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The Binding of Divers Detergent Anions to Bovine Serum Albumin*

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ABSTRACT: The binding of long-chain alkyl sulfates and sulfonates (C_8 to C_{14} sulfates and C_8 to C_{12} sulfonates) to bovine serum albumin (BSA) has been studied at pH 5.6 and two temperatures, 2 and 23°. Although all ligand–protein complexes are shown to undergo a small conformation change at molal ratios greater than the saturation value for the high-energy binding sites, only dodecyl and myristyl sulfate–protein complexes exhibit massive disorganization at high molal binding ratios. The total number of available binding sites on the native macromolecule is shown to be approximately ten for all ligands investigated. However, these ten sites are not all of the same energy. The number of sites in the highest energy set for the sulfates increases with hydrocarbon tail length, but the interaction energy varies only slightly. The sulfonates, on the other hand, bind not only to different numbers of sites but also with different energies when the length of the hydrocarbon tail is altered. It is apparent that the binding affinities are strongly dependent on the character of the polar portion of the ligand and are greatly influenced by the difference between the CS bond in sulfonates and COS bonds in sulfates. A mathematical model is described which predicts protein stabilization as well as disorganization in the presence of bound species, and some experimental data presented herein are analyzed by means of this theoretical treatment.

The intramolecular forces responsible for maintaining secondary and tertiary structure of proteins can be altered by environmental changes such as increasing the temperature of the system, binding small molecules, or increasing the residual electronic charge on the macromolecule. Disruption of these forces results in a conformation change of the protein. For example, when small organic molecules are bound to proteins, a ligand–protein interaction may be substituted for a structure stabilizing segment–segment or side-chain–side-chain interaction, and conformational changes may occur. It is the aim of the present series of investigations to provide a systematic study of protein binding and its effects on the stability of secondary and tertiary structure. Thus, through an understanding of the binding forces and the manner in which they alter the protein conformation, significant information and insight into the nature of protein structure can be obtained.

The first paper of this series (Ray *et al.*, 1966) presented an historical background and the results of binding measurements on a series of related ligands (octane, octanol, octyl sulfate, octyl sulfonate, dodecanol, and dodecyl sulfate) to a single protein in the native state, bovine serum albumin (BSA).[1] The results of this preliminary investigation can be summarized as follows. (1) Different numbers of sites on the native BSA molecule are available to the above ligands. (2) The binding energy and the number of sites in the highest affinity set appear to increase

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[1] Abbreviation used: BSA, bovine serum albumin.

with hydrocarbon tail length when the ligand is an alcohol. (3) The addition of a charged group (for example, in comparing octyl sulfate with octanol) appears to increase the binding energy by 1 or 2 kcal. (4) The binding on sites of highest affinity has a marked effect on the ultraviolet difference spectrum between 2500 and 3000 A in the form of a blue shift. This shift is not accompanied by unfolding. (5) The binding of neutral ligands is pH dependent; that of the corresponding sulfates and sulfonates is independent of pH over the range of pH 5.6–6.8. (6) The binding of dodecanol and dodecyl sulfate is dependent on protein concentration at concentrations above about 0.1%.

The work reported in the present paper involves determination of binding isotherms and corresponding interaction energies for octyl, decyl, dodecyl sulfates and sulfonates, and myristyl (tetradecyl) sulfate on BSA in both native and unfolded conformations. Viscosity and optical rotatory dispersion (ORD) data over a wide range of ligand concentrations are included and demonstrate the effects of binding on the optical properties of protein aromatic groups and on molecular shape. Extension of this investigation to other homologs as well as to additional proteins which differ from BSA in structural stability and binding properties is currently underway.

Experimental Section

Materials. Crystalline BSA was obtained from Nutritional Biochemicals Corp. Solutions of approximately 5% by weight BSA in glass-distilled water 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. The protein solutions were stored at 2° and used in less than 2 weeks. Concentrations were determined from optical density measurements at 2790 A in a Cary 14 spectrophotometer using $\epsilon_{1\%}^{1\,cm}$ 6.67. All calculations were carried out assuming a protein molecular weight of 69,000.

Alkyl sulfates and sulfonates were a special grade obtained from Mann Research Laboratories. They were prepared from starting materials which were molecular weight homogeneous when examined chromatographically and the products were free of impurities when subjected to thin layer chromatographic analysis. All compounds were recrystallized from boiling ethanol before use. The binding isotherm for Mann sodium dodecyl sulfate to BSA is identical with a previously published isotherm from this laboratory (Ray *et al.*, 1966) in which a special high-purity sample of the alkyl sulfate was used (supplied through the courtesy of Dr. K. Mysels, University of Southern California). Phosphate buffers were prepared from Fisher Certified reagent grade sodium phosphates, monobasic and dibasic.

Visking dialysis tubing was used in all equilibrium dialysis experiments and was treated by boiling 1 hr in distilled water followed by repeated washing. Early in the present work it was observed that prolonged exposure of untreated membranes to the phosphate buffer resulted in leaching an ultraviolet-absorbing material from the tubing. This contaminant produced anomalies in the binding isotherm suggestive of protein denaturation.

Methods. Equilibrium dialysis procedures have been described in an earlier publication (Ray *et al.*, 1966). In the present work binding isotherms were determined at both 2 and 23° in phosphate buffer, 0.033 ionic strength, at pH 5.6. A few selected experiments were carried out in NaCl, 0.033 ionic strength. All measurements were made with 0.1% BSA since previous work in this laboratory has shown that the dependence of binding on protein concentration for long-chain alkyl compounds vanishes below approximately 0.2% BSA (Ray *et al.*, 1966). Because of the large free-detergent concentration range which was covered, the relative volumes of protein and salt solutions were varied in order to maximize the accuracy of the analytical technique.

Reversibility of binding was established for all ligand–protein complexes except at $\bar{\nu} > 80$ for dodecyl sulfate where gross unfolding occurs. In this single case a small displacement of the steep part of the binding isotherm to lower equilibrium concentrations was noted.

