Conventional Synthesis of the Tetrapeptide. A. Carbobenzoxyglycyl-L-valine Methyl Ester.—To 7.13 g. (0.0425 mole) of L-valine methyl ester hydrochloride in 30 ml. of methylene chloride at 0° was added 12 ml. (0.085 mole) of triethylamine, followed by a solution of 14 g. (0.042 mole) of carbobenzoxyglycine *p*-nitrophenyl ester in 50 ml. of methylene chloride. After standing 24 hr. at room temperature the clear yellow solution was washed with *N* ammonium hydroxide until colorless, then with *N* hydrochloric acid and water. The dried product was an oil weighing 10.3 g. (75%). Thin layer chromatography on silicic acid in chloroform–acetone (10:1) gave one spot. R_f 0.75, by the hypochlorite–KI spray for amides.[12] The oil was used directly for the next step.

B. **Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine Methyl Ester.**—Carbobenzoxy-glycyl-L-valine methyl ester (5.1 g., 15.8 mmoles) was hydrogenated for 2 hr. at 25° in 50 ml. of methanol containing 15.8 ml. of *N* HCl and 500 mg. of 5% palladium-on-carbon. The product was dissolved in 20 ml. of methylene chloride, cooled to 0° and 4.4 ml. of triethylamine was added. Carbobenzoxy-L-alanine *p*-nitrophenyl ester (5.44 g., 15.8 mmoles) in 20 ml. of methylene chloride was added and the mixture was stirred for 3 days at 25°. The solution was washed with NH_4OH, HCl and water and dried. The solution of carbobenzoxy-L-alanylglycyl-L-valine methyl ester was evaporated to dryness and the oil (3.43 g., 55%) was dissolved in methanol for hydrogenation. The product was then coupled with 2.88 g. (7.65 mmoles) of carbobenzoxy-L-leucine *p*-nitrophenyl ester in a manner similar to that described for the previous step. The yield of crude carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine methyl ester was 2.80 g. (64% from the protected tripeptide), m.p. 155–157°. For analysis the product was recrystallized twice from ethyl acetate–petroleum ether; m.p. 160–161°. $[\alpha]^{21}D$ −31.8° (*c* 2, ethanol),

Anal. Calcd. for $C_{25}H_{38}N_4O_7$: C, 59.25; H, 7.57; N, 11.07. Found: C. 59.21; H, 7.26; N, 11.08.

C. **Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine.**—Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine methyl ester (662 mg., 1.3 mmoles) was dissolved in 2.5 ml. of ethanol and 0.28 ml. of 5 *N* NaOH was added. After 60 min. at 25° the solution was diluted with water and extracted with ethyl acetate. The aqueous phase was acidified with HCl and the product extracted into ethyl acetate. Evaporation of the dried solution gave 520 mg. (81%). m.p. 194°. Two recrystallizations from ethanol–water raised the m.p. to 199–200°, $[\alpha]^{21}D$ −23.8° (*c* 2, ethanol).

Anal. Calcd. for $C_{24}H_{36}N_4O_7$: C, 58.52; H, 7.37; N, 11.37. Found: C, 58.55: H, 7.00; N, 11.30.

D. **L-Leucyl-L-alanylglycyl-L-valine.**—Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine (493 mg., 1.0 mmole) was dissolved in 50 ml. of ethanol and hydrogenated for 24 hr. in the presence of 50 mg. of 5% palladium-on-carbon. The mixture was filtered and evaporated to dryness. The residue was redissolved in 2 ml. of ethanol and filtered again and the tetrapeptide was then precipitated with dry ether: yield 343 mg. (96%), $[\alpha]^{21}D$ +17.5° (*c* 2, ethanol); R_f 0.71 (propanol–H_2O), 0.73 (*sec*-butyl alcohol–formic acid),[14] 0.49 (*sec*-butyl alcohol–ammonia[15]); amino acid ratios[13]: leucine 0.98, alanine 1.00, glycine 1.00, valine 1.01.

Anal.[16] Calcd. for $C_{16}H_{30}N_4O_5$: C, 53.6; H, 8.4; N, 15.6. Found: C, 52.7: H, 8.1; N, 14.9.

Acknowledgments.—The author wishes to express his sincere appreciation to Dr. D. W. Woolley for his interest and advice during this work. The author also is grateful to Dr. John Stewart for many helpful suggestions and discussions and to Miss Angela Corigliano and Miss Gretchen Grove for their technical assistance.

