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Photophysics of a Neurotransmitter: Ionization and Spectroscopic Properties of Serotonin

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ABSTRACT The neurotransmitter serotonin plays a modulatory role in the regulation of various cognitive and behavioral functions such as sleep, mood, pain, depression, anxiety, and learning by binding to a number of serotonin receptors present upon the cell surface. The spectroscopic properties of serotonin and their modulation with ionization state have been studied. Results show that serotonin fluorescence, as measured by its intensity, emission maximum, and lifetime, is pH dependent. These results are further supported by absorbance changes that show very similar pH dependence. Changes in fluorescence intensity and absorbance as a function of pH are consistent with a pK_a of 10.4 ± 0.2. The ligand-binding site for serotonin receptors is believed to be located in one of the transmembrane domains of the receptors. To develop a basis for monitoring the binding of serotonin to its receptors, its fluorescence in nonpolar media has been studied. No significant binding or partitioning of serotonin to membranes under physiological conditions was observed. Serotonin fluorescence in solvents of lower polarity is characterized by an enhancement in intensity and a blue shift in emission maximum, although the solvatochromism is much less pronounced than in tryptophan. In view of the multiple roles played by the serotonergic systems in the central and peripheral nervous systems, these results are relevant to future studies of serotonin and its binding to its receptors.

INTRODUCTION

Serotonin (5-hydroxytryptamine, or 5-HT) is a biogenic amine that acts as a neurotransmitter in the central and peripheral nervous systems (Jacobs and Azmitia, 1992). It is present in a variety of organisms, ranging from humans to species such as worms that have primitive nervous systems (Hen, 1992), and mediates a variety of physiological responses in distinct cell types. It is believed to play a role in the regulation of various cognitive and behavioral functions, including sleep, mood, pain, depression, anxiety, aggression, and learning (Wilkinson and Dourish, 1991; Cases et al., 1995; Yeh et al., 1996). Disruptions in serotonergic systems have been implicated as a critical factor in mental disorders such as schizophrenia, depression, infantile autism, and obsessive compulsive disorder (Lopez-Ibor, 1988; Tecott et al., 1995). Serotonin exerts its diverse actions by binding to distinct cell-surface receptors, which have been pharmacologically classified into many groups (Peroutka, 1993).

Serotonin is a derivative of the naturally occurring amino acid tryptophan (Fig. 1), which is intrinsically fluorescent. Although the fluorescence of tryptophan and its parent indole has been extensively studied (Beechem and Brand, 1985; Eftink, 1991; Eftink et al., 1995; Yu et al., 1995), very little is known about the fluorescence or absorbance characteristics of serotonin itself. In view of the multiple roles played by the serotonergic systems in the central and peripheral nervous systems, serotonin fluorescence could prove to be a convenient tool for physiological and biochemical studies involving the neurotransmitter and its receptors. With this goal in mind, we have characterized the spectroscopic (fluorescence and absorption) properties of serotonin and their modulation with the ionization state of serotonin. Our results show that serotonin fluorescence, as measured by its intensity, emission maximum, and lifetime, is pH dependent, with a characteristic ionization constant. These results are further supported by absorbance changes with pH.

Serotonin mediates its physiological actions by binding to its receptors, which are G-protein coupled integral membrane proteins that span the membrane several times (Peroutka, 1993). The binding site of serotonin is believed to be located in one of the transmembrane domains (Chanda et al., 1993; Wang et al., 1993). We thus investigated the fluorescence characteristics of serotonin in nonpolar environments to enable us to mimic the changes that could take place in serotonin fluorescence on binding.

MATERIALS AND METHODS

Materials

Serotonin hydrochloride, L-tryptophan, and *N*-acetyl-L-tryptophanamide were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a millipore Milli-Q system (Bedford, MA) and used throughout. The buffers used were 10 mM sodium acetate/150 mM NaCl (pH 3–5), 10 mM [2,(*N*-morpholino)ethanesulfonic acid]/150 mM NaCl (pH 5–7), 10 mM [3-*N*-morpholino)propanesulfonic acid]/150 mM NaCl (pH 7–9), 10 mM [3,(cyclohexylamino)propanesulfonic acid]/150 mM NaCl (pH 9–10),

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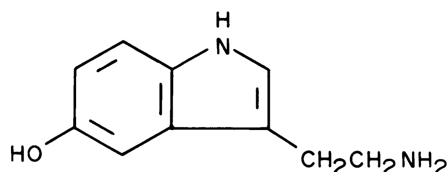

FIGURE 1 Chemical structure of serotonin.

50 mM [3,(cyclohexylamino)propanesulfonic acid]/150 mM NaCl (pH 11–12), and NaOH/150 mM NaCl (pH > 12). The concentrations of serotonin used were 10 and 100 μM for fluorescence and absorbance measurements, respectively.

