Effects of *n*-Alkyl Ligands on the Difference Spectra of Bovine and Human Serum Albumin†

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ABSTRACT: The ultraviolet difference spectra of bovine and human serum albumins, produced by binding various alkyl sulfates and sulfonates and fatty acids, resemble one another in some respects but differ radically in others. Where resemblances are found, the human albumin is characterized by generally larger effects. When 1 or 2 equiv of ligands is bound both proteins produce difference spectra characteristic of tyrosine and phenylalanine red shifts. The maxima are more marked when the proteins are defatted. When larger amounts of detergent are bound, the bovine albumin shows a trough at 293 nm, attributed to a tryptophan blue shift; in the human albumin there is a peak instead. With the unfolding ligand, dodecyl sulfate, the initial peak at 232 nm goes through a maximum at $\bar{\nu} \sim 9$ ($\bar{\nu}$ refers to molal ratio) and becomes a trough at $\bar{\nu} \geq 30$; with the human albumin this reversal does not occur until at least twice as many equivalents are bound. With the bovine albumin the initial tyrosine red shift is reversed to a blue shift when as few as 4–6 equiv of longer chain ligands are bound; this effect is attributed to local disorganization. With the human albumin the disorganization requires $\bar{\nu} \geq 20$. Increasing levels of the various bound ligands produce spectral perturbations which are characteristic of both protein and ligand, although broad resemblances in certain features remain. The results have been correlated with parallel studies of fluorescence changes due to binding in order to draw limited inferences as to the environment of the binding sites on the native proteins. The difference spectrum obtained by comparing unbound deionized bovine albumin to defatted bovine albumin is typical of tyrosine side chains; it disappears when 1 equiv of lauric acid is added to the defatted protein. If sulfates and sulfonates are added instead of fatty acids the difference spectrum is affected but not abolished. Changes due to defatting in difference spectra obtained with the other ligands cannot be abolished by adding lauric or oleic acid. In particular the "disorganization" indicated by the trough at 287 requires more dodecyl sulfate in both defatted and refatted bovine albumin than in the original protein.

The binding of even small numbers of equivalents of normal hydrocarbon derivatives (alcohols, fatty acids, *n*-alkyl sulfonates and sulfates) by bovine serum albumin selectively and characteristically alters the ultraviolet (uv) absorption spectrum of the three aromatic amino acid side chains in the protein (Polet and Steinhardt, 1968; Reynolds *et al.*, 1968). When up to about 10 equiv of detergents (sulfates or sulfonates of long-chain hydrocarbons) is bound by the bovine albumin, the two lowest energy absorption bands of tryptophan in the bovine albumin (280-nm region) are shifted toward shorter wavelengths while the band due to the higher energy transition at about 230 nm is inconsistently shifted in the opposite direction.[1] The two tyrosine bands are more consistent: both are red shifted when only a few equivalents of ligands are bound. When fatty acid anions rather than detergent sulfates or sulfonates are bound, the setyrosine red shifts are larger, and tryptophan absorption is not affected at all (Reynolds *et al.*, 1968).

If the conformation of the protein is drastically altered (as shown by large changes in optical rotatory dispersion and viscosity) by acid or by the binding of very large amounts of certain long-chain detergents, larger and qualitatively different effects have been observed. All of these effects are summarized by Steinhardt and Reynolds (1969).[2]

The spectra of model compounds containing these chromophores (*N*-acetylamides or esters of the simple amino acids) are shifted toward the red when they are transferred from aqueous solutions to most organic solvents (representative shifts are shown as difference spectra in Figure 1). Consistent red shifts would be readily explained by postulating that some of the chromophores were originally in contact with solvent, and that hydrocarbon-like ions or molecules, when bound, shield them from this aqueous contact. The blue shift of tryptophan in the bovine albumin appears to indicate the opposite, that at least one tryptophan of this molecule is buried initially but on complexing with long-chain detergents is exposed to solvent. The red shift in the high-energy (~230 nm) transition is inconsistent with this interpretation, unless the two tryptophans of bovine albumin behave in contrary ways.

Because in bovine albumin the tryptophan rather than the tyrosine transitions appeared anomalous, and because human serum albumin differs from the bovine variety in having a single tryptophan instead of the two in bovine albumin, we have compared the effects of binding alkyl derivatives on the uv absorption spectra of both proteins. The present paper also refines the earlier observations on bovine albumin, particularly those on the native (folded) protein, and shows that there are differences between successive binding sites in this respect and that the difference spectra due to binding of the two proteins differ as drastically as do the corresponding changes in fluorescence of the two proteins (Steinhardt *et al.*, 1971). However, these differences are not readily attributable to the difference in the number of trypto-

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[1] In an earlier paper the $S_0 \rightarrow S_3$ transition was incorrectly referred to as the $S_0 \rightarrow S_2$ transition (Steinhardt *et al.*, 1971).

[2] Acid pH (<4) produces difference spectra which resemble the effects of binding large quantities of dodecyl sulfate. The effects are not additive; in fact the bound detergent appears to oppose the effect of acid on the difference spectra and is therefore obviously still bound at pH 4.

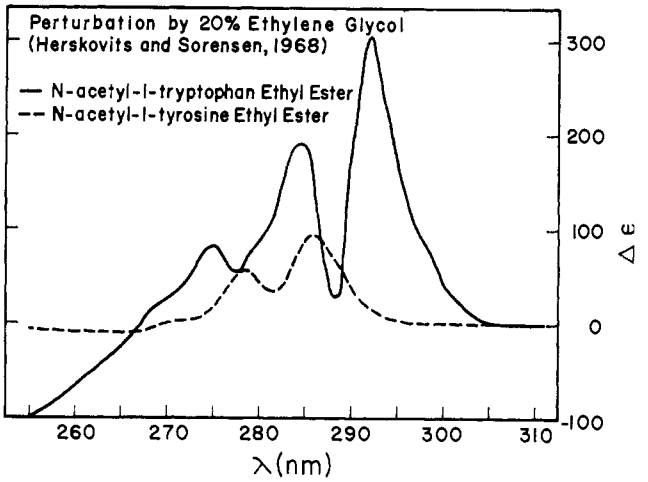

FIGURE 1: Difference spectra (red shifts) of selected model compounds containing tyrosine and tryptophan, produced when aqueous solutions of the substances are placed in the reference beam and 20% ethylene glycol solutions of the same solutes in the sample beam of a spectrophotometer (from Herskovits and Sorensen, 1968a).

phans. It will be further shown that removing the single equivalent of bound fatty acid associated with deionized crystalline protein affects the results, in some respects irreversibly. Large effects of temperature and ionic strength on the difference spectral will be reported elsewhere.

Experimental Section

Materials. Bovine serum albumin (Nutritional Biochemicals lot 9385) and human serum albumin (Pentex lot 24) were deionized on a mixed-bed resin as described earlier (Reynolds *et al.*, 1967). The defatted proteins were prepared by the method of Chen (1967) after deionization on a Dintzis column.

