[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF WHEATON COLLEGE]

The Interaction of Cobalt with Native Proteins

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The interaction of three purified native proteins, bovine serum albumin, pepsin and α-casein with cobaltous chloride in *p*H 6.5 acetate buffer has been found to be small. The free energy of binding has been evaluated for bovine serum albumin and α-casein. Cobalt–protein complexes at higher *p*H values are also discussed.

Introduction

Metal ion–protein complexes containing metals such as copper, zinc, manganese, calcium and others occur naturally. These combinations of small ions and protein molecules have been the subject of much investigation. Klotz and his associates have studied exhaustively the chemistry of copper–protein complexes using carefully purified homogeneous proteins.[1-3] They have shown that cupric ions interact primarily with carboxyl groups of the protein at *p*H 4.8 to 6.5, although some copper–nitrogen linkages may be formed at the higher *p*H.

Recently it has been shown that many well-characterized peptidases are metal–proteins in which the metal is essential and reasonably specific.[4] Thus glycylglycine dipeptidase requires Co^{++} for its activity. Smith[5] has shown that this enzyme must combine with the substrate through a cobalt ion coördinated with both moieties. Since little is known of the interaction of the cobaltous ion with purified, well-characterized protein preparations the following study was undertaken.

Experimental

The interaction of the cobaltous ion with proteins was studied by the equilibrium dialysis technique[6] and by spectrophotometric means.

In the dialysis studies the protein–ion complex at equilibrium is separated from a solution containing non-complexed ion by a cellophane membrane that is permeable to ions but not to protein. The semimicro method of Weber[7] was used. A 2-ml. portion of a solution containing 1% protein and 0.2 *M* acetate buffer was placed in a cellophane bag prepared from commercial sausage casing. This was placed in 2 ml. of a solution containing 0.2 *M* acetate buffer and cobaltous ions. A control tube containing buffer and metal ion was necessary in order to correct for the binding by the casing. The tubes were placed in an ice-bath and shaken for 18 hours. Following attainment of equilibrium the exterior portion of each tube was analyzed for cobalt. Corrections were not made for the Donnan effect, for it is small and within the error of the analytical method.

Analysis for cobalt was made colorimetrically using nitroso-R-salt. A measured volume of a solution containing up to 8.5×10^{-4} millimole cobalt was treated with 2 ml. of a *p*H 5.2 buffer and 2 ml. of 0.1% nitroso-R-salt solution and diluted to 50 ml. The buffer contained 250 g. of sodium acetate trihydrate and 92 ml. of glacial acetic acid per 500 ml. of solution. Reagents were measured accurately since the intensity of the color produced depends upon the buffer and reagent concentrations. A blank containing buffer and nitroso-R-salt was used. Spectrophotometric measurements were made at 420 mμ, using a Beckman quartz spectrophotometer. Proteins as bovine serum albumin must be absent as they interfere with full color development.

Absorption spectra were obtained with a Beckman spectrophotometer. Molecular extinction coefficients, ε, were calculated from the usual expression,

$$\log_{10} I^0/I = \epsilon dc$$

where c is expressed in moles per liter and d is the thickness of the absorption cell in centimeters.

The bovine serum albumin and pepsin were crystallized samples from Armour and Company. The α-casein was prepared from raw skim milk by the method of Warner.[8] Binding measurements were carried out with a 1% solution of these proteins.

Reagent grade $CoCl_2 \cdot 6H_2O$ was used as a source of the cobalt ion. It was checked for purity by an electrolytic method.[9]

Results and Discussion

The spectra of cobaltous chloride in water and in sodium acetate at *p*H 6.5 are shown in Fig. 1. Some

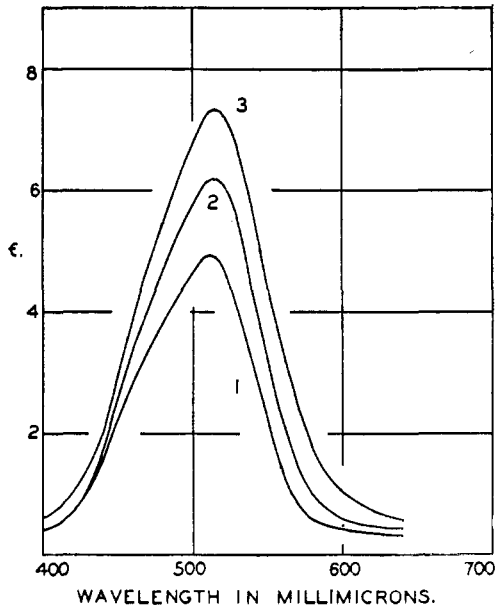

Fig. 1.—Cobalt absorption spectra: 1, 0.1 *M* $CoCl_2$; 2, 0.1 *M* $CoCl_2$ in 0.2 *M* sodium acetate at *p*H 6.5; 3, 0.1 *M* $CoCl_2$ in 0.2 *M* sodium acetate with 6% bovine serum albumin at *p*H 6.5.

