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Effect of pH on the Binding of *N*-Alkyl Sulfates to Bovine Serum Albumin*

Jacqueline A. Reynolds,† Joan P. Gallagher, and Jacinto Steinhardt

ABSTRACT: The binding of octyl and dodecyl sulfates to the sites of highest affinity of native bovine serum albumin is independent of solution pH (and therefore protein charge) over the range 4.8–6.8. This result is consistent with the current view that the energy of interaction is primarily hydrophobic rather than coulombic. The free energy of interaction between decyl sulfate and native bovine serum albumin, however, increases with decreasing pH, possibly as the result of an alteration in the interaction of the anionic end group of this ligand with a charged group on the protein, an interaction which is restricted to this particular ligand for steric reasons. The observed effects of pH are interpreted in terms of the effects on numbers of binding sites, and the binding constants, of native and denatured bovine serum albumin.

The effect of pH on the initial unfolding transition of bovine serum albumin, induced by the binding of certain long-chain detergents, such as dodecyl sulfate, at molar ratios slightly greater than 10–15 is to require progressively higher values of free ligand concentration as the pH is increased. This phenomenon cannot be accounted for entirely by assuming a *single* pH-dependent equilibrium constant between native and unfolded bovine serum albumin in the absence of ligand, but it is unlikely that the affinity of unfolded protein to these ligands depends on pH. At pH 6.8 and above the first unfolding transition can be observed only at free ligand concentrations above those at which a second, more extensive transition occurs.

It has been shown that the free energy of binding of a number of anions to serum albumin involves other than coulombic forces, and is primarily entropic in origin (see, for example, Kauzmann, 1959; Ray *et al.*, 1966; Reynolds *et al.*, 1967, 1968). Thus, native bovine serum albumin binds 4–10 moles of alkyl sulfates, sulfonates, carboxylates, and alcohols (the number depending on the chain length) with negative free energies which exceed 5 kcal/mole of ligand—far larger than would be expected from ionic interactions alone. Markus *et al.* (1964) have implicated cationic sites in the binding of dodecyl sulfate to human serum albumin, but again the free energy of interaction is large and must contain a major contribution from the nonionic entropic term.

Nevertheless, there is a substantial effect of solution pH on certain characteristics of the interaction between detergent-like molecules and bovine serum albumin (*e.g.*, Decker and Foster, 1966). The present work deals with these pH effects when two nonunfolding ligands (octyl and decyl sulfate; Reynolds *et al.*, 1967) and one unfolding ligand (dodecyl sulfate) are bound to bovine serum albumin.

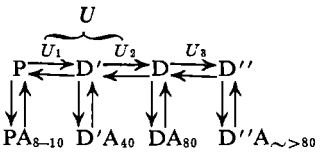
The purpose of this study has been twofold: first, to elucidate the mechanism of large pH effects on binding–unfolding equilibria which are determined only slightly by charge interactions; and second, perhaps more important, to further test the ability of the model equilibria proposed in an earlier paper (Reynolds *et al.*, 1967) to predict or account for the effects of pH described here. In order to accomplish the latter, an effort is made to express the pH effects in terms of the

* From the Department of Chemistry, Georgetown University, Washington, D. C. 20007. *Received October 23, 1969.* This work was supported by Grant GM 12085 (NIH) to one of us (J. S.).

† Present address: Department of Biochemistry, Duke University, Durham, North Carolina.

intrinsic binding constants K and J, of native and denatured proteins, respectively, for ligands, the number of sites, n or m, for these ligands on the folded and unfolded forms, and the equilibrium constants U ($= U_1U_2$) which express the ratios of unfolded to folded protein in the uncomplexed form (these constants are defined later).

The ligand-induced unfolding of bovine serum albumin by dodecyl sulfate occurs in the following steps:



At pH 5.6, and with our preparation of bovine serum albumin, D′ is not observed and the transition may be treated as going directly from P to D. D′′ represents a more highly disorganized state (Reynolds *et al.*, 1967) which exists at high free ligand concentrations where there is micellar binding (the formation of mixed micelles at the surface of the protein in which a hydrophobic region of the macromolecule is incorporated into a micelle-like structure composed of several detergent molecules).

Experimental Section

Materials. Solutions (5%) of bovine serum albumin (Nutritional Biochemicals Corp.) were deionized on a mixed-bed ion-exchange column (AG 501-X8, Bio-Rad Laboratories) containing a 1 in. layer of Dowex 501-X4 at the bottom. Deionized protein solutions were stored at 2° and used in less than 2 weeks. Concentrations were determined spectrophotometrically on the basis of $\epsilon^{1\%}_{1\,cm}$ 6.67 and a protein molecular weight of 69,000. Alkyl sulfates were a special grade made for us by Mann Research Laboratories. They were free of impurities when examined by thin-layer chromatography. Phosphate and acetate buffers were prepared from Fisher Reagent grade chemicals. Phosphate buffers were used at pH values ≥ 5.6 and acetate buffers at pH values ≤ 4.8. Both were at the same ionic strength, 0.033, except in one experiment at pH 3.8 in which the ionic strength was 0.004.

