Binding-Induced Alterations in Ultraviolet Absorption of Native Serum Albumin*

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ABSTRACT: When bovine serum albumin combines with up to 15–17 equiv of anionic detergents (normal aliphatic sulfonates and sulfates) on its highest energy sites characteristic ultraviolet difference spectra are produced: a *blue shift* with minima at λ 293 and 287 mμ, and a *red shift* with a maximum at λ 232 mμ. From these spectral changes it is concluded that for both sulfonates and sulfates, tryptophan residues are at or very near the highest energy binding sites. For the sulfates, with the exception of the shortest chain used, additional sites with slightly lower affinity must be at or close to available tyrosine residues. Octanol, an uncharged ligand, produced at λ 230 mμ an effect very similar to that of the anions, although it has less effect on the tyrosine absorption and hardly any effect on the tryptophans. This observation suggests strongly that at least one more chromophore (histidine or phenylalanine) is involved in the binding at high-energy sites. It is proposed that changes at this residue cause the small changes in optical rotation at 233 mμ. The shortest chain compounds, even though they produce a strong tryptophan effect, do not change the rotation. Combination with larger amounts of the detergents produces no further effects except in the case of the two longest chain sulfates (dodecyl and tetradecyl) which alone produce the same massive disorganization of the protein that is produced by low pH (1.65) and by 8 M urea. Changes in peptide absorption are involved only in the latter massive disorganization. When no massive unfolding occurs, no detectable changes in viscosity accompany any of the changes in $\Delta\epsilon$ although all the ligands induce small viscosity changes at $\bar{\nu}$ values just above those which suffice to complete the changes in ϵ.

Protein–detergent interactions have been much studied since Anson (1939) first demonstrated that a commercial detergent, largely composed of sodium dodecyl sulfate, was a more potent protein denaturant than urea or guanidine, *i.e.*, is often effective at much lower concentrations than the latter. Shortly thereafter, Steinhardt *et al.* (1941, 1942) showed that pure dodecyl sulfate and dodecylsulfonate, as well as certain other sulfur analogs to the long-chain fatty acids, were characterized by extremely high affinities to protein (Steinhardt *et al.*, 1941, 1942) and acted as efficient catalysts to protein hydrolysis by dilute acid (Steinhardt and Fugitt, 1942). Sodium dodecyl sulfate has since been used frequently when a potent disorganizer of the native structure of proteins is required. Nevertheless, it is firmly established that in very low concentrations, dodecyl sulfate (as well as some of the lower fatty acids) protects some proteins against the disorganizing action of extremes of pH or of high concentrations of urea (Lovrien, 1963; Meyer and Kauzmann, 1962; Markus *et al.*, 1964; Duggan and Luck, 1948; Teresi and Luck, 1952). Much of the evidence for both stabilization or disorganization is based on changes in ultraviolet absorption or in optical rotation, which are further investigated in this paper. Except for the work of Foster and collaborators with serum albumin and dodecyl benzenesulfonate, and work of Jirgensons (1962) on changes in optical rotation produced by decyl sulfate at more elevated temperatures, little has been done with other homologous sulfates or sulfonates. In two recent communications from this laboratory (Ray *et al.*, 1966; Reynolds *et al.*, 1967), portions of a systematic study of the nature of binding forces, and of the effects of binding on protein stability, have led to the following generalizations pertinent to the purpose of the present paper. (1) With ligands containing hydrocarbon chains exceeding six carbon bonds, the *affinities* of a small number of high-energy sites for sulfates do not differ greatly, but the *number* of such high-energy sites is larger, the longer the hydrocarbon chain, reaching eight or nine with dodecyl and tetradecyl sulfates. (2) While the free energy associated with the hydrocarbon moiety constitutes the largest part of the total binding free energy, the affinities of the high-energy sites are strongly affected by the nature of the polar end group. The affinities of sulfates are larger than of sulfonates, and both are larger than those of homologous alcohols. Carboxylate groups (Reynolds *et al.*, 1968) are intermediate between sulfonates and alcohols. (3) Only two of the nine detergent salts studied produce massive disorganization of the proteins, manifested by large changes in viscosity and in optical rotation. Both of these are sulfates, but it is known from work in Foster's laboratory that some substituted aromatic sulfonates (octyl and dodecyl benzenesulfonate) are also effective in unfolding (*e.g.*, Decker and Foster, 1966). *Small* changes in

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viscosity occur with all the detergents when molal ratios ($\bar{\nu}$) exceed 10. These changes do not extend over wide ranges of $\bar{\nu}$. *Large* changes, which increase with $\bar{\nu}$, occur only with dodecyl and tetradecyl sulfates. Small changes in optical rotation (a decrease in the depth of the 233-mμ trough) occur between $\bar{\nu} = 0$ and 10 with all of the detergents except those which have only six carbon atoms (these do not affect rotation at all) and octylsulfonate, which does not complete the small initial effect until $\bar{\nu}$ is much larger than 10 (Figures 1 and 2). Only the two massive *unfolders* (dodecyl and tetradecyl sulfates) bring about further changes in rotation at $\bar{\nu}$ well in excess of 40. (4) None of the above effects has a strong temperature dependence between 2 and 25°. This finding is consistent with the common attribution of the binding affinity to hydrophobic and electrostatic interactions rather than to involvement of covalent bonds. All of the effects described in both the previous and the present paper are fully and instantaneously reversible.

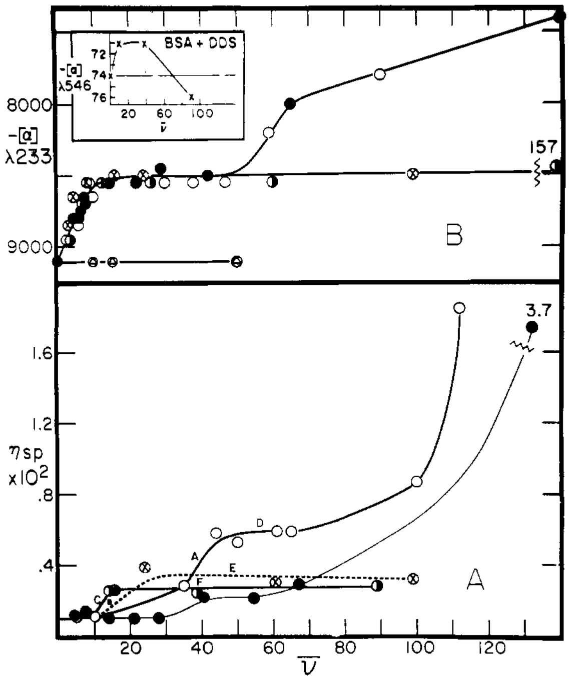

FIGURE 1: Specific viscosities (A) and specific rotation (B) of various BSA–detergent sulfate complexes as a function of molal ratio $\bar{\nu}$. (B) At the Cotton effect trough (233 mμ). The insert shows results with dodecyl sulfate at 546 mμ. (⊜) Hexyl, (◑) octyl, (⊗) decyl, (○) dodecyl, and (●) myristyl.

