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Differences between Bovine and Human Serum Albumins: Binding Isotherms, Optical Rotatory Dispersion, Viscosity, Hydrogen Ion Titration, and Fluorescence Effects*

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ABSTRACT: Human serum albumin (HSA) offers advantages over bovine serum albumin (BSA) in studying the effects of ligand binding to the protein on such optical properties as difference spectra, fluorescence, and optical rotatory dispersion (ORD) because, although the compositions of the two proteins are very similar, human serum albumin (HSA) contains only one tryptophan residue, rather than two, as BSA. The binding isotherms of HSA to a number of alkane derivatives were obtained as a necessary prerequisite to the optical experiments; they are reported here, as well as (a) the effects of binding on the ORD and viscosity of solutions of HSA, and (b) portions of its hydrogen ion titration curve. HSA differs only in detail from BSA in some of these properties. HSA appears to unfold in either one stage or in an essentially continuous process while BSA has been shown to exist in two distinguishable unfolded states at pH 4.8–5.6. At 6.86 both appear to unfold in a single or mixed process. Comparative hydrogen ion titration data demonstrate that the number of basic groups accessible to solvent after exposure to pH 4 is larger in HSA than in BSA. When the fluorescence behavior of the two proteins is compared more radical differences appear. The lone tryptophan of HSA has only about three-quarters the fluorescence emission as the average per chromophore of the two tryptophans of BSA. Tyrosine fluorescence has an abnormally low quantum yield in both proteins, but in HSA disorganization by binding "unfolding" ligands (dodecyl sulfate and myristyl sulfate) strongly raises the tyrosine emission. The two proteins differ strikingly in that the tryptophan fluorescence in HSA is enhanced and in BSA is quenched by combination with long-chain ($n \geq 10$) alkyl derivatives. Binding even small amounts shifts the tryptophan fluorescence spectrum to shorter wavelengths in both proteins; short-chain ligands cause the same shifts but produce little or no quenching or enhancement. BSA quenching requires binding of only 1 or 2 equiv but HSA enhancement requires between 4 and 6. With very large amounts bound, either protein is severely disorganized by unfolders and tryptophan fluorescence is strongly quenched. The relative quenching is the same in both proteins. This observation, and others involving polarization measurements, suggest that considerable structure remains in the unfolded proteins. The fluorescence effects of binding to BSA differ at pH 5.6 and 6.86, but in HSA are essentially the same. Energy transfer from tyrosine to tryptophan occurs to a greater extent in HSA and is greatest when complexed with unfolding ligands. It appears that distinct classes of binding sites exist in the native proteins; the distances between some of them is inferred, as well as the occurrence of rapid diffusion of bound ligand between occupied and unoccupied binding sites, and the existence of internal bonds which reduce fluorescence in uncomplexed HSA.

The complexing of bovine serum albumin (BSA)[1] with alkyl ligands, including long-chain detergents and fatty acids, is accompanied by changes in the absorbance of its tryptophan and tyrosine residues (Bigelow and Sonnenberg, 1962; Polet and Steinhardt, 1968; Gallagher *et al.*, 1970). Some of these changes are paradoxical in that the $S_0 \rightarrow S_1$ transition of the tryptophan band differs in sign (blue shift) from the $S_0 \rightarrow S_2$ transition of the same chromophore (red shift). More complex changes involving both tyrosine and tryptophan occur when the protein is unfolded at $\bar{v} > 10$. Since BSA contains two tryptophans, which may lodge in different environments, and human serum albumin contains only one, further systematic study of the spectral perturbations of these proteins, with the purpose of elucidating the nature of the binding sites, appeared to be more promising if carried out with HSA

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[1] Abbreviations used are: BSA, bovine serum albumin; HSA, human serum albumin; MRW, mean residue weight; $\bar{v}$, moles of ligand bound to protein per mole of protein.

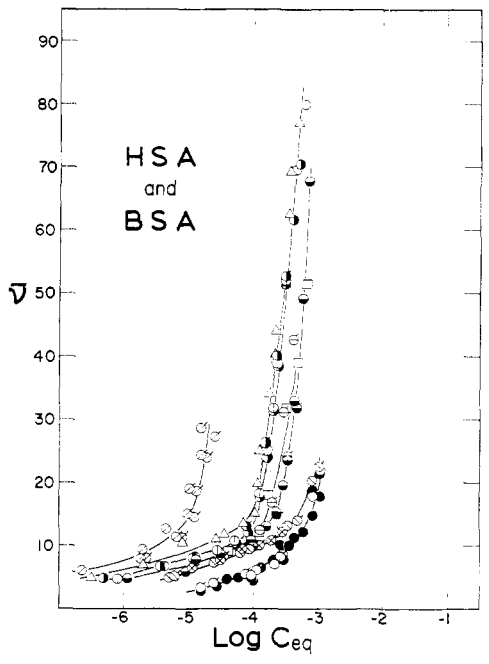

FIGURE 1: Effect of binding various detergent sulfates and dodecylsulfonate by 0.1% HSA at pH 5.6 (phosphate) or 6.86 (phosphate) where noted. Tagged symbols indicate lot 24 HSA, untagged lot 30 HSA. Filled symbols indicate 25°, unfilled 2°. (○) Octyl sulfate; (Ⓢ) decyl sulfate; (⊗) dodecylsulfonate; (Ⓓ) dodecyl sulfate; (⊖) dodecyl sulfate, pH 6.86; (⌀) myristyl sulfate; (△) BSA lot 8167, dodecyl sulfate, pH 5.6; (□) BSA lot 8167, dodecyl sulfate, pH 6.86. Molal ratio $\bar{\nu}$ is expressed as a function of log C_{eq}, the equilibrium concentration of ligand not bound to protein.

rather than with BSA. Except for the difference in tryptophans, and a higher content of valine in HSA,[2] the amino acid compositions of the two proteins are practically identical.

This paper first reports the binding isotherms of HSA at 2 and 25° at pH 5.6 with several alkyl ligands and a fatty acid, as well as the dependence of the ORD and viscosity on the extent and type of the ligand bound. Slight differences between these physicochemical functions of the two proteins, including differences in their titration curves, have been found. In the later pages much more striking differences in the fluorescent properties (including relative intensity) are reported, as well as opposing effects on these properties (quenching or enhancement of each chromophore) when they are complexed with alkyl ligands.

Experimental Section

Materials. Solutions of crystallized BSA (lot 8167, Nutritional Biochemicals) or of HSA (Pentex lots 24 and 30) were deionized on a Dintzis column before use. Stock solutions of deionized protein were stored at 2° and used within 2 weeks. Because solutions of dissolved crystallized HSA (lot 24) became cloudy at room temperature, most stock solutions of deionized HSA were stored in phosphate buffer (μ = 0.033) at either pH 5.6 or 6.86 which kept them clear. The concentrations of the solutions were determined spectrophotometrically as previously described (Reynolds *et al.*, 1967) using the following extinction coefficients at λ_{279}: $\epsilon^{1\,cm}_{1\%\,HSA}$ = 5.3 and $\epsilon^{1\,cm}_{1\%\,BSA}$ = 6.67. The native BSA contained 1.0 equiv of fatty acid/mole of protein and the HSA 0.27 equiv/mole when measured by a modification (Chen, 1967) of the method of Dole (1956).

The alkyl ligands used were a special grade prepared for us by Mann Research. Their purity has been described earlier (Reynolds *et al.*, 1967). The laurate isotherm was determined with the ^{14}C isotope (New England Nuclear).

All measurements were made in phosphate buffers (reagent grade) at 0.033 ionic strength, at either pH 5.60 or 6.86.

Methods. Binding isotherms were determined for HSA at 2 and 25° by the equilibrium dialysis method used for BSA (Reynolds *et al.*, 1967). The ^{14}C isotope of laurate was used at 23° (Reynolds *et al.*, 1968) and assayed with a scintillation center.