Methods

We performed steady-state fluorescence measurements with a Hitachi F-4010 spectrofluorometer, using 1 cm path-length quartz cuvettes and slits with a nominal bandpass of 5 nm. Corrected spectra were recorded in all cases. All experiments were done at 25°C. Background intensities of samples in which fluorophores were omitted were negligible in most cases and were subtracted from the respective sample spectrum to cancel out any contribution that was due to scattering artifacts. Absorption measurements were carried out in 1-cm path-length cuvettes with a Hitachi U-2000 UV–visible absorption spectrophotometer after appropriate baseline corrections. All experiments were done with multiple sets of samples. To check for serotonin fluorescence reversibility on acidification, we added measured aliquots of acetic acid to the high pH samples to bring their pH down to 4.25 ± 0.25 and mixed the solutions well. The pH and fluorescence of these samples were immediately recorded.

For experiments involving membrane binding, 640 nmol of total lipid [either dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) alone or a mixture of 60% DOPC and 40% dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) (mol/mol)] in chloroform was dried under a stream of nitrogen while being warmed gently (~35°C). After further drying under a high vacuum for at least 3 h, 1.5 ml of 10 mM [3-(*N*-morpholino)propanesulfonic acid]/150 mM sodium chloride buffer, pH 7.2, was added and vortexed for 3 min to disperse the lipids. The lipid dispersions so obtained were sonicated for 10 min (in bursts of 2 min) with a Branson 250 sonifier. The samples were then centrifuged at 15,000 rpm for 20 min to remove titanium particles. To incorporate serotonin into the small unilamellar vesicles (SUVs) thus formed, we added a small aliquot containing 6.4 nmol of serotonin from a stock solution in water to the preformed vesicles and mixed the solution well. Samples were kept in the dark for 16 h before fluorescence was measured. Background samples were prepared in the same way, except that serotonin was not added to them.

Quantum yield measurements

The fluorescence quantum yields (Q_x) of serotonin were determined as previously described (Parker and Rees, 1960; Chen, 1965):

$$Q_X = Q_S(F_X/F_S)(A_S/A_X), \tag{1}$$

where the subscripts *s* and *x* refer to the reference standard and the sample, respectively, *F* is the wave-number-integrated area of the corrected emission spectrum at constant slit openings, and *A* is the absorbance at excitation wavelength (always less than 0.1 to avoid inner filter effect). We calculated the areas of the corrected emission spectra, using the built-in computer of the spectrofluorometer. Both tryptophan (Q_s = 0.13) (Eftink, 1991) and *N*-acetyl-L-tryptophanamide (Q_s = 0.14) (Szabo and Rayner, 1980) were used as reference standards, and we checked the internal consistency of the results by measuring the quantum yield of one with respect to the other. Solutions were freshly prepared and degassed by bubbling high purity nitrogen before use.

Time-resolved fluorescence measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays by a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single-photon counting mode. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (16 ± 1 in. of mercury vacuum) and is run at 22–25 kHz. Lamp profiles were measured at the excitation wavelength, with Ludox used as the scatterer. To optimize the signal-to-noise ratio, 5000 photon counts were collected in the peak channel. The excitation wavelength used was 296 nm, which corresponds to a peak in the spectral output of the nitrogen flash lamp. Emission wavelength was set at 337 nm. We performed all experiments by using slits with a nominal bandpass of 10 nm or less. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts that occurred during the period of data collection. The data stored in a multichannel analyzer were routinely transferred to an IBM PC for analysis. Intensity decay curves so obtained were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i), \tag{2}$$

where α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i. The decay parameters were recovered by a nonlinear least-squares iterative fitting procedure based on the Marquardt algorithm (Bevington, 1969). The program also includes statistical and plotting subroutine packages (O'Connor and Phillips, 1984). The goodness of the fit of a given set of observed data and the chosen function was evaluated by the reduced χ^2 ratio, the weighted residuals (Lampert et al., 1983), and the autocorrelation function of the weighted residuals (Grinvald and Steinberg, 1974). A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value (generally not more than 1.4). Mean (average) lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors by the following equation (Lakowicz, 1983):

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \tag{3}$$

Global analysis of lifetimes

The primary goal of the nonlinear least-squares (discrete) analysis of fluorescence intensity decays discussed above is to obtain an accurate and unbiased representation of a single fluorescence decay curve in terms of a set of parameters (i.e., α_i, τ_i). However, this method of analysis does not take advantage of the intrinsic relations that may exist among the individual decay curves obtained for the same system under different conditions. A condition in this context refers to temperature, pressure, solvent composition, ionic strength, pH, excitation–emission wavelength, or any other independent variable that can be experimentally manipulated. This advantage can be derived if multiple fluorescence decay curves, acquired under different conditions, are simultaneously analyzed. This is known as the global analysis, in which the simultaneous analyses of multiple decay curves are carried out in terms of internally consistent sets of fitting parameters (Knutson et al., 1983; Beechem, 1989, 1992; Beechem et al., 1991). Global analysis thus turns out to be very useful for the prediction of the manner in which the parameters recovered from a set of separate fluorescence decays vary as a function of an independent variable and helps to distinguish among models proposed to describe a system.