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Beryllium Binding by Bovine Serum Albumin at Acid pH

BY SIDNEY BELMAN

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The binding of beryllium by bovine serum albumin was investigated, at three temperatures, in the pH region 5 and below, where beryllium salts are soluble and dialyzable. Reversible association was studied by the method of equilibrium dialysis in solutions containing nitrate ion at an ionic strength of 0.15. The amount of binding increased with increasing pH and temperature. The constancy of the intrinsic association constant (K = 36.2) and the variation of n, the number of binding sites, suggest that environmental conditions modify the structure of the protein so that a varying number of a single class of binding sites become available for reaction. Considerations of the effect of pH and temperature on beryllium ion hydrolysis and albumin structure support this interpretation. Esterified serum albumin exhibited no beryllium binding which is evidence for the carboxyls as the reacting groups. The results of potentiometric titrations agreed with those obtained from equilibrium dialysis. Albumin denatured by dodecyl sulfate showed greatly increased beryllium binding which was attributed to the electrostatic effect of the bound anion. The coöperative behavior of the beryllium binding was ascribed to the structural effects induced by dodecyl sulfate bound to albumin. Although the beryllium species in equilibrium with the protein is unknown, the data suggest that Be^{2+} is one reacting species.

Introduction

The interaction of beryllium with proteins is important for several reasons. Beryllium toxicity in the lung disease, berylliosis, is of unexplained mechanism[1,2] but may involve interaction of beryllium with tissue proteins.[3] In addition, studies of metal–protein complex formation can be used to elucidate protein structure.[4]

Beryllium–protein interactions have been qualitatively examined by several procedures. Thus, beryllium precipitates serum and nucleoproteins[5–10] and inhibits heat denaturation of trypsin.[11] Binding in calf serum at pH 7.5 at about 10^{-9} *M* beryllium is indicated by the dialysis experiment of Feldman, *et al.*[12]

In another study the strong inhibition of alkaline phosphatase by beryllium was used by Schubert and Lindenbaum[13] to measure the formation constants between this metal and chelating agents. The agreement of their values with those in the literature demonstrated that alkaline phosphatase binds beryllium.[14]

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(14) These authors err, however, in assuming that a 1:1 complex forms between beryllium and chelating agent. Meek and Banks[15] have shown that sulfosalicylic acid forms a 2:1 complex with beryllium at alkaline pH. The data in Fig. 1 and 3 of Schubert and Lindenbaum's paper can be used to determine which complex is formed. Theoretical curves based on data in Fig. 1 were plotted for the 1:1 and 2:1 complex of sulfosalicylic acid. The curve for the 2:1 complex coincided with the experimental curve in Fig. 3. The curve for the 1:1 complex did not. Furthermore the calculation of the formation constant for the 2:1 complex was $K = 1.74 \times 10^{-9}$, which is to be

Kosel and Neuman[16] used potentiometric titration to show interaction of $BeSO_4$ with several proteins; the binding sites were believed to be the ionized carboxyl groups.

The need for quantitative binding studies is evident. The insolubility of beryllium ions at pH's above 5 is a major difficulty. Even in dilute solutions, where no noticeable precipitate is present, the colloidal nature of hydrolyzed beryllium ion presents serious problems at these pH's. In the pH region 5 and below, however, beryllium ions are soluble and dialyzable at concentrations which permit the expression of the data in terms of standard binding equations. The method of equilibrium dialysis employed in these studies was therefore limited to this pH region. Potentiometric titrations have been used as an adjunct in the interpretations of the equilibrium dialysis results.

The present paper demonstrates that the carboxyl groups of bovine serum albumin are its major binding sites for beryllium ion. The influence of pH, temperature and dodecyl sulfate on these binding sites are correlated with their effect on the structure of this protein.

Experimental

Materials.—The bovine serum albumin was a crystalline product from Armour and Co. The protein was freed of bound anions by passing a 4–5% aqueous solution over an ion-exchange column containing Dowex-1-OH^- and Dowex-50-H^+.[17] Protein concentration was determined with the Beckman model DU spectrophotometer, using $E^{1\%}_{280}$ = 6.6 and a molecular weight of 69,000.[18]

Esterified bovine serum albumin was prepared according to Fraenkel-Conratt and Olcott.[19] About 85% of the carboxyls were esterified as determined by titration.

Inorganic reagents and buffers were analytical reagent or C.P. chemicals. Beryllium perchlorate was prepared by dissolving metallic beryllium in perchloric acid. Isotopic beryllium-7 (Be^7) was obtained from Oak Ridge.

Sodium dodecyl sulfate was supplied by E. I. du Pont de Nemours and Co. and recrystallized from alcohol.

The dialysis bags were 18/32 Visking sausage casings which had been soaked in 0.1 M HNO_3 for several days and then washed with water to remove competing metals.[20]

Apparatus.—A Farrand fluorometer was used for the fluorometric determination of beryllium.[7,21] Solutions containing the γ-emitting isotope Be^7 were analyzed with a scintillation counter obtained from Radiological Services, Inc.

Hydrogen ion activities were measured at the experimental temperatures with a Beckman model G pH meter.

The dialysis tubes were rocked in a water-bath which was kept to within ±0.10° of the desired temperature.

Equilibrium Dialysis.—Binding experiments were conducted at 4.0°, 25.0° and 37.3°. The buffers used were pH 2.5, glycine; pH 3.5–4.3, HCOOH; pH 4.5–5.0, HOAc at 0.05 M. The pH of the protein solutions was measured at the end of each experiment.