It is the purpose of the present paper to report in detail the effects of binding these detergents on the light-absorption properties of the protein. This study also distinguishes between those small changes brought about by binding from those due to large conformation changes induced by certain of the detergents, or by other agents such as acid or urea, in order to gain an increased understanding of the molecular events involved in binding and in the changes in properties which accompany it.

Experimental Section

Materials. The protein used was crystalline BSA[1] from Nutritional Biochemicals of several different lot numbers (the results were consistent for the different lots). The solutions were deionized with a mixed-bed resin (AG 501-X8 Bio-Rad) above a small amount of acid resin (Dowex 50W-X8). All of the solutions consisted of 0.1% BSA dissolved in phosphate buffer (Fisher Certified Reagent) at pH 5.6 and ionic strength 0.033. It has been shown that the deionizing procedure suffices to remove most of the fatty acid present in the original material. About 1 equiv may remain, but there is reason to believe that this may be partially displaced by the ions studied in this paper. The effect on ϵ_λ of combining fatty acid ions is reported elsewhere (Reynolds *et al.*, 1968).

The sodium salts of alkyl sulfates and sulfonates were of special highly purified grade made for us by Mann Research Laboratories (see Reynolds *et al.*, 1967). The octanol used was puriss grade obtained from ICN; the amino acids and dipeptides were obtained from Nutritional Biochemicals.

Methods. All measurements were made at room temperature, usually 23°; solutions of known $\bar{\nu}$ were prepared by adding the amount of detergent or octanol required, as calculated from the isotherms determined earlier (Ray *et al.*, 1966; Reynolds *et al.*, 1967). It is estimated that $\bar{\nu}$ is known within ± 1 in the low binding range; the uncertainty is 10% at $\bar{\nu}$ above 10.

Difference spectra were obtained with a Cary Model 14 spectrophotometer, using paired cells of either 2-cm or 1-mm path length for the two wavelength ranges at 280 and 230 mμ. A slide wire with a tenfold expanded optical density scale was used for the difference spectra with small $\Delta\epsilon$ values; the normal scale (OD = 1 at full scale) was used for the larger differences. The maximum slit width was 0.5 mm. A few experiments (some of the data presented in Figure 8 only) were made with 5-cm and 2-mm path length cells at 280 and 230 mμ; the maximum slit width for these experiments was 2 mm. The absence of significant light scattering is attested by the applicability of Beer's law over these differences in path length.

The reference cell contained no detergent; the latter was always placed in the sample cell. Compensation for the detergent proved unnecessary with the low concentrations used.

In a few experiments HCl was substituted for detergent. The ionic strength was then kept at 0.033 with NaCl in sample and reference solutions.

Difference spectra with amino acids and dipeptides were carried out with HCl since low concentrations of detergents are without effect on the optical properties of small peptides.

Circular dichroism measurements were made in the same Jasco Model UV-5 spectropolarimeter used in the optical rotatory dispersion work reported earlier (Reynolds *et al.*, 1967).

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[1] The abbreviation used in this paper that is not defined in *Biochemistry 5*, 1445 (1966), is: BSA, bovine serum albumin.

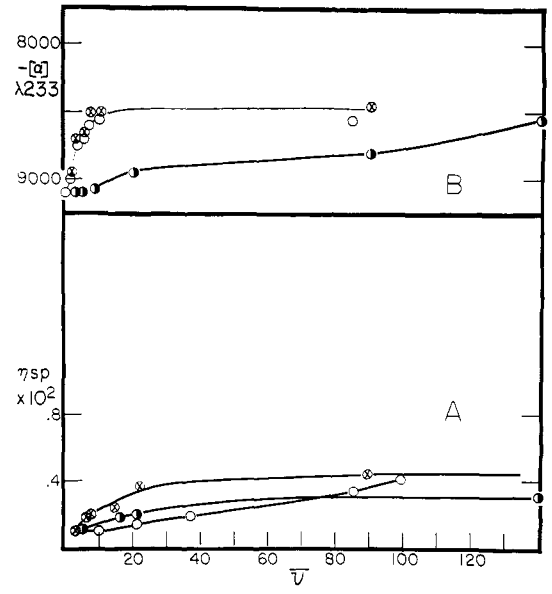

FIGURE 2: Viscosities (A) and specific rotation (B) of BSA–sulfonate complexes as a function of molal ratio $\bar{\nu}$. (B) At Cotton effect trough (233 mμ). (◐) Octyl, (⊗) decyl, and (○) dodecyl.

Results

Small spectral changes due to binding by the protein of all the ligands used have been observed in measuring difference spectra between 330 and 215 mμ. The ligands used were normal (unbranched) alkyl sulfates (C_6, C_8, C_{10}, C_{12}, and C_{14}), alkylsulfonates (C_6, C_8, C_{10}, and C_{12}), and 1-octanol. The difference spectra can be divided into two regions: the wavelength range near 280 mμ, where only the aromatic side chains of the protein absorb,[2] and the wavelength range near 230 mμ, where, in addition to the aromatic amino acids, histidine, cystine, and the peptide group all contribute to the absorption. This makes identification of chromophores contributing to the difference spectra in the latter region a more difficult problem than in the neighborhood of 280 mμ.

Difference Spectra Due to Binding. The most striking of the results is that all of the detergents produce characteristic difference spectra even when they are bound in very small amounts ($\bar{\nu}$ less than 5). The effect increases as $\bar{\nu}$ increases up to about 15–17, where with most of the ligands the effect is complete, although $\bar{\nu}$ may continue to increase considerably higher as the concentration is raised. However, with dodecyl and tetradecyl sulfate, which cause unfolding, further changes in absorption at higher $\bar{\nu}$ may be observed.

