 5.8, the pH was 6.2. A phosphate buffer of pH 6.8 and ionic strength 0.05, brought up to 0.25 in ionic strength by the addition of sodium chloride, was employed in the determinations with conalbumin.

The value of x, the distances of the peak from the center of rotation, were determined and an allowance of 0.025 cm. was made for stretching of the rotor at 60,000 r.p.m.[22,23] A plot of ln x *versus* t was made, where t is the time of sedimentation at maximum speed plus 1/3 the acceleration time.[24-27] The slope of this graph divided by ω^2 (where ω is the angular velocity in radians per second) yields s, the sedimentation constant. Initial and final temperature of the rotor were noted and the assumption was made that the rotor temperature rose linearly with time.

Values of s_{20w} were calculated according to the usual relationship.[27,28] Viscosities and densities of the solutions were estimated from tables in Svedberg and Pedersen[28] and International Critical Tables. The partial specific volumes (V) of the proteins at 20° were measured. A change in V of +0.0005 was assumed for each degree rise in temperature.[28]

Diffusion.—Experiments were carried out at 3.8° in a Neurath-type diffusion cell[29] using the same water-bath and optical recording devices as for the electrophoresis work. The concentration gradient of the diffusing protein was photographed periodically over a 72-hour period and the curves were projected with enlargement and traced manually onto graph paper. An average curve for each enlarged tracing of the scanned dn/dx *versus* x pattern was constructed from the upper and lower edges of the pattern. The area under this average curve was determined by counting squares. Calculations of the diffusion constant were made by the maximum ordinate method and by the maximum ordinate-area method.[29] An average value was computed for each method and the average value from both methods determined.

Diffusion experiments were made at three concentrations of iron–conalbumin in a buffer composed of 0.2 M sodium chloride, 0.05 M sodium acetate and adjusted to pH 6.2 with acetic acid. The diffusion of iron-free conalbumin was studied at two protein concentrations in a buffer of pH 6.8 containing 0.25 M sodium chloride and 0.01 M total phosphate. The protein solutions were dialyzed for 48 hours against buffer on a rotating dialyzer before placing them in the diffusion cell.

Partial Specific Volume.—Partial specific volume determinations were made at 20 ± 0.01° with pycnometers of 64.46- and 33.44-ml. capacities which were calibrated with air-free distilled water. Great care was taken to duplicate weighing conditions. Protein concentrations were determined on aliquots of the solutions as described for the light scattering experiments.

When the partial specific volume is essentially independent of solute concentration, it is sufficient[28,30] to calculate the apparent specific volume, V_1^a, for several concentrations and take the average value as equal to the partial specific volume. V_1^a is defined as

$$V_1^a = \frac{m_0 - (m - gv)}{\rho_0 gv}$$

where m_0 is the mass of the contents of the pycnometer when filled with the solvent, g is the concentration of the solute in g. per cc., v is the volume of the pycnometer and ρ_0 is the density of the solvent.

Results

The yield of conalbumin in later preparations compared favorably with the 44% yield reported by Warner and Weber.[7] In one preparation, 28.64 g. of 4 times recrystallized iron–conalbumin was obtained from 4300 ml. of egg white (12 dozen eggs). This represents a yield of 42% of the conalbumin estimated from electrophoresis patterns to have been present in the egg white.

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(25) E. G. Pickels, "Methods in Medical Research," Vol. 5, Year Book Publishers, Chicago, Ill., 1952, p. 107.