A standard solution of $Be(NO_3)_2$ was diluted with buffer to give the desired concentration and the requisite amount of $NaNO_3$ was added to maintain an ionic strength of 0.15. The maximum $Be(NO_3)_2$ concentration was limited to 0.04 M. Higher ionic strengths produced precipitation of native serum albumin in the presence of $Be(NO_3)_2$, even at 4°.

The pH was adjusted for each concentration before dilution to final volume. This was necessary because the hydrolysis of Be^{2+} is a function of its concentration and the pH therefore increased on dilution. Tracer Be^7 was then added in a volume which did not exceed 0.2% of the final volume.

Three milliliters of an approximately 2.5% buffered serum albumin solution in a dialysis bag was equilibrated with 5.0 ml. of the $Be(NO_3)_2$ solution in rubber-capped glass tubes. Controls indicated that no absorption of beryllium occurred on the bags or glass walls and that equilibrium was established in 20 hr. The tubes were routinely equilibrated for 40 hr. Both inside and outside solutions were analyzed for Be^7.

Potentiometric Titrations.—The titrations were carried out at room temperature (27°), pH 2.5, 3.5 and 4.5, with the aid of microburets. A solution of 0.5 M $BeClO_4$ was slowly added to 10.0 ml. of 2.5 × 10^{-4} M serum albumin in water until the pH increased about 0.05–0.10 unit and the 1 N $HClO_4$ was added until the pH was restored to its initial value; 10 ml. of water replaced serum albumin solution in the control. The hydrolysis was a slow process requiring 15–20 min. for equilibrium to be reached.

Results

Calculation of Binding Parameters.—The application of the law of mass action to multiple equilibria involving ion–protein associations gives the following relationship between r, the moles of ion bound per mole of protein, and (A) the concentration of free ion.[22]

$$r/(A) = Kn - Kr \quad (1)$$

This equation is based on the assumption that there are n binding sites with equal intrinsic association constants K and that there is no interaction between bound ions.

Under these conditions plots of $r/(A)$ *vs.* r will be linear and the values for n, K and $nK = K_1$, the association constant for the first site, can be obtained from the slope and intercepts.

The results of the dialysis experiments are plotted according to this function in Fig. 1–3. The total concentration of free dialyzable beryllium is taken to be Be^{2+} because the true concentrations of the various ionic species of beryllium are unknown because of hydrolysis. The lines were calculated by the method of least squares to obtain the parameters n, K and nK.

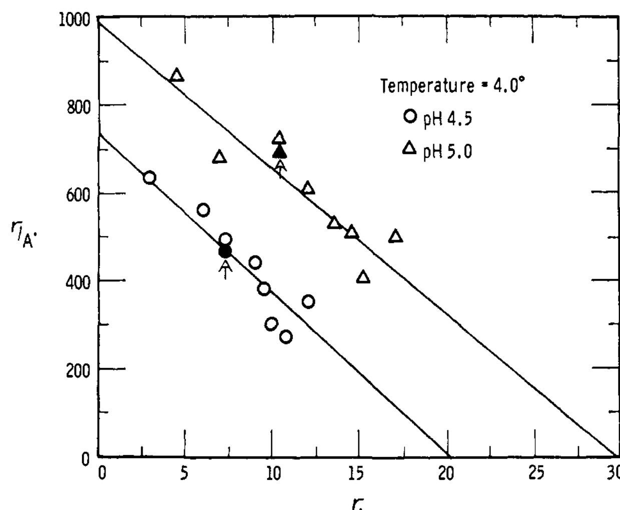

Fig. 1.—The effect of pH on the beryllium binding by bovine serum albumin at 4.0°. The symbols are experimental and the lines are calculated by the method of least squares. The filled symbols are the result of reversal experiments.

The reversibility of the reaction of 4 and 37.3° and all pH's was demonstrated by placing a protein-containing bag, which had been equilibrated with the highest concentration of $Be(NO_3)_2$, into a fresh solution of buffer plus $NaNO_3$ to maintain μ = 0.15. The new equilibria are given by the arrows in Fig. 1 and 3.

Reversibility and general linearity of the graphs lends support to the employment of eq. 1.

Effect of pH and Temperature.—The beryllium binding by bovine serum albumin was observed to be modified by pH and temperature (Fig. 1–3). This is clearly shown in Fig. 4 which gives the results at the

compared with the K = 1.12 × 10^{-9} obtained by Meek and Banks corrected for ionic strength and temperature.