ORD measurements were made with a Jasco Model ORD/UV-5 spectrophotometer.

Viscosity measurements were made in 50-stoke Cannon-Fenske viscometers with flow times of 200–600 sec with photoelectric timing reproducible to ±0.01 sec. Measurements were made at 2 and 23°; the temperature was controlled to better than ±0.01°. All solutions were prepared with filtered (Millipore) phosphate buffer.

Titration curves were obtained with a Radiometer Titrator TTT 1c and Titrigraph SBR 2c using 8 ml of 0.5–1.3% protein solutions in 0.033 M KCl and adding 0.306 M HCl or NaOH in 0.033 M KCl; there was no significant change in ionic strength during the titration since practically all the acid added was combined and solutions were originally at the same ionic strength. No significant contribution to the ionic strength was made by the protein (Tanford, 1961, p 468).

Fluorescence measurements of 0.1% protein solutions at 25° were made with an Aminco-Bowman spectrophotofluorometer and a Hewlett-Packard X–Y recorder Model 7035B. The sample in a 0.3-cm² silica cell was excited with horizontally polarized light to suppress scatter at 90° due to the excitation beam (Chen, 1967a). A 1P28 photomultiplier tube and gratings blazed for 300 nm were used with 0.5-mm slits at the two faces of the cell compartment and a 4-mm slit at the photomultiplier tube.[3] With this phototube, gratings, and polarizer, the emission spectra were almost completely undistorted by instrumental light output, sensitivity, or absorption (Chen, 1967a). The wavelength scales were calibrated and corrected, except in the case of Figure 5 which uses consistent uncalibrated scales for expository purposes only.

Fluctuations in the exciting light intensity were minimized by closely regulating the power supply. When necessary, the photomultiplier gain or recorder amplification was altered to keep each set of spectra of the native protein approximately superimposable.

Equivalents of ligands present were translated to $\bar{\nu}$, the molal ratio bound, on the basis of the isotherms presented in Figure 1, combined with data from other isotherms for BSA determined earlier at 23° with a different lot number (Reynolds *et al.*, 1967). Considerable variation in results with different lot numbers can occur at $\bar{\nu} \geq 20$ (Anderson, 1966); however our own experiments show that this variability occurs significantly only with ligands which unfold. With myristyl sulfate binding is almost quantitative and the problem does

[2] The only other marked differences in composition are in the amide nitrogen and the valine content, of which BSA contains 35 residues (Schultze *et al.*, 1962) and HSA 45 (Phelps and Putnam, 1960, p 143). There is fragmentary evidence that portions of the amino acid sequences differ substantially (Swaney and Klotz, 1970).

[3] Both excitation and emission spectra obtained with a 1-mm slit proved to be indistinguishable from those obtained with a 4-mm slit.

not arise; with dodecyl sulfate (the other unfolder included in these experiments) a new isotherm for BSA of the lot number used in this work (included in Figure 1) proved to be measurably different at high $\bar{\nu}$ from those published earlier. Since we have also established that isotherms obtained with both proteins at both 2 and 25° do not differ within the accuracy of our measurements, we have supplemented the data of Figure 1 with earlier data on the binding of the non-unfolders to BSA as required.

Results

HSA Isotherms. Portions of binding isotherms of HSA for the ligands studied were measured as in our earlier work with BSA (Ray *et al.*, 1966; Reynolds *et al.*, 1967). The results of such measurements with four sulfate half-esters of varying chain length—from C_8 to C_{14}— and with one long-chain sulfonate are shown in Figure 1.[4] For reference purposes binding isotherms of BSA (lot 8167) with dodecyl sulfate at two pH values are included. A few experiments at 25° established that differences between data obtained at 2 and 25° did not exist or were within the experimental error.

Although the binding isotherms for dodecyl sulfate of BSA and HSA are very similar, with BSA a change in pH from 5.6 to 6.86 has a relatively large effect on the "unfolding" region of the isotherms, whereas with HSA the change in pH has only about half the effect.

With the exception of myristyl (C_{14}) sulfate, the ligands in Figure 1 appear to bind to HSA to about the same extent as to BSA. Myristyl sulfate is remarkable in appearing to unfold HSA at much lower equilibrium concentrations than with BSA. Klotz or Scatchard plots of the low $\bar{\nu}$ regions (native protein) indicate that the numbers of high-affinity sites in HSA for each ligand are close to those in BSA and most of the association constants are usually very nearly the same in HSA as in BSA; with the shorter chains however, the association constants are only one-tenth to one-fifth as great. Table I gives the best values for the numbers of high-affinity sites (n) for each nonunfolding ligand and the corresponding association constants (K) in the native protein.[5] For comparison, values for BSA (Reynolds *et al.*, 1967, 1970) are also included.[6] The values for the unfolder dodecyl sulfate were newly determined for the BSA (lot 8167) used in the present study. The table also contains values for the unfolding constant U_1 (in the case of unfolding ligands) and m and J (the numbers of sites in unfolded protein and their intrinsic association constant). The additional constants, U_2 and L, included refer to a postulated second unfolding reaction (Decker and Foster, 1967; Reynolds *et al.*, 1970). The values U_1 and U_2 are not critically determined. The value for J in the case of myristyl sulfate is also subject to wide uncertainty since only a small part of the "unfolded" portion of the isotherm was obtained,

TABLE I: Binding Constants for BSA[a] and HSA at 2 and 25°, pH 5.6, μ = 0.033.

Ligand	n		K ($\times 10^{-6}$)		m		J ($\times 10^{-4}$)		L ($\times 10^{-4}$)		U_1		U_2		$-\Delta F°$ (kcal)	
	BSA	HSA	BSA	HSA	BSA	HSA	BSA	HSA	BSA	HSA	BSA	HSA	BSA	HSA	BSA	HSA
Alkyl sulfate half-esters																
Octyl sulfate	4–5	6	0.6	0.050											7.3	6.3
Decyl sulfate	5–6	8	1.4	0.263											7.8	7.3
Dodecyl sulfate[b]	8–9	8	1.2	2.2	40	38		0.11		0.6		0.1		0.1	7.7	8.6
Myristyl sulfate[b]	10–11	9–10	0.9	8.6–6.7		40		10		6.0		0.1		1.0	7.5	8.3
Alkylsulfonates																
Dodecylsulfonate	6	8–10	0.30	0.3–0.1											6.9	7.4
Fatty acid anions																
Laurate 23°	6–7[d]	2		1.6											7.7	8.4
		5[e]	0.23[d]	0.24[e]												
BSA (8167)																
Dodecyl sulfate[b]	7–8		1.4		40		0.15		0.6		0.1		0.1		8.3	

[a] Except for the entry on the final line, BSA parameters are from Reynolds *et al.* (1967, 1970). [b] Unfolder. [c] Data of Goodman (1958). [d] Data of Reynolds *et al.* (1968) at 2°.

[4] $\bar{\nu}$ values for BSA in the figures are usually from earlier papers and are therefore subject to a 6% decrease because 69,000 rather than 65,000 was used as the molecular weight in their calculation. New $\bar{\nu}$ values for BSA determined in this work are also high by 6% since for consistency, they were similarly calculated; 69,000 was correctly used in calculating all values for HSA.

[5] Data for laurate, a fatty acid anion, are taken from Goodman (1958) for $\bar{\nu} \leq 6$, and from our own data (see Methods) at $\nu > 6$. There is fairly good agreement in the overlap region $\bar{\nu} = 6$ to 9 between the two sets of data.

[6] In the case of lot 8167 BSA, only seven to eight high-affinity sites for dodecyl sulfate were found in place of the eight to nine reported earlier for other lot numbers.