The fatty acids (dodecanoic acid and oleic) were purified products from Mann Research (Mann Analyzed grade). The sulfates and sulfonates were special custom syntheses from Mann Research, as previously described (Reynolds *et al.*, 1967). The inorganic salts were Reagent grade.

Methods. Difference spectra were obtained by placing protein dissolved in 0.033 ionic strength phosphate buffer (pH 5.6) in the reference beam of a Cary 14 spectrophotometer, fitted with an expanded-scale (0.0–0.1 optical density) slide-wire, and protein plus a given number of equivalents (1–300) of ligand in the same buffer in the sample beam. The bovine albumin (0.3%) or the human albumin (0.1%) was used in 1-cm path lengths in most of the work in the 280-nm region; 0.1% protein in 2-mm cells was used for the 235-nm region. After tracing the spectrum the cell positions were reversed, and the spectrum was obtained again, to make the results independent of base-line variations. In a few cases n equiv were used in one cell, and $(n - 1)$ equiv in the other, *i.e.*, the effect of adding a single equivalent was measured for systematically varied values of n. Experiments in which appreciable light scattering occurred were rejected.

Equivalents of ligand added were translated into equivalents of ligand bound by making use of previously determined isotherms (Reynolds *et al.*, 1967, 1968; Steinhardt *et al.*, 1971) or by determining additional isotherms, as needed.[3]

Results

Figure 2 shows that a fatty acid (dodecanoic) affects both proteins similarly, but that the spectral shift produced in the human albumin is substantially larger than the effect in the bovine albumin. The difference spectra are simple, consisting of relatively strong tyrosine and phenylalanine red shifts, with no trace of an effect on the tryptophan. They are wholly consistent with the earlier data of Zakrzewski and Goch (1968) on the human albumin, and of Reynolds *et al.* (1968) on the bovine albumin.

Figure 3 displays the difference spectra for both "deionized" (not defatted) proteins binding selected numbers of equivalents of a nonunfolding alkyl ligand anion, octyl sulfate. Much of the data, obtained with other numbers of equivalents bound, have been omitted to avoid cluttering the figure. It is apparent that there are great differences between the data obtained with the two proteins in both uv spectral regions.

Among the obvious differences one may list: (a) greater effects in the human albumin than in the bovine albumin; (b) an absence, in the human albumin, of the troughs at 293 and 288 nm which characterize the bovine albumin difference spectra. The trough at 293 is very characteristic of the blue shift of the tryptophan spectrum incident to transfer from apolar to more polar environments. An inverted effect at 293 nm may be present in the human albumin showing as a peak or shoulder rather than as a trough, at all but the lowest value of molal ratio bound ($\bar{\nu}$); (c) the presence in the human

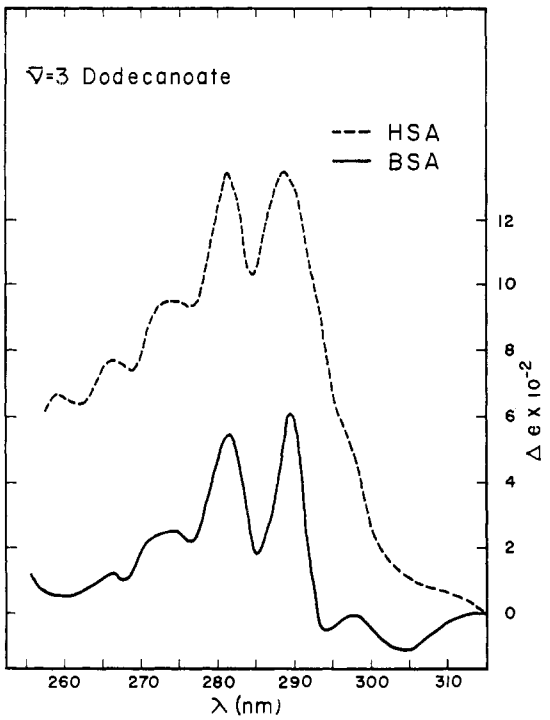

FIGURE 2: Difference spectra of both bovine and human albumin produced when the sample beam traverses a solution of protein combined with 3 equiv of dodecanoate ion at pH 6.8 phosphate buffer at an ionic strength of 0.033.

[3] For consistency with earlier papers we have continued to use 69,000 for the molecular weight of both proteins, although bovine albumin has a lower molecular weight (65,000). Thus with bovine albumin, for example, 1 equiv must be read as 0.94 equiv, etc., and the values of $\Delta\epsilon$ given must be reduced by 6%.

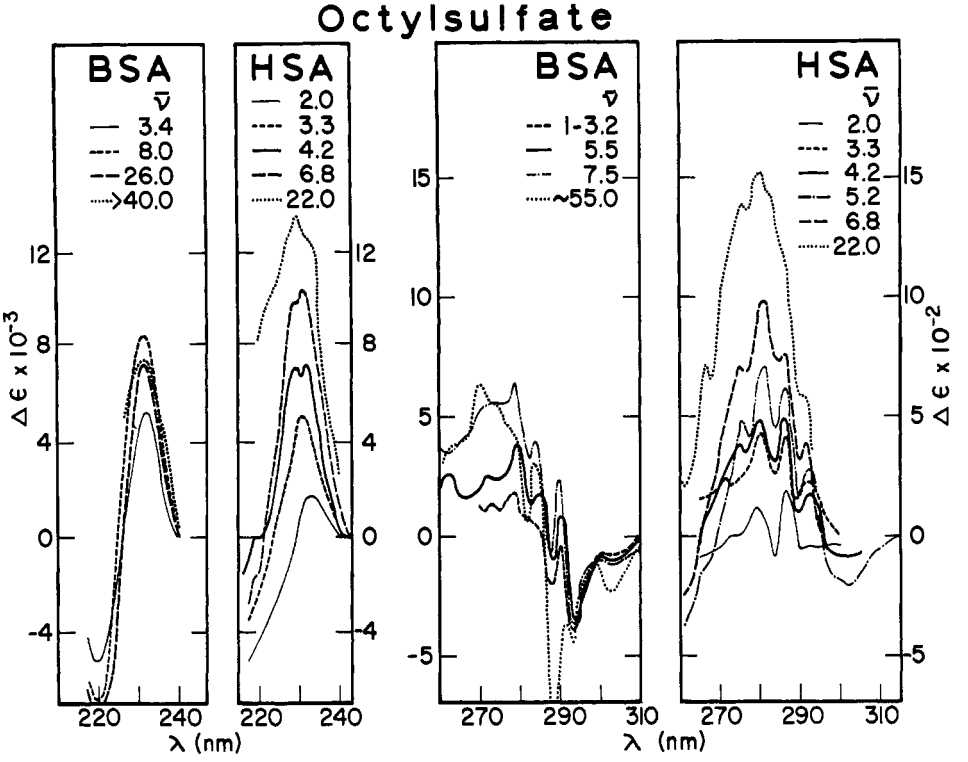

FIGURE 3: Difference spectra of both deionized bovine and human albumin in two different ultraviolet spectral regions produced by binding the indicated numbers of equivalents of octyl sulfate at pH 5.6. Note that there is a tenfold difference between the ordinates of the panels representing the shorter wavelengths and those on the other panels.