We have obtained fluorescence decays as a function of pH. The physical model under investigation is that there exist two distinct populations, namely, the ionized and unionized forms of serotonin, that give rise to the observed decay patterns, either as pure components or as mixtures. The global analysis, in this case, thus assumes that the lifetimes are linked

among the data files (i.e., the lifetimes for any given component are the same for all decays) but that the corresponding preexponentials are free to vary. We accomplish this by using a matrix mapping of the fitting parameters in which the preexponentials are unique for each decay curve whereas the lifetimes are mapped out to the same value for each decay. All data files are simultaneously analyzed by the least-squares data analysis method using the Marquardt algorithm (as described above) utilizing the map to substitute parameters appropriately while minimizing the global χ^2. The program used for the global analysis was obtained from Photon Technology International (London, Western Ontario, Canada).

RESULTS

One can effectively use the intrinsic fluorescence of serotonin to follow its behavior under various conditions. We have used serotonin's fluorescence here to monitor its ionization. Fig. 2 shows the effect of pH on serotonin fluorescence when samples are excited at 309 nm, which is the isosbestic point for serotonin (see below). The samples were excited at the isosbestic point to avoid complications in fluorescence values caused by differential absorbances of the two forms of serotonin (ionized and un-ionized). There is a plateau in the fluorescence intensity of serotonin up to pH 8. Above pH 8 the intensity decreases drastically until it reaches a negligible value near pH 12. This decrease in fluorescence is partly due to a reduction in quantum yield with increasing pH (Table 1), and the rest is due to a decrease in absorbance (see Fig. 4). We attribute this drop in serotonin's fluorescence to its ionization. In such a case the intensity change with pH should be reversible. We tested this theory by acidifying the high-pH samples with acetic acid and measuring fluorescence of these "pH-reversed" samples again. Fig. 2 shows that the change in serotonin fluorescence is indeed reversible. The apparent pK_a value (all pK_a values reported are apparent pK_a) derived from Fig. 2 is 10.2.

TABLE 1 Quantum yields of serotonin as a function of pH

pH	Quantum Yield
2.15	0.270
3.20	0.270
4.55	0.270
5.40	0.260
6.33	0.270
7.56	0.280
8.34	0.280
9.36	0.280
10.36	0.230
10.50	0.180
10.80	0.090
11.20	0.080
11.50	0.035
12.02	0.015
12.50	0.003
12.85	0.002

Excitation wavelength 277 nm.

Fig. 3 shows the change in serotonin fluorescence emission maximum with pH. The emission maximum remains steady at 337 nm until ~pH 8. When the pH is increased further, the emission maximum undergoes a red shift, with a sharp increase occurring after pH 11. This change in emission maximum is also reversible on acidification of the high-pH samples (data not shown), indicating that it is caused by ionization. It is interesting to note here that the maximum of emission for tryptamine is 356 nm at pH 5.0 and that it shifts further to 363 nm at pH 10.5 (Eftink et al., 1995). The additional hydroxyl group in serotonin is probably responsible for its blue-shifted emission maximum.

Inasmuch as the apparent ionization process detected by fluorescence changes may reflect the behavior of serotonin only in the excited state, the absorbance of serotonin was also monitored as a function of pH. Fig. 4 shows the effect of pH on the absorption spectrum of serotonin. As pH is

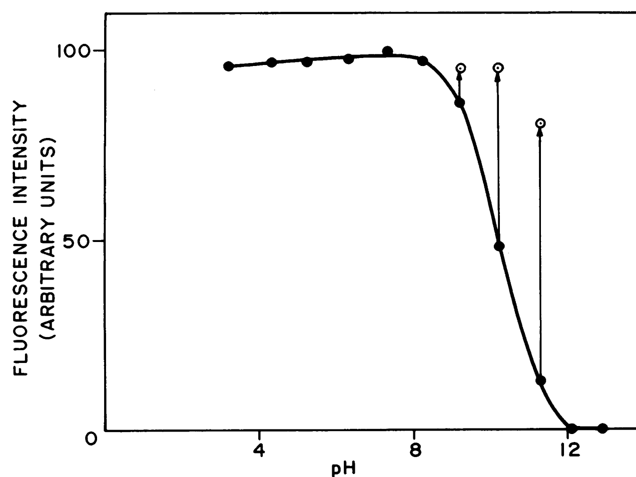

FIGURE 2 Effect of pH on serotonin fluorescence. The concentration of serotonin was 10 μM. The excitation wavelength was 309 nm (isosbestic point), and emission was collected at 336 nm. The vertical arrows represent acidification of the high-pH samples by acetic acid followed immediately by measurement of fluorescence. After pH reversal, all four samples had a pH of 4.25 ± 0.25. Fluorescence values shown are corrected for dilution on acidification. See Materials and Methods for details.

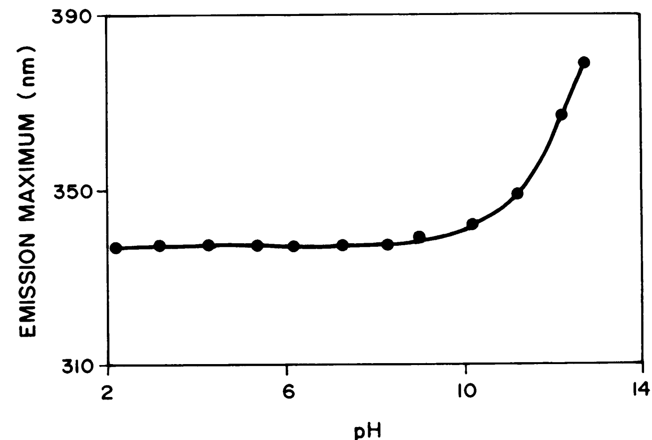

FIGURE 3 Dependence of serotonin fluorescence emission maximum on pH. The concentration of serotonin was 10 μM. The excitation wavelength was 277 nm. See Materials and Methods for details.

