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ARTICLE *in* CHEMISTRY & BIODIVERSITY · JULY 2004

Impact Factor: 1.52 · DOI: 10.1002/cbdv.200490069 · Source: PubMed

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Characterization of Serotonin in Protein and Membrane Mimetic Environments: A Spectroscopic Study

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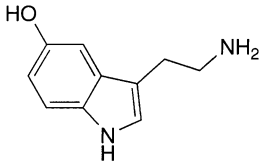
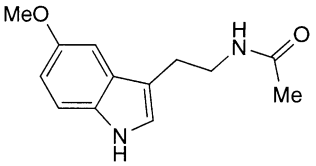
As a first step toward using the photophysical properties of serotonin to probe its interactions with biological target sites, we have examined its interactions with human serum albumin (HSA), chosen as a surrogate for the actual receptor proteins in physiological systems, and with sodium bis(2-ethylhexyl)sulfosuccinate (AOT)/heptane/water reverse micelles, chosen as a biomembrane mimetic environment for the transmembrane portion of the receptor protein. Although the emission maximum of serotonin is relatively insensitive to the polarity of the local environment, which is attributed to lack of solvent dipolar reorientation of the 5-hydroxyindole chromophore, the fluorescence anisotropy (r) served as a useful and sensitive parameter from which the binding constants (K) and *Gibbs* energy changes (ΔG) were estimated for serotonin–HSA and serotonin –AOT reverse micellar interactions. Fluorescence-decay studies of serotonin show double-exponential kinetics in homogeneous aqueous solvent due to the structural heterogeneity arising from different rotamers of serotonin. In contrast, upon binding to HSA, a single-exponential fluorescence-decay profile was observed indicating the occurrence of a single structural species of serotonin in the protein environment. Furthermore, far-UV-circular-dichroism (CD) spectroscopic data indicate that the secondary structural features of HSA remain essentially intact after binding to serotonin. This preliminary research can be expected to open the door to extensive future studies on interactions of serotonin with relevant target proteins and associated cell membranes involved in its diverse physiological functions.

Background and Introduction. – Serotonin (5-hydroxytryptamine) is a biogenic amine found in many mammalian tissues as well as in lower organisms. Apart from acting as an important neurotransmitter, it displays remarkable hormonal activities and is involved in the contraction of smooth muscle, stimulation of respiratory and circulatory chemoreceptors and nerve endings, tachycardia, blood-pressure elevation, pain sensation, *etc.* Disruptions in serotonergic systems can lead to mental disorders such as *Alzheimer*'s disease, schizophrenia, infantile autism, and depression [1].



Serotonin



Melatonin

Kishi et al. [2] examined the fluorescence-emission behavior of serotonin in H_2O and D_2O at various pH values. Then, detailed characterization of its photophysical properties and their modulation by ionization and polarity of the medium were reported by *Chattopadhyay et al.* [3]. *Ouyang* and *Vogel* [4] explored the interactions of

serotonin and melatonin (a structural analog of serotonin) with the calcium-binding protein calmodulin (CaM) *via* fluorescence, far- and near-UV CD, NMR spectroscopy, and other techniques. Such ventures [2–4] have prompted our present research aimed at detailed photophysical characterization of serotonin in environments, which are relevant to those of its binding sites (in physiological systems) such as proteins and membranes.

Serotonin exerts its diverse physiological actions by targeting specific receptor proteins in the biomembranes. As an initial step in our spectroscopic studies on serotonin–protein interactions, we chose human serum albumin (HSA) as a model for the receptor protein of serotonin in biological systems. The chromophoric moiety of serotonin (5-hydroxy-1*H*-indole) has a red-shifted absorption edge with respect to tryptophan (the common intrinsic fluorophore of proteins such as HSA), which permits selective photo-excitation of serotonin in presence of the single tryptophan in HSA [5]. In the present study, we have exploited the intrinsic absorption- and fluorescence-emission properties of serotonin to explore its binding to HSA. In this context, we include results of preliminary fluorescence-decay measurements of serotonin in this environment, in addition to steady-state-fluorescence data. We also examined the far-UV-CD spectra to ascertain possible influence of serotonin binding on the secondary structure of HSA.

