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A spectroscopic survey of substituted indoles reveals consequences of a stabilized¹L_b transition

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Abstract

Although tryptophan is a natural probe of protein structure, interpretation of its fluorescence emission spectrum is complicated by the presence of two electronic transitions, ¹L_a and ¹L_b. Theoretical calculations show that a point charge adjacent to either ring of the indole can shift the emission maximum. This study explores the effect of pyrrole and benzyl ring substitutions on the transitions' energy via absorption and fluorescence spectroscopy, and anisotropy and lifetime measurements. The survey of indole derivatives shows that methyl substitutions on the pyrrole ring effect ¹L_a and ¹L_b energies in tandem while benzyl ring substitutions with electrophilic groups lift the ¹L_a/¹L_b degeneracy. For 5- and 6-hydroxyindole in cyclohexane, ¹L_a and ¹L_b transitions are resolved. This finding provides for ¹L_a origin assignment in the absorption and excitation spectra for indole vapor. The 5- and 6-hydroxyindole excitation spectra show that despite a blue-shifted emission spectrum, both the ¹L_a and ¹L_b transitions contribute to emission. 10⁰ ns fluorescence lifetimes for 5-hydroxyindole are consistent with a charge acceptor-induced increase in the nonradiative rate (1).

INTRODUCTION

As tryptophan is an intrinsic fluorescent probe of protein structure and environment, understanding its photophysics is of paramount importance. Complicating factors include two overlapping, electronic transitions, ¹L_a and ¹L_b, and the solvent sensitivity of the ¹L_a transition, which allows its tuning to an energy lower than that of ¹L_b. This stabilization makes ¹L_a the emitting transition. The resulting emission band is energetically red-shifted, broad and featureless. In a hydrophobic solvent, the tryptophan emission spectrum is blue-shifted, and some vibronic structure may be present suggesting emission from the ¹L_b transition. This has been difficult to prove (2). Emission from ¹L_a is difficult to rule out because the energetic position of the ¹L_a origin is not known, and is hidden by overlap with the ¹L_b transition. Various spectroscopic techniques (2-9), theoretical calculations (10-15) and tryptophan model compounds (3, 6, 16-20) have been applied to resolve this conundrum.

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SUPPLEMENTARY MATERIALS

Figures S1 – S4 can be found at DOI: 10.1562/2006-xxxxxx.s1.

Callis and coworkers have shown through hybrid quantum/classical simulations of tryptophan fluorescence that the emission maximum can be tuned to higher energy (blue-shifted) by placing either a negative charge near the benzyl ring or a positive charge near the pyrrole ring of indole (12, 14). Reversing the charge placement yields a red-shift in emission maximum. Together with the solvent, these point charges create an electric field, constituting an internal Stark effect.

An experimental approach to studying both charge placement and solvent effects on the relative energy of indole 1L_a and 1L_b transitions is given here. Absorption, fluorescence emission and excitation spectra were recorded for fourteen indole derivatives in solution with water and cyclohexane at room temperature and indole in the vapor phase. The focus is on electrophilic substitution on the benzyl ring and methylation at pyrrole sites. A histogram of 1L_a and 1L_b origin absorption maxima for derivatives in cyclohexane reveal that the 1L_b transition is especially sensitive to substitution on the benzyl ring. 1L_b energy decreases while 1L_a energy increases for benzyl substitution, resulting in a large energy separation between transitions. In the case of 5- and 6-hydroxyindole, the 1L_a and 1L_b transitions are essentially resolved. For 6-hydroxyindole in cyclohexane, the Stokes shift is absent, and the emission band is characteristic of emission spectra recorded for tryptophan in a hydrophobic environment, i.e., a blue-shifted emission maximum with resolved vibronic peaks. The fluorescence excitation spectrum for 6-hydroxyindole in cyclohexane demonstrates that some emission emanates from the 1L_a transition even in this case where the 1L_a and 1L_b transitions are resolved. A general pattern of mixed 1L_a / 1L_b fluorescence emission is demonstrated for indole vapor as well. Because of the resolution of the 1L_a and 1L_b transitions for 5- and 6-hydroxyindole in cyclohexane, additional spectroscopic data was collected to illuminate the nature of these transitions. Fluorescence excitation anisotropy measurements, necessarily carried out at low temperature required use of a hydrophilic medium. For 6-hydroxyindole, excitation anisotropy showed that although the 1L_a transition did not completely overlap with the 1L_b , the 1L_a transition possessed sufficient intensity at the red edge of the excitation spectrum to become the emitting dipole. Fluorescence lifetime measurements yielded 10^0 ns lifetimes that are consistent with published results (1, 21-23). An increase in charge transfer to the benzyl ring and an increase in the nonradiative rate are discussed as tandem effects of electrophilic substitution on the benzyl ring.

MATERIALS AND METHODS

All indole derivatives were purchased from Gold Biotechnology, (St. Louis, MO) with the exception of indole, 3-methyl, 5-chloro, 5-fluoro, and 4-hydroxyindoles, and L-tryptophan, which were obtained from Acros Organics (NJ). 7-Hydroxyindole was purchased from Chem-Impex International, Inc. (Wood Dale, IL). Indoles were used without further purification. Deionized water was used in all aqueous solutions. Cyclohexane was 'spectranalyzed' grade (for use in the UV range; Fisher Scientific, Fair Lawn, NJ). *P. aeruginosa* azurin was purchased from Sigma-Aldrich (St. Louis, MO).