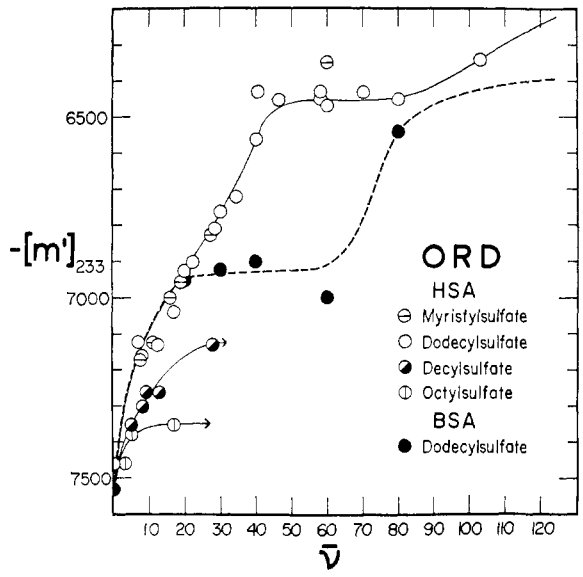

FIGURE 2: Mean residue rotation of 0.1% HSA and BSA complexed with various detergent sulfates as a function of molal ratio $\bar{\nu}$ at the Cotton effect trough (233 nm), pH 5.6, ionic strength 0.033.

because of solubility limitations. The full equation used for two-stage unfolding will be furnished by the authors on request.

Binding-Dependent Optical Rotatory Dispersion (ORD). HSA shows a Cotton effect trough at 233 nm similar to that of BSA. The two proteins have troughs of approximately the same depth, about $-8300°$, corresponding to a mean residue rotation ($[M']_{233}$) of about $-7500°$ (MRW = 114). However, Figure 2 shows that the reduction in the levorotation of HSA resulting from combination with a number of alkyl ligands differs radically from the variation in the ORD of BSA we have previously described (Reynolds *et al.*, 1967). With BSA there is an initial reduction of about 7%, due to binding itself, as $\bar{\nu}$ rises to about 10; almost identical effects are produced by complexes with C_8 to C_{14} sulfates. Among the sulfonates C_8 alone produces a much smaller effect (in terms of $[M']_{233}$ at a given $\bar{\nu}$), and hexyl derivatives produce no effect at all. No further changes occur as $\bar{\nu}$ rises beyond 10 except with the "unfolders" dodecyl and myristyl sulfates (and myristylsulfonate). With the latter $-[M']_{233}$ is greatly diminished at $\bar{\nu}$ values above about 50.

With HSA, the division of changes in $[M']_{233}$ into three

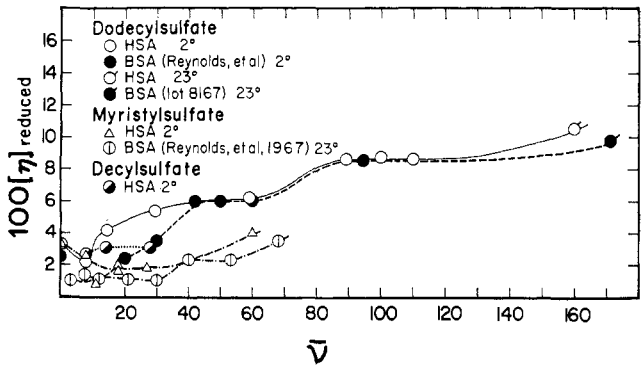

FIGURE 3: Reduced viscosities of 0.2% HSA complexed with various detergent sulfates as a function of molal ratio $\bar{\nu}$ at pH 5.6 (phosphate), ionic strength 0.033, 2 and 23°.

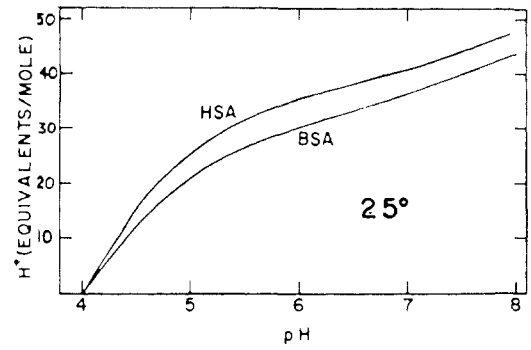

FIGURE 4: Titration curves from pH 4 of HSA and BSA in 0.033 M KCl with 0.306 N NaOH in 0.033 M KCl at 25°. Abscissa is negative.

stages is not observed. $-[M']_{233}$ is reduced from an initial value (for the uncomplexed protein) by nearly the same proportion (5–7%) as with BSA, as $\bar{\nu}$ rises from 0 to 10. With octyl and decyl sulfates, which do not unfold, there are no further changes. With the two unfolders included in Figure 2, where there was a plateau with BSA beginning at $\bar{\nu}$ *ca.* 10, none is found with HSA; $-[M']_{233}$ decreases monotonically with no hint of a pause at the level of $\bar{\nu} = 10$ and only levels off at $\bar{\nu} = 60$ (where for BSA, the second stage of change is beginning). The two unfolders give the same ORD results. The nonunfolders level off at different $-[M]_{233}$ values.

Viscosity. Figure 3 displays the dependence of the reduced viscosity of 0.2% HSA solutions at 2° and pH 5.6 on the extent to which the protein combines with decyl, dodecyl, and myristyl sulfates. The results with decyl sulfate are similar to those found with BSA in experiments conducted at 0.1% protein (Reynolds *et al.*, 1967). The data obtained with dodecyl sulfate differ in three respects from those obtained with BSA (shown as a broken curve):[7] (a) the viscosity goes through a minimum at a value of $\bar{\nu}$ below 10;[8] (b) it rises substantially at $\bar{\nu}$ above 10; and (c) a further rise between $\bar{\nu} = 60$ and 90 brings the viscosity up to a plateau (8.6) where in the Reynolds *et al.* data (1967) BSA showed a substantial further rise. The results obtained with myristyl sulfate are in qualitative agreement with those obtained with BSA: at low $\bar{\nu}$ numbers the viscosities are lower than with dodecyl sulfate.

Titration Curves. The titration curves shown in Figure 4 were obtained by commencing the titration with base after bringing identical weight per cent solutions of both proteins in 0.033 M NaCl to pH 4.0 with HCl. It is clear that to return the pH from 4.0 to 5.5–6.0, 5 equiv more base are required for HSA than for BSA. This difference between the titration curves, however, remains constant at higher pH values up to at least pH 7.6. When 30 equiv of dodecyl sulfate are present, the difference between HSA and BSA rises from 5 equiv to 10, *i.e.*, HSA gains a still greater buffering capacity than BSA under these conditions for incipient unfolding. The extra groups brought into play by the presence of detergent have a midrange (pK?) of 6.5.

Preliminary experiments show that when the initial acidification is much greater, *i.e.*, to pH 2.6, it is BSA rather than HSA that requires more base in returning to pH 8. Clearly the conformation changes accompanying acid unfolding (N →

[7] The results shown for BSA differ at high $\bar{\nu}$ from those published by Reynolds *et al.* (1967) in that lot 8167 fails to show a sharp rise in viscosity near $\bar{\nu} = 100$ and thus resembles HSA at all $\bar{\nu}$ above about 40.

[8] A broad minimum is found with BSA only at low pH (3.8) and much lower ionic strength (0.001) (Reynolds *et al.*, 1970).

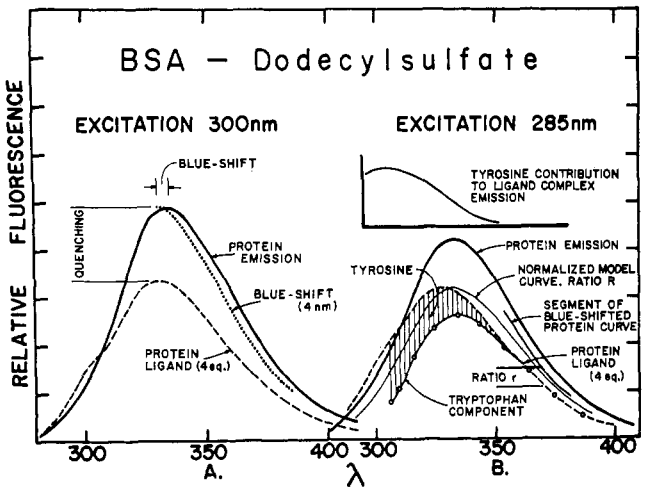

FIGURE 5: Steps in the determination of the contributions of tyrosine and tryptophan to the total emission of BSA and its alkyl complexes and of distinguishing between the effects of shifts in λ_{max} and quenching or enhancement. (A) Excitation at 295 to 305 nm; (B) excitation at 280 to 290 nm. The steps in the analysis are explained in the text.