It is also noteworthy that the existing literature [3][6] shows that serotonin binds to the transmembrane portion of receptor proteins in biomembranes. This raises the possibility that membranes play a role in the binding of serotonin to its receptors, which aroused our interest in the fluorescence-emission properties of serotonin in membrane environments as well. Biological membranes are highly complex because of the presence of integral proteins embedded in the bilayer lipid matrix. Fortunately, much of the physical and chemical understanding of membrane structure can be conveniently obtained through the investigation of appropriate models that mimic, at least in part, the physical and chemical properties of membrane architecture as well as physico-chemical properties relevant to functional aspects of membranes. In this connection, bio-membrane-mimetic organized molecular assemblies, such as micelles, reverse micelles and liposomes have been extensively studied during the past decade [7][8]. Such model systems are able to capture a number of important and essential features of biological membranes, although they lack much of the membrane's complexity. Reverse micellar assemblies, in particular, afford the opportunity to examine molecules in various states of hydration, which can be conveniently modulated by gradual addition of H_2O, thereby varying the water/surfactant molar ratio, w_o [7][8]. In previous reports, we focused attention on the influence of reverse micellar environments on the fluorescence-emission properties of 5-hydroxy-1*H*-indole [5][9], the chromophoric moiety of serotonin, as well as of 5-hydroxytryptophan [10], serotonin's physiological precursor. Here, we have examined the fluorescence spectroscopic behavior of serotonin in sodium bis(2-ethylhexyl)sulfosuccinate (AOT) reverse micellar assemblies.

Experimental. – Serotonin, HSA, *HEPES* buffer, and AOT were purchased from *Sigma*. Serotonin and HSA were used without further purification after ascertaining the purity of the samples by comparing absorption and emission spectra with literature data [3][11]; AOT was used as received. All solvents were of spectroscopic quality (*Merck*) and were used without further purification after confirming the absence of

absorbing or fluorescing impurities. Experiments in homogeneous solvents were performed with fluorophore concentration of 10 μM.

Binding of Serotonin to HSA. In all studies of serotonin–protein interactions, 50 mM *HEPES* buffer soln. prepared in triple-distilled water, pH 7.0, was used. Solns. of HSA at different concentrations were prepared in *HEPES* buffer and, in each case, serotonin was added to maintain [serotonin] at 10 μM. The binding parameter for serotonin with HSA was calculated from the fluorescence anisotropy measurements [9], which were carried out at 25°.

The binding constant K for the association reaction

$$P + A \rightleftharpoons PA \tag{1}$$

(where P is the protein (HSA) and A is the ligand molecule (serotonin)) is given by $K = \frac{[PA]}{[P]_f[A]_f}$ where f denotes free ligand. The number of moles of ligand bound ($[A]_b$) to one mole of protein is defined by v, where

$$v = \frac{[A]_b}{[P]_{total}} \tag{2}$$

The binding parameter has been calculated by *Scatchard*'s procedure [12]. This method is based on the general equation

$$\frac{v}{[A]_f} = -vK + nK \tag{3}$$

where n is the binding-site multiplicity per class of binding site.

Binding of Serotonin in Reverse Micellar Environments. Since serotonin is not fully solubilized in heptane, stock solns. were made in triple distilled H_2O. Small aliquots from the respective stock solns. were added to freshly prepared solns. of AOT (*ca.* 100 mM) in spectrograde heptane. Appropriate amounts of H_2O were subsequently added to make solns. of different w_o values where w_o indicates the $[H_2O]$/[surfactant] molar ratio, with $w_o = 0$ representing the condition existing prior to addition of H_2O to the AOT/heptane mixture. The final fluorophore concentration was kept at *ca.* 10 μM. Assuming that a given fluorophore species is totally solubilized in the micellar phase and is distributed among the micelles according to *Poisson* statistics, the average number of fluorophore molecules per micelle is given by $\langle n \rangle = [F]/[M_T]$ where $[F]$ is the macroscopic fluorophore concentration and $[M_T]$ is the total micellar concentration [8]. According to the available literature data for the aggregation number of AOT/heptane, the typical micellar concentration $[M_T]$ is estimated to be *ca.* 0.002–0.004M; the average number of fluorophore molecules per micelle ($\langle n \rangle$) would then be typically 0.01–0.02 [8]. This ensures that, on average, not more than one fluorophore molecule would be present per micelle [8]. Under such conditions, solubilization of fluorophore molecules should cause negligible perturbation of micelle structure and related properties.