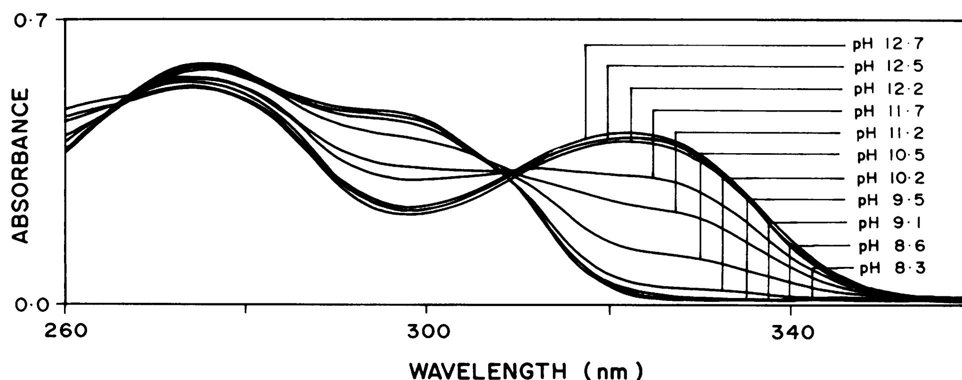

FIGURE 4 Effect of pH on the absorption spectra of serotonin. The concentration of serotonin was 100 μM. See Materials and Methods for details.

increased from 8.3 to 12.7 the absorbance near 297 nm decreases, with a small blue shift in the wavelength of absorption maximum. Concomitantly with this reduction in the absorbance of the 297-nm peak, the absorbance near 325 nm increases with increasing pH. The system also displays a clear isosbestic point at ~309 nm, which could be interpreted as the accumulation of ionized species of serotonin with increasing pH. The absorbance changes shown in Fig. 4 are found to be reversible on acidification of high-pH samples, further indicating that the changes are due to ionization. If the drop in absorbance below 300 nm and its simultaneous increase above 300 nm correspond to the same ionization process, the apparent pK_a values determined from changes in absorbance in these two wavelength ranges with pH should be identical within limits of experimental error. In Fig. 5 the absorbance changes at 297 and 325 nm are plotted as a function of pH. The pK_a values derived from these two curves are in the range of 10.5 ± 0.1. This self-consistency reinforces our conclusion. Further, the pK_a value obtained from absorbance changes is in excellent agreement with that obtained from fluorescence changes.

Fluorescence lifetime serves as a sensitive indicator for the ionization state of a fluorophore (De Lauder and Wahl, 1970; Jameson and Weber, 1981; Beddard, 1983). Table 2 shows serotonin lifetimes as a function of pH. The decays obtained at pH 2.27–10.56 can be fitted to monoexponential functions with lifetimes of 3.61–4.04 ns. The decays obtained at higher pH could no longer be fitted to a monoexponential function. A new component with a much shorter lifetime (0.50–0.90 ns) appears at pH > 10.56. We interpret this as the appearance of a new fluorescent species produced by the ionization of serotonin (also see below). This finding is supported by an increase in the relative contribution of this component with increasing pH. Results obtained by global analysis of the same data are consistent with this interpretation, with lifetimes of 3.80 ns for the protonated species and 0.57 ns for the deprotonated species (Table 2). The fittings of the set of decay profiles analyzed by the global method are presented in Fig. 6 as a pseudo-three-dimensional plot of intensity versus time versus increasing file number. The weighted residual corresponding to each of these fittings is shown in Fig. 7.

The mean fluorescence lifetimes of serotonin were calculated with Eq. 3 for both discrete and global analyses and are plotted in Fig. 8 as a function of pH. As shown in the figure, the mean lifetime remains more or less steady near