Figure 3 shows the saturation effects for sulfonates and sulfates separately. Figure 3b includes data for octanol and Figure 3a includes data at $\bar{\nu} = 8$ for the two unfolders as well.

It is obvious from Figure 3 that the sulfates, the sulfonates, and octanol produce somewhat similar saturation effects in both absorption regions compared with the much larger effects caused by drastic unfolding (shaded background of Figure 3a). It can also be seen that the two unfolders produce the same effect as the nonunfolders at low $\bar{\nu}$, at which no unfolding occurs. All the compounds show small *blue shifts* in the long-wavelength region, with minima at 293 and 287 mμ. The differences between sulfates and sulfonates in this region appear more clearly in Figures 4 and 5. They are: (1) with sulfonates the absolute value of $\Delta\epsilon$ at 287 mμ is smaller than with sulfates; and (2) the smallness of the effect with octylsulfonate. Hexyl sulfate and hexylsulfonate do not differ appreciably.

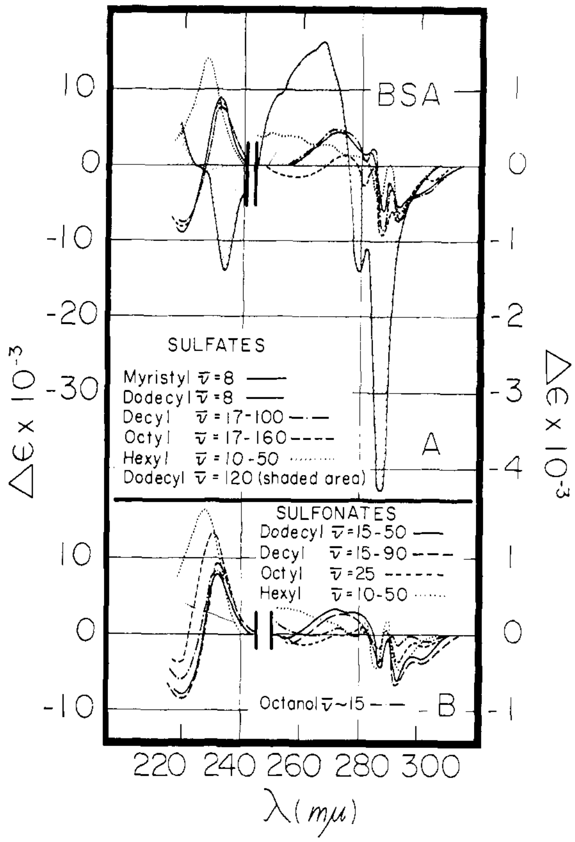

FIGURE 3: BSA difference spectra due to binding sulfates (A) and sulfonates and octanol (B). The contour of the shaded area represents the difference spectrum due to unfolding by combining with 120 equiv of dodecyl sulfate.

In complete contrast, the short-wavelength region of the difference spectra has the appearance of a typical *red shift* with a maximum at 232 mμ and a minimum close to 220 mμ. No differences between sulfates, sulfonates, and octanol are observed, but again the hexyl compounds and octylsulfonate differ from the others: with these the position of the peak is shifted to shorter wavelength, and no corresponding minimum appears to accompany it.

When these results are compared with the changes in optical rotatory dispersion (Figures 1 and 2[3]) it may be shown that of all the features of the spectra, only the appearance of the negative $\Delta\epsilon$ at 220 mμ parallels the optical rotatory dispersion changes; thus (1)

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[2] The absorption of cystine in this region is far smaller than that of the aromatic side chains.

[3] Figures taken from Reynolds *et al.* (1967).

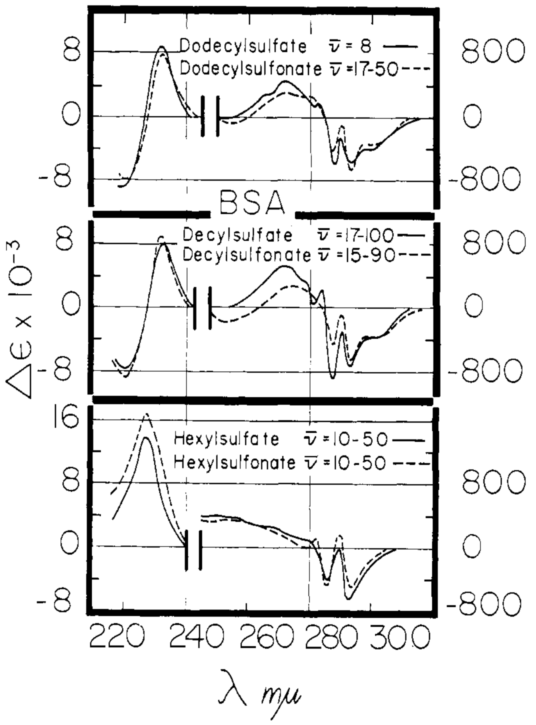

FIGURE 4: Comparison of BSA difference spectra due to binding some sulfates and sulfonates.

sulfates, sulfonates, and octanol produce characteristic differences in $\Delta\epsilon$ between 260 and 300 mμ. They all have the same symmetrical red shift with a minimum near 220 mμ in the low-wavelength range, and they all produce the same change in optical rotatory dispersion (a small decrease of 5–7% in the depth of the Cotton effect trough at 233 mμ without any corresponding change at 200 mμ, the peak of the α-helical Cotton effect). The hexyl compounds (measured at $\bar{\nu}$ above those giving saturation effects on $\Delta\epsilon$) and octylsulfonate at low $\bar{\nu}$ give a larger positive (red shift) difference peak in the far ultraviolet at somewhat lower wavelength, but no corresponding negative part at all. These spectral changes are not accompanied by any changes in optical rotatory dispersion. (2) With octylsulfonate there are indications of an optical density trough at short wavelength (220 mμ) which is deepened at very high $\bar{\nu}$. This deepening is accompanied by a decrease and a shift to 232 mμ of the positive part. It is also paralleled by a very gradual decrease of the absolute value of the 233-mμ optical rotation (Figure 2). With this compound neither the difference spectrum in the short-wavelength range nor the changes in rotation becomes as great as the values reached with the other detergents at $\bar{\nu} = 10$, even when $\bar{\nu}$ approaches 120. The uniquely small difference spectrum around 280 mμ does not change at all beyond $\bar{\nu} = 10$.