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Electrophoresis.—Electrophoresis patterns of conalbumin purified by the electrophoresis–convection method showed it to be homogeneous over the *p*H range of 4.87 to 8.57. Mobilities determined at several *p*H values are listed in Table I. In general, these mobility values fit the *p*H–mobility curve given by Warner and Weber.[7] The value obtained in phosphate buffer confirms their observation that the protein exhibits a much more negative mobility in this buffer than in other buffers of the same *p*H and ionic strength. The fact that the mobility of conalbumin in cacodylate buffer was positive at *p*H 6.4 lends support to the report of Warner and Weber that the isoelectric point is at *p*H 6.8 rather than at *p*H 6.1 as given by Bain and Deutsch.[6]

TABLE I

MOBILITIES OF CONALBUMIN

*p*H	Buffer	Ionic strength	Mobility (cm.^{2}v.$^{-1}$ sec.$^{-1}$ $\times 10^5$)
4.87	Acetate	0.1	2.35
6.40	Cacodylate	.1	0.63[a]
7.00	Phosphate	.1	−1.95
8.57	Veronal	.1	−2.31

[a] This determination was made in a 2-ml. micro-Tiselius cell and the mobility estimate is less precise than the others. The direction of movement, *i.e.*, sign of charge, was definite however.

Light Scattering.—The values of Hc/τ, as obtained for conalbumin at the two wave lengths of incident light employed are plotted as a function of c in Fig. 1, where the best straight line has been drawn

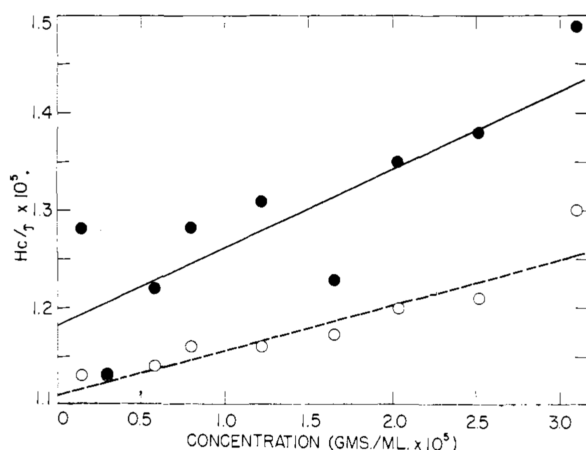

Fig. 1.—Hc/τ *versus* c data (light scattering) for conalbumin at 546 mμ (●) and 436 mμ (O).

by the method of least squares. The intercept of Hc/τ at zero concentration is the reciprocal of the molecular weight, M, according to the Debye equation. The observed intercept values of 1.18 $\times 10^{-5}$ and 1.11 $\times 10^{-5}$ for 546 and 436 mμ, respectively, yield values of M of 84,700 and 90,100. The molecular weights thus determined were multiplied by the Cabannes factor to correct for depolarization. Depolarization factors of 0.0131 (546 mμ) and 0.0403 (436 mμ) yielded values of the Cabannes factor of 0.972 and 0.917, respectively. By this method the molecular weight of conalbumin was found to be 82,200 and 82,600 using incident light of 546 and 436 mμ, respectively. The specific refractive index increment was found to be 0.001964 at 546 mμ and 0.001990 at 436 mμ.

Osmotic Pressure.—The values of P/c, where P is the observed osmotic pressure in centimeters of water, are plotted against the values of c, the concentration in g./100 ml., in Fig. 2. The

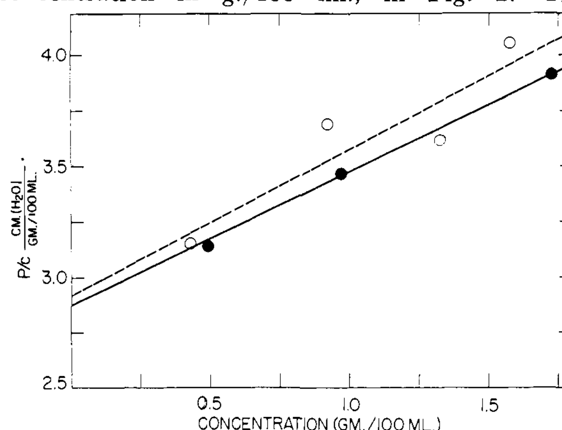

Fig. 2.—P/c *versus* c data (osmotic pressure) for iron–conalbumin (O) and conalbumin (●) at 20°.

method of least squares was used to draw the best straight line through the experimental points. The molecular weights, calculated from the relationship $P/c_{(c=0)} = RT/M$, of conalbumin and iron–conalbumin were 86,500 and 85,000, respectively.