Analysis of the alkyl sulfates and sulfonates has also been described previously (Ray *et al.*, 1966). A complex is formed between methylene blue and the sulfate or sulfonate and subsequently extracted from the aqueous solution into chloroform. The optical density of the chloroform solution is then determined on a Cary 14 spectrophotometer at an appropriate wavelength. It was necessary to increase the concentration of methylene blue solution to 0.7 g/l. and use shorter path-length cells when working at equilibrium concentrations above 10^{-4} M.

Viscosity measurements were made in Cannon–Fenske viscometers having flow times varying from 100 to 600 sec. The constant-temperature bath was thermostated to 4 ± 0.005 and 20 ± 0.002°. All 20° viscosities were determined using solutions from the dialysis experiments; the solvent reference thus contained the equilibrium concentration of alkyl sulfate or sulfonate and the solutions contained 0.1% BSA, bound anion, and free anion. Solutions were prepared for the 4° measurements by calculating the appropriate salt concentrations from the binding isotherms. No evidence of viscosity dependence on rate of shear was noted. Solvent flow times were identical when determined before and after exposure of the viscometers to protein solution using usual cleaning and drying procedures between each determination. The possibility of adsorption of protein on the walls of the capillary affecting measured flow times was eliminated by the following experiment. (a) The viscometer was filled with 8 ml of 0.1% BSA in phosphate buffer at pH 5.6 and the flow time determined. (b) Protein solution (4 ml) was removed quantitatively and replaced with 4 ml of fresh 0.1% BSA solution. Flow times were

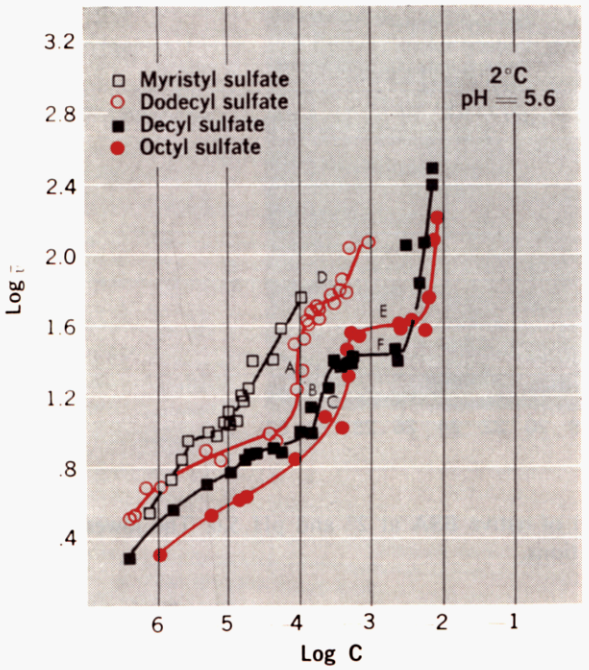

FIGURE 1: Logarithmic plots of binding isotherms of BSA at 2° and pH 5.6 with various detergent sulfates. The letters are explained in the text.

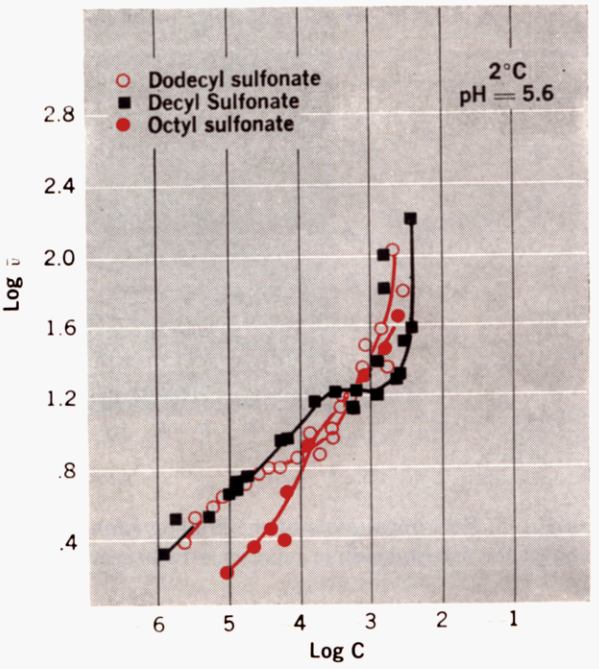

FIGURE 3: Logarithmic plots of binding isotherms of BSA at 2° and pH 5.6 with various detergent sulfonates.

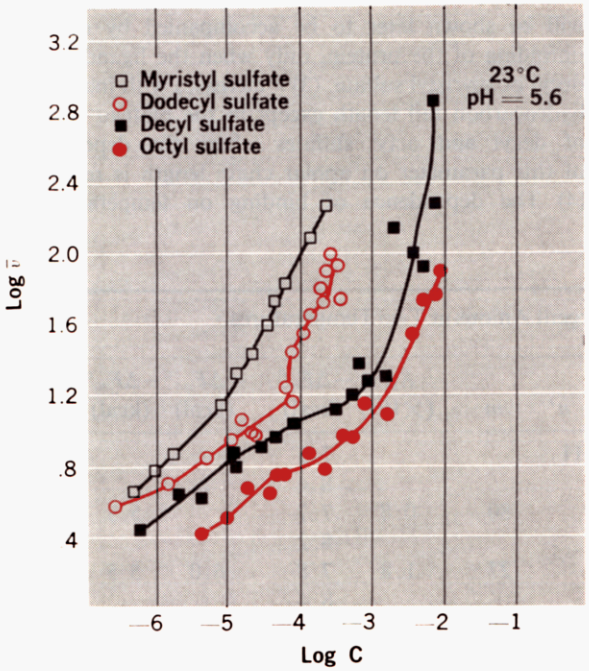

FIGURE 2: Logarithmic plots of binding isotherms of BSA at 23° and pH 5.6 with various detergent sulfates.