Spectroscopic Measurements. Steady-state absorption and fluorescence spectra were recorded with a *Hitachi* model *U-2000* spectrophotometer and model *F-4010* spectrofluorometer, resp. In the case of reverse micellar solns., excitation was performed at 290 nm, while, when working with HSA, excitation of serotonin was carried out at 315 nm, where the endogeneous tryptophan of HSA exhibits little absorption [10]. Generally, a rectangular quartz cuvette of pathlength 10 mm was used for recording fluorescence spectra. In the case of emission measurements of the probe in reverse micellar solns., background fluorescence as well as light scattering from the AOT reverse micellar preparations were removed by subtraction of the spectra recorded with blank solns. The fluorescence-anisotropy (r) values were obtained from the expression $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} are the vertically and horizontally polarized components of probe emission with excitation by vertically polarized light at a given wavelength, and G is the sensitivity factor of the detection system [13]. Each intensity value used in this expression represents the computer-averaged values of ten successive measurements. Fluorescence lifetimes were obtained with an *Edinburgh Instruments* time-correlated single-photon-counting nanosecond fluorescence spectrometer [8]. Data analysis was carried out by a deconvolution method based on non-linear least-square fitting. The goodness-of-fit was estimated on the basis of χ^2 values. The average lifetime ($\langle \tau \rangle$) was evaluated from the equation: $\langle \tau \rangle = (A_1\tau_1 + A_2\tau_2)/(A_1 + A_2)$ where A_1, A_2 and τ_1, τ_2 represent the amplitudes and time constants, resp., of the individual components in biexponential-decay profiles. Fluorescence-decay measurements were performed with an exciting line of the N_2 lamp of the longest available wavelength ($\lambda^{ex} = 316$ nm) falling within the absorption region of serotonin, where the

tryptophan of HSA has no absorption. CD Spectra were recorded on a *Jasco Spectropolarimeter J-600* with a cylindrical cuvette with 1-mm pathlength. The CD profiles reported here were obtained with a spectral bandwidth of 2.0 nm and the signal averaged for four successive scans.

Results and Discussion. – *Serotonin in Protein (HSA). Fig. 1,a* shows the emission spectra of serotonin in HSA at different concentrations. As is evident from *Fig. 1,a* and *Table 1*, the emission maximum remains invariant to the concentration change. To ascertain the binding of serotonin with HSA, the fluorescence anisotropy (*Table 1*) of serotonin was measured with increasing HSA concentration, as presented in *Fig. 2*. The plot shows that the anisotropy of serotonin increases from 0.001 ([HSA] = 0 μM) to 0.019 ([HSA] = 30 μM). The increase in anisotropy of serotonin emission suggests binding of serotonin with HSA.