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TABLE I

BINDING PARAMETERS FROM FIGURES 1–3 AND THERMODYNAMIC CONSTANT FOR THE FIRST BINDING SITE

	T = 4.0°		T = 25.0°			T = 37.3°		
pH	4.5	5.00	4.12	4.58	5.02	4.02	4.50	4.98
n	20	29.5	36.7	23.5	37.8	28.4	25	42.5
K	37	33.6	13.2	37.8	30.9	21.1	40.5	37.6
$K_1 = nK$	740	990	484	890	1164	600	1013	1598
ΔF_1^0, kcal./mole	−3.64	−3.71	−3.68	−4.02	−4.18	−3.86	−4.28	−4.54
ΔS_1^0, e.u.	13.2	13.8	12.3	13.6	14.1	12.8	13.9	14.7

maximum concentration of $Be(NO_3)_2$ = 0.04 M. No demonstrable binding occurred at pH 2.5 and 3.5. Binding begins above pH 3.5 and then increases with increasing pH and temperature.

Suggestions as to the factors responsible for the increased binding emerged from examination of the parameters n and K (Table I). At pH 4.5 and 5.0 and at all temperatures the values of K are reasonably uniform, the average K = 36.2 ± 2.7.

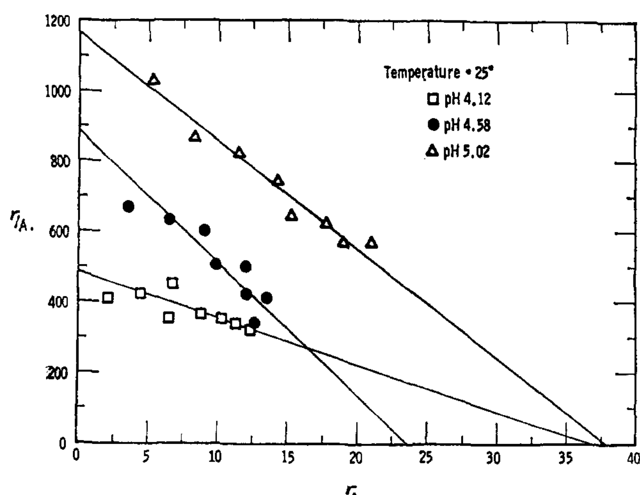

Fig. 2.—The effect of pH on the beryllium binding by bovine serum albumin at 25.0°. The symbols are experimental and lines are calculated.

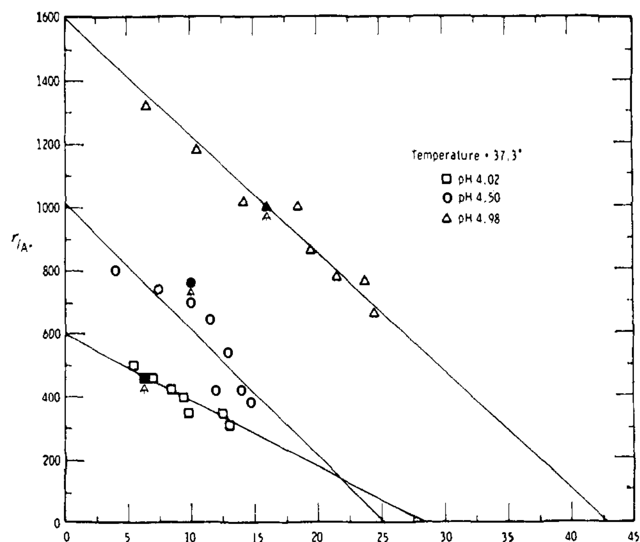

Fig. 3.—The effect of pH on the beryllium binding by bovine serum albumin at 37.3°. The symbols are experimental and the lines are calculated. The filled symbols are the results of reversal experiments.

The significance of the uniformity of K at pH 4.5 and 5.0 is that a single class of sites is reacting with beryllium at these pH's and at all temperatures. The appearance of different sites is therefore not responsible for the increased binding which must then be due to the increased availability of the same class of sites. The rising values of n as the pH and temperature increase at pH 4.5 and 5.0 (Table I) is indicative of this. The binding at pH 4 is inconsistent with that observed at higher pH in that the values of n are greater than at pH 4.5 and decrease with increasing temperature.

This striking dependence on pH and temperature of the beryllium binding to bovine serum albumin, including the anomalous values of n near pH 4 (Table I), probably reflects the marked structural changes known to occur in the serum albumin molecule in the acid pH range.[23–26] A discussion of this relationship will be postponed until the other data are presented.

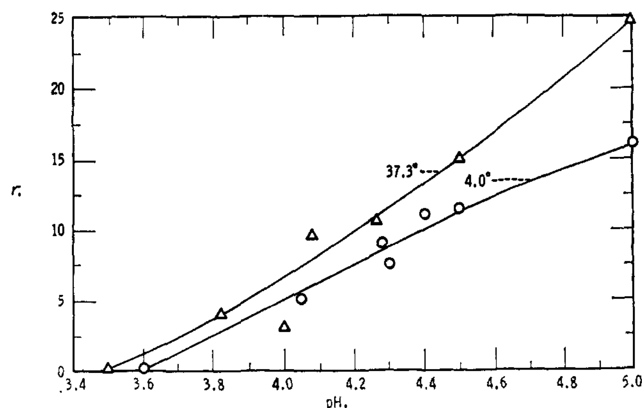

Fig. 4.—The effect of temperature and pH on the beryllium binding by bovine serum albumin at a free beryllium concentration of 0.04 M.