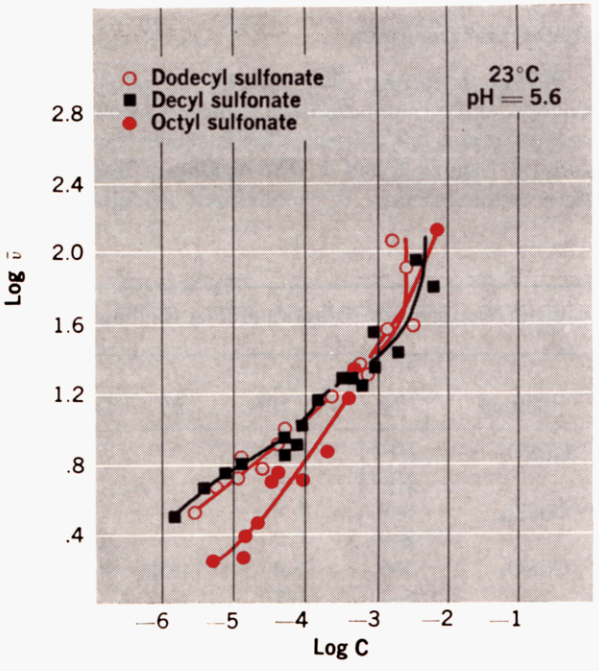

FIGURE 4: Logarithmic plots of binding isotherms of BSA at 23° and pH 5.6 with various detergent sulfonates.

identical with those measured initially. This replacement procedure was repeated several times and flow times were reproducible to within 0.2 sec. All solutions were filtered through Millipore filters before measurements and the protein concentration determined optically by the measurement of optical density at 2790 A in a Cary 14 spectrophotometer.

Optical rotatory dispersion was measured on a Jasco

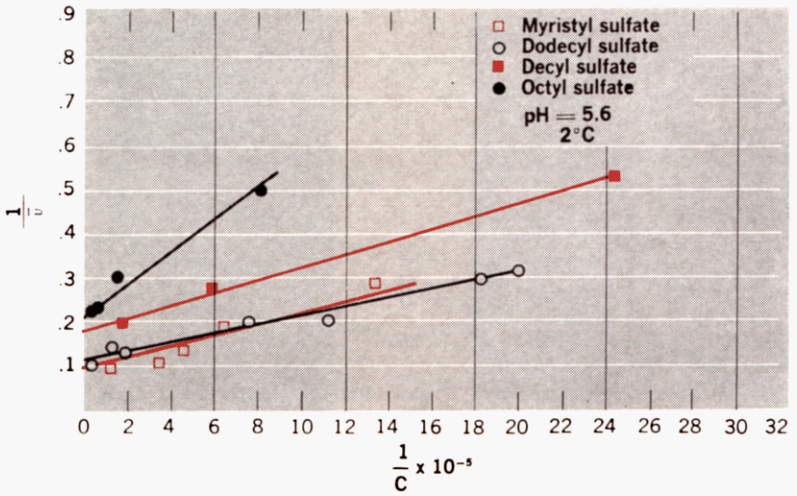

FIGURE 5: Reciprocal plots of the binding isotherms (sulfates) of native BSA at 2° and pH 5.6. The lowest point shown for myristyl sulfate represents two separate determinations.

Model UV-5 spectropolarimeter, using 0.1% BSA in phosphate buffer and appropriate concentrations of alkyl sulfate and sulfonate. The greater part of the data was taken at 2330 A. However, some measurements with dodecyl sulfate were made at 5460 A. Results are reported as $-[\alpha]$, specific rotation.

Results and Discussion

Binding Isotherms. The binding of octyl, decyl, dodecyl, and myristyl sulfate at 2 and 23° as a function of equilibrium salt concentration is shown logarithmically in Figures 1 and 2. The significant features of these isotherms may be summarized as follows. (1) The almost vertical portions of the binding isotherms at 2° labeled A, B, and C in Figure 1 suggest a phase transition when the C_8, C_{10}, and C_{12} sulfates are bound to BSA. (2) There are plateau regions in these same three isotherms labeled D, E, and F in Figure 1. The second apparent transition which is suggested by the vertical portion of the isotherms following the plateau, will be shown later to be accompanied by massive unfolding of the protein, only when the ligand is dodecyl or myristyl sulfate. (3) $\bar{\nu}$ increases with increasing hydrocarbon tail length except in the plateau regions of decyl and octyl sulfates where the dependence of this parameter on apolar chain length is reversed. (4) The dependence of binding on temperature is

TABLE I: Numbers and Affinities of BSA Binding Sites for Detergent Anions at Two Temperatures.[a]

Compd	n	K_n ($\times 10^6$)	n'	K'_n ($\times 10^4$)	$n + n'$	m	K_m ($\times 10^4$)	$-\Delta F_n°$ (kcal)	$-\Delta F_{n'}° - \Delta F_m°$ (kcal)		(°C)
$C_{14}SO_4$	10–11	0.9			10–11			7.5			2
	10–11	1.5						8.4			33
$C_{12}SO_4$	8–9	1.2			8–9	80	0.8	7.7		5.3	2
	8–9	1.2						8.2			23
$C_{10}SO_4$	5–6	1.4	[4–5]	[4.1]	10	27	1.8	7.8	6.0	5.5	2
	6–7	1.7						8.4			23
C_8SO_4	4–5	0.6	[7]	[0.7]	11	42	0.8	7.3	5.0	5.3	2
	4–5	0.6						7.4			23
$C_{12}SO_3$	6	0.3						6.9			2
	6	0.3						7.4			23
$C_{10}SO_3$	5	0.9						7.5			2
	5	0.9						8.1			23
C_8SO_3	3	0.1						6.3			2
	4	0.1						6.8			23
Column 1	2	3	4	5	6	7	8	9	10	11	12

[a] The significance of the symbols is given in the text.