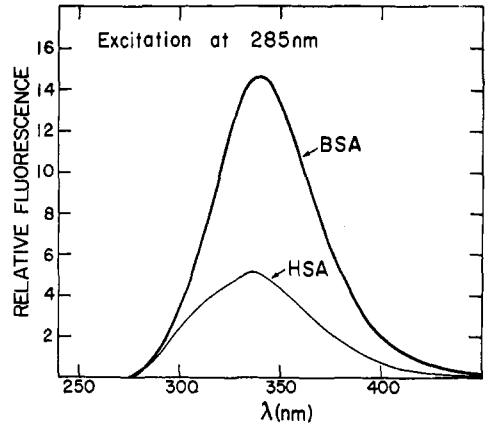

FIGURE 6: The emission spectra of BSA and HSA excited at 285 nm plotted on the same scale.

F transition) which may unmask prototropic groups are not rapidly reversible in at least one of the two proteins. Lovrien and Tanford (1959) obtained identical titration curves 1 sec and 5 min after mixing BSA with acid, but apparently they did not titrate in the reverse direction also. The results described above are not to be compared with normal titration curves of these proteins (Tanford, 1955; Foster and Clark, 1962; Vijai and Foster, 1967) since the titrations are preceded by an unmasking exposure to acid pH.

Analysis of the Fluorescence Spectra. When native uncombined HSA or BSA is excited by radiation with a wavelength near 280 nm the fluorescence contains emission by both tyrosine and tryptophan side chains (Chen, 1967b, p 443). With BSA the tyrosine contribution is much less conspicuous than with HSA. Tryptophan emission comes not only from side chains excited by the external radiation, but also from tryptophan side chains excited by radiationless energy transfer from excited tyrosine side chains (Chen, 1967b). However, when either HSA or BSA is excited by wavelengths between 295 and 305 nm, all of the tryptophan which emits is excited by the incident radiation; tyrosine is not excited, and thus neither emits nor transfers energy. Therefore, by exciting with such wavelengths only a pure tryptophan emission spectrum is obtained, with the position of the energy maximum (λ_{max}) dependent on the properties of the tryptophan environment. When alkyl ligands are bound, and tryptophan only is excited, the spectrum changes in two ways: (a) λ_{max} and the entire fluorescence spectrum are blue shifted without distortion; and (b) the intensity of the emission is either enhanced, or diminished (quenched). Since only tryptophan is excited, there is no difficulty in determining to what extent the changed emission represents a blue shift and/or quenching. The latter is readily determined from the ratio of the intensities at λ_{max} or at any other homologous part of the emission spectrum. The blue shift can be recognized by direct determination of λ_{max}. Since the maxima are broad, the blue shift is more accurately determined by multiplying the emission curve for the protein–ligand complex by whatever factor is required to make the maxima equal, and then averaging at several different levels of the long-wavelength portion of the two curves (protein alone and protein–ligand). This procedure is possible because all the spectra obtained with excitation above 295 nm are fully congruent, *i.e.*, superimposable by shifts along the wavelength axis (after normalization) on a model emission spectrum of a protein tryptophan analog, *N*-acetyltryptophanamide. With HSA, however, with its large tyrosine emission component the curves depart slightly from complete congruency, and the blue shift measured at λ_{max} may be smaller than the blue shift measured at the red end after normalization (the latter is used in this paper). The discrepancy is usually under 20%, and has been disregarded in this first investigation.

When the excitation wavelengths are <295 nm, both tyrosine and tryptophan are excited, and the emission of both may be affected by energy transfer from excited tyrosine to tryptophan. Analysis of each of the emission spectra into tyrosine and tryptophan components is made possible by making use of:

(1) The emission spectrum of a tryptophan peptide model compound (*N*-acetyltryptophanamide) in ethanol.[9]

(2) The emission spectra of HSA and BSA–ligand complexes when only the tryptophan is excited (λ_{exc} > 295 nm); this permits distinction between these changes in emission at particular wavelengths which are due to quenching (or enhancement) and those changes which are due to a shift toward the ultraviolet of the entire spectrum.

(3) The emission spectra of HSA– and BSA–ligand complexes when both the tryptophan and the tyrosine residues are excited (λ_{exc} < 295 nm). Since the tryptophan contribution has been determined (see 2) the contribution to the emission due to tyrosine can be obtained by subtraction.

Details of all the steps in the procedure (which differ from the "matrix" method of Weber [1961]) to which Figure 5B refers, are available on request from the authors.

Relative Fluorescence Intensities. Since BSA contains twice as much tryptophan as HSA a large difference in emission intensity is to be expected; however, the ratio of intensities actually found when the fluorescence of both proteins is plotted with the same amplification is larger than 2 (Figure 6). The ratio is close to 2.7 with either deionized or defatted protein, when the excitation wavelength affects only the tryptophan. Nevertheless the lifetimes of the excited states (6.1 and

[9] The emission spectrum of *N*-acetyltryptophanamide dissolved in ethanol is very similar to that of the tryptophan in BSA, or HSA (when the latter is shifted 3 nm to the blue). The emission spectrum of *N*-acetyltyrosinamide is almost independent of solvent.

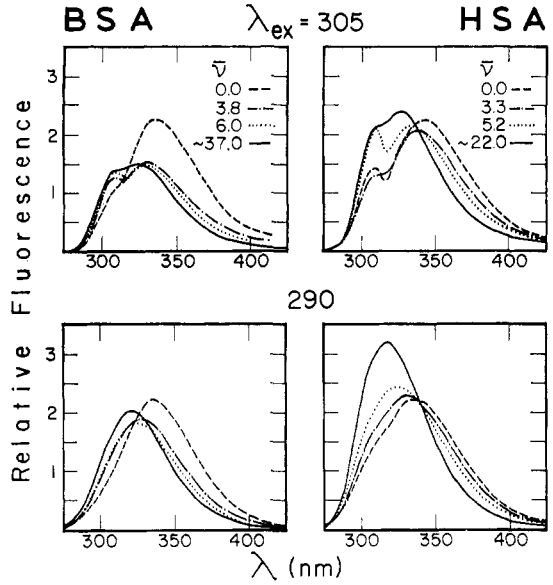

FIGURE 7: The fluorescence of BSA and HSA complexed with various amounts of octyl sulfate. Selected data obtained at two different excitation wavelengths. The wavelengths scales are calibrated and the spectra require practically no correction for differential sensitivity of the measuring system.

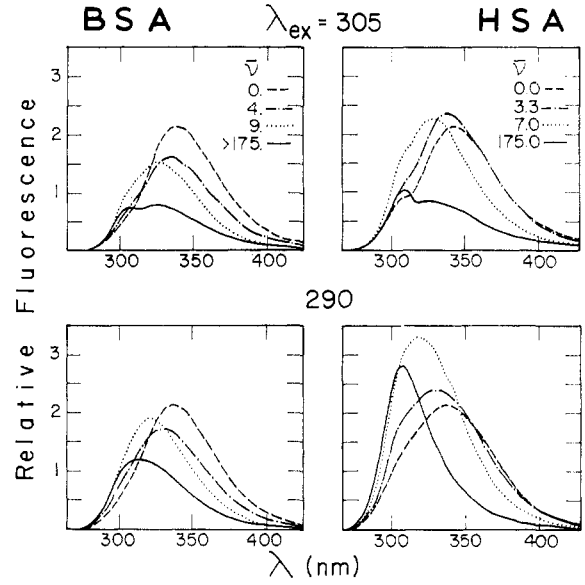

FIGURE 8: A selection of data obtained with dodecyl sulfate complexes of BSA and HSA, similar to those shown for octyl sulfate in Figure 7. Since binding large amounts of dodecyl sulfate causes the proteins to unfold, some of the curves included show the effects on fluorescence (severe tryptophan quenching) of such unfolding.