small degree of interaction of cobalt and acetate is indicated by the increase in the extinction coefficient of 515 mμ. Addition of bovine serum albumin to the buffered cobaltous solution resulted in a further slight increase in the extinction coefficient. Cobaltous–protein interaction was confirmed by binding studies. The extent of binding is shown in Fig. 2 where the number of bound ions per 10^5 g. of protein is plotted as a function of the log of the con-

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(2) I. M. Klotz and H. A. Fiess, *J. Phys. Chem.*, **55**, 101 (1951).
(3) H. A. Fiess and I. M. Klotz, THIS JOURNAL, **74**, 887 (1952).
(4) E. L. Smith, *Ann. Rev. Biochem.*, **18**, 35 (1949).
(5) E. L. Smith and R. Lumry, *Cold Spring Harbor Symposia on Quantitative Biology*, **14**, 168 (1950).
(6) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946); **69**, 1609 (1947).
(7) I. M. Klotz, J. M. Urquhart and W. W. Weber, *Arch. Biochem.*, **26**, 420 (1950).
(8) R. C. Warner, THIS JOURNAL, **66**, 1725 (1944).
(9) D. H. Brophy, *Ind. Eng. Chem., Anal. Ed.*, **3**, 363 (1931).

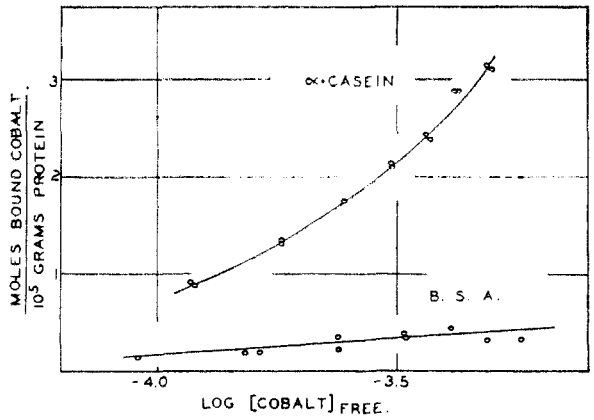

Fig. 2.—The extent of binding of cobaltous ions in acetate solution at *p*H 6.5 by α-casein and by bovine serum albumin.

centration of the unbound metal ion. The binding is small indeed for less than one mole is bound per 10^5 g. of protein. This contrasts markedly with copper interaction with bovine albumin. When the unbound ion concentration is 1.6×10^{-4} *M*, approximately 0.2 mole of cobaltous ion is bound compared with 8.7 moles of cupric ion. It is not known what groups are responsible for cobalt binding by this protein. However Klotz and co-workers[1-3] and Tanford[10] have shown that imidazole and carboxyl groups are active in metal ion binding.

It is customary to express the formation of protein metal-ion complexes by the equation

$$PCo_{i-1} + Co^{++} = PCo_i \qquad (1)$$

where PCo_{i-1} and PCo_i represent complexes with the $i-1$ and i cobalt ions. The association constant, k_1 for the binding of the first cobalt ion may be evaluated conveniently[3] from a graph of r/[free ion] *versus* [free ion] where r equals the moles bound cobalt/10^5 g. protein. The intercept at [free ion] = 0 gives k_1. (A unit molecular weight of 100,000 has been used for the purpose of comparison.)

The thermodynamic relation

$$\Delta F_1^\circ = -RT \ln k_1 \qquad (2)$$

enables one to evaluate ΔF_1°, the energy of binding of the first cobalt ion, as -3.96 kcal. per 10^5 g.

The binding of cobalt by the protein, α-casein, was also studied and the results summarized in Fig. 2. Cobalt α-casein interaction is large when compared with cobalt albumin. ΔF_1° was found to be -4.86 kcal. per 10^5 g.

The cobalt–α-casein complex is an insoluble precipitate. The reversible nature of the complex was demonstrated by the solution of the precipitate on the addition of sodium citrate of the same *p*H. There is evidence that citrate forms a very stable complex with cobalt.