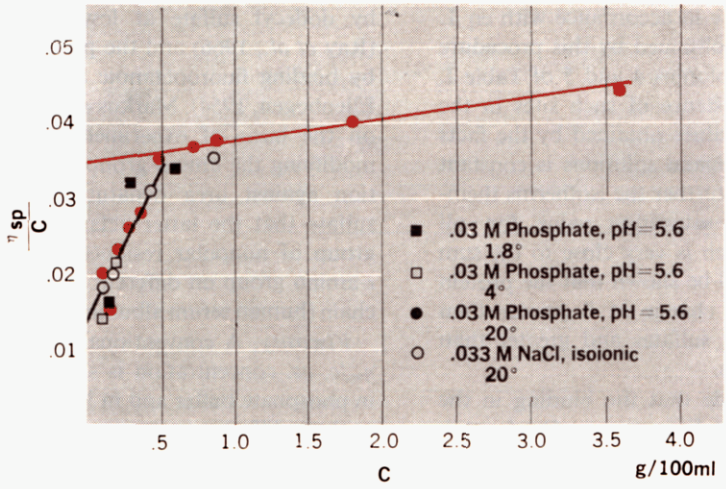

FIGURE 6: The specific viscosities of BSA in 0.03 M phosphate buffer (pH 5.6) as a function of protein concentration at a number of temperatures.

small in the low $\bar{\nu}$ regions. However, pronounced differences between the two temperatures are noted above $\bar{\nu} = 10$ (note the absence at 23° of regions comparable to B, C, E, and F in octyl and decyl sulfate at 2°).

Octyl, decyl, and dodecyl sulfonate isotherms at the same two temperatures are shown in Figures 3 and 4. In contrast to the sulfates these isotherms show less sensitivity to the alkyl chain length and no apparent phase transition except at high equilibrium concentration ($-\log c < 3$). It will be shown that no viscosity increase accompanies this latter vertical portion of the binding isotherms for any of the ligands except the C_{12} and C_{14} sulfates. Apparently the steplike increase in number of ligand molecules bound which occurs above $\log c = -3.0$ for C_8 to C_{12} sulfonates and C_8 and C_{10} sulfate is a condensation-type phenomena, such as micelle formation, rather than a conformation change in the protein itself.

An analysis of the isotherms by means of the well-known equation derived from the law of mass action and applied to multiple equilibria involving sets of identical sites

$$\frac{1}{\bar{\nu}} = \frac{1}{nKc} + \frac{1}{n} \qquad (1)$$

(where $\bar{\nu}$ = average molal ratio of bound ligand to protein, K = intrinsic association constant of each site for the ligand, c = equilibrium concentration of the ligand, and n = number of binding sites in a set) gives a linear plot of $1/\bar{\nu}$ *vs.* $1/c$ (hereafter referred to as a reciprocal plot) from which n and K can be calculated. Figure 5 provides an example of this analysis for the four sulfate ligands in the region $\bar{\nu} \leq 10$.

The application of this equation assumes that the sites bind with equal energy, and that no phase transition takes place in the substrate during binding. Results of the application of eq 1 to the isotherms reported here are shown in columns 2 and 3 of Table I. Below $\bar{\nu} = n$ these reciprocal plots are linear and deviation from linearity at higher $\bar{\nu}$ values can be adequately explained either in terms of a second set of sites or a conformation change in the protein.[2]

While there is some scatter in the data above $\bar{\nu} = n$, an attempt was made to determine the number of sites of lower affinity for decyl and octyl sulfate

[2] In this work, as in the first paper of this series (Ray *et al.*, 1966) the electrostatic interaction factor, wz, suggested by Klotz *et al.* (1946) and Scatchard (1949) has not been applied. This interaction factor may be calculated from the Debye–Hückel theory and is simply the electrostatic interaction energy between the charged protein ion and ions in the solution. Karush (1951) has shown by studying the effect of protein charge on the binding of methyl orange to BSA that w (Experimental Section) is considerably smaller than the theoretical value and, in fact is negligible at protein charges less than 16. Kirkwood and Shumaker (1952) have also shown that dielectric constant increments for some proteins are adequately accounted for without applying this correction. The assumption embodied in the theoretical derivation of w (see, for example, Tanford (1963) or Steinhardt and Beychok (1964)) that the charge distribution on the macro ion can be treated as continuous and long range, rather than as discrete points having the expected short-range effects only, has appeared to be satisfactory when applied to hydrogen ion–protein equilibria. However, the validity of extending this approximation to other ions which are not in equilibrium with receptor molecules in the solvent, is not obvious and has not been borne out experimentally. In addition, it has been shown in this laboratory (Herbert, 1966) that as in the case of other anions binding of alkyl sulfates and sulfonates in unbuffered solutions leads to an alteration in the hydrogen ion equilibrium of BSA as evidenced by pH changes upon addition of the detergent to the aqueous protein solution. If the application of an electrostatic correction factor were physically reasonable, the experimentally determined value of $\bar{\nu}$ should differ with the charge on the macro ion. As has been pointed out, $\bar{\nu}$ is identical in both buffered solutions (where the H^+ ion association must change) and in unbuffered where the charge on the protein has been shown to vary with anions bound.

by plotting $1/(\bar{\nu} - n)$ *vs.* $1/c$ in accordance with eq 1. The values for n' and K_n' obtained by this procedure are shown in brackets in columns 4 and 5 of Table I. Credence is lent to the existence of such sites in the native protein and to the values obtained by the facts that the viscosity of BSA–ligand solutions is constant throughout this region and further the isotherms themselves show an apparent saturation value for all the sulfates at $\bar{\nu} \sim 10$ which is very close to the sum of $n + n'$ in Table I. It will be shown that the protein undergoes a conformation change at $\bar{\nu}$ greater than $n + n'$ in the case of the sulfates and greater than n in the case of the sulfonates.

If the assumption is made that the binding in the plateau regions (D, E, and F in Figure 1) of octyl, decyl, and dodecyl sulfate follows a conformation change in the BSA molecule and is representative of an isotherm peculiar to a perturbed state, this region can also be analyzed by means of eq 1. A reciprocal plot of data between log $c = -4.00$ and -2.50 is approximately linear (note that the plateaus are not absolutely flat and the finite slope indicates a value of K sufficiently small to make eq 1 operative over the plateau region.) The number of sites, m, and the intrinsic association constant, K_m, for this perturbed protein conformation are reported in Table I. It will be shown that the specific viscosity of protein plus ligand is constant throughout this region and differs from that at lower $\bar{\nu}$ values.