albumin of the clear "twin peaks" at about 279 and 289 nm characteristic of a tyrosine red shift—and the presence in the bovine albumin, at large values of $\bar{\nu}$, of both these peaks *inverted into troughs* (hence blue shifts). (d) In the far-uv region, the human albumin is characterized by a gradual shift of the maximum to lower wavelengths (from 232 to 228 nm), while in the bovine albumin the maximum remains at 232 nm at a remarkably constant magnitude. There is evidence of a double peak in the human albumin, absent in the bovine albumin.

Additional differences between the serum albumins of the two species appear when the difference spectra are obtained with ligands which unfold the protein when large amounts are bound. Figure 4 shows results at selected $\bar{\nu}$ values, obtained with such a ligand, dodecyl sulfate.

The results are somewhat similar to those found with octyl sulfate except at the largest $\bar{\nu}$ values where dodecyl sulfate produces much larger and qualitatively different effects. At $\bar{\nu} = 5$ and 120 (the bovine albumin) an exceedingly deep and wide trough which is only partially displayed in the figure (see Polet and Steinhardt, 1968) makes $\Delta\epsilon$ negative at all values of λ between 273 and 310 nm, whereas with octyl sulfate a deep narrow trough was formed at $\bar{\nu} = 55$, over a width of only a few nanometers near $\lambda = 287$. With the human albumin a trough at this wavelength is found only at very high $\bar{\nu}$. It is very narrow and never attains negative values of $\Delta\epsilon$.

In the far-uv region with dodecyl sulfate and the bovine albumin the peak at about 230 nm ($\bar{\nu} \leq 9$) is transformed, when large amounts of dodecyl sulfate are bound, to a deep trough at about the same wavelength. With the human albumin the peak in the far-uv region also reverses to a trough ($\bar{\nu} = 63$). The shift of the peak to lower wavelengths at low values of $\bar{\nu}$ is due to the formation of the positive branch of a Cotton effect, the negative part of which is obscured by the remnant of the original peak. The latter becomes a shoulder on the new peak, then disappears, allowing the new trough to show at high $\bar{\nu}$.

These transformations do not appear when octyl sulfate is bound. $\Delta\epsilon$ never becomes negative at or near 233 nm. Thus the results obtained with the two proteins differ more strikingly when the unfolding ligand is bound. The differences are chiefly incidental to unfolding, but somewhat larger effects are produced by the longer chain detergent even at low $\bar{\nu}$.

Results obtained with sulfonates are so similar to those obtained with sulfates that they are not described in this paper. The wavelengths of peaks and troughs differ slightly. General unfolding is found with myristylsulfonate but not with dodecylsulfonate.

Effects of Various Ligands on Defatted Bovine Serum Albumin. Similar measurements have been made with both proteins with a number of other homologous detergents, including three short-chain sulfates which do not unfold (C_6, C_8, C_{10}) and two longer chain compounds which do (C_{12}, C_{14}), using both column-deionized and charcoal-defatted proteins. Every detergent shows particular idiosyncracies although the underlying patterns are strongly similar. We illustrate both similarities and differences in the case of defatted bovine albumin in Figure 5, which shows the difference spectra produced by a *single equivalent* of each of the detergents on *charcoal-defatted* bovine albumin. The results with defatted bovine albumin are selected because the differences between ligands at the level of 1 or 2 bound are more clear-cut than they are with the merely deionized protein.

The largest effect is produced by the two shortest chains, hexyl and octyl sulfates, even though they are bound less, at a fixed detergent concentration, than are the longer chains.

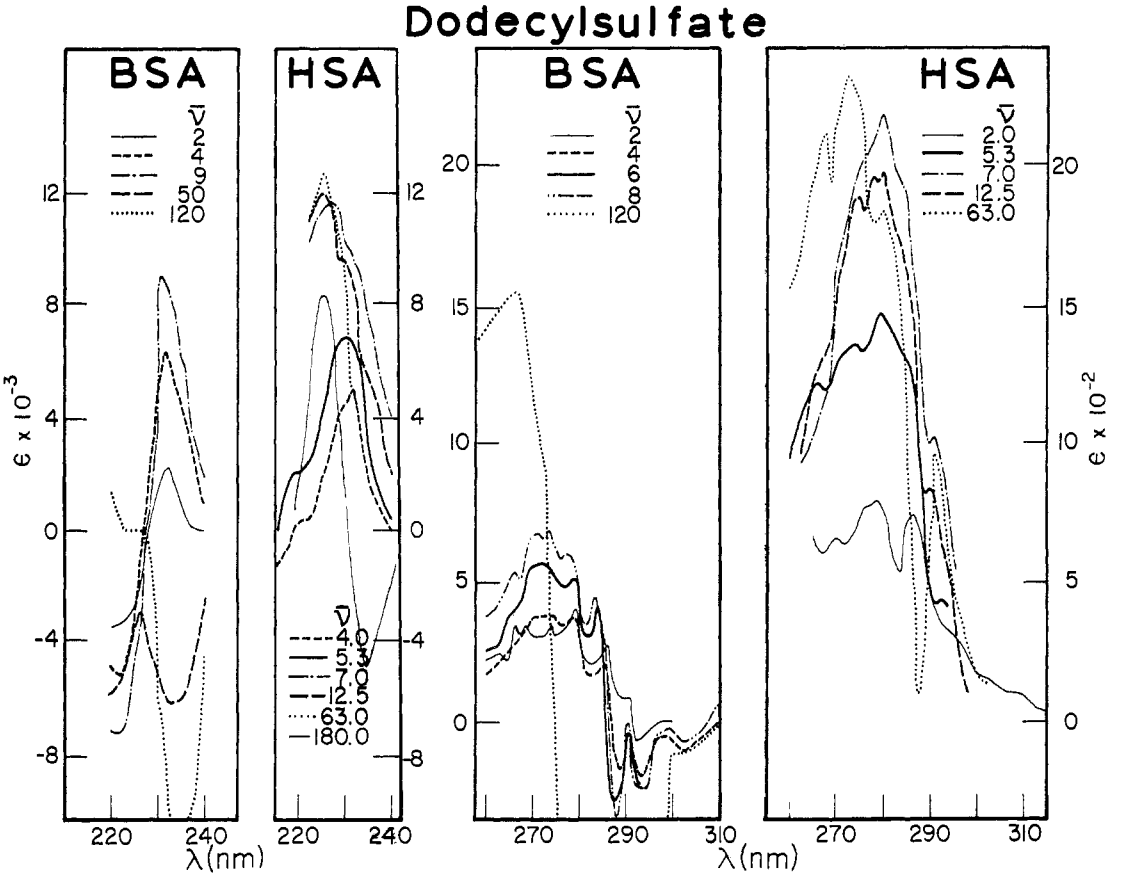

FIGURE 4: Difference spectra of both bovine and human albumin in two different spectral regions produced by binding the indicated numbers of equivalents of dodecyl sulfate at pH 5.6. Note that the ordinate scale for the shorter wavelengths is in units ten times as great as those for the data at other wavelengths.