Potentiometric Titrations.—Potentiometric titrations of $Be(ClO_4)_2$ with $HClO_4$ at constant pH were undertaken to provide additional information on the beryllium binding of serum albumin.

The hydrolysis of beryllium ion may be expressed in a general way by the equilibrium[27]

$$x\text{Be}^{2+} + y(\text{H}_2\text{O}) \rightleftharpoons \text{Be}_x(\text{OH})_y + y\text{H}^+ \qquad (2)$$

$$K_{xy} = (\text{Be}_x(\text{OH})_y)(\text{H}^+)^y/(\text{Be}^{2+})^x$$

where $Be_x(OH)_y$ represents any hydrolyzed polynuclear species. It is evident from eq. 2 that the hydrolysis reaction is a function of pH and beryllium concentration. The dilution of a hydrolyzed beryllium solution results in dissociation of the polynuclear species with a concomitant uptake of hydrogen ions. This effect was measured by determining the amount of acid required to maintain a constant pH.

The results, corrected for volume changes, are given in Fig. 5. The letters T and P indicate turbidity and gross precipitation, respectively.

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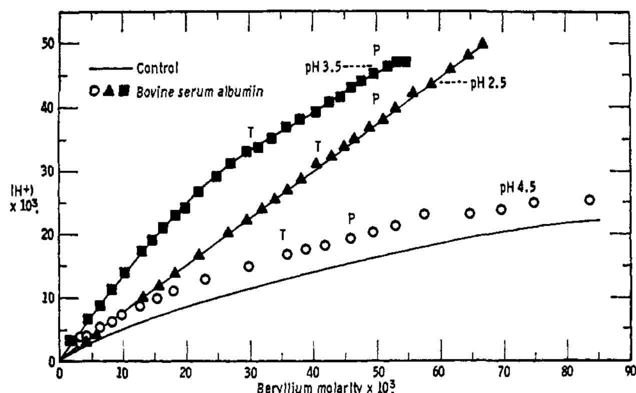
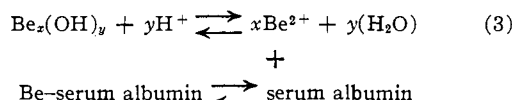


Fig. 5.—Potentiometric titrations at constant pH of diluted $Be(ClO_4)_2$ solutions with 1 N $HClO_4$. The lines are control titrations and the symbols are the results in the presence of bovine serum albumin; T = turbidity, P = precipitate.

According to Feldman and Havill[28] and Kakihana and Sillén[27] the hydrolysis of beryllium ion in dilute solution begins above pH 2.5. One would therefore expect that upon sufficient dilution complete dissociation of the hydrolyzed complex would result, and this would be revealed by a constant ratio of y/x. The linear relationship between hydrogen ion uptake and beryllium concentration at this pH (Fig. 5) therefore indicates that beryllium exists as unhydrolyzed Be^{2+}. At pH 3.5, the decreasing curvature of the graph indicates beryllium hydrolysis which increases with beryllium concentration. The presence of serum albumin had no effect on the curves of pH 2.5 and 3.5.

At pH 4.5 there is considerable hydrolysis. In this case the protein curve is displaced upward revealing interaction with beryllium. The pH dependence of beryllium binding revealed by dialysis experiments is thus substantiated by the titration results.

The additional uptake of hydrogen ions in the presence of protein must result from further dissociation of the polynuclear complex to form a less hydrolyzed beryllium species, represented by Be^{2+}, which is being bound to the protein according to reaction 3.



$$Be_x(OH)_y + yH^+ \rightleftarrows xBe^{2+} + y(H_2O) \quad (3)$$
$$+$$
$$\text{Be–serum albumin} \rightleftarrows \text{serum albumin}$$

The extent of beryllium binding may be estimated by assuming an equivalence between the additional hydrogen ions required and the atoms of beryllium bound. Table II compares this estimated binding with that obtained from dialysis at the same pH. It is significant that the maximum value for r is approximately the same in both experiments. The difference in the two sets of r-values may be partially attributed to the difference in experimental conditions.[29]

The Effect of Denaturation by Sodium Dodecyl Sulfate.—Binding experiments were conducted at 25°, pH 5.0, in the presence of 1% (0.035 M) dodecyl sulfate. Beryllium concentrations >0.003 M precipitated the protein inside the bags. Lower concentrations which did not precipitate the protein were therefore used.

Examination of the curve in Fig. 6 reveals that the data are inconsistent with the assumptions expressed in eq. 1, namely that all sites are independent and equivalent. The rising values of $r/(A)$ with increasing values of r and negative curvature of the function indicate a coöperative phenomenon which would result from an increase in n or K or both as beryllium is being bound to the protein.