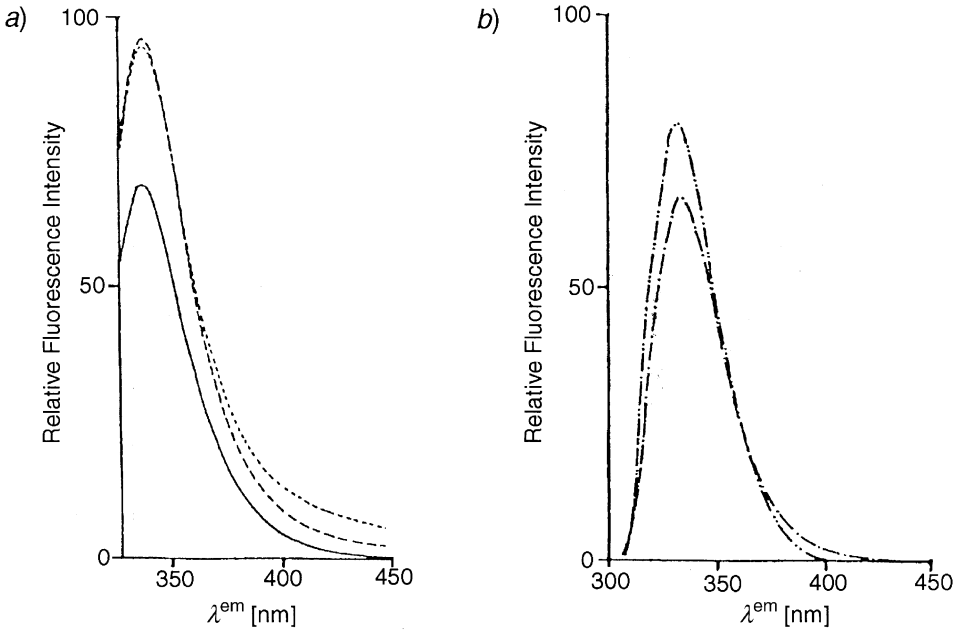


Fig. 1. a) *Fluorescence emission spectra of serotonin* (10 μM) *as a function of HSA concentration*. [HSA] = 0 μM (——), 6 μM (– –), and 27 μM (- - - -); $\lambda^{ex} = 315$ nm, and excitation and emission bandwidths were 5 nm. b) *Fluorescence emission spectra of serotonin* (10 μM) *in AOT reverse micelles as a function of* w_o. $w_o = 0.8$ (– · · –), $w_o = 45$ (– · –); $\lambda^{ex} = 290$ nm, and excitation and emission bandwidths were 5 nm.

The binding parameters $[A]_f$ and $[A]_b$ were calculated from *Eqn. 4:*

$$\frac{r_x - r_o}{r_\infty - r_o} = \frac{[A]_b}{[A]_o} \tag{4}$$

where r_x denotes the anisotropy of serotonin at any HSA concentration where serotonin is not completely bound, r_∞ denotes the anisotropy when serotonin is completely bound, r_o denotes the anisotropy of serotonin in the absence of HSA, and

Table 1. *Fluorescence-Emission Maximum* (λ^{em}_{max}) *and Anisotropy* (r) *Data of Serotonin in Various Milieu*

Milieu	λ^{em}_{max} [nm][a])	Anisotropy (r)[b]) at 25°
H_2O/*HEPES* Buffer	337	0.001
[HSA] (6 μM)	337	0.001
[HSA] (30 μM)	336	0.019
AOT Reverse micelles ($w_o = 0.8$)	333	0.11
AOT Reverse micelles ($w_o = 45$)	335	0.08

[a]) $\lambda^{ex} = 290$ nm in AOT reverse micelles; $\lambda^{ex} = 315$ nm in HSA. [b]) $\lambda^{ex} = 315$ nm, $\lambda^{em} = 335$ nm for both environments.

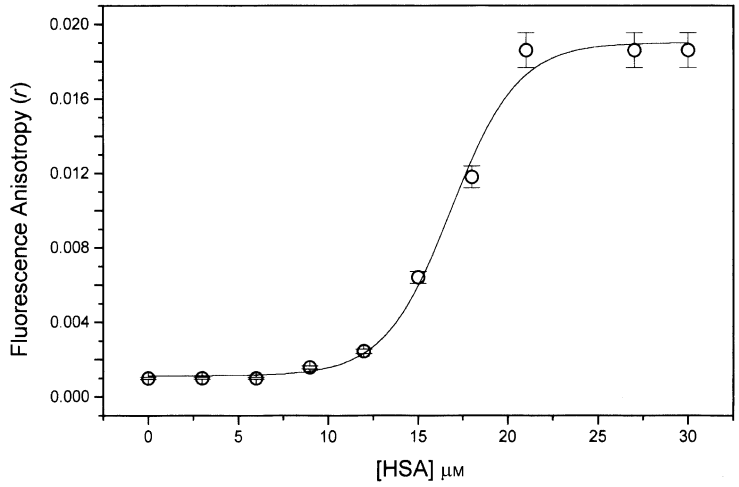


Fig. 2. *Fluorescence anisotropy* (r) *of serotonin* (10 μM) *as a function of HSA concentration*. Each data point indicates the average of three determinations; error bars indicate the standard deviations; $\lambda^{ex} = 315$ nm, $\lambda^{em} = 335$ nm.