Sedimentation.—Calculated s_{20w} values were plotted against concentration (Fig. 3) and the

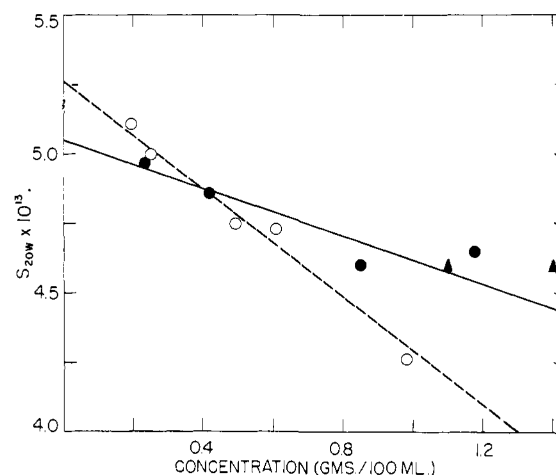

Fig. 3.—s_{20w} *versus* c data for iron–conalbumin (O) and conalbumin (●). Values marked (▲) on conalbumin graph are data of Cann and Phelps.[38]

value at infinite dilution was determined for each form of the protein. Extrapolated values of s_{20w} of 5.05 $\times 10^{-13}$ for conalbumin and 5.26 $\times 10^{-13}$ for iron–conalbumin were obtained. From these values, the corresponding diffusion constants and the partial specific volumes (see below), the molecular weight of conalbumin was found to be 86,000 and that of the iron complex to be 83,000.

Diffusion.—Figure 4 shows the linear relationship obtained when $1/\sqrt{t}$ is plotted against H, the maximum ordinate of the dn/dx *versus* x curve at

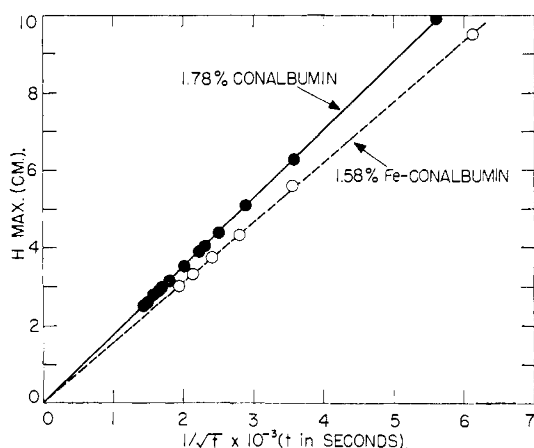

Fig. 4.—Maximum ordinate *versus* $1/\sqrt{t}$ data (diffusion) for typical experiments with iron–conalbumin (O) and conalbumin (●).

time t, for typical determinations with conalbumin and iron–conalbumin.

By the maximum ordinate–area method of calculation, data of Fig. 4 yield values for $D°_{3.8}$ of 3.21×10^{-7} cm.^{2} sec.$^{-1}$ for conalbumin and 3.29×10^{-7} cm.^{2} sec.$^{-1}$ for iron–conalbumin. By the maximum ordinate method applied to the individual patterns obtained during these same experiments, average values for $D°_{3.8}$ of 3.11×10^{-7} cm.^{2} sec.$^{-1}$ for conalbumin and 3.40×10^{-7} cm.^{2} sec.$^{-1}$ for iron–conalbumin were obtained. Values of D estimated at other concentrations of the proteins showed no consistent variation with protein concentration.

For use in the calculations of molecular weights of the proteins from sedimentation data, the average values of $D°_{3.8}$ were corrected to 20° and water as solvent.[28] The factors used in converting $D°_{3.8}$ to D_{20w} were 1.677 and 1.712 in the conalbumin and iron–conalbumin experiments, respectively, and the corresponding values of D_{20w} so obtained are 5.30×10^{-7} and 5.72×10^{-7} cm.^{2} sec.$^{-1}$.