It is possible that the hexyl compounds would behave like octyl sulfonate at $\bar{\nu}$ above those investigated (>50). In any case, the optical rotatory dispersion and ϵ effects are separable with the short-chain compounds only. With longer chains where low $\bar{\nu}$ suffices to produce maximum effects at $\bar{\nu}$ near 10, both effects occur together, and are not accompanied by changes in viscosity (changes in molecular shape or volume).

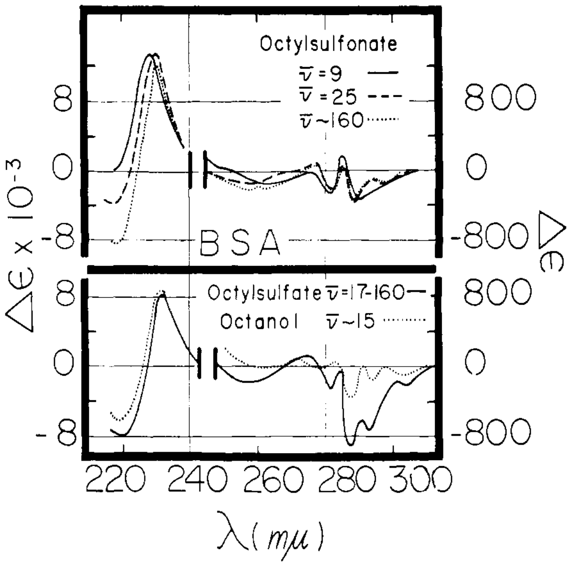

FIGURE 5: BSA difference spectra due to binding octylsulfonate, octyl sulfate, and octanol.

All of the sulfate data in Figure 3a are shown against a background silhouette representing data for protein exposed to such high concentrations of dodecyl sulfate ($\bar{\nu} = 120$) that the maximum disorganizing effect of this unfolder has been produced. The most casual inspection shows that the spectrum due to unfolding is qualitatively as well as quantitatively different from the effects produced by the initial binding. The trough at 293 mμ has become obscured and the trough at 287 mμ has become much deeper. The positive peak is not only larger, but occurs at shorter wavelengths, and the peak at 232 mμ has been transformed into a deep trough.

The saturation effects at $\bar{\nu} = 15$–17 are obviously dependent on binding at high-energy sites only; this binding produces no viscosity change (Figures 1 and 2). They cannot therefore be disorganization dependent. The results with dodecyl sulfate are consistent with those of Bigelow and Sonnenberg (1962), who reported small aromatic side-chain blue shifts at 285–295 mμ with small molal ratios of this detergent, and a more massive qualitatively different blue shift at higher concentrations.[4] The blue shifts have been considered to be criteria for disorganization of the native structure of the protein. Similar blue shifts have been observed when aromatic chromophores are transferred from organic environments to aqueous solutions (Yanari and Bovey (1960) and Leach and Scheraga (1960), among others), and have been ascribed to a change of the $\pi \rightarrow \pi^*$ transition brought about by changes in the polarizability of the solvent. It has been natural, therefore, to ascribe the blue shifts in proteins to unmasking to solvent of "hidden" aromatic chromophores

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[4] These investigators did not determine $\bar{\nu}$, or equilibrium free-detergent concentration.

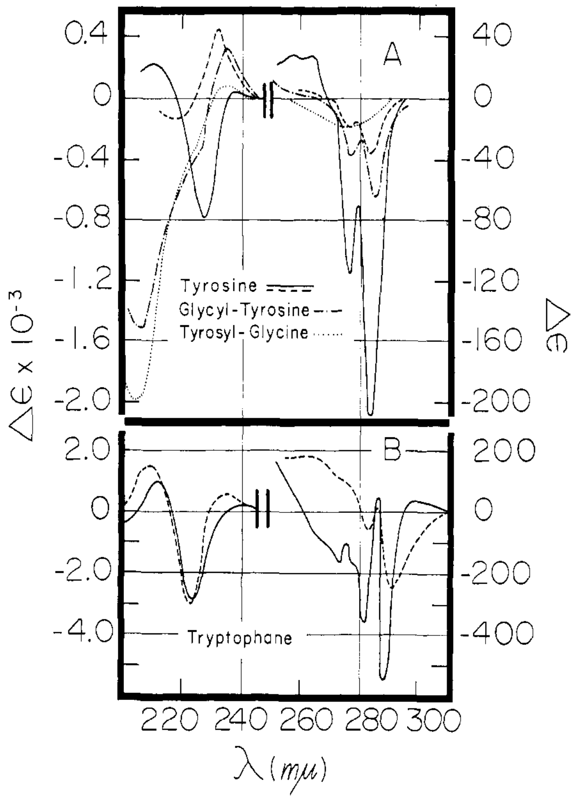

FIGURE 6: Difference spectra of tyrosine and tyrosine dipeptides[6] (A) and of tryptophan (B). The solid lines are solvent difference spectra: water *vs.* water–methanol (1:1). All broken lines are acid difference spectra, pH 1.8 *vs.* 5.6. Ionic strength 0.033.

brought about by detergent-induced disorganization of the protein.

Difference Spectra Due to Massive Unfolding. Such blue shifts have been demonstrated with many proteins when they are denatured by various means; they are, however, always accompanied by still larger blue shifts at 235 mμ which have been ascribed to changes of the second $\pi \rightarrow \pi^*$ transition of tyrosine and tryptophan brought about by the same changes in the environment. The aromatic amino acids are characterized by analogous effects when they are compared in different solvents (Figure 6).[5] In the present study, however, blue shifts at the first band are accompanied by red shifts at the second band. Glazer and Smith (1961) observed this blue shift at λ 235 mμ in several proteins, and pointed out that changes in the peptide absorption, due to conformation changes and changes in environment, must contribute to this difference peak.