5.9 sec) are almost the same. However, as will be shown, when HSA binds 4 to <60 equiv of most alkyl ligands its fluorescence is greatly enhanced. The maximum enhanced fluorescence is almost exactly half of that of uncomplexed BSA, so that under these conditions, average fluorescence efficiency per tryptophan is about the same in the two proteins.[10] The tryptophan emission of both unfolded proteins is severely quenched, but the "enhanced" HSA is quenched more than BSA; thus, at very high $\bar{\nu}$, both proteins have about the same ratio of tryptophan fluorescence (*ca.* 2.7) as in the uncombined state. This unexpected result is considered further in the Discussion.

The fluorescence intensity given by 1.45×10^{-5} M BSA, on excitation at 300 nm, is very nearly the same as that given by 3×10^{-5} M *N*-acetyltryptophanamide, a suitable model for tryptophan in a protein (two tryptophans per molecule). It is therefore tempting to conclude that the tryptophans in BSA are "normal."[11] It follows that the single tryptophan of HSA is partially quenched. If the tyrosines in BSA were also "normal," one would expect the intensity of the tyrosine emission from a 0.1% albumin solution to be similar to that of about 2.4×10^{-4} M *N*-acetyltyrosinamide when excited at 280 nm. Considerably less than this is found (in both BSA and HSA roughly equal to 1×10^{-5} M). There are very nearly the same number of tyrosines in the two proteins, and the tyrosine emission (at 290–300 nm with excitation at 280) in both appears to be the same, although the latter in HSA is substantially higher than in BSA relative to that of tryptophan.

Fluorescence Spectra. Some of the features of individual fluorescence spectra are shown in Figures 7 and 8. The peak or shoulder near 305 nm found in the spectra obtained with excitation at this wavelength is due to incomplete suppression of scattering of the exciting beam. It is observed to increase when binding occurs, or when defatted protein is used. A variable amount of aggregation may be responsible (Andersson, 1966). The scattering is more prominent in experiments with HSA. Excitation at shorter wavelengths (at 280, 285, and 290 nm) rarely shows a detectable shoulder.

Differences between the two proteins when octyl sulfate is bound are shown in Figure 7. When excited at 305 nm (top row) only tryptophan emits and the molecular basis of the differences is especially clear. The BSA tryptophan emission is strongly quenched at $\bar{\nu} = 3.8$. Very little additional quenching, if any, occurs as the amount bound increases to 6.0, and scarcely more when it rises to 37 (nearly 300 equiv are required to attain $\bar{\nu} = 37$). A progressive blue shift is clearly shown. With HSA there is a blue shift but only a slight degree of quenching at $\bar{\nu} = 3.3$. At larger amounts bound the tryptophan emission is enhanced. When the exciting beam is at 290 nm, the analysis displayed in Figure 5 shows that in BSA the tryptophan component is blue shifted and quenched. With HSA the blue shift is accompanied by considerable enhancement at $\bar{\nu} = 22$.

With dodecyl sulfate, an unfolder (Figure 8), a very similar situation prevails (blue shifts accompany tryptophan enhancement in HSA, quenching in BSA), until massive unfolding occurs ($\bar{\nu} = 175$, in this figure). Tryptophan emission is then strongly quenched in both BSA and HSA (upper row) but this strong tryptophan quenching is accompanied by great tyrosine enhancement in the case of HSA. The curve obtained with $\bar{\nu} = 175$ in the lower right-hand panel is very similar to a tyrosine emission curve.

Comparisons with Individual Ligands. Measurements have been made with the two proteins at many values of $\bar{\nu}$, and with many ligands, and the results analyzed into tryptophan blue shifts and quenching (or enhancement), and tyrosine quenching or enhancement. Summaries are presented in Figures 9–12. Since $\bar{\nu}$ runs from 0 to over 175, and since some of the most significant changes occur at low values of $\bar{\nu}$, $\bar{\nu}$ is represented on a logarithmic scale. Since $\bar{\nu} = 0$ cannot be represented on the graphs, the values for $\bar{\nu} = 0$ have been indicated by horizontal

[10] This statement does not imply that the fluorescence intensities of the two tryptophans in BSA are the same.

[11] The quantum efficiency of tryptophan in BSA has been reported as 0.152 (Teale, 1960). Excitation was at 280 nm.

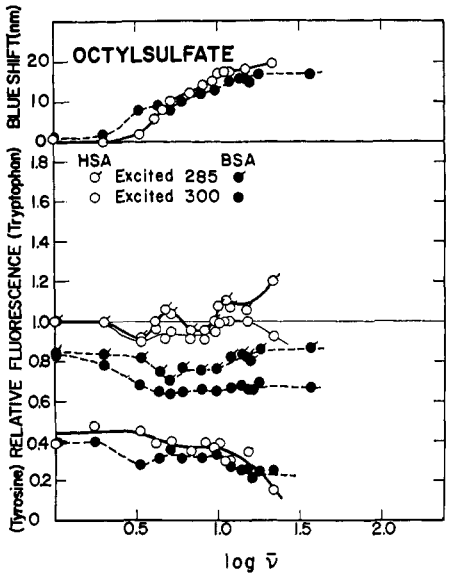

FIGURE 9: Summary of the effects on the fluorescence of BSA and HSA of binding various amounts of octyl sulfate. Closed circles represent BSA and open circles represent HSA. The blue shifts (top of figure) were obtained with λ_{exc} close to 300 nm. The quenching (BSA) or enhancement (HSA) of tryptophan is shown for two excitation wavelengths, *ca.* 300 and *ca.* 285 nm. Data obtained with the latter excitation wavelength, which excited tyrosine as well as tryptophan, are denoted by an attached diagonal. The data for tyrosine emission were all obtained with λ_{max} near 285 nm. Unlike the tryptophan data, they are not given on a scale relative to the uncombined protein (see text).

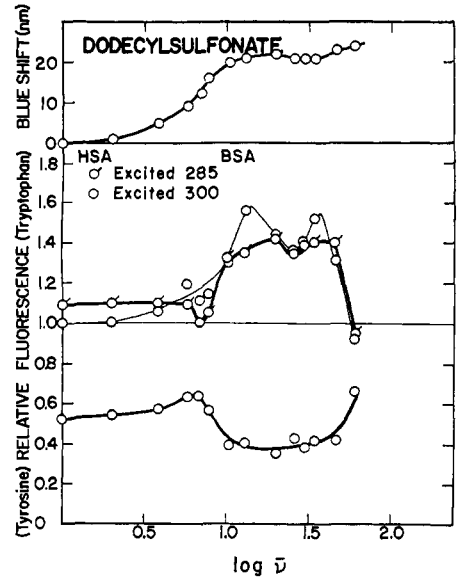

FIGURE 11: Summary of fluorescence changes in HSA due to the binding of dodecylsulfonate. See Figure 9 (figure legend) for explanation. The abrupt drop in tryptophan fluorescence and the sharp rise in tyrosine fluorescence are similar to those of the unfolding ligands, although dodecylsulfonate has been shown to be a non-unfolder of BSA.

ledger lines at the values characteristic of the uncombined protein (0 in the case of the blue shift, 1 in the case of relative tryptophan emission).

Although the results obtained with each ligand differ in detail, the more important distinctions can be displayed by contrasting the data obtained for both proteins with one ligand that does not unfold (octyl sulfate) and one that unfolds (dodecyl sulfate).