Examination of the data in Table I reveals the following factors. (1) For the first set of sites on the native protein when the ligand is an alkyl sulfate, ΔF° varies little with the size of the apolar group. (2) The number of sites in both native and perturbed states (2° isotherm regions labeled D, E, and F in Figure 1) varies significantly in the sulfate series although the sum of $n + n'$ (native protein) is close to ten for all four compounds. (3) Both n and ΔF° display dependence on the alkyl chain length for the alkyl sulfonate series. (4) All sulfonates have lower ΔF° values than their corresponding sulfates.

These observations make it clear that the interaction of alkyl sulfates and sulfonates with BSA cannot be related in a straightforward manner to the size of the apolar portion of the ligand as has been previously postulated for other protein–ligand systems (Steinhardt *et al.*, 1942). If the binding energy were controlled only by the size or stereochemistry of the polar group, removal of a $(CH_2)_2$ group from the alkyl chain should lead to energy changes of approximately the same magnitude in the sulfate and sulfonate series. It can be seen that this does not occur.

It becomes necessary then to consider the role of the ionic polar portion of the ligand in binding. It is clear that the sulfate and sulfonate groups must participate in the protein–ligand interaction with different energy contributions. These ligands are probably multifunctional with one of the functional groups involving the ionic portion of the molecule. Other evidence has been presented for this multifunctional hypothesis in the inference of protein aggregation by dodecyl sulfate at low detergent concentrations (Ray *et al.*, 1966) and the protein aggregation induced by binding fluorooctanoic acid to BSA (Klevens and Ellenbogen, 1954). Markus *et al.* (1964) have suggested, on the basis of experiments involving the effect of modifying the amino groups of the protein on protection against urea denaturation by sodium dodecyl sulfate that the latter acts as a cross-link between a group of nonpolar residues and a positively charged ϵ-amino group on different loops of the folded peptide chain (human serum albumin).

Viscosity. A conventional plot of reduced viscosity, η_{sp}/c, *vs.* concentration is shown in Figure 6 for BSA in phosphate buffer and in NaCl at 0.033 ionic strength at three different temperatures and over a wide protein concentration range. Extrapolation to $c = 0$ of the data above 0.5% BSA leads to an intrinsic viscosity which agrees well with that of previous experimenters (Tanford and Buzzell, 1956; Yang and Foster, 1954) (Note that density corrections have not been applied to this data. Correcting for the effect of protein on solution density leads to $[\eta] = 0.0037$.) However, extrapolation of the experimental points at lower protein concentrations leads to a surprisingly low value of the intrinsic viscosity. The experimental error involved in determining the specific viscosity of a 0.1% BSA solution is approximately $\pm$ 0.006. Thus the apparent extrapolated value of the reduced viscosity is 0.014 $\pm$ 0.006. All precautions were taken to eliminate sources of experimental error in determining the viscosity of protein solutions at low concentrations. The relative flow times of protein and solvent are reproducible in viscometers with flow times of 200 and 400 sec, and, as was pointed out in the Experimental Section, adsorption of protein on the walls of the capillary cannot account for the very low values obtained. However, such low values of specific viscosity cannot be easily explained, and the absolute value may be in error due to an experimental artifact not recognized. Since the primary purpose of these measurements is comparative, *i.e.*, a change in η_{sp} with binding, a final resolution of this problem is not essential for the ensuing discussion. We stress only the *changes* in specific viscosity as anions are bound to BSA and not the absolute values.

The specific viscosity of a 0.1% BSA solution in 0.033 ionic strength phosphate buffer as a function of $\bar{\nu}$ is shown in Figures 7A and 8A. There is no apparent temperature dependence with either sulfate or sulfonate–protein complexes. In addition, a small increase in η_{sp} is noted in regions labeled A, B, and C in Figure 7A, indicating a phase transition in the BSA molecule when C_8, C_{10}, and C_{12} sulfates are bound. The corresponding transition regions in the binding isotherms can be seen in Figure 1 and are also labeled A, B, and C. The $\bar{\nu}$ value at which the plateaus designated D, E, and F in both Figures 1 and 7A begin are approximately the same. The magnitude of this change in η_{sp} increases as the alkyl chain length increases although the difference between octyl and decyl sulfate is within experimental error. A

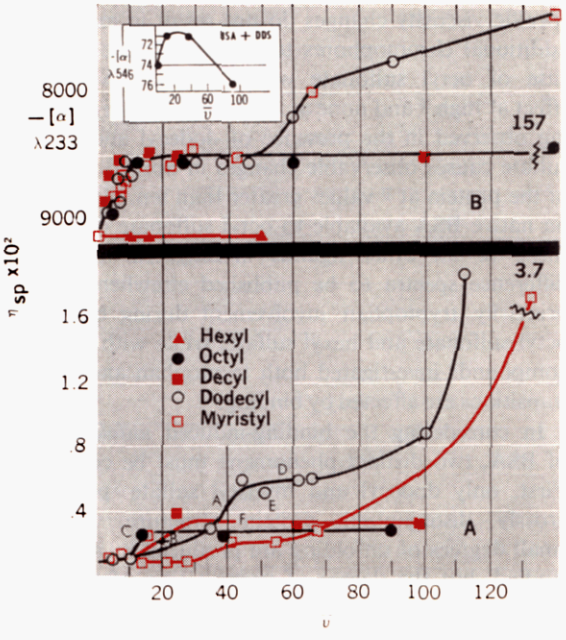

FIGURE 7: Specific viscosities (A) and specific rotation (B) of various BSA–detergent sulfate complexes as a function of molal ratio $\bar{\nu}$. (B) At the Cotton effect trough (233 mμ). The insert shows results with dodecyl sulfate at 546 mμ.