Visking dialysis tubing was treated before use by boiling approximately 1 hr in distilled water, followed by repeated washing.

Methods. Equilibrium dialysis procedures and analysis of alkyl sulfates have been described previously (Ray *et al.*, 1966; Reynolds *et al.*, 1967). At the temperature of these experiments, 2°, no appreciable hydrolysis of the half-esters occurred during the maximum time, 72 hr, required for the attainment of equilibrium. Viscosity measurements were made in Cannon–Fenske viscometers having flow times from 100 to 600 sec. The constant temperature bath for viscosity measurements was thermostated to 2° ± 0.005.

Numerous studies of bovine serum albumin in Foster's laboratory (see particularly Sogami and Foster, 1968) have shown the protein to be microheterogeneous. Sogami and Foster have reported large variations in the breadth and position of the N → F transition as the result of alterations in the degree of microheterogeneity. In the authors' laboratory similar perturbations of the unfolding transition of bovine serum albumin which is induced by dodecyl sulfate binding have been noted when results obtained with different lots of bovine serum albumin are compared. Figure 1 shows three different binding isotherms (dodecyl sulfate) of different lot numbers of bovine serum albumin at the same pH and ionic strength. The highest energy sites are apparently unaffected, but the breadth of the transition and the free ligand concentration at which the transition occurs vary with the protein preparation as reported previously by Anderson (1966) for the interaction of alkyl benzenesulfonates with bovine serum albumin. Consequently, the studies carried out on pH effects on binding which are reported here relate to one lot number of bovine serum albumin (9385) subjected to identical deionizing and storage procedures.

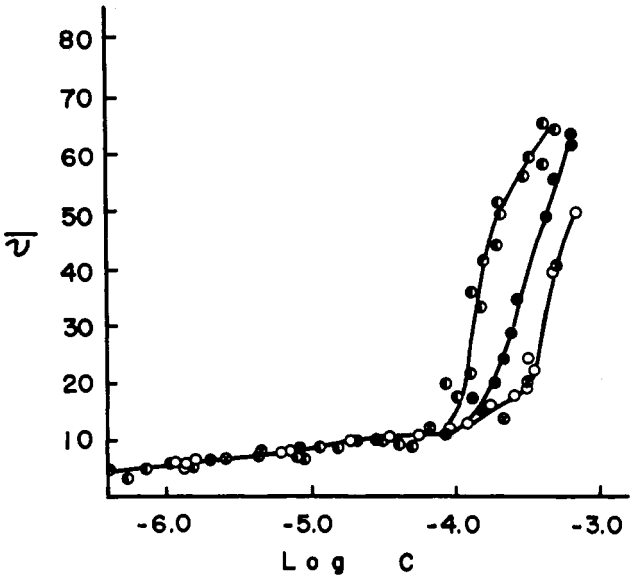


FIGURE 1: Binding of dodecyl sulfate at 2° in pH 5.6 phosphate buffer at 0.033 ionic strength by four different batches of crystalline bovine serum albumin: (○) resin-deionized Nutritional Biochemicals lot 5776; (⊗) charcoal defatted lot 5776; (●) lot 9385; (◐) another unidentified lot number.

In view of the demonstration by Gallagher and Steinhardt (1969) that defatting bovine serum albumin by the charcoal method of Chen has no discernible effect on the binding isotherm for these ligands with the native form of bovine serum albumin, only one experiment with defatted bovine serum albumin (Figure 1) is reported here.

Results

Binding to the Native State of Bovine Serum Albumin. Figures 2, 3, and 4 show partial binding isotherms at 2° for octyl, decyl, and dodecyl sulfate to native bovine serum albumin at pH 4.8, 5.6, and 6.8. Within experimental error n and K (the number of sites and intrinsic association constant, respectively) for octyl and dodecyl sulfates are independent of pH. However, the *decyl sulfate* isotherm shows a systematic decrease in the number of sites occupied at any given free ligand concentration as the pH is increased. Over this pH range bovine serum albumin undergoes no pH dependent transitions, and the intrinsic viscosity of the protein as a

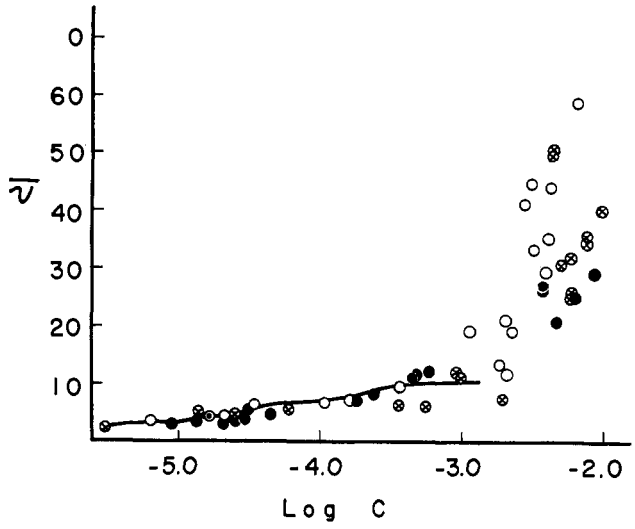


FIGURE 2: Octyl sulfate–bovine serum albumin binding isotherms at 2°, ionic strength 0.033: (●) pH 4.8 (acetate); (○) pH 5.6 (phosphate); (⊗) pH 6.8 (phosphate).