Eisenberg and Edsall (1963) have shown that when human serum albumin (which has one tryptophan and 18 tyrosines) is unfolded by acid both the blue shift minima (287 and 235 mμ) deepen together with a factor of proportionately 3.28, as unfolding progresses, and hence are caused by the same chromophore. With carbonic anhydrases (six or seven tryptophans and eight or nine tyrosines), Edsall *et al.* (1966) observed, again, that both blue shifts increase together as unfolding proceeds but that here a larger proportionality factor (5–7) is obtained. All our *unfolding* difference spectra (Figure 7) have the same general features but the two blue shifts do not increase in a parallel fashion. The Δε at 235 mμ, when due to unfolding by dodecyl or tetradecyl sulfate at high concentrations, is three times greater than Δε at 287 mμ. When the difference spectrum is due to unfolding by acid (pH 1.65), this factor is over 4.5. At higher pH values the factor is much smaller, being 1 or even less at pH 3.7 and 4, where the N → F transition is complete (Foster *et al.*, 1965). Changes due to the N → F transition are obviously much smaller than those due to the greater disorganization produced at pH 1.65. It should be noted that all of the difference spectra known to be due to unfolding are quantitatively and qualitatively different from the small effects, due to binding, in which *blue* shifts at one band appear together with *red* shifts at the second band, and which are the principle subject of this paper. Green's (1962) observation that the binding of biotin by avidin results in a red shift at 235 mμ does not parallel the binding results reported here, since in his work a red shift also occurs in the 280-mμ region. Green concluded that binding biotin resulted in shielding the chromophores involved from the aqueous environment. Results similar to Green's have been observed in this laboratory with other hydrocarbon ligands, and are reported elsewhere (Reynolds *et al.*, 1968).

Discussion

The results reported would acquire particular significance if they could be shown to give information about the nature of the binding sites, or perhaps, less ambitiously, to shed light on small changes in conformation, which accompany binding, or the larger changes which we call unfolding.

Chromophore Assignments. We may tentatively assign various features of the data to contributions of at least two chromophores on the basis of the following analysis which makes use of the extensive data on model compounds collected by Wetlaufer (1962) and, more recently, by J. W. Donovan (to be published). The trough at 293 mμ is unambiguously due to a tryptophan blue shift; the trough at 287 mμ corresponds to additive effects of both tryptophan and tyrosine blue shifts, as are the broad peaks at about 277 mμ.

Among the detergent ions that do not cause massive unfolding the sulfonates and both hexyl compounds seem to affect mainly tryptophan and little or no tyrosine. With these compounds saturation of the 287 and 293 mμ troughs is reached at $\bar{\nu} \sim 10$. The sulfates appear to affect both tryptophan and tyrosine, since the trough at 287 mμ becomes deeper than the one at 293 mμ and also the second smaller tyrosine trough at 280 mμ is clearly visible. With the sulfates the tryptophan effect again reaches saturation at $\bar{\nu} \sim 10$, but the tyrosine maximum effect is reached only at $\bar{\nu} = 15$–17.

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[5] The large Δε at 205 mμ is to be expected for small peptides because of interaction of the titrated carboxyl group with the peptide group. This effect is negligible for large peptides and proteins (Saidel, 1955).

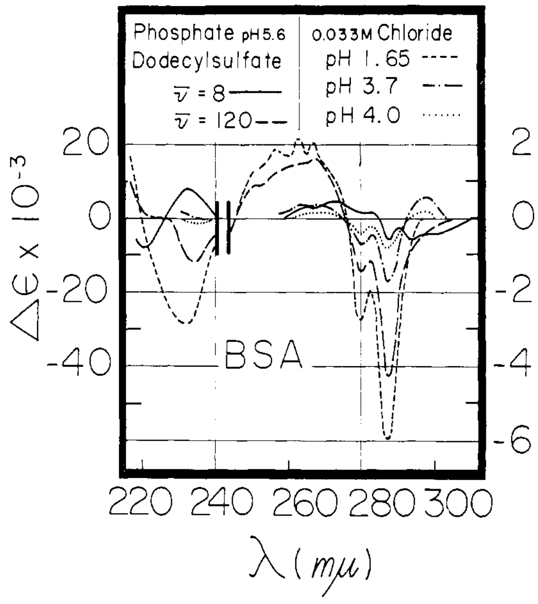

FIGURE 7: Comparison of the difference spectra of BSA caused by detergent binding, detergent unfolding, the N → F transition (pH 4.0 and 3.7), and unfolding at lower pH. The ionic strength is 0.033.

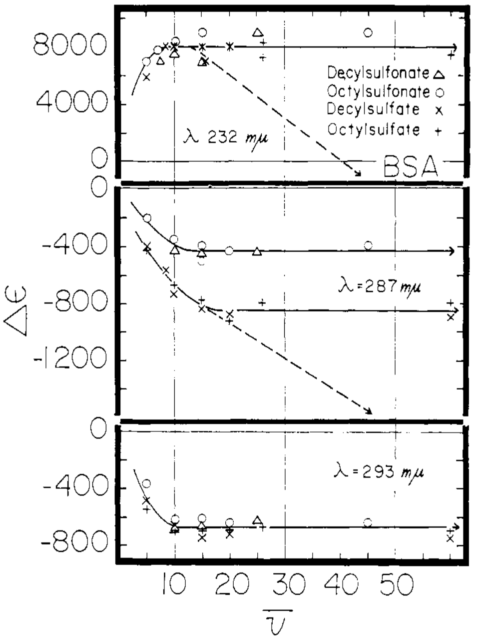

FIGURE 8: Difference in absorption of BSA at three different wavelengths (λ 232 mμ, top; λ 287 mμ, middle; and λ 293 mμ, bottom) due to binding sulfates and sulfonates, as a function of the molal ratio $\bar{\nu}$. The dashed lines represent data for the two unfolders dodecyl and tetradecyl sulfate.

The approach to these saturation effects can easily be seen in Figure 8.