(a) Figure 9, octyl sulfate: There is a progressive blue shift of the tryptophan fluorescence in both proteins from $\bar{\nu} = 2$ to almost 20. It is greater in HSA than in BSA. The changes which occur between $\bar{\nu} = 10$ to 20 and $\bar{\nu} = 40$ to 60 are small compared to those which occur in the range $\bar{\nu} = 2$ to 8 and in the case of the unfolders at $\bar{\nu}$ over 60.

Even the smallest amounts bound to BSA result in strong quenching which does not increase further until $\bar{\nu}$ exceeds 40. When tyrosine is excited (λ_{exc} 285 nm) the apparent quenching

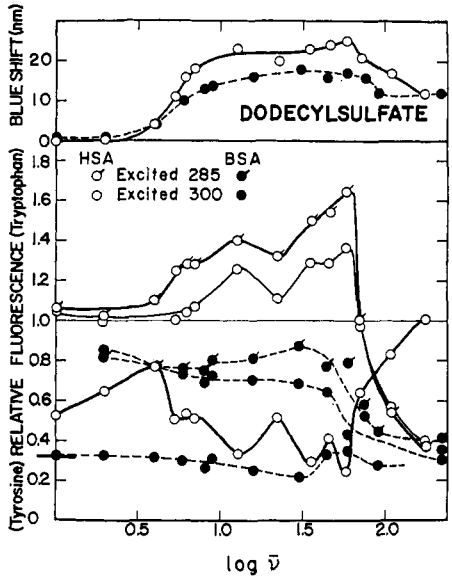

FIGURE 10: Summary of the effects on the fluorescence of BSA and HSA of binding various amounts of an unfolding ligand, dodecyl sulfate. The arrangement of the data and the conventions used are the same as in Figure 9.

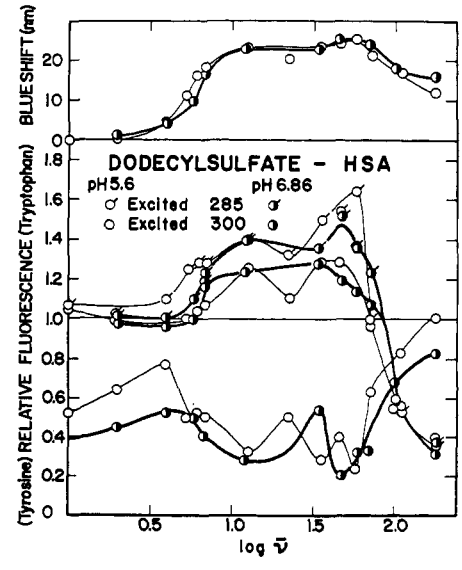

FIGURE 12: Comparison of the fluorescence changes in HSA brought about by binding an unfolding ligand, dodecyl sulfate, at two different pH values, 5.6 (solid circles) and 6.86 (divided circles).

is smaller, probably because radiationless transfer of energy from excited tyrosine to tryptophan occurs, counteracting some of the quenching.

With HSA, ligand binding enhances the tryptophan fluorescence. When fewer than 8 equiv are bound, apparent transfer of energy from tyrosine occurs when the latter is excited (λ_{exc} 285 nm), and the enhancement of the tryptophan is larger.

(b) Figure 10, dodecyl sulfate: Initiation of the tryptophan blue shift requires a higher $\bar{\nu}$ than in the case of octyl sulfate. HSA gives a smaller blue shift at binding ratios up to those at which massive unfolding is known to occur (with myristyl sulfate, another unfolder, this difference does not occur).

With excitation at 285 nm, very substantial transfer of excitation from tyrosine to tryptophan occurs with HSA, but is less evident with BSA at $\bar{\nu} < 40$. Review of all the data shows that transfer of energy from tyrosine to tryptophan occurs with unfolding to a much greater extent than with nonunfolders, in both proteins.

A sharp drop in the tryptophan fluorescence of HSA occurs as $\bar{\nu}$ approaches 100. Enhancement changes to quenching, until the tryptophan emission is only 0.4 of that in the uncomplexed protein. There is a parallel drop in the tryptophan fluorescence of BSA to only 0.3 of the value in uncomplexed protein. The viscosity and ORD data show that the protein retains structure; therefore the quenching found is not that characteristic of fully unfolded protein (random coil).

Preliminary experiments (J. Cassatt and J. Steinhardt, unpublished data) have shown that the great drop in fluorescence in HSA when it unfolds can be reversed by simple dilution with 1 equiv of buffer, and that the conformation change occurs with a half-period of about 0.1 sec. This is not far from the value 0.18 sec, found by Lenz and Steinhardt (1969) with BSA, using changes in optical density rather than fluorescence.

As the fluorescence drops sharply at the right-hand side of the figure, the tyrosine emission of HSA only rises.

Other features of the data obtained with the non-unfolder, octyl sulfate, are also found with the unfolder.

Decyl sulfate, a non-unfolder, gives results much like those obtained with octyl sulfate, but the effects on the fluorescence are larger. The effects with the fatty acid laurate ion are also small; no clear effects on the tryptophan emission are seen until at least seven or eight sites on the protein are filled, although the blue shifts start, as in the others, at lower amounts bound. The enhancement of HSA emission is also larger, but starts to fall at lower values of $\bar{\nu}$.

Data have been obtained with other non-unfolding ligands: hexyl sulfate with HSA, octylsulfonate with BSA; they have almost negligible effects on the tryptophan and tyrosine emissions although blue shifts occur. Data obtained with HSA with one other ligand, dodecylsulfonate, are shown in Figure 11 and suggest that this ligand may unfold HSA, although it does not do so to BSA (Reynolds *et al.*, 1967). The suggestion arises from the great fall in tryptophan fluorescence and the concomitant rise in tyrosine that occurs at high $\bar{\nu}$, which have appeared elsewhere only where other criteria indicate substantial unfolding.

Effect of pH on Fluorescence Changes Caused by Binding. No changes in the fluorescence of our model compound occur when the pH is changed from 5.6 to 6.86. With the serum albumins differences due to binding were sought with a single ligand, dodecyl sulfate. The results with HSA are shown in Figure 12, and are obviously not large. With BSA the data obtained at pH 6.86 are characterized by larger blue shifts (especially at low $\bar{\nu}$) and by much less tryptophan quenching than was found at pH 5.6. In fact, when excited at 285 nm, the tryptophan emission of BSA, like that of HSA, is actually enhanced at $\bar{\nu}$ values between 8 and 40.

To summarize, the differences in the fluorescence properties between the two proteins are as follows. (a) Tryptophan fluorescence is enhanced in HSA while it is quenched in BSA, usually even when only 1 or 2 equiv of ligand are bound. (b) BSA fluoresces more strongly per tryptophan than HSA. (c) Although blue shifts occur in both proteins when 2 or more equiv are bound, no substantial effect in the enhancement (or quenching) of tryptophan in HSA occurs until at least 4, more often 6, equiv are bound (1 or 2 equiv suffice to quench BSA). (d) Large quenching effects occur in both proteins when they are unfolded by long-chain detergents. With HSA these effects are accompanied by large increases in tyrosine emission. In both proteins there is a partial reversal of the blue shift when unfolding occurs. (e) Transfer of energy from tyrosine to tryptophan occurs to a greater extent in HSA than in BSA; tyrosine emission, relative to that of tryptophan, is also larger in HSA. (f) Short-chain ligands cause blue shifts, but have very little quenching or enhancement effects on tryptophan fluorescence. (g) Change in pH from 5.6 to 6.86 has little, if any, effect on HSA fluorescence, but produces a readily discernible effect on the fluorescence of BSA.