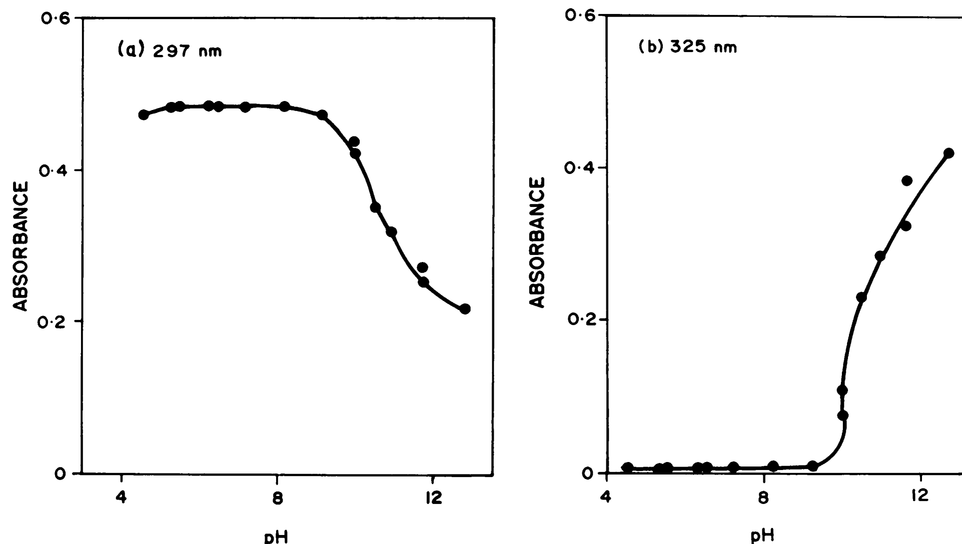

FIGURE 5 Effect of pH on the absorbance of serotonin at 297 nm (*a*) and 325 nm (*b*). All other conditions are as for Fig. 4.

TABLE 2 Lifetimes of serotonin as a function of pH

pH	α_1	τ_1 (ns)	α_2	τ_2 (ns)
2.27	1.00 (1.00)	3.82 (3.80)	–	–
3.46	1.00 (1.00)	3.79 (3.80)	–	–
4.50	1.00 (1.00)	3.77 (3.80)	–	–
5.26	1.00 (1.00)	3.85 (3.80)	–	–
6.52	1.00 (1.00)	3.95 (3.80)	–	–
7.32	1.00 (1.00)	3.94 (3.80)	–	–
8.88	1.00 (1.00)	3.93 (3.80)	–	–
9.31	1.00 (1.00)	3.88 (3.80)	–	–
9.82	1.00 (1.00)	4.00 (3.80)	–	–
10.28	1.00 (1.00)	3.89 (3.80)	–	–
10.56	1.00 (1.00)	4.04 (3.80)	–	–
10.82	0.74 (0.37)	3.45 (3.80)	0.26 (0.63)	0.57 (0.57)
11.04	0.55 (0.50)	3.71 (3.80)	0.45 (0.50)	0.54 (0.57)
11.42	0.39 (0.26)	3.52 (3.80)	0.61 (0.74)	0.68 (0.57)
11.63	0.29 (0.16)	3.33 (3.80)	0.71 (0.84)	0.68 (0.57)
11.80	0.16 (0.10)	3.59 (3.80)	0.84 (0.90)	0.90 (0.57)
11.85	0.16 (0.09)	3.35 (3.80)	0.84 (0.91)	0.74 (0.57)
12.00	0.08 (0.04)	3.15 (3.80)	0.92 (0.96)	0.68 (0.57)
12.15	0.05 (0.04)	3.59 (3.80)	0.95 (0.96)	0.55 (0.57)
12.84	0.03 (0.04)	4.48 (3.80)	0.97 (0.96)	0.50 (0.57)

Excitation wavelength 296 nm, emission wavelength 337 nm. Numbers in parentheses indicate values for global analysis.

3.80 ns until pH 10.56, after which there is a sharp and continuous decrease (~65%) in the mean lifetime as the result of ionization. Fig. 8 also shows that the mean lifetimes calculated from the components obtained by discrete analysis are in excellent agreement with those calculated from the global analysis. This finding supports the model used for the global analysis (discussed in Materials and Methods) and confirms the validity of the global approach in this case. We note here that the pK_a value for serotonin, as judged by examination of Fig. 8, is somewhat higher than what we obtained from analysis of fluorescence intensity or absorbance with pH. The reason for this apparent discrepancy is not clear to us. However, the greater uncertainties involved in measurements of fluorescence lifetime as opposed to intensity measurements could contribute to this discrepancy.

For serotonin to exert its diverse physiological actions, it has to bind to specific receptors in the membrane. The ligand-binding site for many membrane-bound receptors lies in the extramembranous domain of the protein. This is true for members of the superfamily of chemically gated ion channel receptors such as the nicotinic acetylcholine receptor, the γ-aminobutyric acid (GABA) $GABA_A$ receptor, and the glycine receptor (Karlin et al., 1986; Changeux and Revah, 1987; Changeux et al., 1987; Ochoa et al., 1989; Ortells and Lunt, 1995; Smith and Olsen, 1995). However, for the G-protein coupled heptahelical receptor family (all serotonin receptors belong to this family, except the 5-HT_3 receptor, which belongs to the former class), this is not quite true. The conserved residues in these receptors are primarily within the hydrophobic regions and not in the hydrophilic loops containing the transmembrane segments. Several polar residues within the transmembrane segments are among those conserved.