The result with hexyl sulfate (peaks at 280.5 and 288, troughs at 276.5 and 283.5) is very much like that of *N*-acetyltyrosine ethyl ester (Figure 1) or, except for being much smaller, like the difference spectrum of the bovine albumin produced by binding fatty acids; the latter consistently appear to perturb tyrosine side chains only (Reynolds *et al.*, 1968); there is no sign of a tryptophan shift. The result with octyl sulfate (which binds more nearly quantitatively) differs in only two respects: (a) it is shifted downward as a whole; (b) the short-wavelength end is shifted more strongly negative than the long-wavelength end.

The effect of the next longer chain, decyl sulfate, is very much smaller, but shows a small tryptophan blue-shift trough at 293 nm which is totally absent with the C_6 compound and doubtful with C_8, both of whose difference spectra are essentially that of tyrosine; in addition, the decyl spectrum is very slightly shifted toward shorter wavelengths and the trough at 276–277 is less well marked. The shorter wavelength regions (260–275), which may contain elements contributed by phenylalanine, show no clear trend.

The two unfolding ligands (C_{12} and C_{14}) give difference spectra much like that of decyl, except for a displacement upward. They are almost identical at $\lambda = 283$; at shorter wavelengths C_{12} shows a more positive effect with the result that the relative height of the two main peaks is reversed.

The magnitude of the effects produced at 260–300 nm by a single equivalent of ligand is a substantial fraction of the effect produced by much larger amounts (unless unfolding occurs). Thus with hexyl sulfate the second equivalent *diminishes* the effect, increasingly so at $\lambda \leq 293$; and further additions are almost without effect. With octyl sulfate only small effects modify the results given by 1 equiv until more than 5 are present, *except* for the development of a trough at 293–294. With *decyl* sulfate only small additional effects are produced until the fourth equivalent is added—the effect of the latter is confined to making the entire difference spectrum less positive. The fifth equivalent has essentially no effect, but at 10 equiv, a conspicuous trough at 288 develops together

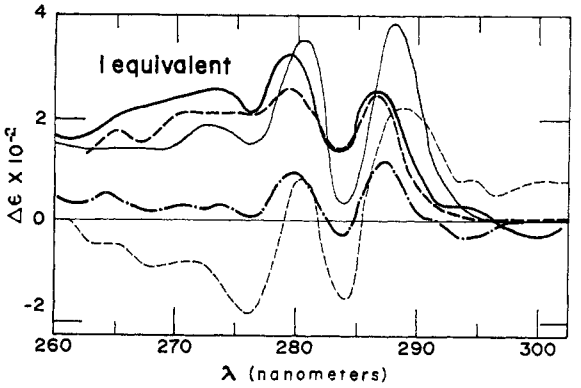

FIGURE 5: Difference spectra of defatted bovine albumin produced by the presence of a single equivalent of five *n*-alkyl sulfates: (—) hexyl; (– – –) octyl; (–·–·–) decyl; (—) dodecyl (unfolder); (- - - - -) myristyl (unfolder).

with a lesser one at 281—*both are very suggestive of inversions of the peaks produced by the first equivalent (tyrosine red shift) very slightly shifted toward the red.*

With dodecyl sulfate, however, the second and third equivalents of ligand substantially reinforce the effect of the first, in addition to introducing the characteristic (tryptophan blue-shift) trough at 293 nm. The fourth ligand is without much additional effect, but the fifth introduces the 287- to 288-nm trough which appears to constitute a reversal of the peak at this wavelength produced by the first equivalent. With the next 5 equiv, there are increases in phenylalanine contributions, and further clear inversion of the two peaks which were seen at 1 equiv; the trough at 288 has become quite deep, possibly containing the trough of a tryptophan red shift from the second tryptophan of the bovine albumin, as well as the trough of a tyrosine blue shift (the inverted peak referred to above).

With myristyl sulfate (C_{14}), as with dodecyl, the second equivalent almost doubles the height of the peaks produced by the first, and adds a trough at 293. The third equivalent does little more than deepen the trough. The fourth equivalent strengthens all of the features noted. The fifth produces entirely new effects in addition to slightly deepening the 293-nm trough: (a) the peak at 288–287 begins to diminish; at 10 equiv it becomes a deep trough at 287.5; (b) the long-wavelength side of the peak at 279.5 diminishes, becoming quite small at 10 equiv; (c) only small effects are visible at 275–280 nm.

It is clear that successive ligands bound (1) occupy either different sites on the bovine albumin or (2) different distributions from sets of highly individual sites; or (3) a given effect may be produced by any of a number of sites the effects of which are not necessarily additive.

All of the results just described refer to regions above 260 nm. In the much more intensely absorbing wavelength region which lies below 240 nm, the effects of the various ligands on the absorbance of the bovine albumin can be described more simply. Figure 6A shows that in the presence of 2 equiv of hexyl, decyl, and myristyl sulfates, $\Delta\epsilon$ rises identically with all these ligands as λ decreases until a maximum (or, in the case of hexyl, a shoulder) is formed at 233 nm. At $\lambda \leq 233$ the results with hexyl sulfate diverge from the others, being essentially featureless. Decyl and myristyl sulfate descend to a well marked trough at 221–22 nm. Decyl has a larger effect in the trough region than myristyl, although the latter is an unfolder, and decyl is not.

At 5 equiv (Figure 6B) the results differ not only in showing more marked differences, but in showing that the peak for hexyl sulfate shifts to 227 nm, and that the data for decyl and myristyl are very similar throughout. Since both of the latter affect the mean residue rotation of the bovine albumin at the 233-nm Cotton effect trough at 2 and 5 equiv per mole but hexyl sulfate does not (Polet and Steinhardt, 1968), it is clear that the effect on α_{233} is associated with the process producing the trough at 222 rather than with the peak at 233 nm, *i.e.*, with an *absorption* process having a maximum near 228.