$[A]_o$ is the initial serotonin concentration. The binding constant K of serotonin − HSA complexation is determined by plotting $v/[A]_f$ *vs.* v and the best-fit straight line of the experimental points was drawn, which is given in *Fig. 3*. The slope of the plot gives a binding constant of *ca.* 6.4×10^5 M^{-1}. The linearity found in the *Scatchard* plot suggests the existence of only one class of binding sites ($n = 1$) with a unique binding constant (*Fig. 3*). The standard *Gibbs* energy change, determined according to the relationship $\Delta G^\circ = -RT \ln K$, was found to be −33.20 kJ/mol.

Preliminary measurements of serotonin decay have been carried out in aqueous buffer (*HEPES*) and HSA environments. The times (τ) for serotonin decay are given in *Fig. 4* and *Table 2*. The instrument response, *i.e.*, the N_2-flash-lamp profile is the response of the instrument to a dilute scattering solution (*Ludox*) with a zero lifetime (shown by ++++ in *Fig. 4*). The decay-time values were obtained by deconvolution of the experimental profile (not shown), which is a convolution of the fluorescence-decay curve and the N_2-flash-lamp response. The fitted function curve (the solid line in *Fig. 4*) is the best computer fit for the points of the fluorescence-decay curve obtained

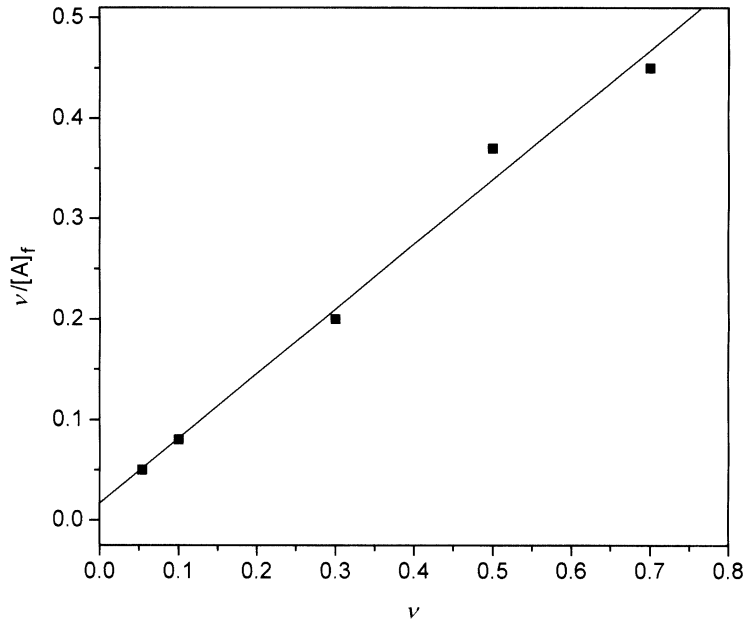


Fig. 3. Scatchard *plot for the binding of serotonin to HSA*. See *Eqn. 2* for the definition of ν, which was obtained by fluorescence anisotropy (*Fig. 2*) at 25°. The binding constant is derived from the slope of the plot.

Table 2. *Fluorescence-Decay Characteristics of Serotonin in Aqueous Solution* (*HEPES* buffer) *and in Protein* (HSA). [Serotonin] = 10 μM, λ^{ex} = 316 nm, λ^{em} = 340 nm.

Sample	τ_1 [ns]	A_1	τ_2 [ns]	A_2	χ^2	$\langle\tau\rangle$ [ns]
Serotonin/HSA	3.609	100	–	–	0.733	3.609
Serotonin/*HEPES*	4.03	96.25	0.605	3.75	0.600	3.9

after deconvolution [13]. It is noteworthy that serotonin exhibits double-exponential fluorescence decay in homogeneous aqueous buffer, which may be attributed to the structural heterogeneity owing to the existence of different rotamers of serotonin. Upon binding with HSA, serotonin shows single-exponential fluorescence decay (*Fig. 4*), indicating the presence of a single structural species of serotonin in this environment.