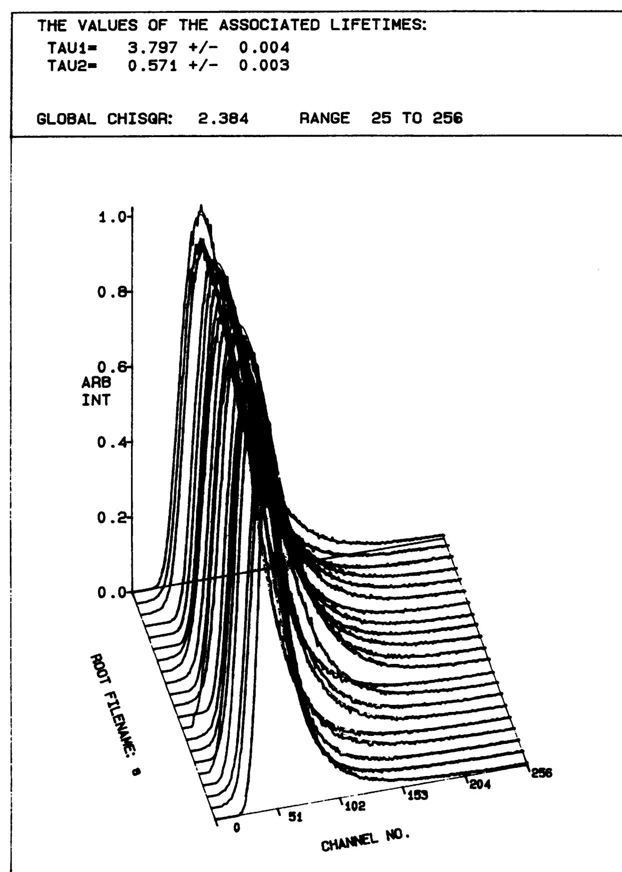

FIGURE 6 Global fittings of the set of decay profiles of serotonin obtained as a function of pH. See Materials and Methods for details.

Rhodopsin and the β-adrenergic receptor serve as representative members for the study of the structure and function of the G-protein coupled receptor family (Strosberg, 1991; Donnelly and Findlay, 1994; Strader et al., 1995). It is known that the agonist binding site for the β-adrenergic receptor is in the membrane-embedded region of the receptor, similar to the retinal binding site in rhodopsin (Dixon et al., 1987; Strader et al., 1987). Subsequent studies using the β-adrenergic, α-adrenergic, and muscaranic receptors have demonstrated that the location of the binding site inside the membrane is a common feature of all these receptors (Ostroski et al., 1992). The agonists for these receptors contain an amine group that is believed to form a complex with the negatively charged aspartate residue in the third transmembrane domain. This is believed to constitute one of the epitopes necessary for high affinity binding (Wang et al., 1993). For serotonin receptors, mutagenesis and molecular dynamics studies have shown that the ligand-binding site is located in a transmembrane domain (Chanda et al., 1993; Peroutka, 1993; Sylte et al., 1993; Wang et al., 1993). Because the microenvironmental polarity experienced by serotonin in such a domain would be significantly lower than in the bulk aqueous phase, we investigated the fluorescence characteristics of serotonin in environments of

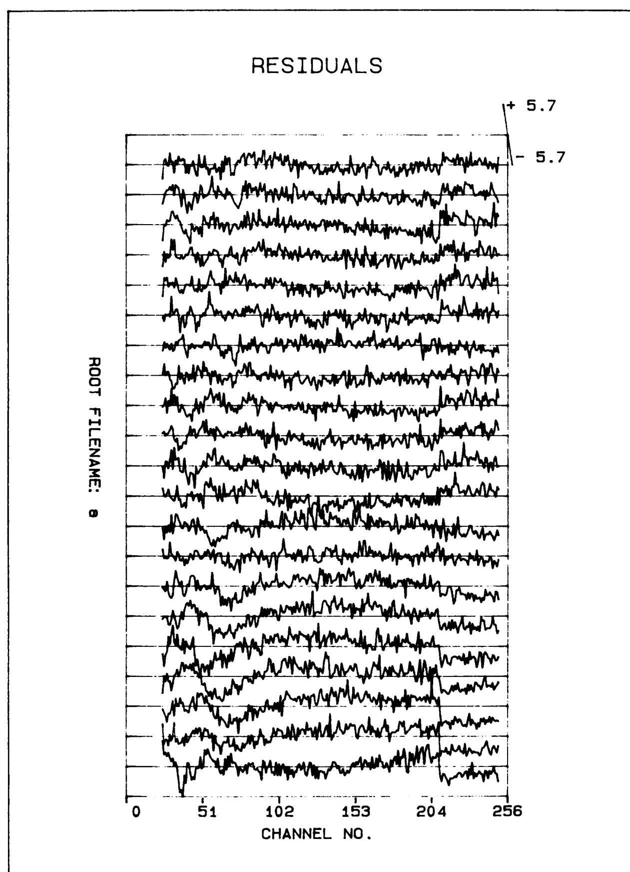

FIGURE 7 The weighted residuals corresponding to the global fittings shown in Fig. 6.