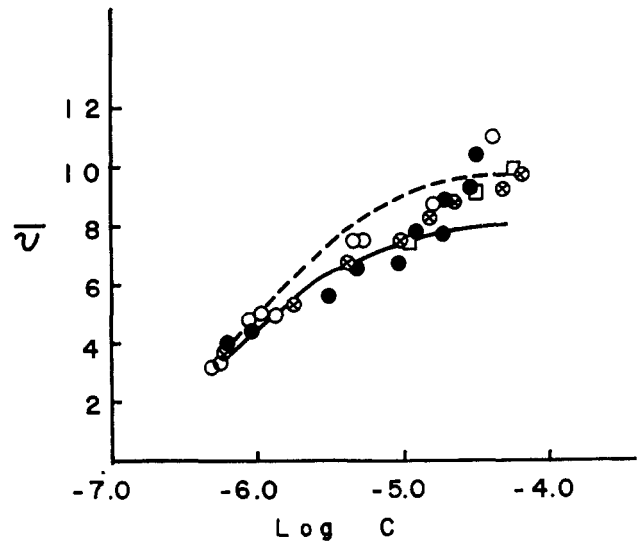


FIGURE 4: Dodecyl sulfate–bovine serum albumin binding isotherms at 2°, ionic strength 0.033: (●) pH 4.8; (○) pH 5.6; (⊗) pH 6.8; (——) eq 2 with $n = 8$, $K = 1.2 \times 10^6$; (– – –) $n = 10$, $K = 10^6$.

function of $\bar{\nu}$ between $0 \geq \bar{\nu} \leq 10$ is constant with all three ligands.

The solid lines in Figures 2 and 4 are calculated from the n and K values shown in Table I using the law of mass action

$$\bar{\nu} = \frac{nKC}{1 + KC} \qquad (1)$$

where $\bar{\nu}$ = moles of ligand bound/mole of protein, and C = free ligand concentration, and n and K are as defined above. Equation 1 assumes that there is no interaction between identical sites (Reynolds *et al.*, 1967). If the number of sites in the set of highest affinity is assumed to be unchanged at all three pH values with decyl sulfate, the association constants vary as shown in Table I. With all three ligands and with all pH values plots of log $n/(n - \bar{\nu})$ *vs.* log C are linear with slopes of 1, provided the values of n listed in Table I are used.

At this point it should be noted that the net charge $\bar{h}$ on the protein varies from −8.5 at pH 6.8 to +8.0 at pH 4.8 at this ionic strength if no buffer anions are bound (Tanford *et al.*, 1955). This variation apparently does not affect the number of sites or the free energy of interaction with anions when the ligand is octyl or dodecyl sulfate. The special case of decyl sulfate will be considered later.

It has been shown previously (Reynolds *et al.*, 1967; Polet and Steinhardt, 1968) that neither octyl nor decyl sulfate induces a measurable alteration in bovine serum albumin conformation at pH 5.6 over the entire range of free ligand

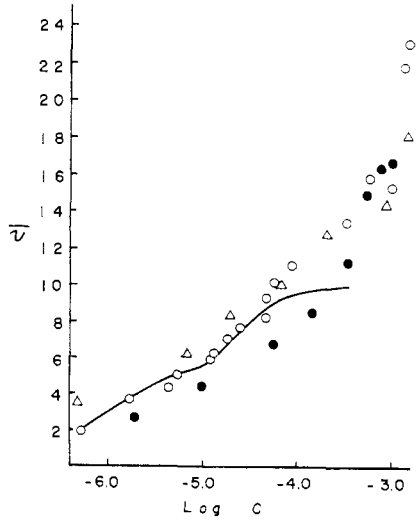


FIGURE 3: Decyl sulfate–bovine serum albumin binding isotherms at 2°, ionic strength 0.033: (△) pH 4.8 (acetate); (○) pH 5.6 (phosphate); (●) pH 6.8 (phosphate).

TABLE I: Association Constants (K) and Number of Sites (n) in Folded and Unfolded Forms.