The cobaltous ion also formed a precipitate with the phosphate or the hydrogen phosphate ion at *p*H 5.5. Since an essential point of difference between bovine albumin and α-casein is the presence of a large number of phosphoserine groups in the α-casein, the binding of cobalt to this protein may well occur through these groups.

Spectra studies with the protein pepsin and cobalt showed a very low degree of binding. Binding studies were not made because of the difficulty of accurate analysis in the low binding range.

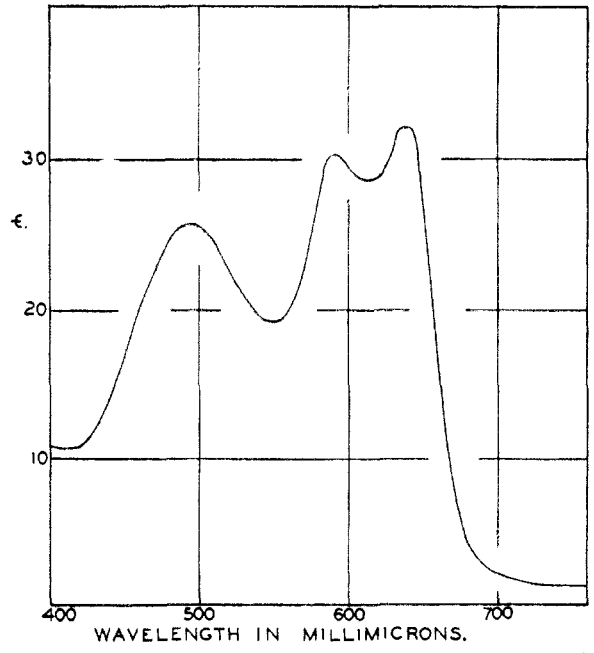

Fig. 3.—Cobalt absorption spectrum: 0.01 *M* $CoCl_2$ containing 4.4% bovine serum albumin at *p*H 9.0. The solution was prepared in an inert (N_2) atmosphere.

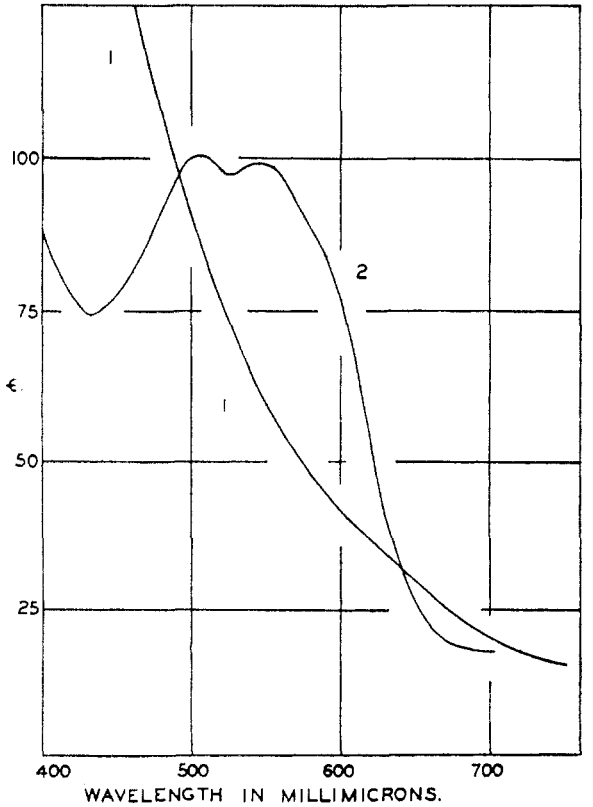

Fig. 4.—Cobalt absorption spectra: 1, 0.01 *M* $CoCl_2$ containing 0.25% bovine serum albumin and 2.5% NaOH; 2, 0.004 *M* $CoCl_2$ containing 1.0% α-casein and 5.0% NaOH. Prepared in a nitrogen atmosphere.

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Behavior at Higher *p*H Values.—Cobaltous hydroxide precipitates from dilute solutions at *p*H values between 6.8 and 7.5. The color of the hydroxide may be blue, or rose, for X-ray analysis has shown two isomeric forms.[11] A fresh precipitate of the hydroxide is readily oxidized to the brown hydroxide by the air at higher *p*H values. Bovine serum albumin appears to form a soluble complex with cobaltous hydroxide. The characteristic spectrum of Fig. 3 was obtained after forming the complex in a nitrogen atmosphere. When the complex was prepared in air some oxidation of cobalt was indicated by a decrease in optical density above 560 mμ and an increase at lower wave lengths.