With the two unfolding detergents, dodecyl and tetradecyl sulfates, which produce large viscosity and optical rotatory dispersion changes at higher $\bar{\nu}$, the tryptophan contribution at higher $\bar{\nu}$ is not further enlarged and is obscured or possibly reversed. The tyrosine blue shift, manifested as a contribution to the trough at 287 mμ, and to the broad peak at 277 mμ, becomes greater and greater. Thus the maximum effect on the two tryptophans of BSA is manifested at $\bar{\nu}$ below 10, where no viscosity changes are detectable, but the effects on the 19 tyrosines in the molecule are only marginally manifested unless there is massive unfolding. It should also be noticed that as acid unfolding proceeds at lower and lower pH a tryptophan trough is never observed. Thus the two tryptophans do not seem to be buried in the interior of the native molecule. With detergent unfolding the $\Delta\epsilon$ at 293 mμ decreases, as unfolding progresses, to a shoulder on the larger tyrosine trough, at $\bar{\nu}$ between 40 and 60. At $\bar{\nu} > 80$ no shoulder is detectable. Thus the tryptophan effect seems to be characteristic of binding only. It is not surprising that this binding-induced effect decreases and disappears when the protein unfolds since the unfolded protein is without high-energy sites (Foster and Aoki, 1958; Reynolds *et al.*, 1967).

With this conclusion in mind and with the model compound data of J. W. Donovan (to be published) and of our own laboratory (Figure 6) we now attempt to assign the red shift at 232 mμ. For the nonunfolders this effect reaches saturation at $\bar{\nu}$ below 10 (Figure 8). With unfolding by either of the two unfolders the maximum of the peak obtained at low $\bar{\nu}$ is depressed, and high $\bar{\nu}$ results in its gradual obliteration, and eventual transformation into a trough. It is hard to avoid the conclusion that this *reversal* of the 232-mμ red shift by the unfolding ligands is due to the same process which sharpens and deepens the tyrosine blue shift at 287 mμ when combination with large amounts of these detergents causes the protein to undergo radical disorganization. The blue shifts at 287 and 232 mμ are thus both due to tyrosines. A contribution to the 232-mμ *trough* may also be made by the $n \rightarrow \pi^*$ transition of the peptide bond (Glazer and Rosenheck, 1962; Rosenheck and Doty, 1961).

However, in the case of difference spectra due to binding, a comparison of the saturation effects in the two wavelength regions shows that these two effects are not necessarily due to transitions in the same chromophore. Thus: (1) sulfonates, sulfates and octanol show characteristic differences in their long-wavelength (270–300 mμ) spectral shifts (Figures 4 and 5). Sulfates and sulfonates have the same tryptophan effect, but the tryptophan effect with octanol is much smaller, in fact, almost negligible. The sulfates have a large tyrosine peak but the sulfonates show only a small or possibly no tyrosine effect at all. Nevertheless, with all the compounds mentioned the red shift at the short-wavelength range (230–235 mμ) is identical. (2) Both hexyl compounds have a difference spectrum at the longer wavelength range similar to that of the sulfonates, but at 230–235 mμ the effect appears to be very different from that produced by the other ligands. (3) With octyl sulfate (not an unfolder) the spectrum around 230 mμ undergoes slow changes up to $\bar{\nu} \sim 200$, while the spectrum around 280 mμ does not change after $\bar{\nu}$ reaches 10.

In the wavelength region of the red shift there is strong absorption not only of the aromatic amino acids,

TABLE I: Absorption Data of Amino Acids and of BSA at $\lambda \sim 220$ mμ.

Residue	λ	ϵ	No. of Residues/ Molecule of BSA (mol wt 69,000)[a]
Tryptophan	218	30,000[b]	2
Tyrosine	222	8,500[b]	19
Phenylalanine	208	8,000[c]	28
Histidine	212	6,000[c]	18
Cystine	212 sh	1,300[c]	17
BSA	225	600,000	

[a] Data from Stein and Moore (1949). [b] Data from Edelhoch (1967). [c] Data from Wetlaufer (1962).

but also of histidine, cystine, and the peptide group; all except the peptide group absorb because of $\pi \rightarrow \pi^*$ transitions; the peptide absorption alone is due to an $n \rightarrow \pi^*$ transition. Table I gives a comparison of the contribution to the absorption of each of these compared with the absorption of the protein itself.

The cystine contribution to the absorption is too small to cause such large spectral changes as are found near 232 mμ. The phenylalanine and histidine absorptions are at shorter wavelengths. If, however, more than one chromophore contributes to the difference spectrum in this region, the lower wavelength part could then well be caused by either phenylalanine or histidine (Donovan, 1965, and to be published). This idea is compatible with the fact that the hexyl compounds and octyl sulfonate show only a positive peak in their difference spectrum and lack the trough at lower λ, and with the fact that binding of these substances is not accompanied by optical rotatory dispersion changes.

Perturbation of the $n \rightarrow \pi^*$ transition of the peptide bond by changes in solvent would cause a red shift with decreasing polarity of the solvent (Glazer and Rosenheck, 1962; Litman and Schellman, 1965). A covering of the peptide linkages with the hydrocarbon tail of the detergents could cause such a shift, but it should be accompanied by an increase of the rotation at the 233-mμ Cotton effect (Litman and Schellman, 1965). If we assumed more than one kind of chromophore to cause the spectral shift, then the negative $\Delta\epsilon$ at $\lambda \sim 220$ mμ might be the result of changes in the $n \rightarrow \pi^*$ transition. Such a shift would be paralleled by a decrease of the rotations and would imply that peptide linkages from the interior of the molecule are exposed to water. Many peptide groups would have to be involved to cause an $\Delta\epsilon$ as large as -8000. This seems unlikely to occur without other signs of changes in conformation. A change of the $n \rightarrow \pi^*$ transition can also be eliminated by means of circular dichroism measurements. The latter can be used to resolve the effects due to the peptide group which contribute to the rotation at 233 mμ, *i.e.*, the parallel polarized $\pi \rightarrow \pi^*$ transition at $\lambda \sim 210$ mμ and the $n \rightarrow \pi^*$ transition at $\lambda \sim 222$ mμ. If the spectral and rotational changes are results of changes in the $n \rightarrow \pi^*$ transition we should be able to detect this change in the ellipticity band at 222 mμ. The results of such measurements (Figure 9), however, show a small decrease in ellipticity for both bands when the BSA–detergent complex is formed, an effect which is more easily explained if a change in ellipticity of another chromophore has occurred, influencing both bands equally. The weak ellipticity band between 260 and 280 mμ (Legrand and Viennet, 1964; Beychok, 1966) remains unchanged. Furthermore, neither the absorption maximum at 190 mμ nor the optical rotation at 200 mμ (due to the antiparallel polarized $\pi \rightarrow \pi^*$ transition of the peptide group) undergoes any changes when the detergent complex is formed. Finally, with the hexyl compounds and octylsulfonate it is still more obvious that the $n \rightarrow \pi^*$ transition is not involved: the large spectral changes are not accompanied by changes in optical rotation.