Results with Deionized (Undefatted) Protein. The presence of bound fatty acid changes the results slightly from those just described. Thus, for example, at 293 nm a single equivalent of each ligand produces troughs which we have identified with tryptophan blue shifts. Hexyl sulfate is no longer significantly more effective than the other ligands and has a pronounced trough at 293 besides a trough at 284.5 between peaks at 289 (large) and 282 (small), instead of approximately

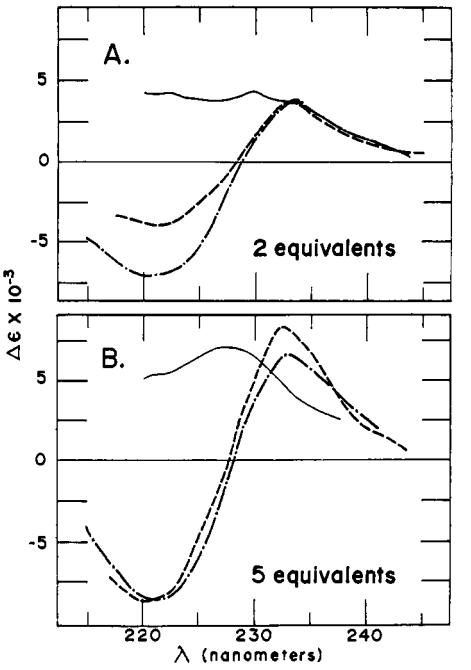

FIGURE 6: Difference spectra in the far-ultraviolet region produced by three of the five ligands represented in Figure 5: (—) hexyl sulfate, (-·-·-) decyl sulfate, and (- - - - - -) myristyl sulfate (unfolder).

equal peaks at 281 and 288 with a trough 285.5. Both peaks for decyl sulfate are displaced to shorter wavelengths, the one at the right from 289 to 286 nm. Its trough at 282.5 almost overlaps the peak of hexyl sulfate at 282. The data for dodecyl sulfate show a further displacement of the peaks to shorter wavelengths; the trough at 282.5 is filled in so well that the peaks are no longer striking. All that is left of the striking peak at 289 (hexyl sulfate) is a slight shoulder at that wavelength. The data for myristyl sulfate are very similar to those for dodecyl sulfate except for a diminution in their magnitude. The shoulder at 289 has disappeared.

Table I summarizes the salient differences between the difference spectra of the bovine and human serum albumins at two levels of ligand.

Although the data obtained with undefatted bovine albumin show a smaller range of effects than those obtained with the defatted protein, the several ligands show idiosyncrasies here also. Thus the second equivalent of hexyl sulfate is strongly negative, as with the defatted protein. The next 2 equiv greatly deepen the 293-nm trough, but raise $\Delta\epsilon$ strongly at $\lambda \leq 280$, in a manner suggestive of phenylalanine. Larger quantities (up to 10) have very little if any effect. With decyl sulfate any quantity over 1 equiv has only a small effect, except in slowly deepening the 293-nm trough and, up to 4 equiv, increasing the phenylalanine region (260–275 nm).

Since the "undefatted" and "defatted" bovine albumin used in this investigation differed in lipid content by almost exactly 1 equiv/mole, one would expect that the difference spectrum between the two (with concentrations carefully equalized by *nonspectrophotometric methods*) would be the same as that given by the addition of one equivalent of fatty acid to defatted bovine albumin, *i.e.*, a classical tyrosine spectrum. Figure 7 (lower left) shows that this expectation is realized. Furthermore if 1.1 equiv of dodecanoate ion (or 2 of oleate) is added to the solution in the cuvet which contained the defatted protein, the "tyrosine" difference spec-

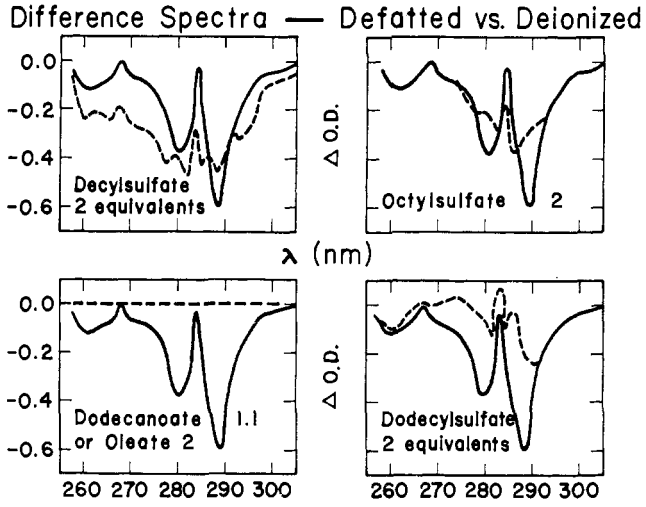

FIGURE 7: Lower left: difference spectrum of the defatted bovine albumin against the undefatted deionized bovine albumin. The horizontal line was obtained after adding 1.1 equiv of laurate or 2 equiv of oleate to the defatted sample. Other three panels: the difference spectrum shown in the lower left panel is repeated in each of these, together with the result of adding 1 equiv of nonfatty acid *n*-alkyl ligands to the defatted sample.

TABLE I: Differences between Bovine and Human Serum Albumins in Sign of Tyrosine and Tryptophan Difference Spectra at Two Different Levels of Presence of Ligand.

	$\bar{\nu}$ = 1 or 2		$\bar{\nu}$ = 5–10	
	Tyrosine	Tryptophan	Tyrosine	Tryptophan
	Dodecanoate			
Bovine	Red shift[a]		Red shift[a]	
Human	Red shift[a]		Red snift[a]	
	Hexyl Sulfate			
Bovine	Red shift[a]	Blue[b]	Red shift[c]	Blue shift
Human	Red shift[a]		Red shift[a]	Red shift
	Octyl to Myristyl Sulfates			
Bovine	Red shift[a]	Blue shift	Red shift[d]	Blue shift
Human	Red shift[a]		Red shift[a]	Red shift

[a] Tyrosine red shift peaks at both 232- and 280- to 290-mμ region. [b] Weak. [c] Peak at *ca.* 228 nm indicates high-energy band of tryptophan. [d] Red shift changes to blue shift between 5 and 10 equiv.

trum entirely disappears—a very good base line is all that is seen.

However, the situation is more complex than it seems. We are not dealing simply with occupancy or vacancy of a site which perturbs a particular tyrosine residue. When any of the alkyl sulfates which give, at 1 or 2 equiv, a tyrosine difference spectrum with defatted bovine albumin (Figure 5) are substituted for dodecanoate, the tyrosine difference spectrum is not obliterated, but is merely distorted—a straight horizontal base line is never recovered except when fatty acids are added (Figure 7). However, we have shown that a single fatty acid anion produces a much greater perturbation than a single alkyl sulfate anion—it is therefore not surprising that "refatting" with alkyl sulfate does not restore the original state in which lipid was involved. Tyrosine residue(s) are affected, but perhaps not the same ones or not in the same way.