To ascertain the possible influence of serotonin binding on the secondary structure of HSA, we performed far-UV-CD studies in the presence of different concentrations of serotonin. The CD-spectral profiles, shown in *Fig. 5*, indicate that binding of serotonin induces no significant perturbation in the secondary structure of HSA.

Serotonin in AOT Reverse Micelles. The emission profiles of serotonin in AOT/heptane reverse micelles at different H_2O/surfactant molar ratios (w_o) are shown in *Fig. 1,b*. The λ^{em}_{max} values for serotonin change only slightly from 333 nm ($w_o = 0.8$) to 335 nm ($w_o = 45$, *Table 2*). From this insensitivity in the emission parameter, we can infer either that the microenvironment of 5 HT remains practically invariant with w_o, or that a change in microenvironment does take place but is not revealed by λ^{em}_{max} values due to the insensitivity of this parameter to the environmental polarity.

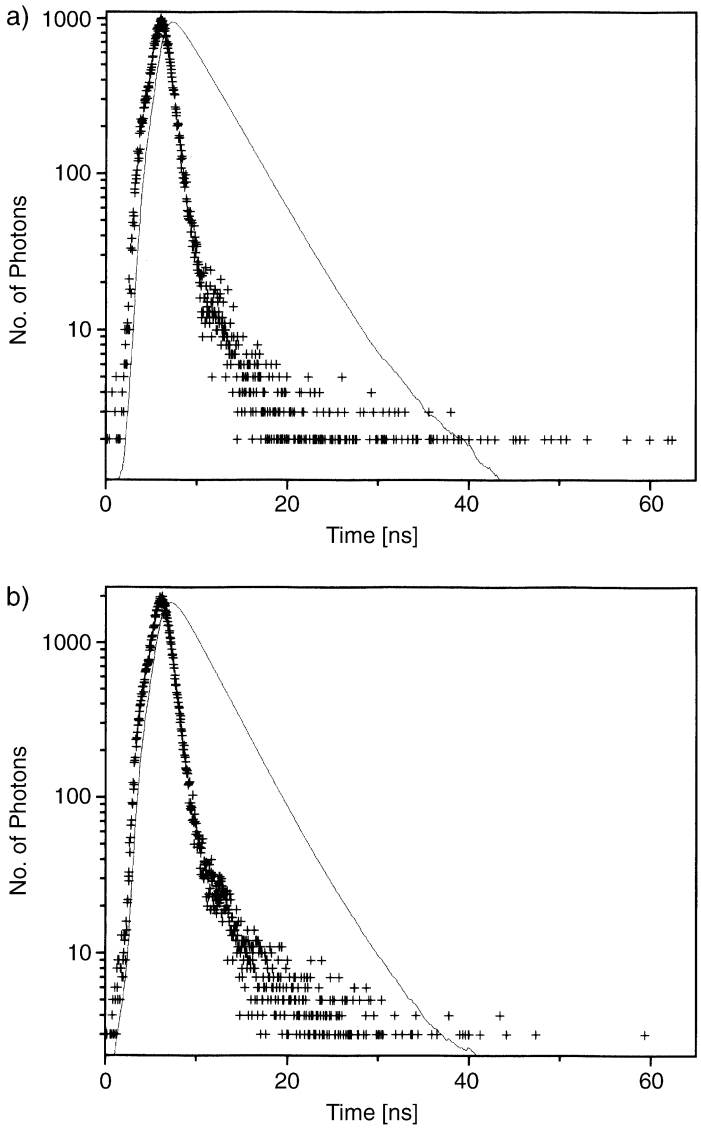


Fig. 4. *Fluorescence-decay profiles of serotonin in* a) *aqueous buffer solution and* b) *bound to HSA*. The solid curves represent the best fits of the experimental points to a biexponential-decay curve, $F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$. The lamp profiles are shown by unconnected points (+++++). The decay studies were performed with a N_2-flash-lamp excitation $\lambda^{ex} = 316$ nm and $\lambda^{em} = 340$ nm for both milieu.