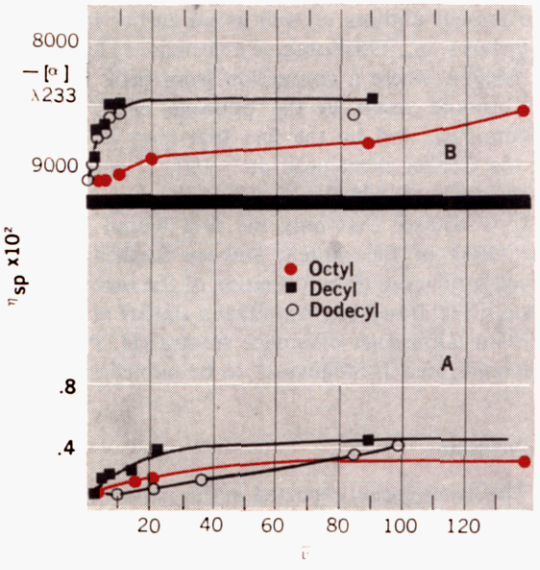

FIGURE 8: Viscosities (A) and specific rotation (B) of BSA–sulfonate complexes as a function of molal ratio $\bar{\nu}$. (B) At Cotton effect trough (233 mμ).

second transition is observed with myristyl and dodecyl sulfate only when both are bound in a molal ratio greater than 80 and indicates massive disorganization of the protein. Tanford *et al.* (1966) have recently shown that BSA unfolded by 6 M guanidine hydrochloride containing β-mercaptoethanol to break the disulfide bonds has an intrinsic viscosity of 0.522 dl/g. The maximum reduced viscosity observed in the present work at 0.1% BSA is 0.370 dl/g at $\bar{\nu}$ = 130 with myristyl sulfate.

In Figure 8A a small increase in η_{sp} is observed with all three sulfonates which takes place slightly above the n calculated by application of eq 1 to the binding isotherms. There is no evidence of a higher degree of unfolding as binding is increased when the sulfonates are used as ligands, even in the apparent transition region of Figures 3 and 4, previously noted.

It might be argued that the increase in η_{sp} observed at B and C in Figure 7A and for all sulfonates in Figure 8 is the result of some experimental anomaly which brings the abnormally low viscosity observed for 0.1% protein up to the expected value. This is unlikely for two reasons. (1) The viscosity transitions for octyl, decyl, and dodecyl sulfate occurs at the same anion equilibrium concentration as the apparent transitions in the binding isotherm shown in Figure 1. (2) The small viscosity increase for all seven protein–ligand complexes differs significantly with the bound anion.

The changes in viscosity noted on these two figures cannot be attributed to a simple increase in molecular weight due to bound ligand since it is apparent that binding 20 moles of anion/mole of protein results in an increase of η_{sp} for octyl sulfate but not for the heavier ligand, dodecyl sulfate. Thus, the binding of all alkyl sulfates and sulfonates investigated induce small conformational changes in the native BSA molecule, the magnitude of which is dependent on the ligand. Dodecyl and myristyl sulfate alone, when bound at high molal ratios, induce an almost total disorganization of the secondary and tertiary structure.

Optical Rotatory Dispersion. The change in levorotation in the Cotton effect trough which occurs at 2330 A for many proteins has been previously used to determine the alteration in helix content (Schecter and Blout, 1964). An investigation of this parameter in the system BSA–alkyl sulfate or sulfonate has been conducted and the results are presented in Figures 7B and 8B. There is a continual decrease in levorotation as $\bar{\nu}$ is increased to approximately ten for all ligands except octyl sulfonate and hexyl sulfate. At higher binding values there is no further change in rotation with the exception of a decrease between $\bar{\nu}$ = 45 and 60 when the ligand is dodecyl or myristyl sulfate. This second decrease in levorotation corresponds to a viscosity increase and suggests that additional chromophores are made available by the protein configuration change. Above $\bar{\nu}$ = 65, it will be noted that a change in slope occurs in the $-[\alpha]$ *vs.* $\bar{\nu}$ curve for these two sulfates. This corresponds to the large increase in η_{sp} in the same region and is intrepreted as significant disorganization of the secondary structure.

The initial change in $-[\alpha]$ at low binding values is not accompanied by a change in viscosity and probably does not represent a change in helix content. This conclusion is further supported by the fact that

the present authors as well as an earlier investigator (Markus *et al.*, 1964) observe a decrease in levorotation at 5460 A where a conversion from helix to random coil should result in the opposite effect (insert in Figure 7B); and by the fact that there is no change in the Cotton effect peak at ~1900 A corresponding to the decrease at 2330 A discussed above.

It is notable that only the first bound ions affect the ORD of the protein and no further change is noted until gross disorganization of the macromolecule takes place. It will be shown that a similar phenomenon exists in ultraviolet difference spectra (H. Polet, J. A. Reynolds, and J. Steinhardt, to be published).

Conclusions

The investigation reported in this paper encompasses two distinct types of protein properties. Short-range forces have been studied by means of optical measurements, available number of binding sites, and protein–ligand interaction energies. Macroscopic shape changes have been evaluated by means of viscosity measurements.

In the region of binding to *native* BSA (defined by a constant specific viscosity identical with that of protein with no ligand present), the optical property $-[\alpha]_{2330}$, changes in an approximately linear manner as $\bar{\nu}$ is increased from 0 to 10 and remains constant at higher $\bar{\nu}$ values except and until gross unfolding occurs. Only octyl sulfonate and hexyl sulfate do not follow this pattern. The latter ligands show no change in optical rotation up to $\bar{\nu} = 10$ and a continuing small decrease in levorotation at higher binding values. In conjunction with the above observations, it has been noted that the specific viscosity of BSA plus alkyl sulfonate undergoes a change when $10 > \bar{\nu} > n$. The conclusions to be drawn from these observations can be summarized as follows. (1) *Native* BSA binds approximately ten anions of alkyl sulfate on one or more sets of sites depending on whether n is equal to or less than 10. Binding to each site progressively perturbs one or more chromophores. The ligand is envisaged as being multifunctional in that there is a specific interaction between it and more than one active area on the protein. For example, the alkyl chain of the ligand may interact with an apolar portion of a protein segment and the ionic head of the ligand with a charged group. Note that the value of n for C_{10} and C_8 sulfates in Table I is less than 10, but a second set of sites is indicated. The total number of sites in all sets on the native protein appears to be approximately ten for the alkyl sulfates. (2) *Native* BSA binds approximately ten anions of dodecyl and decyl sulfonate. The optical properties of these BSA complexes reported here suggest that at least the chromophoric portion of these sites is the same for all six ligands (excluding octyl sulfonate). The ten suggested native BSA sites for the C_{10} and C_{12} sulfonates are not of equal energy since the first set consisting of n sites is less than ten (See Table I). (3) The small perturbations in the macroscopic shape of BSA evidenced by small specific viscosity changes do not result in exposure of additional chromophores to the ligand (except in the case of octyl sulfonate which shows a very small effect at high $\bar{\nu}$ and none at low). Only the large unfolding observed in the presence of dodecyl and myristyl sulfate causes observable changes in optical properties of the protein at $\bar{\nu}$ values greater than ten. (4) The sites on native BSA available to octyl sulfonate differ from those of all other ligands investigated. Ultraviolet difference spectra to be published elsewhere indicate primarily tryptophan involvement during binding of octyl sulfonate and hexyl sulfate, while with the other compounds investigated both tryptophan and tyrosine transitions are affected by binding.