Although adding an equivalent of laurate to defatted bovine albumin makes it spectrophotometrically identical with undefatted crystallized protein (Figure 7, lower left), one may show that the defatted protein, even after restoration of fatty acid, is irreversibly changed from the protein that has not undergone the defatting procedure. Figure 8 shows that defatting accentuates the tyrosine peaks at 279 and 286.5 produced by binding dodecyl sulfate, and that no trough appears in defatted protein at 287 nm until $\bar{\nu} \geq 5$ although there is a clear indication of the latter trough at $\bar{\nu} \geq 2$ with normal protein. The tryptophan blue shift in normal bovine albumin appears only at $\bar{\nu} \geq 4$ also. At low $\bar{\nu}$ the phenylalanine peaks in defatted bovine albumin are more marked than in normal bovine albumin. With *refatted* protein, most of the features of the defatted protein still appear; in particular the 289-nm trough does not appear until $\bar{\nu}$ is well over 4. Refatting also affects the phenylalanine region.

Refatting at acid pH (similar to that at which defatting was done) restores a small part of the 289-nm trough characteristic of normal protein, when decyl sulfate (3 equiv) is used.

Of the two peaks given by dodecanoate, the one at 289–290 nm is most affected by defatting. The ratio of the heights of the two peaks A_{281}/A_{289} is 1.38 at 2 equiv bound by defatted protein. It is 1.25 with undefatted protein. These ratios are predictable from the slopes of the direct spectra at these wavelengths.

Results with Human Serum Albumin. Although differences exist between defatted and nondefatted human albumin similar to those found in bovine albumin the effects of varying the ligand are shown only for deionized human albumin at three different levels of added ligand, 2, 4, and 10 equiv.[4] The dodecanoate spectrum at 2 equiv corresponds to the familiar tyrosine difference spectrum, as in the bovine albumin and as previously reported (Reynolds *et al.*, 1968; Zakrzewski and Goch, 1968), but the two small peaks given by the two alkyl sulfates are shifted to shorter wavelengths. At 4 equiv (Figure 9B) all the ligands give essentially the same difference spectrum, except for (a) differences in amplitude, which are not correlated simply with the length of alkyl chain; and (b) the appearance of a small peak or shoulder at 293 nm (instead of a trough as with the bovine albumin) in some but not all of the data. Dodecanoate data are omitted because $\Delta\epsilon$ is much larger—almost three times as great as any other; its spectrum of course, does not show the similarities just noted for the others.

At 10 equiv (Figure 9C) the similarity of the spectra produced by the various ligands is even plainer. On shifting the spectra vertically they nearly superimpose throughout the long-wavelength half of the diagram; at shorter wavelengths they then diverge only slightly.

In the region of stronger absorbance below 240 nm (Figure 10), this description must be modified. At the level of 2 equiv octyl sulfate produces only a progressively more negative $\Delta\epsilon$ ($\lambda \leq 232$ nm). Decyl and dodecyl sulfate also show this drop in $\Delta\epsilon$, but are both characterized by a clear maximum at 232–233 nm; the data for both ligands are almost indistinguishable. Note that dodecanoate produces a much larger effect, peaked at 233–234. These results are consistent

[4] Some human albumin preparations have been turbid after defatting.

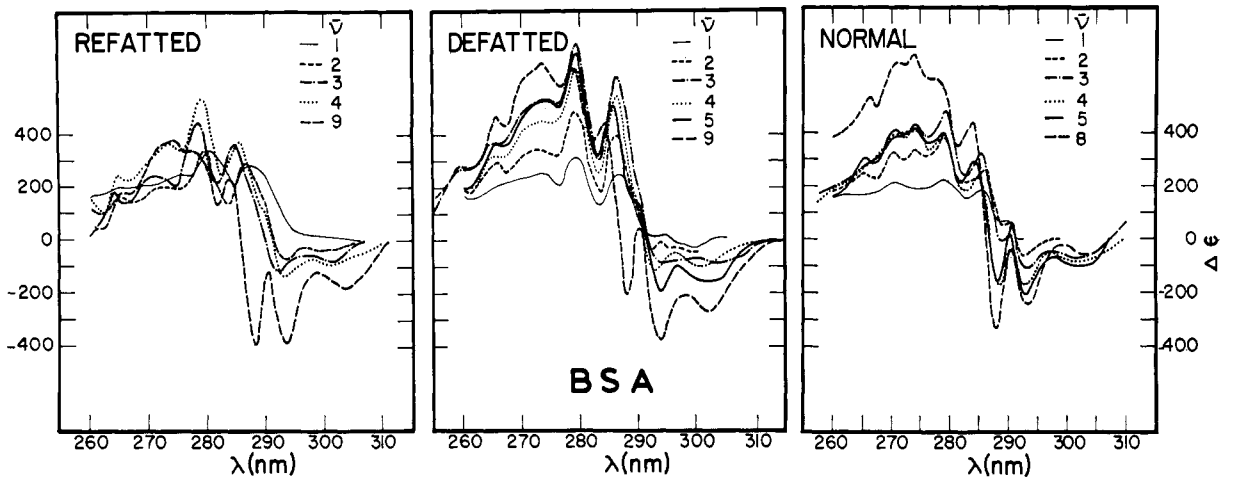

FIGURE 8: Comparison of the difference spectra (to dodecyl sulfate) of the normal (deionized) bovine albumin (right-hand panel), the defatted bovine albumin (center panel), and the refatted bovine albumin (left-hand panel). The differences are described in the text.

with those of Figure 9 at longer wavelengths, in showing that occupancy of the two highest affinity sites does not perturb the single tryptophan of the human albumin. With 6 equiv present (Figure 10B) both octyl and dodecyl sulfates give larger perturbations, and there is an indication of a chromophore which has an absorption at shorter wavelengths than that of tyrosine, presumably tryptophan. Reference to Figures 3 and 4 shows that at still higher levels the tryptophan perturbation increases further, especially with large amounts of unfolding ligands, which cause the sign of the tyrosine perturbation to reverse (Figure 4). This reversal can be seen in the longer wavelength region (incipient trough at 289 nm) at much lower amounts bound than in the short-uv region.

The data obtained with the human albumin (Figures 9 and 10) are simpler than those given by the bovine albumin. The complexity of the results with the bovine albumin (an unexplained change in sign between the perturbations of the high- and low-energy bands of tryptophan, a greater effect on the difference spectra of the presence of traces of lipid, and more marked idiosyncrasies of the perturbations caused by successive extents of binding by different ligands) may be related to the presence of two tryptophans in the bovine albumin, one in an essentially aromatic sequence, and one in an aliphatic one (Brown *et al.*, 1971; Swaney and Klotz, 1970).