To clarify this ambiguity, fluorescence-anisotropy measurements have been carried out. *Fig. 6* shows the anisotropy (r) plotted against w_o when $\lambda^{ex} = 315$ nm. Interestingly, at $w_o = 1$, the value is quite high ($r \approx 0.1$, *Table 1*) compared with a maximum theoretical value of 0.4. Such a high anisotropy value means that the serotonin

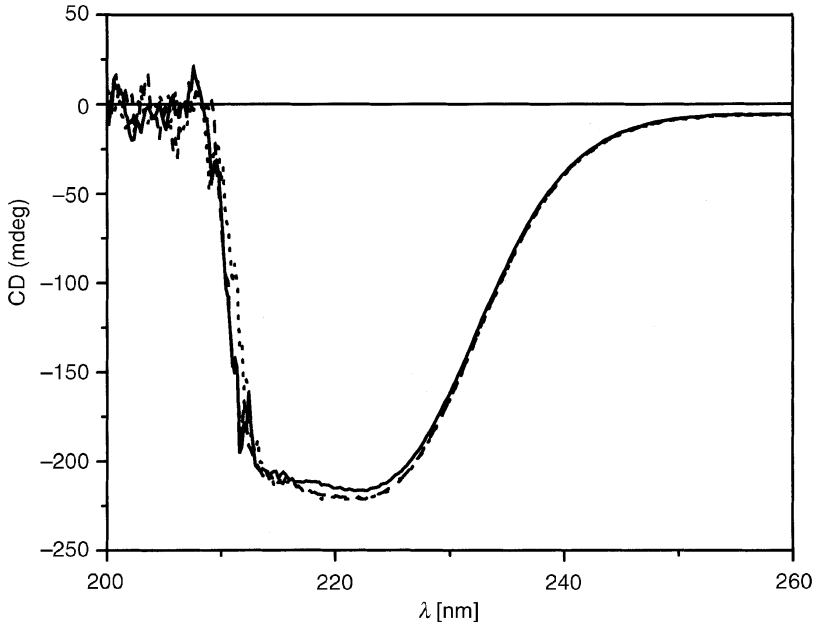


Fig. 5. *Far-UV-CD spectra of HSA at representative concentrations of serotonin.* [HSA] = 20 μM; [serotonin] = 0 μM (—), 10 μM (– –), and 20 μM (- - - -).

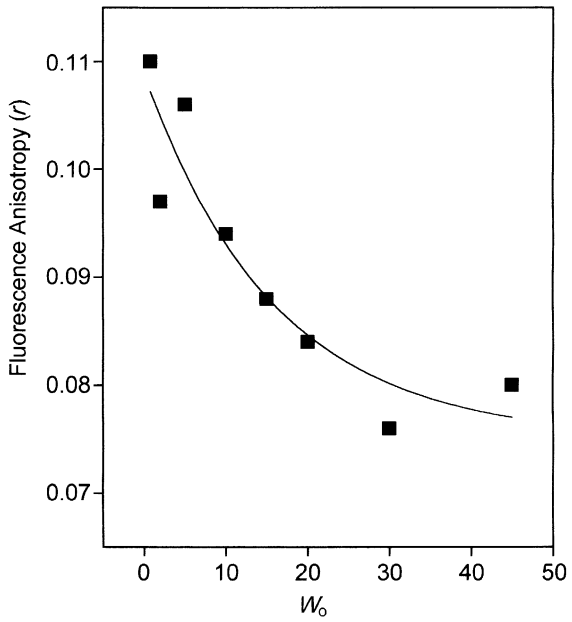


Fig. 6. *Fluorescence anisotropy* (*r*) *of serotonin* (10 μM) *in AOT reverse micelles as a function of* w_o. $\lambda^{ex} = 315$ nm, $\lambda^{em} = 335$ nm.

molecules are solubilized in motionally constrained environments of reverse micelles, *i.e.*, mainly in the interfacial region proximal to the polar head groups, where rotational diffusional motions are inhibited [14]. As w_o increases r rapidly decreases, which can be attributed to the increase in mobility of the surrounding H_2O molecules and/or the partitioning of more and more probe out of the interface into the bulk aqueous region. At and above $w_o = 15$, very little change in anisotropy (r) is observed, indicating that there is no appreciable further change in the average mobility of serotonin molecules. It is noteworthy that, even at the highest w_o examined ($w_o = 45$), r is still higher (≈ 0.07) than that obtained for serotonin in bulk water (≈ 0.001). The binding constant of serotonin in AOT reverse micelles has been calculated from fluorescence-anisotropy (as mentioned before in the case of HSA) values and is found to be *ca.* 7.4×10^5 M^{-1}. The corresponding *Gibbs* energy change is estimated as -35.17 kJ/mol, which indicates the spontaneity in the incorporation of serotonin in AOT reverse micelles.