Partial Specific Volume.—A plot of m against w indicated that no change in dm/dw occurs with solutions of iron–conalbumin within the concentration range employed. Table II summarizes the data for three concentrations of iron–conalbumin and one concentration of iron-free conalbumin. The partial specific volume of both forms of the protein was found to be 0.732 ± 0.002.

TABLE II

PARTIAL SPECIFIC VOLUME DATA ON Fe CONALBUMIN AND CONALBUMIN

	Large pycnometer v = 64.46 cm.^{3} m_0 = 64.3440 ρ_0 = 0.99823		Small pycnometer v = 33.44 cm.^{3} m_0 = 33.3792 ρ_0 = 0.99823	
g	m	V_1[a]	m	V_1[a]
		Fe Conalbumin		
0.0149	64.6031	0.7315	33.5151	0.7285
.0197	64.6831	.7342	33.5598	.7271
.0316	64.8934	.7315	33.6576	.7378
		Conalbumin		
0.0170	64.6427	.7287	33.5297	.7365

The pycnometers and procedure used were checked by determining the partial specific volume of ovalbumin. A value of 0.748 was obtained with each pycnometer. This value agrees well with literature values of 0.749 and 0.745.[31–33]

The partial specific volume of conalbumin was also calculated from its amino acid content[34] according to the method of Cohn and Edsall.[35,36] A value of 0.731 was obtained, in excellent agreement with the experimentally determined value.

Frictional Ratio.—The frictional ratios for both forms of the protein have been calculated using the constants obtained by the above determinations. These ratios were calculated by equations given by Svedberg and Pedersen[23] which involve: (a) s_{20w} and M_e (the molecular weight determined by other than sedimentation velocity, here taken to be 85,000), (b) D_{20w} and M_e and (c) s_{20w} and D_{20w}. The values are shown in Table III.

Summary and Discussion

The physical constants of the two forms of conalbumin are summarized in Table III.

Values for some of the constants given above have been reported earlier. Kegeles and coworkers, as reported by Bain and Deutsch,[6] found D_{20w} for conalbumin, presumably prepared by the method of the latter authors, to be 5.66×10^{-7} cm.^{2} sec.$^{-1}$, a value about 6% higher than that reported here for conalbumin and 1% lower than that reported for iron–conalbumin. Bain and Deutsch also reported a s_{20w} value of 5.4 S and a molecular weight of 87,000. It is of interest to note that their determination was made on 0.7% solutions of the conalbumin in 0.15 M NaCl in the standard Svedberg oil turbine ultracentrifuge at about 220,000 × gravity. They also state that "under the conditions we have used, this substance is separated as a flavoprotein." This and the low isoelectric point of their protein, indicate that they were probably dealing with what is now known as the iron complex. Their value of 5.4 S is about 3% greater than our value of 5.26 S for iron–conalbumin. This deviation is in agreement with the recent work of Shulman[26] who determined sedimentation constants of other proteins with both oil turbine and Spinco ultracentrifuges and found that the latter gave values 2–3% lower than the oil turbine instrument. Similar results have been reported by other investigators.[22,23,36] This may explain the somewhat higher molecular weight reported by Bain and Deutsch.

Cann and Phelps,[38] using a Spinco instrument, found that conalbumin purified by electrophoresis–convection, had a sedimentation constant of 4.6 S for protein concentrations of 1.1 and 1.4%. They pointed out the large difference between this value and that of Bain and Deutsch. This difference is

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TABLE III

SUMMARY OF THE PHYSICAL CONSTANTS OF CONALBUMIN AND IRON–CONALBUMIN

Protein	Molecular weight Osmotic pressure	Light scattering	Sedimentation diffusion	s_{20w}, sec.$^{-1}$ $\times 10^{13}$	D_{20w}, cm.^{2} sec.$^{-1}$ $\times 10^7$	V, cm.^{3}	Specific refraction increment	f/f_0 a	b	c
Conalbumin	86,500	82,400	86,000	5.05	5.30	0.732	0.001964 (546 mμ) .001990 (436 mμ)	1.365	1.376	1.375
Iron–conalbumin	85,000	....	83,000	5.26	5.72	.732		1.310	1.277	1.295

understandable in light of the present work since they were dealing with the iron-free form, while Bain and Deutsch used the iron complex. In addition, their value would have been higher at a lower protein concentration. Actually, the value reported by Cann and Phelps fits very well on the s_{20w}–concentration curve for conalbumin reported herein (see Fig. 3).