Proteins react with cobaltous chloride under the conditions of the biuret reaction to form a violet-red complex which is rapidly oxidized to a brown cobaltic complex.[12] The brown complex has no characteristic absorption maxima in the visible spectrum but shows increasing absorption with decreasing wave length (Fig. 4). When the protein was bovine serum albumin the oxidation occurred in an inert atmosphere as well as in air. By contrast, α-casein formed a stable violet-red complex in a nitrogen atmosphere (Fig. 4), and the typical oxidized solution in air. Since α-casein contains but two cystine units per 10^5 g. of protein, while the same unit of bovine serum albumin contains 22.5 cystine units it appears probable that the disulfide groups may oxidize the cobaltous ion in alkaline solution. Only a fraction of the cobaltous complex is oxidized by α-casein in an inert atmosphere because of the low cystine content.

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WHEATON, ILLINOIS

[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY OF PRINCETON UNIVERSITY]

Microwave Absorption and Molecular Structure in Liquids. VIII. Dielectric Relaxation in Some Long-Chain Esters[1,2]

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Dielectric constants and losses between 3 and 90° at wave lengths of 1.25, 3.22 and 10.0 cm. and 577 m. have been measured for ethyl, isoamyl, cetyl and octadecyl acetates, tetradecyl palmitate, decyl, tetradecyl and cetyl stearates, tristearin, distearin and monostearin, and ethylene dimyristate, dipalmitate and distearate. Refractive indices, densities and viscosities have also been measured. The critical wave length, at which the loss is a maximum, increases with molecular length and with viscosity, as observed previously for alkyl bromides. The viscosities of the esters are slightly lower than those of alkyl bromides of about the same molecular length, but the critical wave lengths are only about half as large, showing greater ease of dielectric relaxation. Orientation of polar molecular segments, presumably, occurs by rotation around the carbon–carbon bonds.

In previous papers of this series the results of investigations of twenty-seven alkyl bromides and related compounds in the pure state and a number of specially selected systems of organic halides in non-polar solvents have been reported and discussed. The work on the pure polar liquids has provided information on the effects of molecular size and shape upon dielectric relaxation and has served to contrast and compare the processes of dielectric relaxation and viscous flow. In the work on dilute solutions, in which it has been possible to vary the environment of the polar molecules, extreme departures from the classical Debye proportionality between the viscosity and the dielectric relaxation time have been observed. These departures have been attributed to intermolecular forces and the effects of the solvent on differently shaped orienting units. The intermolecular forces have been associated with the interaction between the solute and permanent local, as well as induced, dipoles in the solvent molecules. The present paper, which discusses measurements upon a series of long-chain esters of high viscosity, is a continuation of the work on pure liquids.

(1) This research has been supported in part by the Office of Naval Research.

(2) This paper represents a part of the work submitted by Dr. P. L. McGeer to the Graduate School of Princeton University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(3) Ethyl Corporation Fellow in Chemistry, 1949–1950; McCay Fellow in Chemistry, 1950–1951.

Purification of Materials

Table I lists the melting or boiling points, refractive indices, and densities of the compounds studied. The three glycerides, kindly loaned by the Procter and Gamble Company, were used without further purification. The ethylene diesters obtained from the Matheson Co. were used after recrystallizing several times from a benzene–methanol mixture. The ethyl and isoamyl acetates were dried over finely divided potassium carbonate for 24 hours and repeatedly distilled in a 4-foot column. The cetyl and octadecyl acetates, also obtained from the Matheson Co., were subjected to two vacuum distillations and then recrystallized several times from an ether–methanol solvent. The remain-

TABLE I

PHYSICAL CONSTANTS OF MATERIALS

	M.p., °C.	t, °C.	d_4^t	n_D^t
Ethyf acetate	77–77.2	50	0.863	1.37257 (20°)
	(b.p.)	25	.894	
Isoamyl acetate	141.5–141.7	70	.823	1.40169 (18°)
	(b.p.)	27	.866	
Cetyl acetate	18.3	60	.810	1.41846 (80.3°)
		30	.830	
Octadecyl acetate	32.6	60	.830	1.42957
		30	.851	
Decyl stearate	35.4	80	.8423	1.42967
Tetradecyl palmitate	46.4	80		1.42987
Tetradecyl stearate	50.1	80		1.43279
Cetyl stearate	56.8	80	.816	1.43400
		60	.829	
Monostearin	72.4	90		1.43963
Distearin	76.7–77.5	90		1.43771
Tristearin		90		1.43711
Ethylene dimyristate	61.7	80	.8600	1.43168
Ethylene dipalmitate	69.1			
Ethylene distearate	75.3			