Compound	pH	n	$K \times 10^6$	kcal/mole F	n'	$K' \times 10^4$	$\bar{h}$[a]
$C_{12}SO_4$	4.8	8–10	1–1.2	7.7			+8.0
	5.6	8–10	1–1.2	7.7			−1.0
	6.8	8–10	1–1.2	7.7			−8.5
$C_{10}SO_4$	4.8	5–6	4.0	8.3			+8.0
	5.6	5–6	1.4	7.8	4–5	4.1	−1.0
	6.8	5–6	0.4	7.1			−8.5
C_8SO_4	4.8	4–5	0.6	7.3	6–7	0.7	+8.0
	5.6	4–5	0.6	7.3	6–7	0.7	−1.0
	6.8	4–5	0.6	7.3	6–7	0.7	−8.5

[a] Tanford *et al.*, 1955.

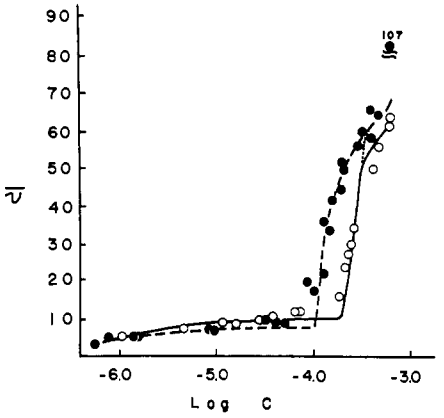


FIGURE 5: Dodecyl sulfate–bovine serum albumin binding isotherms at pH 5.6, 2°, ionic strength 0.033: (●) unidentified early lot number; (○) lot 9385; (——) eq 2 and 3 with $n = 10$, $K = 10^6$, $m = 80$, $J = 6 \times 10^3$, $U = 5 \times 10^{-7}$; (– – –) $n = 8$, $K = 1.2 \times 10^6$, $m = 80$, $J = 8 \times 10^3$, $U = 5 \times 10^{-7}$.

concentration investigated—including the "cooperative"[1] region in the binding isotherms at which $\bar{v}$ rises sharply (log $C = -2.2$ to -2.4 with octyl sulfate). At pH 4.8 and 6.8 no change in the intrinsic viscosity of bovine serum albumin solutions is observed in the presence of these two ligands at these high concentrations. The "cooperativity" noted in the binding isotherm is probably due to mixed micellar binding at the surface of the protein.

The Binding-Induced Transition in Bovine Serum Albumin–Dodecyl Sulfate Complexes. Figure 5 shows the complete binding isotherm (including transition region) of dodecyl sulfate with bovine serum albumin at pH 5.6. The position on the abscissa of the sharp rise in the isotherm is a function of the preparation used as described in the Experimental Section.

The model proposed by Foster and Aoki (1958) and expanded in detail by Reynolds *et al.* (1967) for binding-induced conformational changes in proteins employs the following relationships:

$$\mathrm{F}(C) = \frac{U\sum_{r=0}^{m} C^r J^r \dfrac{m!}{r!(m-r)!}}{\sum_{s=0}^{n} C^s J^s \dfrac{n!}{s!(n-s)!}} = U\frac{(1+JC)^m}{(1+KC)^n} \qquad (2)$$

where F(C) = ratio of protein in state 2 to that in state 1 at any given free ligand concentration C, and $U = D/P$ (ratio of unbound protein in state 2 to unbound protein in state 1). As shown earlier, U may be regarded as the product of two other constants, U_1 and U_2. C = free ligand concentration, m = total number of sites in state 2, J = intrinsic association constant between ligand and protein in state 2, n = total number of sites in state 1, K = intrinsic association constant between ligand and protein in state 1, r = number of occupied sites in state 2, s = number of occupied sites in state 1, and

$$\bar{v} = \frac{C}{1+\mathrm{F}(C)}\left[\frac{nK}{1+KC} + \frac{\mathrm{F}(C)mJ}{1+JC}\right] \qquad (3)$$

The solid lines in Figure 5 have been calculated from these equations with the numerical values indicated in the figure as the closest fit to the experimental data at pH 5.6. Figure 6 shows that if m, n, K, and U are kept constant but J is increased by the rather small factor 1.38 the transition point is displaced from log $C = -3.7$ to log $C = -4.0$. In Figure 7, the effect of varying U widely from 10^{-3} to 5×10^{-7}, again keeping m, n, K, and J constant at values given in the figure legend, is shown. It is apparent that only a very small shift in the position of the transition is brought about by a very large change in the equilibrium constant for the transition of uncomplexed native to uncomplexed perturbed state. If U is assumed to be 5×10^{-3}, and $n = 10$, $K = 10^{+6}$, $m = 80$, and $J = 8.3 \times 10^3$, the calculated binding curve is identical with the solid line in Figure 5 except that it joins the dashed line as shown at log $C = 3.4$. Thus, it is not possible to decide from these experimental data alone whether the difference between protein preparations represents small differences in the association constant for ligand in the sites in state 2 or large differences in the equilibrium constant, U.