Having excluded the $n \rightarrow \pi^*$ transition of the peptide group, it is necessary to attempt to decide between other effects which may possibly cause the spectral shifts. Local conformation changes due to binding, which cause changes in the environment of the aromatic chromophores, cannot alone fully explain the effects. Such spectral shifts are well known from studies of model compounds in a variety of solvents or from observations of proteins in different states of "folding." Exposure to water of chromophores buried within the protein would cause blue shifts in both ranges. The opposite process would cause red shifts, as would covering of the accessible chromophores with the hydrophobic tail of the detergents, *but they would cause red shifts for both absorption regions* as in the case of the binding of biotin by avidin reported by Green (1962).

Before discussing charge effects as possible causes for the spectral changes we first exclude the effects of the protonation which occurs in the presence of buffer when detergent anions are bound. Binding of anions with no buffer present causes the pH of a protein solution to rise by an amount equal to $0.87w\bar{\nu}$, where w, the electrostatic interaction factor, has a value of about 0.05 corresponding to the ionic strength of 0.033 in these experiments. When buffer is present the pH cannot rise and instead the protein is protonated to an extent determined by the appropriate shift in its titration curve. Since these experiments were done at pH 5.6, where the titration curve is not steep, fewer protons are added at the values of $\bar{\nu}$ of interest here than are required to give any spectral change (see Figure 7). At $\bar{\nu} = 10$ the number of protons bound compares to the almost negligible protonation which would be caused by dropping the pH from 5.6 to 5.1. Detergent difference spectra in 0.033 M NaCl appeared to be almost identical with those in buffer.

If we make a quantitative comparison of the acid-induced difference spectra of tyrosine and tryptophan (Figure 6) with the difference spectra of BSA due to binding detergents we find a surprising similarity of magnitude and sign of the effects. $\Delta\epsilon$ for 19 tyrosines

$+\Delta\epsilon$ for 2 tryptophans equals -550 at 293 mμ and -800 at 287 mμ when pH 1.8 is compared with pH 5.6. It is noteworthy that the tyrosine undergoes a red shift at the short-wavelength range but a blue shift at 287 mμ (Wetlaufer, 1962). Tryptophan, however, has blue shifts in both absorption bands (Figure 6). The summation at 230 mμ of both effects in the stoichiometric proportion characteristic of BSA results in a red shift with $\Delta\epsilon$ at 232 mμ $+9000$ and $\Delta\epsilon$ at 220 mμ -8000.

However, this is an effect which parallels anion binding by the protein rather than protonation; at pH 1.8 the protein shows only the blue shifts characteristic of massive disorganization. Furthermore, it is difficult to understand how binding anions can produce an effect similar to that of protonation, since: (1) we add rather than remove negative charges when anions are bound: (2) even a binding-induced rearrangement of the molecule, which might bring positive charges close to the aromatic ring, should not be expected to have the same effect as the vicinal charge effect produced by titration of a carboxyl group which is covalently bound close to the ring. As Figure 6 shows, the vicinal charge effect produces qualitatively different and much smaller spectral changes when the ionized group is transferred from the α-carbon of the aromatic amino acid to an adjacent residue. Glycyltyrosine has the same characteristic bands in the acid difference spectrum as tyrosine; with tyrosylglycine, however, these spectral effects are much smaller. Tryptophan is distinguished from tryptophan dipeptides in an analogous manner.

Leach and Scheraga (1960), as well as Yanari and Bovey (1960), believe that both anions and cations may interact with the π electrons of the aromatic ring and that either may cause red or blue shifts. The sign and size of such spectral shifts in proteins have not been extensively studied. Since octanol has no charge the octanol shift, even though it is very small, cannot be explained in this way. The direct charge interaction theory implies that the spectral shifts in the short-wavelength range include effects due to one or more other chromophores than tryptophan and tyrosine.

In summary, the spectral shifts due to binding are obviously of a complex nature. Local conformation changes and/or local changes in environment of the aromatic side chains will not suffice to explain them. Either one or both of the charge effects discussed above are also required. Bigelow and Sonnenberg (1962) have made the same suggestion. The spectral differences around 230 mμ suggest strongly that another chromophore, phenylalanine or histidine, undergoes spectral changes in addition to those of tryptophan and tyrosine.

Binding Site Structure. The conclusions enumerated in this Discussion acquire pertinence to the broader purpose of the series of investigations to which the present work belongs to the extent that they yield information about the nature of the binding sites, or alternatively about the lability of the protein as affected by ligand binding. We therefore restate a number of the conclusions of this paper in terms of binding site structure. (1) Tryptophan residues are at, or very near, the binding sites of highest affinity for certain hydrocarbon derivatives. Since at least 8 equiv is required to produce saturation effects, it appears that four anions can exert an effect on each tryptophan residue. The only alternative to this conclusion, unacceptable for other reasons, is that binding this number of ligand ions causes an extensive disorganization of the three-dimensional structure. (2) There are other sites which have only slightly lower affinities, at or near the available (surfaced) *tyrosines*. These sites are not high-energy sites at all for the shorter chain ligands, and do not appear to contribute to the difference spectra obtained with sulfonates. This conclusion is consistent with the dependence of the number of the highest energy sites on chain length and polar head, already reported elsewhere. Each available tyrosine residue may be at a site which binds a single ligand ion, at least in the case of the sulfates. (3) Unfolding, which occurs only with the longest chains, parallels the exposure of the buried tyrosines to the solvent, but the number of sites in the unfolded protein is far larger than the number of tyrosines. There are thus other sites of lower affinity *in the unfolded protein*, as well as the possibility of micellar aggregation on bound ligand. The quantitative description of the relation of binding to unfolding already published (Reynolds *et al.*, 1967) requires only slight modification to conform to the model which is presented here.