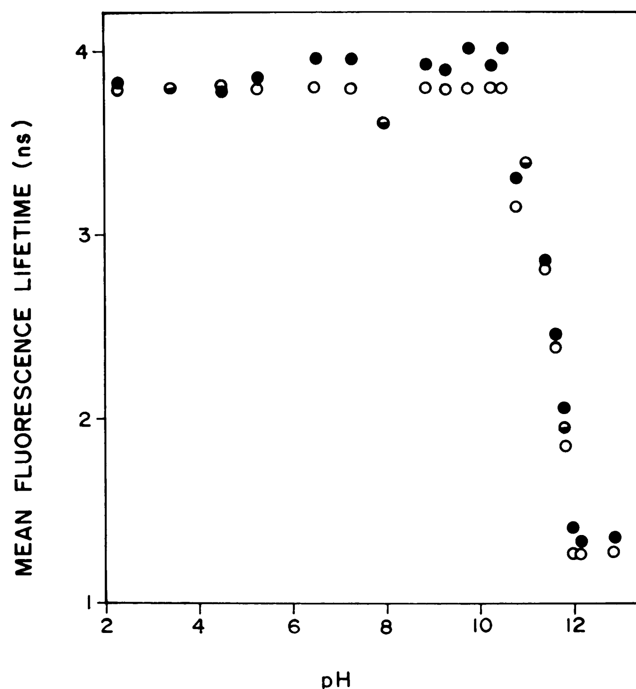

FIGURE 8 Mean fluorescence lifetime of serotonin as a function of pH obtained by discrete lifetime analysis (●) and by global lifetime analysis (○). The excitation wavelength used was 296 nm, and emission was set at 337 nm. Mean lifetimes were calculated from Table 2 by use of Eq. 3. See Materials and Methods for details.

lower polarity. Incubation of serotonin with SUVs of DOPC (see Materials and Methods) did not result in appreciable partitioning or binding of serotonin to the membrane, as evidenced by a lack of change of fluorescence emission maximum (Table 3) or polarization values (not shown). This is consistent with the findings in an earlier report in which it was shown that serotonin does not bind to lecithin dispersions (Krishnan and Balaram, 1976). Because serotonin is positively charged at pH 7, we wanted to check whether it binds to negatively charged membranes. For this, we used SUVs containing 40 mol % DOPG along with DOPC. We could not detect any binding in this case either (Table 3). The inability of serotonin to bind to membrane vesicles could be attributed to the insufficient hydrophobicity of serotonin. These results indicate that the binding of serotonin to its receptor(s) involves some type of specific polar interaction, as indicated above. The fluorescence characteristics of serotonin in solvents of lower polarity (both protic and aprotic) are shown in Table 3. In solvents such as methanol, acetonitrile, and 2-propanol, fluorescence intensity of serotonin is enhanced (Fig. 9 and Table 3). This enhancement is accompanied by a blue shift of 2–5 nm in the wavelength of maximum emission and an increase in fluorescence lifetime.

DISCUSSION

The spectroscopic and ionization properties of serotonin under various conditions and the modulation of its fluorescence characteristics when it is transferred to nonpolar environments (which could mimic binding to its receptors) have been the focus of this report. Our results show that serotonin's fluorescence (intensity, emission maximum, and lifetime) depends on its ionization state with a characteristic pK_a of 10.4 ± 0.2. That these fluorescence changes are not

TABLE 3 Solvent effects of serotonin fluorescence

Medium	Dielectric Constant*	Emission Maximum (nm)	Relative Intensity#	Lifetime§ (ns)
2-Propanol	18.3	335	1.80	4.28
Methanol	32.6	334	1.69	4.04
Acetonitrile	37.5	332	1.78	4.10
Buffer (pH 7.3)	–	337	1.00	3.94
DOPC SUV (pH 7.3)	–	337	–	–
DOPC/40% DOPG SUV (pH 7.3)	–	337	–	–

*From Lide, 1992.

#Calculated by measuring fluorescence intensity at the respective emission maximum when excited at the excitation maximum.

§Excitation wavelength 296 nm, emission wavelength 337 nm. All decays fitted to monoexponential functions.

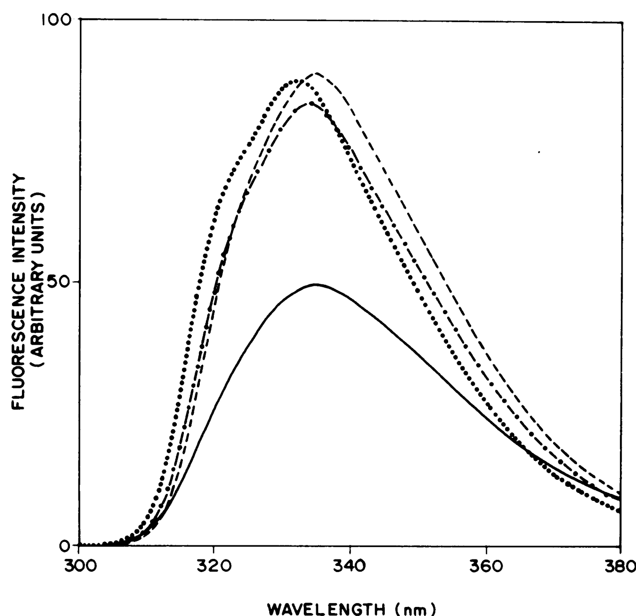

FIGURE 9 Corrected fluorescence emission spectra of serotonin in buffer (pH 7.30) (———), methanol (— · — ·), acetonitrile (····), and 2-propanol (— — —). The excitation wavelengths used were 275 nm (acetonitrile), 276 nm (methanol), and 277 nm (buffer and 2-propanol). The concentration of serotonin was 10 μM in all cases.

due solely to excited-state processes is supported by corresponding absorbance changes with pH.