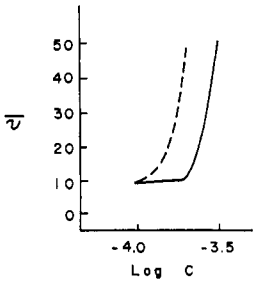


FIGURE 6: Theoretical binding isotherm (eq 2 and 3) for the bovine serum albumin–dodecyl sulfate transition region: $n = 10$, $K = 10^6$, $m = 80$, $U = 5 \times 10^{-7}$; (——) $J = 6 \times 10^3$; (– – –) $J = 8 \times 10^3$.

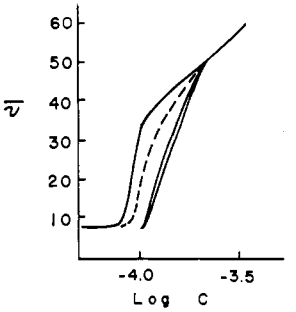


FIGURE 7: Theoretical binding isotherm (eq 2 and 3) for the bovine serum albumin–dodecyl sulfate transition region: $n = 8$, $K = 1.2 \times 10^6$, $m = 80$, $J = 8.3 \times 10^3$; (——) $U = 10^{-3}$; (– – –) $U = 5 \times 10^{-5}$; (═══) $U = 5 \times 10^{-7}$.

[1] The term "cooperative" is used here as a matter of convenience to refer to that portion of the binding isotherm in which $\Delta\bar{v}/\Delta \log C$ increases sharply. This phenomenon may be the result of a conformation change exposing a set of binding sites not available on the native protein, or it may be the result of multiple binding to one site (condensation phenomenon) such as the formation of a mixed micelle between a hydrophobic region on the protein surface and a number (>1) of detergent-like ligands.

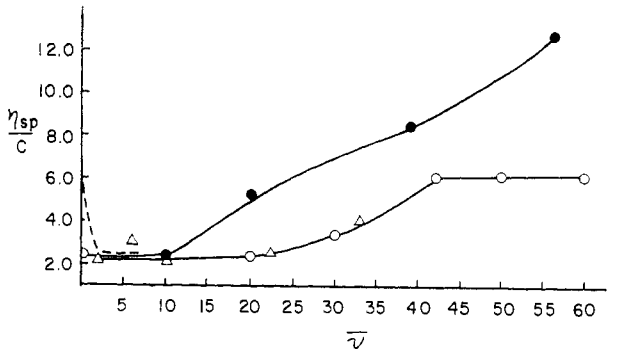


FIGURE 8: Reduced viscosity of 0.1% bovine serum albumin–dodecyl sulfate complexes at 2°, ionic strength 0.033: (△) pH 4.8; (○) pH 5.6; (●) pH 6.8; (- - -) pH 3.8, ionic strength 0.001.

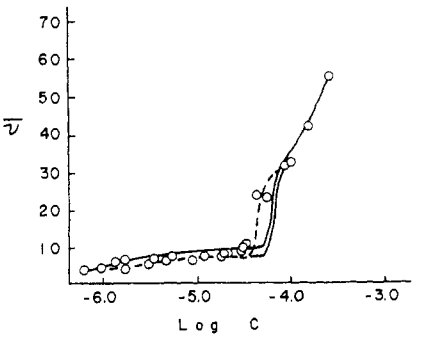


FIGURE 9: Theoretical dodecyl sulfate–bovine serum albumin binding isotherms (eq 2 and 3) at pH 4.8, 2°, ionic strength 0.033: (——) $n = 10$, $K = 10^6$, $m' = 40$, $J' = 5 \times 10^4$, $U_1 = 2.5 \times 10^{-7}$, $m = 80$, $J = 8.3 \times 10^3$, $U_2 = 2.5 \times 10^{-7}$; (- - -) $n = 8$, $K = 1.2 \times 10^6$, $m' = 40$, $J' = 5 \times 10^5$, $U_1 = 2.5 \times 10^{-7}$, $m = 80$, $J = 8.3 \times 10^3$, $U_2 = 2.5 \times 10^{-7}$; (═══) $n = 8$, $K = 1.2 \times 10^6$, $m = 80$, $J = 8.3 \times 10^3$, $U = 1$.

Figure 8 presents the reduced viscosities[2] of 0.1% bovine serum albumin solutions as a function of $\bar{v}$ (dodecyl sulfate) for pH values 4.8, 5.6, and 6.8. As demonstrated previously (Reynolds *et al.*, 1967) η_{sp}/C at pH 5.6 is constant at $\bar{v} \leq 10$ and increases from $\bar{v} = 10$ to 42 reaching a plateau between $\bar{v} = 42$ and 60. Above $\bar{v} = 60$, η_{sp}/C increases gradually to 16 ml/g at $\bar{v} = 110$. At pH 6.8 the viscosity of the protein–detergent complex increases at a greater rate than at lower pH, and there is no obvious plateau region (in $\bar{v}$) which would correspond to a second set of sites on a stable perturbed state of bovine serum albumin. It is apparent that at pH 6.8 a greater degree of unfolding sets in at lower $\bar{v}$ values.[3] The binding isotherm also indicates that state 2 (D′) referred to in the mathematical analysis exists, if at all, in mixture with state 3 (D). At pH 5.6 state 3 is present only at free dodecyl sulfate concentrations greater than 5×10^{-4} M.