The steady-state fluorescence-emission data show that the fluorescence-emission maximum (λ_{max}^{em}) of serotonin is rather insensitive to solvent polarity. We can explain this in terms of the lack of significant solvent-dipolar-relaxation effects around the photoexcited molecule [13]. The observed insensitivity of the λ_{max}^{em} parameter of serotonin is consistent with previously reported theoretical studies based on CNDO/S calculations on the energies of the lowest-lying electronic states designated by the *Platt* [15][16] levels ^1L_a and ^1L_b. The ^1L_a state interacts more strongly with solvents than ^1L_b, which is the emitting state in nonpolar solvents. Existence of a relatively small energy gap (*Fig. 7*) in tryptophan (also indole and its derivatives other than the 5-substituted

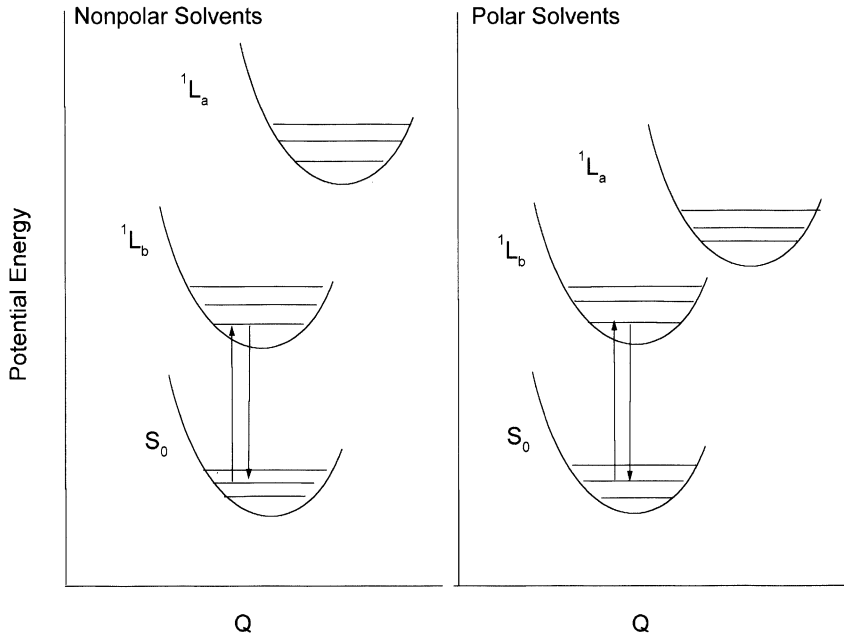


Fig. 7. *Schematic potential-energy diagrams that describe the effects of solvents on the $\pi\pi^*$ singlet electronic states of serotonin* (*Platt* labels, ^1L_a and ^1L_b). Q refers to an intramolecular coordinate in 5-hydroxyindole, probably the N–H stretch [17].

one) permits crossing and inversion of the two levels upon going over to polar solvents, resulting in emission from the solvent-sensitive ^1L_a state [9][16]. In serotonin, where the $^1L_a/^1L_b$ gap of the 5-hydroxy-1*H*-indole chromophore is relatively large, emission always occurs from the ^1L_b state, which remains below ^1L_a in all solvents (*Fig. 7*).

Concluding Remarks. – It is expected that the preliminary studies of serotonin in model environments we describe here will constitute the initial steps for extensive utilization of its photophysical properties in both *in vitro* and *in vivo* situations of direct physiological relevance. Particularly, extension of the present research to functionally active proteins and natural membranes in relation to their interactions with serotonin would be of considerable interest.

One of us (*A. B.*) would like to thank CSIR, India for providing a Junior Research Fellowship.

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Received February 5, 2004