In considering the binding-induced *unfolded* states of BSA, two distinct phenomena must be considered. First, only dodecyl and myristyl sulfate produce a grossly disorganized protein structure. Secondly, small degrees of unfolding are produced by all ligands studied, but the degree of perturbation of the native structure appears to differ with the anion bound.

The driving force for any degree of unfolding brought about by anion binding may be one or a combination of the following: (a) electrostatic repulsion between the charges of bound species, including the net charge of the protein; (b) penetration of the hydrocarbon tail into the apolar regions of the protein and the resultant replacement of conformation-stabilizing segment–segment interactions by ligand–segment interactions; (c) binding-induced changes in the protein–hydrogen ion equilibrium resulting in an increase in electrostatic repulsion between charged species which are an intrinsic part of the primary protein structure (see Tanford (1963) for a discussion of the N–F transition in BSA induced by pH changes); and (d) a favorable ratio of the number of binding sites and association constants in the native form to those in the unfolded form (first suggested by Foster and Aoki (1958) and further developed and extensively discussed in the Appendix of this paper).

Not one of these can be chosen or eliminated on the basis of the experiments reported herein. It is obvious that if electrostatic repulsion alone were considered, disruption of the protein structure should be approximately the same for all seven ligands since their charges (although not necessarily their charge distributions) are identical. If the second type of denaturing force were singly operative, dodecyl sulfonate should produce the same degree of unfolding as dodecyl sulfate and this obviously does not occur. It is possible, of course, that the stereochemistry of binding these two ligands is not the same and the apolar tail of the sulfonate does not penetrate the protein in a manner conducive to unfolding.

Alterations in details of hydrogen ion equilibrium are not easily evaluated. However, we have shown that binding of large anions is not dependent on the protein charge, at least in the region characterized by small viscosity changes. It is possible that at higher binding large changes in this equilibria cause a transition similar to that already observed when the pH

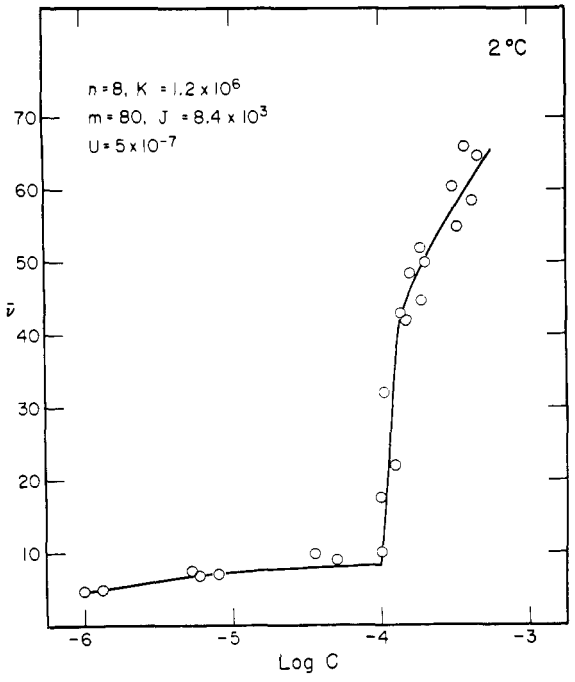

FIGURE 9: The binding isotherm of BSA–dodecyl sulfate at 2° fitted by eq 3 and 4, with the parameters given on the figure.

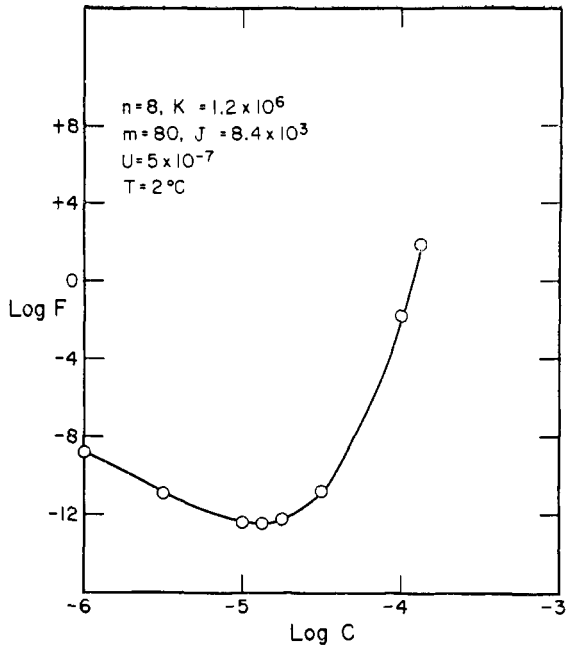

FIGURE 10: Stabilization by dodecyl sulfate. Variation in the ratio of the logarithm of the ratio of denatured to native protein as a function of equilibrium concentration of ligand.

of BSA is lowered below 4.3. If this were the case, all seven ligands would be expected to produce large conformational changes such as are observed with dodecyl and myristyl sulfate, since extremely high binding ratios are apparently reached in each case. It must be noted, however, that the apparently high binding ratios may be the result of micelle formation, in which case the above argument may not be valid.

A reasonable hypothesis is that the controlling factor in configuration changes induced by anion binding is the ratio of the number of sites available in two or more states of the protein. The failure of five of the seven ligands studied to grossly unfold the BSA molecule under the experimental conditions employed may be due to low numbers of sites for these ligands on the unfolded protein. This implies that some binding sites in unfolded protein are not available for all ligands of this group.