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(29) The titrations were conducted with perchlorate instead of nitrate, and the temperature and ionic strength were not held constant.

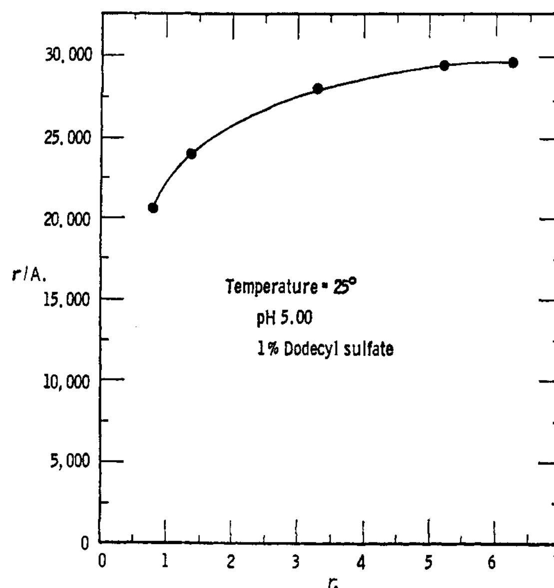

Fig. 6.—The beryllium binding by bovine serum albumin in 1% dodecyl sulfate.

Another important difference between native- and dodecyl sulfate-treated serum albumin is that the latter has a much greater affinity for beryllium. For example, the binding of 6.3 mmoles of beryllium/mole of albumin occurs at 2.12×10^{-4} M beryllium in dodecyl sulfate. This binding value would be obtained at 7.1×10^{-3} M beryllium for native protein. This striking difference must be largely due to the electrostatic influence of the high negative charge of the dodecyl sulfate albumin complex. These experiments were conducted at a molar ratio of dodecyl sulfate/albumin = 105 where all the detergent is bound to the protein.[30]

TABLE II

COMPARISON OF BERYLLIUM BINDING DETERMINED BY POTENTIOMETRIC TITRATION WITH THAT OBTAINED BY EQUILIBRIUM DIALYSIS

Free beryllium concn., mole/l.	Moles beryllium bound/mole albumin = r Titration pH 4.50, 27°	Dialysis[a] pH 4.58, 25°
0.0082	8.4	6.0
.0130	10.0	8.0
.0172	14.0	9.5
.0215	17.5	10.0
.0360	21.0	13.0
.0414	19.0	
.0557	23.5[b]	

[a] The maximum value of r (= n, Table I) = 23.5. [b] At beryllium concentration above 0.0360 the values of r did not greatly change and were in the neighborhood of 20.

The Ionized Carboxyls as the Sites for Beryllium Binding.—The pH dependence of the binding in the region where the carboxyls become ionized and the large number of binding sites (n = 42, pH 5.0, 37.3°) suggest that the carboxyls are the beryllium binding sites. In addition, esterified albumin pH 5.0 at 4 and 37.3° exhibited no beryllium binding. This loss of binding may, however, be a result of an electrostatic effect owing to the high positive charge of esterified albumin.

Interaction with imidazoles or amino groups is unlikely since beryllium has a low affinity for such groups and it would require a very strong affinity to

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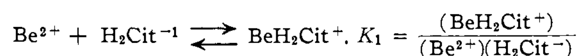
compete with H^+ at the experimental pH's. Interaction with the tyrosines is also unlikely since the ultraviolet absorption spectrum of bovine serum albumin, pH 4.5–5.0, containing 0.1 *M* $BeSO_4$ is identical with that of protein alone. Combination with the sulfhydryl group was tested by the effect of beryllium on the copper reaction with bovine albumin. Klotz, *et al.*,[31] have shown that dilute solutions of copper react with bovine albumin to give an absorption maximum at 375 mμ which is due to copper–sulfhydryl interaction. This band was depressed or abolished by other metals which reacted with the sulfhydryl groups. This band, which was obtained with a solution of 3×10^{-4} *M* albumin, 0.003 *M* CuOAc, pH 4.5, was not depressed by 0.01 *M* $BeSO_4$. The sulfhydryl group is therefore not involved.

At higher concentration of copper, the copper–carboxyl interaction gives a green solution with a maximum absorption near 800 mμ.[32] If beryllium reacts with the carboxyl groups, this band should be affected. The role of carboxyls could not, however, be tested in this way since precipitation occurred when beryllium was added to the copper–albumin solution at pH 4.5–5.0.