At pH 4.8, η_{sp}/C is identical with that at pH 5.6 at $\bar{v} \leq 33$. Above this value bovine serum albumin precipitates and viscosity measurements are meaningless. However, the binding isotherm at pH 4.8 shown in Figure 9 does not contain any obvious anomalies such as sharp discontinuities as the result of protein precipitation. Calculated binding isotherms are shown as dashed and solid lines in Figure 9. Two curves were computed by assuming two distinct protein transitions at $\bar{v}$ below 60 with identical equilibrium constants, D′/P (U_1) and D/D′ (U_2), where P represents unbound protein in state 1, D′ unbound protein in state 2, and D unbound protein in state 3. n, K (state 1) and m, J (state 3) were taken to be identical with the values at pH 5.6. m', J' (state 2) were chosen to give the best fit as 40 and 5×10^4, respectively. Decker and Foster (1966) have observed similar bovine serum albumin–dodecyl benzenesulfonate complexes ($n = 11$, $m' = 38$, and $m = 76$) and Putnam and Neurath (1945) also demonstrated the presence of $PA_{m'}$ and PA_m where $m' = {}^1/_2 m$ with bovine serum albumin–dodecyl sulfate complexes. The first authors, however, have shown that at pH values above 6.5 the amount of $PA_{m'}$, as determined by electrophoretic analysis, decreases with increasing pH. The third calculated curve in Figure 9 shows that if the intermediate species, $PA_{m'}$, is not allowed for, U (D/P) must be increased to an unacceptable number in order to fit the experimental data. Thus, if $U = 1$, there would be an equal amount of bovine serum albumin in the perturbed and native states at $\bar{v} = 0$. The fact that the viscosity of bovine serum albumin at pH 4.8 is identical with that at pH 5.6 excludes this possibility since $U = 5 \times 10^{-7}$ at pH 5.6.

Binding of Dodecyl Sulfate to Bovine Serum Albumin at Low pH. In the pH interval 4.0 to 4.5 bovine serum albumin undergoes a small expansion ($[\eta] = 4.4$ ml/g in the expanded state as opposed to 3.7 ml/g in the native state (Tanford *et al.*, 1955). At pH below 4.0 the protein expands further and $[\eta]$ increases by a larger amount.

Figure 10 is a comparison of the transition region of bovine serum albumin induced by dodecyl sulfate binding at a number of pH values, including two (pH 4.1 and 3.8) within the pH interval in which expansion occurs. It is apparent that the free ligand concentration at which the first transition occurs continues to decrease with decreasing pH as it has already been shown to do in going from pH 6.8 to pH 4.8. If we propose the following equilibria at pH 4.1 or 3.8 where

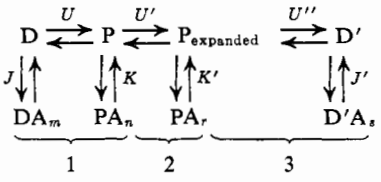
[2] It should be noted that η_{sp}/C as presented here and in preceding publications is calculated by assuming C = protein concentration in g/ml. At high values of $\bar{v}$ in detergent solutions the concentration in g/ml of protein plus bound detergent is actually higher. For example, at $\bar{v} = 100$ for a dodecyl sulfate–bovine serum albumin complex 1 g/ml of protein = 1.4 g/ml of protein-bound dodecyl sulfate. Thus, if no conformational change takes place at $\bar{v} = 100$, the measured viscosity of the complex is 1.4 times that of the uncombined protein if the concentration of protein rather than protein–complex is used in the calculation, and this "apparent" increase in viscosity would not reflect a conformational change in the protein itself. Alternatively stated:

$$[\eta] = \bar{v}(\bar{v}_p + \delta_{H_2O}\bar{v}^\circ_{H_2O} + \delta_{\text{bound detergent}}\bar{v}^\circ_{\text{bound detergent}})$$

where the δ's are densities. An increase in $[\eta]$, then, reflects an increase in $(\delta_{H_2O}\bar{v}^\circ_{H_2O} + \delta_{\text{bound detergent}}\bar{v}_{\text{bound detergent}})$. If the protein undergoes no conformational change $\delta_{H_2O}\bar{v}^\circ_{H_2O}$ should remain constant, but $\delta_{\text{bound detergent}}\bar{v}^\circ_{\text{bound detergent}}$ will increase as binding increases. At low $\bar{v}$ values, the contribution from this term is small, but at high $\bar{v}$ it can be quite large. It is important, therefore, when postulating conformational changes in detergent–protein systems to determine the extent of the contribution from $\delta_{\text{bound detergent}}\bar{v}^\circ_{\text{bound detergent}}$ to $[\eta]$. The viscosity data presented in this paper for the first unfolded state of bovine serum albumin–dodecyl sulfate at $42 \geq \bar{v} \leq 60$ is clearly larger than any contribution made by the increased mass of the detergent–protein complex. However, the earlierre ported small increases in η_{sp}/C for 0.1% bovine serum albumin in the presence of nonunfolding detergents (Reynolds *et al.*, 1967) probably reflects the contribution of $\delta_{bd}\bar{v}^\circ_{bd}$ rather than any small conformational change in the protein.