The average molecular weight reported here for both forms of the protein by the three independent methods of measurement is 84,000 ± 2000. The agreement between the values obtained by the three different methods is strong evidence for the homogeneity of the preparations used and the reliability of the molecular weight reported. It is of interest that the extrapolated values of s_{20w}, the values of D_{20w}, and those of the frictional ratios all indicate the protein molecule to be of a more spherical shape when in the form of the iron complex; however, the greater dependence of the s_{20w} value on concentration in the case of the iron–conalbumin would indicate the reverse relationship to hold.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Structural Specificities in the Interactions of Some Organic Ions with Serum Albumin. II

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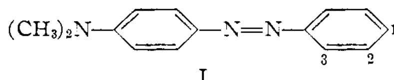
Previous observations of marked differences in the interaction of human albumin with isomeric molecules (I), containing an anionic substituent, –X, in positions 1, 2 and 3, respectively, have been ascribed to differences in the distances between $(CH_3)N$– and –X. The validity of this view has been bolstered by further studies with molecules of the type II in which chains of varying length and rigidity have been interposed between the ring and carboxyl group.

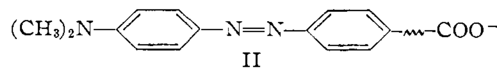
Introduction

It was suggested recently[2] that it might be feasible to establish distances between side chains of proteins through spectrophotometric studies of interactions with ions of fairly rigid structure containing two substituents a known distance apart. Thus, it was shown that with human serum albumin, azo compounds of the structure I behaved



differently if an anionic substituent, -X, was in position 1 or 2 as contrasted to position 3. If –X is a $-COO^-$ group, for example, the spectrum of the complex of protein with the *meta* or the *para* dye (carboxyl in 2 or 1 position, respectively) showed a peak near 480 mμ, whereas that of the complex with the *ortho* compound had a maximum near 430 mμ. Evidence was presented that two side chains on the protein formed bonds with I when $-COO^-$ was at 1 or 2, but that only one of these was involved when the carboxyl was at 3. The critical feature seems to be the distance between the N of $(CH_3)_2N$– and the O of $-COO^-$, which is approximately 12 Å. for either the *m*- or *p*-carboxyl, but near 9 Å. for the *ortho*. The protein side chains involved in binding apparently are able to span a distance of 12 Å. but not one of 9 Å.

As a further test of this interpretation it seemed appropriate to separate the $(CH_3)_2N$– and $-COO^-$ by a spacing greater than 12 Å. to see if one could exceed the distance which the two protein side chains could span. A number of compounds have been prepared, therefore, in which chains of varying length and rigidity have been interposed between the ring and carboxyl group (structure II),



and the interactions of these dyes with serum albumin have been examined.

Experimental

Reagents.—4-(*p*-Dimethylaminophenylazo)-hydrocinnamic acid (VI) was prepared by the following sequence of reactions. Hydrocinnamic acid (Eastman Kodak Co. sample) was nitrated according to the procedure of Konek and Pacsu,[3] and the *p*-nitrocinnamic acid isolated was hydrogenated in a Parr bomb. The resultant *p*-aminohydrocinnamic acid was diazotized and coupled according to the general procedure of Fieser.[4] The dye thus produced (VI) was recrystallized several times from ethanol–water mixtures. It melted at 194–197° dec., and gave a neutralization equivalent of 306 (calcd. 297). *Anal.* Calcd. for $C_{17}H_{19}O_2N_3$: C, 68.66; N, 14.14. Found: C, 69.06; N,

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