Depolarization of Fluorescence. Since "unfolding" implies an increase in the internal configurational degrees of freedom of the protein, it might be expected that the tryptophan fluorescence of the dodecyl sulfate—or myristyl sulfate—treated proteins might show less polarization than that of the uncomplexed proteins. However, no significant change in polarization is found with the unfolding brought about by binding nearly 200 equiv of either dodecyl or myristyl sulfates. When the proteins are treated with 6 M guanidine hydrochloride, with or without reduction of the disulfide bonds, the polarization does decrease. Thus, the tryptophan side chain in the native protein does not appear to be free to rotate in what must now be regarded as a partially "unfolded" state. Further work is in progress.

The Effect of Defatting. A number of experiments have been performed using BSA and HSA defatted by the charcoal method of Chen (1967c). Although defatting has a very prominent effect on the difference spectra generated by binding a number of the ligands reported in this paper (Gallagher *et al.*, unpublished data), the effects on fluorescence are small in all respects except the following:[12] (a) The fluorescence spectrum of defatted HSA only is shifted toward shorter wavelengths by three nanometers, *i.e.*, it may be in a less polar environment. (b) When light of 300-nm wavelength is used to excite defatted protein, it is scattered more severely than when ordinary deionized protein is used. This effect is confirmed in a light-scattering photometer. The effect is further enhanced by binding dodecyl sulfate and the other ligands used. Thus, defatted serum albumins may be partially aggregated and the aggregation may increase when detergents are bound. Since buffer salts (0.03 M phosphate) were always present the apparent aggregation is not the same as that reported by Foster, *i.e.*, it cannot be prevented by the presence of salt.

Discussion

The present work shows that the two proteins differ quantitatively in their binding behavior and in the way in which

[12] Barenboim *et al.* (1969) has reported that lipid binding raises the quantum efficiency of fluorescence; this effect, if valid, must require more than the single equivalent of fatty acid, on our proteins.

complexing affects their conformations as indicated by ORD, viscosity, and accessibility of prototropic groups. The fluorescence data also show that there are large differences in the immediate environment of tryptophan residues of the two proteins.

Although the amino acid compositions of BSA and HSA are closely similar (except for the extra tryptophan in BSA), the amino acid sequences in the neighborhood of tryptophan differ. Swaney and Klotz (1970) give the HSA sequence in the immediate vicinity of tryptophan as: $Lys^{+}AlaTrpAlaValAla-Arg^{+}$. The primary chain environment of the tryptophan is thus wholly hydrophobic. Since it is well known that the binding of small numbers of alkyl anions to BSA perturbs the absorption of tryptophan, Swaney and Klotz suggested that the highly hydrophobic character of the region and the two positive charges which flank it make it especially suitable for the binding of anions, especially those which contain both apolar and electronegative regions. However the same reasoning would apply to the neighborhood of the "reactive" tyrosine (Sanger, 1960) which is $Arg^{+}TyrThrLys^{+}$ in HSA and $Arg^{+}TyrThrArg^{+}$ in BSA.

Furthermore the regions in BSA which include both tryptophans lack these positive charges, at least on the C-terminal side (Sugae and Jirgenson, 1964): *Trp*SerValAlaGlyAlaSerGlx and *Try*GlyPheLeu. One tryptophan may have a negative charge on the C-terminal side if Glx is actually Glu. Positive charges may however be found on the N-terminal side of both tryptophans.

In the binding studies published earlier (Reynolds *et al.*, 1967) there was a spread in the number of high-affinity binding sites from as few as 4 to 5 for the shorter alkyl chains to as many as 11 for the [^{14}C]sulfate. A similar trend is to be found in data for the binding of fatty acid anions by serum albumins (Goodman, 1958; Reynolds *et al.*, 1968). From this observation a conclusion was tentatively drawn that high-affinity binding involved multiple contacts (at least 2) of randomly located sites with additive free energies (Steinhardt and Reynolds, 1969). There is less evidence of such a trend in the data for HSA in Table I since the total span in number of sites is from 6 to at most 10. The weakening of the trend is primarily due to the larger number of high-affinity sites for the shorter chains of octyl and decyl sulfates in native HSA.

If one had only the HSA ORD data, there would appear to be no basis for distinguishing, as we have previously done, between rotation changes at the trough due solely to binding from those due to conformational changes (unfolding). It would merely appear that HSA suffers a continuous diminution in secondary structure as binding progresses. Another interpretation is equally possible: numerous observations have been offered as indications that unfolding in BSA occurs in two stages (Decker and Foster, 1966, 1967; Reynolds *et al.*, 1970). The first of these occurs at $10 < \bar{\nu} < 40$ and involves only small changes in both the ORD and hydrodynamic properties; the second stage ("massive unfolding") involves pronounced ORD and hydrodynamic effects. It is possible that with HSA a separate first stage of unfolding does not intervene.[13] The viscosity comparisons with BSA offer support for this interpretation.

If no unmasking of hidden groups complicates the data, the pK of the extra acid groups in HSA indicated in Figure 4 appear to be $\simeq$4.5. If this were the only difference, the isoionic points of the two proteins (BSA 5.05, HSA 5.20 at 0.1%) would be reversed. Thus, a somewhat larger number (>5) of accessible basic groups may also be found in HSA. It cannot be assumed, without further investigation, that the titration curves obtained by the methods used here are directly and uniquely related to the groups accessible in the native protein at pH 5.6–6.8. It is more likely that our curves are influenced by an equilibrium, which changes the number of accessible groups as a function of pH. The situation presented by these differential titrations is complex and worthy of the further investigation now under way.

Three kinds of fluorescent effects require explanation: (a) blue shifts in λ_{max} (tryptophan) which increase gradually from $\bar{\nu} = 2$ or 3 to $\bar{\nu}$ as high as 20; essentially similar in the two proteins; (b) large differences in emission intensity (tyrosine and tryptophan) between BSA and HSA; and (c) binding-induced quenching in HSA, enhancement in HSA.

Other divergences, such as differences in tyrosine emission intensity or in efficiency of energy transfer, also require explanation.

Blue shifts would result if there are sets of binding sites which do not differ greatly in their association constants for any given ligand with one of the sites near enough to an exposed tryptophan for the hydrocarbon tail of the ligand to cover the tryptophan and remove it from contact with the solvent. This hypothesis would account for the blue shift, and for a progressively higher probability of having the emission of the tryptophans in all the molecules shifted, *i.e.*, a gradually increasing shift. However, the hypothesis requires that the population of molecules consists of a varying mixture of two kinds (one with the emission appropriate to a solvent contact, one with the blue-shifted emission appropriate to an apolar contact). The emission spectrum of such a mixture (*e.g.*, half the sites occupied) should be broader than that of a homogeneous population, and it might be expected to show a flattened peak, or even a double peak, depending on the size of the maximum blue shift. There is no sign of any of these features in any of the data, even when the slit width at the photomultiplier tube is reduced from 4 to 1 mm (peak width 9 nm). The blue shift occurs without broadening or flattening of the spectra.

We conclude therefore that, when there is only partial site occupancy, ligands can diffuse from site to site in times appreciably less than 3–6 nsec, the approximate lifetime of the various excited states. Only a few sites nearest the one which perturbs tryptophan need be nearer than about 10 Å away from it, if the activation energy required is that of a diffusion-controlled process (Weber, 1970).[14] λ_{max} will shift to progressively shorter wavelengths as the fraction of the lifetime during which the tryptophan-perturbing site is occupied increases with $\bar{\nu}$. Thus, the existence of the gradual blue shift may yield several pieces of information. (a) In the native protein there are a number of sites which have nearly the same affinity to ligand as the perturbing site (the same K_{int}). (b) At least a few of these sites are within about 10 Å of the perturbing site. (c) In spite of the high association constants, ligands diffuse from occupied to unoccupied sites in times substantially lower than 10^{-8} sec. (d) Tryptophan at the perturbing site is in a relatively polar environment, which is changed to an apolar one when an alkyl ion is bound.

[13] This hypothesis is not sufficient to account for the distinctly different plateaus given by the two non-unfolders.