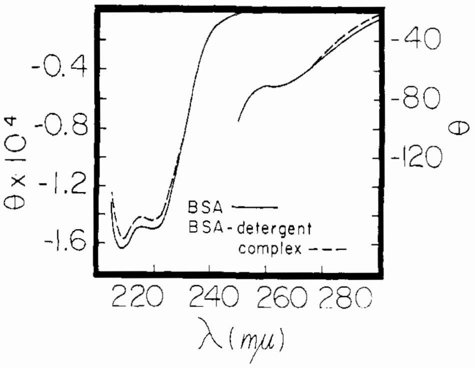

FIGURE 9: Ellipticity (deg $\times$ cm^2 $\times$ dmole^{-1}) of BSA plus dodecylsulfonate (or decyl sulfate) at $\bar{\nu} \sim 20$, pH 5.6 phosphate buffer; ionic strength 0.033.

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In models of both the α and β isomers of the fructofuranoside and fructopyranoside the monoanion can exist in a conformation in which the O^- of the phosphoryl group is close to the anomeric hydroxyl at C-2 and the phosphoryl OH is close to the ring oxygen. The potential efficiency of bifunctional catalysis of mutarotation has been amply demonstrated by Swain and Brown's (1952) experiments with α-pyridone, while Cunningham and Schmir (1966) have shown that phosphate monoanion can act as a bifunctional catalyst in the hydrolysis of the iminolactone, *N*-phenyliminotetrahydrofuran.

The experiments of Gottshalk (1943) suggest that 80% of fructose is in the pyranose configuration at 22°. While both F-1-P and fructose can exist in the pyranose form, FDP cannot. If we assume that phosphorylation at C-1 does not affect the equilibrium between pyranose and other forms of fructose, and that the effect of phosphorylation at C-6 is to prevent the formation of the pyranose form, the concentrations of the other forms should increase by a factor of five. The rate of exchange of FDP should then be five times that of F-1-P. Experimentally we find a ratio of 5.6 for the two rates (see Table I).

Phosphorylation at C-6 of fructose appears to facilitate exchange by increasing the concentration of the free ketone form, while phosphorylation at C-1 appears to facilitate exchange and, presumably, addition reactions by intramolecular catalysis.

Phosphorylation of fructose at C-1 and C-6 may affect the course of the enzymatic reaction in ways other than by increasing electrostatic binding of the substrate to aldolase. The phosphate on the C-6 of fructose increases the concentration of the free keto form by a factor of 5 by preventing the existence of fructose in the pyranose form. Secondly the phosphoryl group at C-1 can act as a bifunctional catalyst in the conversion of the cyclic to the keto form and subsequently to facilitate the formation of a Schiff base with the amino group of the enzyme.

Enzymatic Exchange. Horecker and his collaborators (Grazi *et al.*, 1962a,b; Horecker *et al.*, 1963; Lai *et al.*, 1965) have shown that DHAP forms a Schiff base with the ε-amino group of a lysine residue in the active site of aldolase (see also Cash and Wilson, 1966). The occurrence of an ^{18}O exchange with two substrates of aldolase at rates faster than the catalytic rate suggests strongly that for this enzyme, as for acetoacetate decarboxylase (Hamilton and Westheimer, 1959; Fridovich and Westheimer, 1962) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Rose and O'Connell, 1967), the formation of a Schiff base is an obligatory part of the reaction. This inference is substantiated by the observation that the potent inhibitor of the cleavage reaction, xylitol 1,5-diphosphate, also inhibits the exchange. Further there is a parallel loss of exchange and cleavage activity following treatment of aldolase plus DHAP with $NaBH_4$. The latter treatment has been shown to result in the covalent attachment of no more than three molecules of DHAP (as the glyceryllysine derivative) to aldolase (Lai *et al.*, 1965; Ginsburg and Mehler, 1966; Kobashi *et al.*, 1966). Measurement by a variety of techniques has shown that there appear to be three binding sites for DHAP per aldolase molecule (Ginsburg, 1966; Kobashi *et al.*, 1966; Castellino and Barker, 1966) and it would thus appear that the only effect of the DHAP–$NaBH_4$ procedure is to modify the active sites needed for enzymatic function. We conclude, therefore, that the participation of these active sites of the enzyme is required for the catalysis of the exchange reaction.

The exchange, under equilibrium conditions, proceeds faster than the initial rate of the forward reaction (see Table II). Further, aldolase partially degraded with carboxypeptidase retains its catalytic effect on the oxygen exchange of FDP even though this treatment reduces the rate of cleavage of FDP to about 5% of that found with the native enzyme. These data exclude the possibility that the exchange results from cleavage of the hexose diphosphate, rapid nonenzymatic equilibration of the [2-^{18}O]DHAP with the normal water of the medium, and resynthesis of a [2-^{16}O]hexose diphosphate.

The rate of the aldolase-catalyzed ^{18}O exchange of [2-^{18}O]F-1-P is about 3% that of [2-^{18}O]FDP, and is about twice as fast as the initial rate of F-1-P cleavage at the same concentration. Carboxypeptidase treatment of aldolase markedly reduces the rate of cleavage of FDP without, however, affecting F-1-P cleavage (Drechsler *et al.*, 1959). In the exchange reaction the difference in the catalytic activity of native aldolase acting on F-1-P and of carboxypeptidase-treated aldolase on FDP is consistent with the finding of Rose *et al.* (1965) that the principal effect of the carboxypeptidase treatment is to slow the rate of the last step in the cleavage of FDP, the protonation and release of DHAP from the DHAP–enzyme anion.

The work of Hartman and Barker (1965) and of Ginsburg and Mehler (1966) suggests that Schiff base formation *per se* does not contribute to the binding of FDP. Rutter (1964) has indicated that aldolase can be inactivated in the presence of FDP by borohydride, but no experimental details were given, nor was the product of the reaction identified, so that it is not certain that this inactivation resulted from the reduction of an FDP–enzyme complex. Our observation that the rate of exchange of the carbonyl oxygen of FDP and F-1-P is faster than the rate of cleavage strongly supports the hypothesis that Schiff base formation occurs subsequent to binding but before cleavage and release, and that the formation of the Schiff base is readily reversible. The slow rates of exchange and cleavage of F-1-P relative to those of FDP would reflect, therefore, a slow rate of Schiff base formation. This may be due either to a lower concentration or reactivity of the carbonyl of F-1-P (see above) or, as Rose (1966) has suggested, to the formation of aldolase–F-1-P complexes which are incapable of further reaction.

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