Discussion

The experiments reported in this paper were undertaken in the expectation that results obtained with the simpler chromophore system (human serum albumin) would be less ambiguous or contradictory than those of the bovine albumin (two tryptophans) and help in the interpretation of the latter. This expectation has not been realized, but the new data have lead to the development of substantial new knowledge about the binding sites of the two proteins and the nature of their environments. (1) The difference spectra, and data on fluorescence reported earlier (Steinhardt *et al.*, 1971), agree in showing profound differences between bovine and human serum albumins with respect to the environment of the tryptophans and the way these environments change when alkyl ligands are bound.[5] (2) They also furnish consistent evidence that the binding sites for the one or two most strongly bound detergent ions ($\bar{\nu} = 1$ or 2; $K \geq 10^6$) perturb tyrosine rather than tryptophan in both proteins (*i.e.*, the red or blue shifts of tryptophan fluorescence are absent until more numerous sites are occupied). These two highest affinity sites were not observed by Polet and Steinhardt (1968) who worked with higher molal ratios bound but they were detected by Goodman in his measurements of isotherms (1958). Their existence is reflected in the onset of a blue shift in tryptophan fluorescence of both proteins only at $\bar{\nu} \geq 2$, but is hard to reconcile with the quenching of the bovine albumin fluorescence with a single equivalent of long-chain ligands. The tryptophan perturbing sites are only slightly lower affinity sites ($K = 10^6$ for C_{10}–C_{14} chains). Lower affinities are involved in additional tyrosine-perturbing sites discussed in point 4 below. (3) The difference spectra, but not the fluorescence data, point strongly to partially irreversible changes in the bovine albumin caused by the much used defatting procedure of Chen (1967). (4) The data presented suggest that the initial tyrosine red shifts are reversed to blue shifts when more than a few equivalents of *n*-alkyl ligands are bound by either protein, at lower $\bar{\nu}$ than is necessary for massive unfolding with, *e.g.*, dodecyl sulfate. We have interpreted the reversal (exposure of buried tyrosines to solvent) as an indication of microdisorganization, and suggest that the extent of this disorganization is reflected in the depth of the trough at 287–288 nm. This view is, of course, inconsistent with a simple two-state model of denaturation. The blue shifts merge progressively into the difference spectrum of massively denatured protein, described by Polet and Steinhardt (1968), dominated by deep troughs at 287 and 232 nm. When the 287-nm trough, which we have interpreted as a tyrosine blue shift, begins to appear at moderately low values of $\bar{\nu}$ (4 or 5 with some ligands), there is no sign of such a blue shift in the much larger low-wavelength transition band which affects the peak near 232 nm; its contribution, if any, therefore does not outweigh the large combined tyrosine and tryptophan red shifts *in this region* until $\bar{\nu}$ exceeds 60. There is no sign of the initiation of tyrosine blue shifts in the fluorescence data, although the massive unfolding effects into which

[5] The two tryptophans in bovine albumin are found in radically different amino acid sequences (Brown *et al.*, 1971; and personal communication). Herskovits and Sorensen (1968a,b) and Herskovits and Laskowski (1962) have drawn semiquantitative conclusions as to the partial exposure of tyrosines and tryptophans of bovine albumin to solvent. See also Donovan (1969).

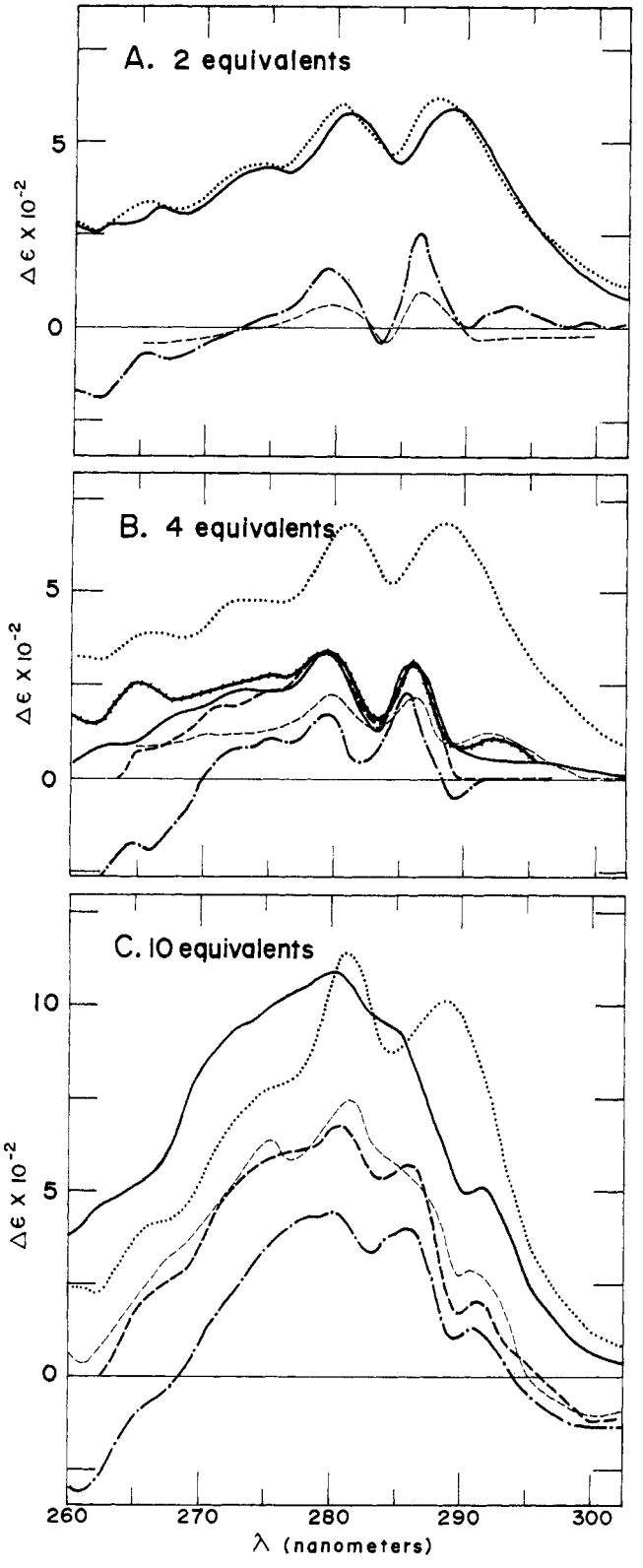

FIGURE 9: Difference spectra of the deionized human albumin produced by 2, 4, and 10 equiv of several *n*-alkyl ligands. (- - - -) Octyl sulfate, (-·-·-·-) decyl sulfate (2, 3, and 10 equiv), (—) dodecyl sulfate; and (.....) dodecanoate.