[14] The partial enthalpies (ΔH) of both on and off reactions may be large, but the difference between them (ΔE) must be less than about 5 kcal for diffusion over distances of 10 Å to occur (Weber, 1970). Actually, the equilibrium ΔH is usually small also (Ray *et al.*, 1966; Reynolds *et al.*, 1967).

The difference in sign of the binding-induced changes in fluorescence intensity in the two proteins can be related to the great difference in efficiency of the tryptophan emission in the two species: HSA in its uncomplexed state is obviously severely quenched relative to BSA. Uncomplexed BSA is only slightly quenched relative to *N*-acetyltryptophanamide, our model for this amino acid in peptide linkage.[15] Since preliminary measurements indicate that in both proteins binding is accompanied by progressively diminishing lifetimes of excitation, we conclude that in HSA some of the protein exists as a species with a very low (or zero) quantum efficiency; on binding ligands, there is a progressive reversion of this fraction to the form with a normal quantum efficiency (related to its measured lifetime).

In BSA a single equivalent of ligand bound appears to be sufficient to quench the tryptophan. With HSA three or four seem to be required to initiate enhancement. Although other changes than the initial ones occur, the only other large effect (with unfolding ligands) is the severe quenching of tryptophan fluorescence that occurs when massive unfolding sets in at $\bar{\nu} > 100$. In HSA this is accompanied by the same kind of increase in tyrosine fluorescence which is found when ribonuclease is denatured.[16]

The very low quantum yield of tyrosine in the two proteins is not uncommon (Teale and Weber, 1959) and has been ascribed to H bonding to COO^- or >C=O (Teale, 1960; Cowgill, 1965). In HSA this "native" quenching of tyrosine diminishes sharply under conditions which cause some loss of the native structure (high equivalents of dodecyl or myristyl sulfates bound). High concentrations of urea have been shown to increase quantum yields in other proteins. This phenomenon is not found when BSA is unfolded by the detergents. The fact that on unfolding one finds equal degrees of tryptophan quenching, in both proteins, relative to that of the uncomplexed proteins, is also unexpected, *i.e.*, the large difference in quantum efficiency appears to persist after unfolding. Clearly, much of the original structure conditioning the tryptophan environment remains in detergent-unfolded protein. The experiments with viscosity and ORD have already pointed in less specific ways to the same conclusion.

BSA and HSA both contain the same amount of tyrosine, although the ratio of tyrosine to tryptophan is doubled in HSA. It is not surprising therefore that the analyses comprised in Figures 6–12 invariably show a higher tyrosine component in HSA than in BSA on a scale relative to the tryptophan or total protein fluorescence. A more significant difference between the two proteins may be in the amount of tyrosine excitation which is transferred to tryptophan. On binding an unfolder, the transferred increment tends to be larger in HSA than in BSA, although exceptions are found. In general, the transferred energy is not accompanied by decreased tyrosine emission.

We defer attempts to define the binding-site characteristics in the two proteins on the basis of the foregoing until detailed difference spectra due to binding have been published.

[15] We find little difference between the fluorescence spectra of *N*-acetyltryptophanamide and tryptophan. See footnote 9.

[16] However, the denaturation of thyroglobulin in 30% propylene glycol by urea or dodecyl sulfate *enhances* the fluorescence to equality with *N*-acetyltryptophanamide itself. This seems to be a special case of the effect of enhancement by lowering the dielectric constant. The tryptophan is shielded from the change in solvent until the denaturing agent is added (Steiner *et al.*, 1964, pp 355–366).

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A Proton Magnetic Resonance Study of Single-Stranded and Double-Helical Deoxyribooligonucleotides*

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ABSTRACT: We report the results of a 100-MHz proton magnetic resonance study of the following deoxyribooligonucleotides: d(T-G), d(A-C), d(T-T-G-T-T), d(A-A-C-A-A), and a mixture of the last two. We conclude that ribo- and deoxyribodinucleotide monophosphates have a different conformation in solution. We describe a simple model that is qualitatively successful in predicting the relative shielding of base protons in ribo- and deoxyribodinucleotide monophosphates of analogous sequence. The model assumes that the basic contrast between RNA and DNA geometry, arising from steric hindrance by the 2′-OH to base overlap in the former, is maintained in single-stranded oligomers. We also find that the nuclear magnetic resonance results on the single-stranded pentanucleotides are consistent with a right-handed helical conformation, with the bases in the anti conformation about the glycosidic link. Double-helix formation produces dramatic upfield shifts of the base protons. For example, all thymine methyl resonances are shifted roughly 0.6 ppm to higher field on converting from coil to helix. We conclude that this does not arise solely from the influence of base stacking as found in single-stranded oligonucleotides. We suggest that perturbation of the electronic structure of the carbonyl bond adjacent to the methyl group could explain the observed effect.

Nucleic acids form ordered structures in solution. The extent of ordering varies from highly regular double and triple helices to a moderate preference for selected conformations of oligonucleotides. Many interactions combine to give minimum free energy to a particular form. Among these may be cited base stacking, hydrogen bonding, solvent properties, and electrostatic interactions (for reviews of these topics, see Zimm and Kallenbach, 1962, and Felsenfeld and Miles, 1967), and the preferred torsional angles of the polynucleotide chain (Sundaralingam and Jensen, 1965; Haschemeyer and Rich, 1967; Lakshminarayanan and Sasisekharan, 1969; Sundaralingam, 1969; Arnott, 1970).

Oligonucleotides offer particular advantages for study of nucleic acid conformation. The virtue of studying oligomers rather than polymers is that the smaller molecules are more accessible to detailed structural studies, through optical, magnetic resonance, and crystalographic techniques, and they also provide more tractable models for theoretical calculations. The conformation of single-stranded oligonucleotides is influenced by all the interactions listed above, excepting only hydrogen bonding and possibly electrostatic effects. Furthermore, double-helix formation by oligomers should reflect the influence of virtually all the important interactions.

One topic of current interest is the difference in conformation between ribose and deoxyribose nucleic acids (Arnott, 1970; Fang *et al.*, 1971). Solution studies of single-stranded materials have so far been more extensive with the ribonucleic acid oligomers and polymers (Ts'o *et al.*, 1969b, with references therein; Ts'o *et al.*, 1969a; Chan and Nelson, 1969; Hruska and Danyluk, 1968; Jardetsky, 1960; Prestegard and Chan, 1969; Cantor *et al.*, 1970; Inners and Felsenfeld, 1970; Hruska *et al.*, 1970; Schweizer *et al.*, 1971). The measurements we report here refer exclusively to deoxyoligonucleotides. Two chain lengths are considered: dinucleotides and complementary (in the Watson–Crick base-pairing sense) pentanucleotides at sufficient concentration to form double helix in solution.

Nuclear magnetic resonance has been used widely to obtain information on the conformation of nucleotides in solution. In this paper we summarize the results of a 100-MHz proton magnetic resonance study of the following deoxyribooligonucleotides: d(T-G), d(A-C), d(T-T-G-T-T), d(A-A-C-A-A), and a mixture of the last two, which are complementary in sequence. The results with the two deoxyribose dinucleotide monophosphates are compared with those of a similar study (Ts'o *et al.*, 1969a) on their ribose analogs. Since these two sets of compounds differ primarily in the 2′-hydroxyl function, significant differences in the base stacking reflect the influence of that group on conformation.

In measurements on the pentanucleotides, we compared the magnetic resonance spectrum of the equimolar mixture of the complementary strands with the spectra of the two separate components. Even though these studies are incomplete, since all individual resonances could not be assigned, some well-defined effects of double-helix formation are apparent.

Experimental Section

Materials

The deoxyribose oligomers were synthesized in our laboratory by published procedures from Khorana's laboratory: Schaller *et al.* (1963), Ralph and Khorana (1961), Schaller and Khorana (1963), Khorana and Vizsolyi (1961), Kössel

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