[3] This statement, although true, may be misleading. The viscosity at a given C_A where $\bar{v} > 10$ is *lower* at pH 6.8 than at pH 5.6.

there is evidence for a conformational change in the protein without bound ligand



(m, n, r, and s are the numbers of identical binding sites and J, K, K', and J' are the respective association constants; U' the counterpart of U when P is in the expanded state) then it is apparent that we may have up to four different protein conformations present at any one ligand concentration. No mathematical analysis of this situation has been attempted. However, it should be noted that at pH 3.8 and at low ionic strength (0.004) the viscosity of the protein with dodecyl sulfate bound varies as shown in Figure 8 by the dashed curve. The protein at this pH has a higher viscosity (due to expansion) than native bovine serum albumin (at pH 5.6), but η_{sp}/C is reduced to that of the native protein (at pH 5.6) when 3 moles of dodecyl sulfate per mole of bovine serum albumin are bound. This can only be the result of the conformational stabilization of the folded form due to binding, predicted in detail by Reynolds *et al.* (1967), which must occur for a two state process such as the equilibria designated (2) above.

Suppression of the N → F transition by increasing ionic strength is a well documented phenomenon (Yang and Foster, 1954). The difference in the binding isotherms at pH 3.8, μ = 0.004 and 0.033, can only result from an alteration in U.[4]

Discussion

Number of Sites on, and Ligand Association Constant to, Native Bovine Serum Albumin. A naive but plausible analogy to the equation for the hydrogen ion equilibria of proteins: $pH = p(Ke^{-0.87wZ}) + \log r/(n-r)$ in which w is the electrostatic constant $= (\epsilon^2/DRkT)(1/b - \kappa/(1 + \kappa a))$[5] and Z is the net protein ion charge, might lead one to expect that the apparent *association* constant for anions might depend on the extent of protonation of the protein ion, and therefore on the solution pH. It is well established, for example, that in the hydrogen ion titration of some proteins, Z must take into account the binding of anions as well as of hydrogen ions.

However the data presented show that the effect of pH on the native protein is negligible both with respect to the number of binding sites or the association constant when the ligand is octyl or dodecyl sulfate. However, decyl sulfate interaction with native bovine serum albumin has a pronounced pH effect—K varies from 4×10^6 at pH 4.8 to 4×10^5 at pH 6.8. Previous optical studies (Reynolds *et al.*, 1967; Polet and Steinhardt, 1968) strongly suggest that these three ligands are bound at least in part on the same protein sites. Therefore, the alterations in the interaction between decyl sulfate and bovine serum albumin with changing pH must be attributed to the effect of ligand chain length on the exact position of each bound ligand on the surface of the protein, and the binding region itself must contain some charged group which titrates over the pH range 4.8 to 6.8. A reasonable model is set forth below.

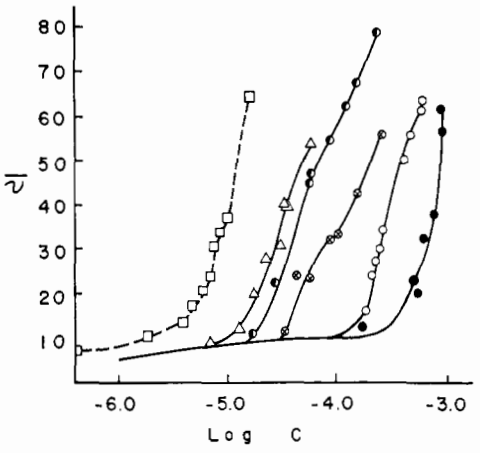


FIGURE 10: The effect of pH on the binding of dodecyl sulfate by bovine serum albumin at 2° and 0.033 ionic strength: (●) pH 6.8 (phosphate); (○) pH 5.6 (phosphate); (⊗) pH 4.8 (acetate); (◐) pH 4.1 (acetate); (△) pH 3.8 (acetate); (□) pH 3.8 (acetate), ionic strength 0.004. The lines drawn through the data are not theoretical curves. Data obtained at pH 6.2 and 7.5 are almost indistinguishable from the results at pH 6.8, but data obtained at pH 8.3 are still further to the right.