An alternative but scarcely useful explanation is that the sites on both native and unfolded configurations of the BSA molecule are so different for the ligands investigated that no accurate comparison can be made with respect to electrostatic repulsion, disruption of the apolar portion of the protein, or changes in the H^+ equilibrium.

Appendix

In considering the equilibrium processes of protein–ligand interaction and protein conformational change induced by this interaction the following mathematical formulation has been found useful. An essentially equivalent model and similar terminology was applied by Foster and Aoki (1958) to the N–F transition of bovine plasma albumin. An important conclusion resulting from this earlier work was that the above-mentioned transition could be shifted by the binding of anions. The treatment which follows employs a model equivalent to that of Foster and Aoki but explicitly predicts both stabilization and disorganization of protein structure by ligand association. In addition, it provides an explicit means of determining the equilibrium constant between two different protein states when the number of binding sites and association constants is known for each state and for a particular ligand. It may be considered a general model for the denaturation of proteins which contain masked binding sites for any ligand including hydrogen ion.

If a protein is considered to exist in two distinct conformational states and undergoes ligand association in each state, the equilibrium processes may be expressed as follows

$$P + A \underset{}{\overset{k_1}{\rightleftharpoons}} PA_1 \qquad D + A \overset{j_1}{\rightleftharpoons} DA_1$$

$$PA_1 + A \overset{k_2}{\rightleftharpoons} PA_2 \qquad DA_1 + A \overset{j_2}{\rightleftharpoons} DA_2$$

$$\vdots \qquad\qquad \vdots$$

$$PA_{n-1} + A \overset{k_n}{\rightleftharpoons} PA_n \qquad DA_{m-1} + A \overset{j_m}{\rightleftharpoons} DA_m$$

where P = protein activity in state 1, D = protein activity in state 2, A = activity of ligand, n = number of ligand sites of constant energy in state 1, m = number of ligand sites of constant energy in state 2, k_i = association constant for protein–ligand interaction in state 1, and j_i = association constant for protein–ligand interaction in state 2.

The individual k_i and j_i values are related to the intrinsic association constants K and J as follows

$$k_i = \frac{i}{n + 1 - i}K$$

$$j_i = \frac{i}{m + 1 - i}J$$

The total concentration of protein present at any equilibrium position may be expressed by means of the following enumeration

$$i = \sum_0^n PA_i = P\sum_{s=0}^n A^s K^s \frac{n!}{s!(n - s)!} \quad (2a)$$

$$j = \sum_0^m DA_i = D\sum_{r=0}^m A^r J^r \frac{m!}{r!(m - r)!} \quad (2b)$$

The following simplifications and assumptions have been employed in the above-mentioned mathematical statements. (1) Activity coefficients are approximately one, thus allowing substitutions of concentrations for activities. (2) There are sets of sites of equal energy on the protein in each conformational state. (3) Electrostatic interaction need not be considered. This parameter can be explicitly added to eq 2a and b, however, in the form $K = K'e^{-wz^2}$. (4) The protein can exist in state 2 in the absence of bound ligand. (5) The sites for binding on the native protein are destroyed when the macromolecule undergoes a phase transition, and are replaced by a new set of sites on the protein in the less organized state.

The ratio of protein present in the two states may be obtained by dividing eq 2a by b, thus obtaining

$$\mathrm{F}(A) = \frac{U\sum_{r=0}^m A^r J^r \frac{m!}{r!(m - r)!}}{\sum_{s=0}^n A^s K^s \frac{n!}{s!(n - s)!}} \quad (3)$$

in which $U = D/P$. All parameters in eq 3 can be determined experimentally except U which is obtained by a computer fit to the experimental binding isotherm. U is the equilibrium constant between states 1 and 2 of the protein (uncombined with ligand), and RT ln U is the free-energy change involved in this equilibrium.

Equation 3 permits us to write

$$\bar{\nu} = \frac{A}{1 + \mathrm{F}(A)}\left[\frac{nK}{1 + KA} + \frac{\mathrm{F}(A)mJ}{1 + JA}\right] \quad (4)$$

An examination of the function F(A) shows the conditions under which a minimum will obtain (stabilization of the protein structure in state 1) and also the conditions under which a transition from state 1 to 2 is predicted.

Stabilization Conditions

$$K \gg J,\ n \leq m \quad (a)$$

$$K \geq J,\ n > m \quad (b)$$

Note that the above is a specific statement of a general condition for stabilization stated earlier by Decker and Foster (1966) that $nK > mJ$.

Transition Conditions

$$m > n,\ K \geq J \quad (a)$$

$$m \leq n,\ K \ll J \quad (b)$$

The above-described model has been applied to the 2° binding isotherms for octyl, decyl, and dodecyl sulfate to bovine serum albumin. Only the regions below log $c = -2.5$ in Figure 1 were used, and state 1 was defined as that protein conformation which corresponded to the lowest specific viscosity plateau seen in Figure 7A. State 2 differed for all three ligand-protein complexes and corresponded to that conformation existing at the first step in the three viscosity curves shown in Figure 7A. In all three calculations the function, F(A), went through a minimum at low anion concentration. The computer values for U are not identical in these cases and indeed should not be since the final states are different. Figure 9 is an example of the computer fit to the binding of dodecyl sulfate to BSA, and Figure 10 is a plot of log F(A) *vs.* log A for the same system. An obvious experimental test of this model would be a determination of U for a protein–ligand system in which several ligands produced the same final protein state. In this case U should be independent of the ligand used. There are indications in the work of Decker and Foster (1966) and our own that dodecyl sulfate and dodecyl benzene sulfonate represent such a pair since both bound on 76–80 sites in the unfolded state.

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CORRECTION

In the paper "Some Properties and Mechanism of Action of the β-Hydroxy-β-methylglutaryl Coenzyme A Reductase of Yeast," by Mary E. Kirtley and Harry Rudney, Volume 6, January 1967, the following correction should be made.

On p 230, right-hand column, under the heading *Assay of HMG-CoA Reductase*, third line down, 0.2 mM TPN^+ should read 0.2 mM TPNH.