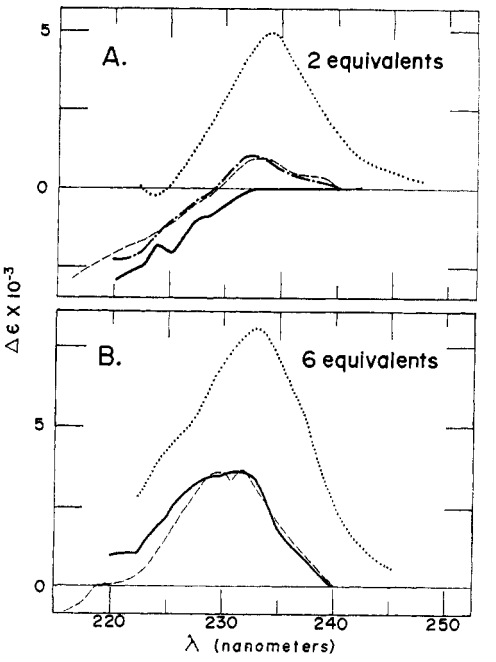

FIGURE 10: Far-ultraviolet difference spectra of human albumin produced by 2 and 6 equiv of *n*-alkyl ligands: (- - - -) octyl, (-·-·-) decyl, (——) dodecyl, and (.....) dodecanoate.

they merge at high values of $\bar{\nu}$ are clearly shown (Steinhardt *et al.*, 1971). (5) Evidence has been presented to support the assignment of the difference peak at 232 nm to tyrosine and the peak at about 228 nm to tryptophan. Therefore the small differences in rotatory dispersion at the 233-nm Cotton effect trough due to binding (Polet and Steinhardt, 1968) must be attributable to effects on tyrosine, and therefore possibly to the tyrosines responsible for the effects discussed in point 4 above. (6) The sign of the tryptophan effect (red shift) in the human albumin is, unlike the case of the bovine albumin, consistent in both the low-energy and high-energy transitions; the proteins therefore differ by the sign of $\Delta\epsilon$ at 293 nm. It is possible that the sign in the bovine albumin is due to the dominance of a blue shift in the second tryptophan of that protein, absent in the human albumin.[6] (7) The differential difference spectra (changes produced by the binding of successive ions of the various ligands) suggest that a variety of binding sites are involved which differ among themselves in their perturbing effects on the tyrosine and tryptophans, even with fully folded protein at low values of $\bar{\nu}$. Thus, for example, although one or two equivalents of all alkyl detergents and fatty acid anions perturb mainly tyrosine, the perturbations produced by the fatty acids are so much greater than the others that it is logical to expect to find them on different sites than those occupied by small numbers of bound alkyl sulfates or sulfonates. With larger numbers, it has been clear since the work of Reynolds *et al.* (1968) and of Zakrzewski and Goch (1968) that fatty acids produce entirely different perturbations than alkyl sulfates or sulfonates and thus may occupy different sites than those occupied by equal numbers of the latter. The fact that these classes of ligands have different free energies of binding at the same value of $\bar{\nu}$ (>2) at which the difference spectra diverge supports this view.

Since the optical perturbations caused by the various ligands at each level of binding are far from being equal, *i.e.*, measurable in modular units, it is not surprising that considerable effort to generate the experimental difference

[6] When human albumin is disorganized by acid, $\Delta\epsilon$ has the same sign in both bands; the values in each band change in strict proportionality to one another (Eisenberg and Edsall, 1963).

spectra by linear computer manipulations of the model difference spectra (Figure 1) (including changes in sign and small wavelength shifts) have all failed. Other factors, such as, for example, differences in charge proximity to the chromophores, are needed to explain how in the bovine albumin the effects of the two electronic transitions of tryptophan in the 280-nm region differ in sign from the effects of perturbations of the higher energy transition visible at about 230 nm.[7]

Since the fluorescence emission blue shift does not reach a maximum until $\bar{\nu}$ exceeds 15, effects on tryptophan absorbance might be expected to manifest themselves up to comparable values. No such persistence has been found. Possibly the tryptophan trough at 293 nm is engulfed by the deeper tyrosine blue shift trough centered at 287 which develops as $\bar{\nu}$ exceeds 10.

(8) The difference spectra of the human albumin diverge most strikingly from those of the bovine albumin in the complete absence of a trough at 287 nm with every ligand (except dodecyl sulfate at high $\bar{\nu}$). This trough has been attributed here to a tyrosine blue shift, a sign of the onset of disorganization of at least part of the tertiary structure of the bovine albumin. Although the resemblance of the human albumin difference spectra to tyrosine red shifts is gradually lost as $\bar{\nu}$ exceeds about 10 with every ligand (except fatty acids) a trough (negative $\Delta\epsilon$) appears only when 60 or more equivalents of dodecyl sulfate are bound (Figure 4; also Gallagher and Steinhardt, unpublished results at $\bar{\nu}$ up to 200). Our identification of this trough in the bovine albumin with disorganization is thus supported, but it appears that not all forms of disorganization produce it, *e.g.*, myristyl sulfate has some of the same disorganizing effects on the human albumin as does dodecyl sulfate (unmasking of new binding sites, and changes in ORD) (Steinhardt *et al.*, 1971), but the difference spectra differ widely, and myristyl sulfate affects the viscosity to a considerably lower extent than does dodecyl sulfate.[8] The development of the 286.5-nm trough in the human albumin by dodecyl sulfate at very high $\bar{\nu}$ is not accompanied by changes in other regions of the difference spectrum which are hardly altered at $\bar{\nu}$ beyond about 20. (9) The fluorescence changes on binding with the bovine and human albumins also differ strikingly in that the unfolding ligands *enhance* the human albumin fluorescence at $\bar{\nu}$ values above about 4, whereas the bovine albumin fluorescence is *quenched* on binding even 1 equiv of the same ligands. Thus the fluorescence of the human albumin appears to be affected at amounts bound which produce the tryptophan red shift shoulder in the difference spectrum. However, with the bovine albumin a tryptophan blue shift is detectable only at $\bar{\nu} \geq 2$, and we have seen that in this protein fluorescence quenching begins at or below $\bar{\nu} = 1$. It may be postulated that one of the two tryptophans in the bovine albumin is quenched but its absorbance band is not shifted at very low $\bar{\nu}$ values, and the other tryptophan suffers a shift when $\bar{\nu}$ reaches 2 or 3. If this is true then the easily quenched tryptophan is the one (in an aliphatic sequence) that is absent from the human albumin (Brown *et al.*, 1971).

[7] Such charge effects are shown for the amino acids themselves by Polet and Steinhardt, as well as by unpublished data of these authors on di- and tripeptides containing tryptophan and tyrosine.

[8] Fluorescence changes in human albumin, caused by binding the two unfolding ligands, differ more than with bovine albumin. The principal differences are: (a) the blue shift of the emission spectrum does not set in with myristyl sulfate until $\bar{\nu} = 6$; (b) the enhancement of fluorescence with this ligand is weaker and in fact passes through a quenching region at $\bar{\nu} = 4$–6 when excited at 300 nm; (c) the tyrosine emission is also lower (Steinhardt *et al.*, 1971).

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