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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 $\bar{\nu} > 10$. 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; $\bar{\nu}$, moles of ligand bound to protein per mole of protein.

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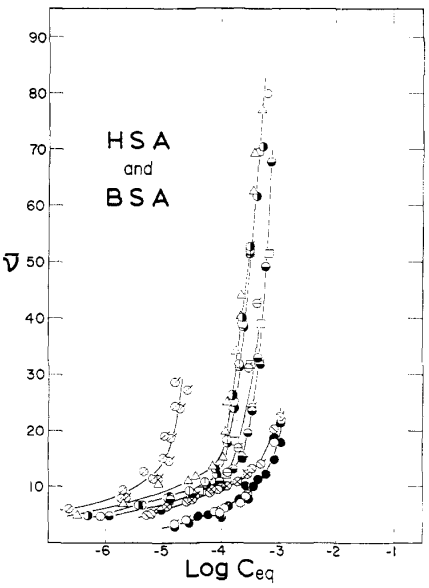

FIGURE 1: Effect of binding various detergent sulfates and dodecylsulfonate by 0.1% HSA at pH 5.6 (phosphate) or 6.86 (phosphate) where noted. Tagged symbols indicate lot 24 HSA, untagged lot 30 HSA. Filled symbols indicate 25°, unfilled 2°. (○) Octyl sulfate; (⊘) decyl sulfate; (⊗) dodecylsulfonate; (⊕) 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 ($\mu = 0.033$) 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.

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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, $\mu = 0.033$.

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[c]	0.23[d]	0.24[c]												
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.

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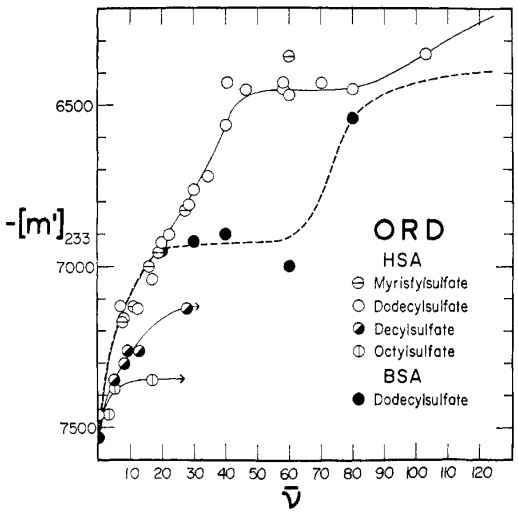

FIGURE 2: Mean residue rotation of 0.1% HSA and BSA complexed with various detergent sulfates as a function of molal ratio $\bar{\nu}$ at the Cotton effect trough (233 nm), pH 5.6, ionic strength 0.033.

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

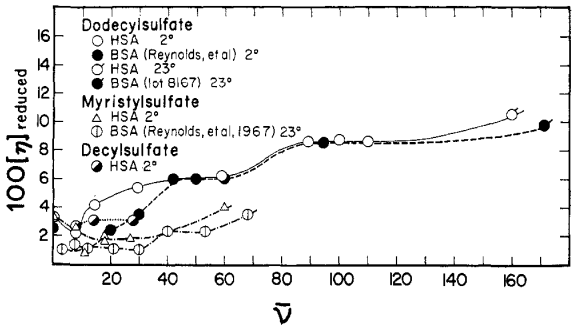

FIGURE 3: Reduced viscosities of 0.2% HSA complexed with various detergent sulfates as a function of molal ratio $\bar{\nu}$ at pH 5.6 (phosphate), ionic strength 0.033, 2 and 23°.

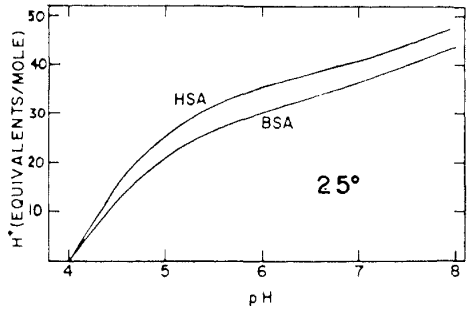

FIGURE 4: Titration curves from pH 4 of HSA and BSA in 0.033 M KCl with 0.306 N NaOH in 0.033 M KCl at 25°. Abscissa is negative.

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).

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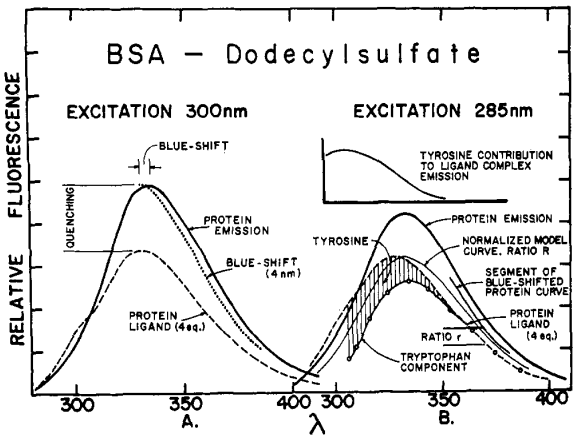

FIGURE 5: Steps in the determination of the contributions of tyrosine and tryptophan to the total emission of BSA and its alkyl complexes and of distinguishing between the effects of shifts in λ_{max} and quenching or enhancement. (A) Excitation at 295 to 305 nm; (B) excitation at 280 to 290 nm. The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

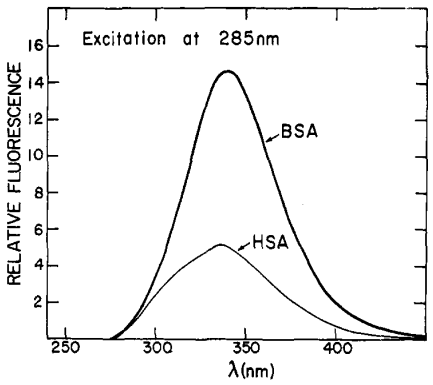

FIGURE 6: The emission spectra of BSA and HSA excited at 285 nm plotted on the same scale.

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 ($\lambda_{exc} > 295$ 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 ($\lambda_{exc} < 295$ 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.

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