(1) The *major* contribution to the free energy of association between C_{12} and C_8 sulfates and bovine serum albumin is assumed to be multiple interactions of the hydrocarbon tail with hydrophobic regions on the protein. The number of such interactions is assumed to increase with chain length. Ionic interactions contribute a very small fraction of the free energy for these two ligands.

(2) The C_{10} sulfate is assumed to have exactly the correct length and "conformation" to interact with both hydrophobic and certain specific ionic interactions not available to the other long-chain ligands, the latter interactions increasing as the pH is lowered. These ionic interactions therefore contribute a larger part of the total free energy of association when the ligand is decyl sulfate than when the shorter or longer chain alkyl sulfate is bound. The postulated ionic interactions can arise from a protein COO^- group which originally repels the SO_4^- at high pH and which may be discharged by protonation. Positive "specific" ionic interactions, if they exist, can only be with protonated histidine residues; there are approximately 16 of these in bovine serum albumin. The difference in the free energy of association of decyl sulfate to native bovine serum albumin between pH 6.8 and 4.8 is 1.2 kcal/mole of ligand, a reasonable value for the postulated model.

Transition of Bovine Serum Albumin Due to Dodecyl Sulfate Binding. Application of the simple model for binding-induced unfolding presented here and in previous publications (Reynolds *et al.*, 1967) to the data at pH 4.8 and 5.6 for bovine serum albumin–dodecyl sulfate interactions shows

[4] U' refers to the equilibria $[PH_x]/[DH_x]$ and thus may depend on pH *and* ionic strength. Electrostatic disruptive forces should increase U' as the pH falls. It is thus not necessary to regard the stabilization by salt as a reduction in the radius of gyration of a fully unfolded peptide bearing charges.

[5] In terms of the Debye–Huckel theory. The meaning of the symbols in the parentheses is given in Tanford (1961), Beychok and Steinhardt (1964), and other reviews of protein titration curves.

clearly that lowering the solution pH results in the appearance of an intermediate set of binding sites ($m' = 40$) such as has been observed by other workers with dodecyl sulfate (Putnam and Neurath, 1945) and with alkyl benzenesulfonates (Decker and Foster, 1966).

The J and J' values proposed by Decker and Foster to fit their binding data for alkyl benzenesulfonate binding are larger than those for dodecyl sulfate–bovine serum albumin complexes by a factor of 10. Thus, the transition from P to D with these aromatic ligands occurs at far lower log C than is observed when it is induced by the alkyl sulfate. With the alkyl benzenesulfonate–bovine serum albumin system D′/P and D/D′ were chosen as 10^{-27} and $10^{-48.5}$, respectively, at pH 6.55 in 0.1 M LiCl. The much smaller value of these two parameters compared to the dodecyl sulfate–bovine serum albumin system at pH 5.8 ($\geq 5 \times 10^{-7}$) cannot be due to the ligand but may be the result of the difference in pH, ionic strength, and supporting electrolyte.

At pH 6.8 viscosity data indicate that the transition to state 3 where $m = 80$ and $J = 8.3 \times 10^3$ is not distinguishable as a separate step from the second transition which occurs at $C = 5 \times 10^{-4}$. The protein goes directly to a more highly disordered state, D″, at log $C = -3.2$ or to a mixture of states D and D″ in the transition region. This situation could result from a large pH associated decrease in U_1 and thus in U (at pH 6.8, U would have to be 5×10^{-5}) which allows the second, more extensive transition to occur before the first stage of unfolding is completed. However, a small reduction in J (at pH 6.8 J would have to be 2×10^3) would have the same effect. Neither alternative can be chosen on the basis of existing data. However, any model which assumes that the equilibrium constant, U, is independent of protein charge and which must, therefore, postulate a small reduction in the free energy of binding of dodecyl sulfate to the 80 sites on bovine serum albumin in state 3 requires that all 80 sites contain the same charged group titrating between pH 5.6 and 6.8. Since the total number of histidines and α-amino groups in bovine serum albumin is at most 18, this a highly unrealistic model. It is more reasonable to assume that U (representing the unfolding equilibrium in uncomplexed bovine serum albumin) is strongly pH dependent over the entire range investigated and that J (representing the binding equilibrium of unfolded protein) is essentially constant at all three pH values discussed here in detail. The intermediate state containing 40 sites is observable only at pH 4.8.

Interaction of Nonunfolding Ligands with Bovine Serum Albumin at log $C > -3.0$. Neither octyl nor decyl sulfate unfolds bovine serum albumin even at the highest $\bar{v}$ value examined. However, the binding isotherms of both these ligands show "cooperativity" which is independent of pH. The equilibrium concentration of ligand at which this "cooperative" binding occurs is higher for octyl than decyl sulfate in accord with the assumption that mixed micellar binding takes place. The same phenomenon has been observed with other homologous nonunfolding ligands such as alkyl sulfonates and fatty acid anions (Reynolds *et al.*, 1967, 1968). In all cases the equilibrium concentration at which "cooperative" binding is observed is a decreasing function of hydrocarbon tail length as would be expected.

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