The evidence presented above indicates that the ionized carboxyls are the groups which interact with beryllium at pH 5.0 and below in agreement with Kosel and Neuman.[16] It would be desirable to compare the association constant of beryllium with a model compound (such as acetate) with those obtained with serum albumin. An approximation may be the reaction of beryllium with citric acid as determined by Feldman, *et al.*[33]

These authors measured the reaction of Be^7 in NH_4ClO_4, $\mu = 0.15$, $T = 34°$ in the pH range 3.0–4.0. The beryllium concentration was sufficiently dilute ($<10^{-5}$ *M*) so that all the beryllium was present as Be^{2+}. They described the reaction in terms of various ionic forms of citric acid. Beryllium did not react with citric acid molecules but did react with citrate ions. Three formation constants were obtained for the formation of (BeH_2Cit^+), (BeHCit) and ($BeCit^-$). The first association



$$Be^{2+} + H_2Cit^{-1} \rightleftharpoons BeH_2Cit^+,\ K_1 = \frac{(BeH_2Cit^+)}{(Be^{2+})(H_2Cit^-)}$$

involves a single carboxyl group and may be taken as our model reaction. The value $K = 25$ obtained by these authors is in reasonable agreement with the value of $K = 36.2$ for the beryllium–serum albumin reaction. This agreement may, however, be fortuitous since the concentration of the ionic species, Be^{2+}, in the protein experiment is unknown and is assumed to equal the total free beryllium.

Thermodynamic Results.—Table I gives the thermodynamic data for $nK = K_1$. The free energy of binding is usually given for the first association, $\Delta F_1°$, in order to avoid a decision as to *n*, the number of sites. The values of $\Delta F_1°$, for the Be–serum albumin reaction is similar to the binding energies of anion and cation interactions with serum albumins, whose $\Delta F_1°$'s are in the neighborhood of −5000 cal./mole.[34–36] The free energy of binding is mostly due to changes in entropy with the apparent enthalpy contribution small and sometimes close to zero. The entropy changes are in the range of 8–24 cal./deg./mole within which the entropy values of the Be–serum albumin reaction lie. The thermodynamics of this reaction is therefore comparable to that of other ion–serum albumin reactions.

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Discussion

Consideration of Beryllium Hydrolysis.—The increase in binding with increasing pH and temperature has been interpreted by us to signify an increase in the number of available carboxyls reactive with beryllium. An alternative possibility is suggested by the nature of beryllium hydrolysis which increases with beryllium concentration, pH and temperature. If some hydrolyzed beryllium species preferentially reacted with bovine albumin, an increase in pH and temperature might increase the concentration of this species and lead to increased binding values.

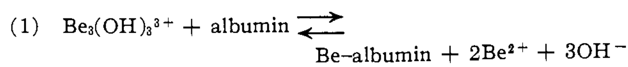
Kakihana and Sillén[27] have recently studied the hydrolysis of beryllium in the pH 2–6 range, beryllium concentration 0.0001–0.048 *M*, $\mu = 3$ (ClO_4^-), $T = 25°$. Their analysis of the data indicated the presence of two major species of beryllium, Be^{2+} and $Be_3(OH_3)^{3+}$, and two minor species, $Be(OH)_2$ and $Be_2(OH)^{3+}$.

The binding experiment in the presence of dodecyl sulfate (Fig. 6) was conducted at low concentration of beryllium, where, according to Kakihana and Sillén, 75–88% of the total free beryllium is present in the unhydrolyzed form Be^{2+}. This suggests, therefore, that this form is capable of reacting with protein. The experiments with esterified albumin indicate that anionic forms of beryllium, such as $BeO_2^{=}$, are not responsible for the binding. The results of the potentiometric titration experiments are consistent with this in that they suggest that a less, rather than more, hydrolyzed form is reacting with bovine albumin. Since beryllium hydrolysis increases with concentration,[27] one would not expect linearity of the binding curves, if only one type of beryllium species were reacting. Attempts to define the reactive species as Be^{2+}, in terms of Kakihana and Sillén's data, led to a further inconsistency in that the values for $r/(A)$ increased with increasing values of r. A possible explanation is that all beryllium species react with bovine albumin and that unknown compensating factors are responsible for the apparent linearity of the graphs.[37]

Although the effect of temperature on the hydrolysis of beryllium has not been measured, the temperature effect is most easily explained in terms of protein changes rather than of beryllium hydrolysis. There is evidence that small temperature differences affect the metal-binding properties of serum albumin. Gurd[38] has shown that the zinc binding of human serum albumin increased in going from 25 to 37° and precipitated the protein at these temperatures. The binding at 25° was the same as at 0° where no precipitation occurred. The precipitation of protein was a complicating feature of the binding so that it is not clear whether the zinc is in true equilibrium with the protein. These experiments were conducted under conditions

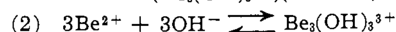
(37) If $Be_3(OH)_3^{3+}$ reacts with bovine serum albumin and the complex is broken up in the reaction, the equilibrium would still be formulated in terms of Be^{2+}.