It was previously observed, from studies of the pH dependence of the fluorescence of tryptophan and its derivatives, that the fluorescence of compounds that have an amine group near the indole ring is more quenched when the amine group is protonated (Beechem and Brand, 1985). Interestingly, this is not true for serotonin fluorescence (Fig. 2). This could be due to the distance of the amine group from the indole ring in serotonin because the amount of quenching depends on the distance of the protonated amine from the indole ring. However, tryptamine (in which the amine group is located in an identical position) fluorescence increases with deprotonation of its amine group (Eftink et al., 1995). This points out the involvement of the hydroxyl group in pH-dependent fluorescence changes in serotonin. An examination of the chemical structure of serotonin (Fig. 1) shows that there are two ionizable groups in the molecule, i.e., the phenolic hydroxyl group and the amine group. The ionization constants of both these groups are such that their pK_a values lie between 9 and 10 (Windholz, 1983). Thus, the spectroscopic changes reported here have contributions from both of these processes. In fact, the fluorescence and the absorbance of tryptamine have been shown to be pH dependent, with a pKa of 10.2–10.3 (Eftink et al., 1995), closely resembling the pK_a of serotonin (5-hydroxytryptamine) reported in this paper.

The variation of fluorescence lifetime of serotonin with pH can be further interpreted by use of the rotamer model of the biexponential decay of tryptophan and its analogs originally proposed by Szabo and Rayner (1980) and recently confirmed by analysis of conformational heterogeneity of tryptophan in crystals of erabutoxin b (Dahms et al., 1995). According to this model, serotonin will exist predominantly in conformation I (or in the equivalent conformation II) at $pH < pK_a$ (Fig. 10). This is due to the stabilization gained from the energetically favorable electrostatic interaction between the π electron cloud of the 5-hydroxyindole ring and the positively charged quarternary nitrogen atom. At $pH > pK_a$, serotonin will be mainly in conformation III. This is because of the loss of the positive charge on the nitrogen atom (which was responsible for the favorable interaction with the 5-hydroxyindole nucleus) and also because of steric crowding. Based on our lifetime data (Table 2), we thus suggest that the longer lifetime corresponds to rotamer I (or II), whereas the short lifetime only seen at higher pH (and whose contribution increases with increasing pH) corresponds to rotamer III. This is in agreement with the characteristics of tryptophan rotamers because, in the case of tryptophan also, the rotamers in which the positively charged quarternary nitrogen atom is close to the indole ring have been assigned a similar long lifetime (Szabo and Rayner, 1980).

Our results also show that serotonin fluorescence is rather insensitive to solvent polarity. This is somewhat surprising because serotonin is a derivative of tryptophan, whose fluorescence is known to be extremely solvatochromic (Lakowicz, 1983; Eftink, 1991). In this respect, serotonin appears to resemble tyrosine more closely, which does not exhibit any appreciable solvatochromism (Lakowicz, 1983; Ross et al., 1992), than tryptophan. Any role of the phenolic hydroxyl group (present both in serotonin and tyrosine and

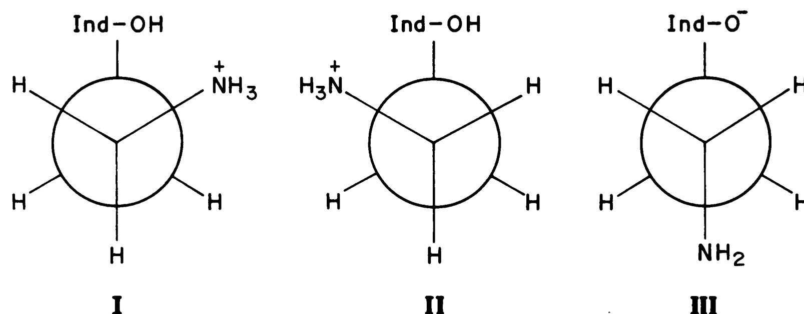

FIGURE 10 Newman projections of three rotamers along the C_α—C_β bond of serotonin. Rotamers I and II represent equivalent conformations and are the predominant species at $pH < pK_a$. Rotamer III represents the major species at $pH > pK_a$. See text for details.

conspicuously absent in tryptophan) in the lack of solvatochromism raises an interesting possibility: It was previously shown that substitution in the aromatic ring at the 5 or 3 position can alter the relative positions, separation, or both of the ^1L_a and ^1L_b states of indole (Strickland and Billups, 1973; Andrews and Forster, 1974). The stabilization of these excited states for serotonin in solvents of varying polarity could be different, thus accounting for its altered solvatochromism.

In summary, we have characterized the photophysical properties of the neurotransmitter serotonin and their modulation by ionization and polarity of the medium. In view of the multiple roles played by the serotonergic systems in the central and peripheral nervous systems, these results could be relevant to future studies of serotonin and its binding to its receptors.

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