(1) $Be_3(OH)_3^{3+} + \text{albumin} \rightleftharpoons \text{Be–albumin} + 2Be^{2+} + 3OH^-$



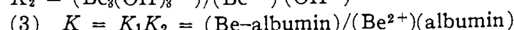
$$K_1 = \frac{(\text{Be–albumin})(Be^{2+})^2(OH^-)^3}{(Be_3(OH)_3^{3+})(\text{albumin})}$$

(2) $3Be^{2+} + 3OH^- \rightleftharpoons Be_3(OH)_3^{3+}$



$K_2 = (Be_3(OH)_3^{3+})/(Be^{2+})^3(OH^-)^3$

(3) $K = K_1K_2 = (\text{Be–albumin})/(Be^{2+})(\text{albumin})$



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where zinc does not hydrolyze; the temperature effect was therefore ascribable to the protein. In addition, irreversible alterations in the protein at 37° was revealed by the insolubility of the Zn–protein precipitate when brought to 0°.

The effect of pH is more difficult to evaluate. The uniformity of K at pH 4.5 and 5.0 at the three temperatures indicates that changing beryllium hydrolysis is not responsible for the increased binding but that more carboxyls become available as the pH is increased. However, if the slightly increased charge on the protein ($Z_p = +3$ at pH 4.5, and -3 at pH 5.0[39]) were exerting an electrostatic effect, then a less hydrolyzed species, *i.e.*, Be^{2+}, might be more extensively bound at higher pH.

The values for n, however, decrease from pH 4 to pH 4.5 and then increase at pH 5.0. These changes are not those expected if beryllium hydrolysis were the major factor; but they may be related to the reactivity of serum albumin in this pH region.[40]

The considerations discussed above suggest that the changes in beryllium binding are more likely a result of changes in protein structure rather than a result beryllium hydrolysis. Although the absolute values of n and K are probably functions of beryllium hydrolysis and hydrogen ion competition, they serve to indicate that the carboxyl reactivity of serum albumin is affected by pH and temperature.

Beryllium Binding as a Reflection of Structural Changes Due to Environmental Conditions.—The serum albumins exhibit marked structural changes in the pH region below 5. The large increase in viscosity[23,41] and optical rotation[41] which occurs below pH 3.5 is a result of the expansion of the molecule which arises from the high positive charge at these pH's. In the pH interval 3.5 to 5 the molecule undergoes a transitional phase which is characterized by viscosity changes,[23] electrophoretic heterogeneity[2,5,42] salting out behavior[24] and marked changes in rate of heat denaturation.[43]

According to Tanford, *et al.*,[23] certain critical bonds are broken in the pH region 4.3 to 4.0 which results in a loosening of the normal structure of the molecule. Below pH 4.0 a single expandable form exists which would readily respond to environmental conditions such as temperature.

The electrophoretic heterogeneity observed by Aoki and Foster[25] consisted of a normal slow form and a fast isomerized form whose concentration increased from 0 near pH 5 to 100% near pH 3.5. The fast form strongly bound anions and was precipitated at high salt concentration.[24] At pH 4 both forms were present in about equal amounts. Schmid and Polis[44] have observed several electrophoretic peaks which were most pronounced at pH 4. The number and relative areas of these peaks were affected by various anions and metal cations.

The beryllium binding of bovine serum albumin with respect to pH and temperature is another indication of the complex behavior of this protein in the acid pH range. The several forms which are present as a function of environmental conditions are characterized by the number of carboxyl groups available for beryllium binding. This suggests an interpretation of the binding consistent with the behavior of albumin if one assumes that the binding site involves two reacting groups, perhaps two carboxyls.

Below pH 3.5 the molecule is expanded and the pairs of reacting groups are too widely separated to react with beryllium. Above this pH the chelate sites become close enough for reaction. The availability of the chelate site is apparently a function of the various isomerized forms which are pH and temperature dependent.[45,46] Increased beryllium binding and protein precipitation were also observed at higher nitrate concentrations[47] which would displace the isomerization reaction.

The Effect of Dodecyl Sulfate.—Detergents such as decyl or dodecyl sulfate produce a partial unfolding or swelling of serum albumin,[48,49] which is reversed by removal of detergent. This reversibility indicates a flexible protein whose structure may be readily modified.

The coöperative binding of beryllium in 0.035 M dodecyl sulfate (Fig. 6) must reflect these structural changes. Many chelating carboxyls are initially too widely separated to react. As beryllium is bound, the flexible protein molecule becomes more compact so that additional sites are brought into juxtaposition to form new binding sites.

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(39) The values for Z were taken from the paper of Tanford, *et al.*,[23] who give these values for bovine albumin at 25°, $\mu = 0.15$ in KCl. Since nitrate is bound more than chloride, Z_p is probably more negative, but the nitrate binding is not accurately known.

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(47) In addition to pH and temperature, an increase in the anion binding may also affect the structure of bovine albumin.[24] An experiment at $\mu = 0.45$ ($NaNO_3$), pH 4.5–5.0, 4°, gave greater binding values than at $\mu = 0.15$. Precipitation occurred at >0.04 M (Be^{2+}) at 4°; and when brought to room temperature, precipitation occurred at >0.02 M (Be^{2+}).

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