All indoles, where soluble, were made up at a concentration of 1 mg/ml and subsequently diluted to a concentration of 0.1 mM for absorbance measurements. For less soluble indoles, concentration was determined by absorbance using an extinction coefficient of $4900 \text{ M}^{-1} \text{ cm}^{-1}$ at the 1L_b 0-0 maximum, and then diluted to 0.1 mM for recording of the absorption spectrum if necessary. Indole solutions were diluted to 0.01 mM for fluorescence measurements. The path length for the indole vapor measurements was 1 cm. For the absorbance measurement, the indole vapor concentration was 4 mg./cm.^3 ; for fluorescence measurements, the indole vapor concentration was 0.2 mg./cm.^3 .

Steady state fluorescence spectra and fluorescence anisotropy measurements were acquired on a PTI QM-4/206 SE Spectrofluorometer (PTI, Birmingham, NJ) with right angle detection of fluorescence. Thermostating of the cuvette holder with a constant temperature bath set to 75° C allowed for fluorescence measurements on indole vapor. The excitation wavelength for indole emission spectra in Figure 1 is 285 nm except for 5-fluoro and 5-chloroindole in water ($\lambda_{\text{ex}} = 290$ nm). Solvent blank spectra were subtracted where necessary. For the fluorescence anisotropy measurements, 5- and 6-hydroxyindoles were dissolved in a solution of poly(ethylene glycol) (400 molecular weight average)/ethylene glycol (1:2 v/v). Solutions were then frozen to a glass at 77K in a 3mm diameter quartz tube. The sample compartment was purged with dry nitrogen to eliminate fogging. Anisotropy was collected every 3 nm with a dwell time of 30 sec at each point. Anisotropy, r_0 , is given by (24):

$$r_0 = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

Where I = the measured light intensity, the subscripts give the orientation of the polarizer in the excitation path and the emission path, respectively. The instrument bias correction factor, G , is given by:

$$G = \frac{I_{hv}}{I_{hh}} \quad (2)$$

Fluorescence lifetime measurements were acquired on a Horiba Fluorolog model FL-1000 fluorometer using 281 nm light-emitting diode excitation (Horiba, Inc., Edison, NJ). The instrument response was 1.8 ns long, full-width half maximum. Measurements were corrected for instrument response using a casein scattering suspension. The fluorescent count maximum was set to 20,000 for all measurements.

A Cary 100 Bio UV-vis spectrophotometer (Varian Instruments, Inc., Walnut Creek, CA) with a built-in Peltier/cuvette holder provided for absorbance measurement on indole vapour at 75°C. All spectra are referenced against solvent.

Baseline fits to the $^1L_a/^1L_b$ peak(s) of the absorption spectra were made with the freeware program, Fityk, v. 0.9.4 (25) to remove contribution from the more intense $S_0 \leftarrow S_2$ band. An example of such a fit is given in Figure S1 (see Supplementary Materials). This program was also used to determine the absorption and emission spectra peaks given in Figure 2. A Gaussian band shape was used. Curve fits to the absorption spectra of 5- and 6-hydroxyindole in cyclohexane (Figs. S2 and S3, respectively) are likewise executed with the Fityk program; the band shapes are Gaussian. Trend line fits to the data in Figure 1 are carried out with the Microsoft Excel program.

RESULTS

Energy trends in indole spectra

Substitution at any one of seven indole ring positions (Scheme 1, top) or variation in solvent from hydrophobic (cyclohexane) to hydrophilic (water), can induce energy shifts in the 1L_a and 1L_b absorption transitions, whose vectorial position relative to the indole ring plane is shown in Scheme 1, bottom. Measured spectral energy shifts for indole derivatives in response to both factors are given in the histogram in Figure 1. The identification key for the indole species' acronyms used in Figure 1 is given in Table 1. Absorption maxima for the 1L_a transition in cyclohexane (diamond) and water (filled triangle) and the 1L_b transition band origin in cyclohexane (filled square) are given, as well as the fluorescence emission

maxima for cyclohexane (triangle) and water (filled circle) solutions. Results for fourteen indole derivatives and indole vapor were acquired. Peaks for an additional eight indoles are also included (16). The peaks were ordered in an identity-blind fashion such that a rough linear trend in peak energy, measured in units of wavelength (nm), could be identified for almost all types of maxima. As a result, pyrrole ring derivatives are mostly found on the left side of Figure 1 while benzyl ring derivatives are found on the right.

Aggregation is always a consideration for molecules dissolved in an incompatible solvent. For such a scenario, red shifts in emission would be expected due to fluorescence reabsorption. Absorption band red shifts would also be expected for aggregated molecules. The spectral results for methyl indoles on the left side of Fig. 1, taken from (16), show large emission red shifts in aqueous solution. However, the 1L_a absorption maxima for the molecules in water are not greatly red shifted relative to their maxima for molecules in cyclohexane. To first approximation, aggregation is not apparent.

Only the absorption spectra for indoles in cyclohexane showed sufficient vibronic structure to locate the 1L_b lowest energy or 0-0 band (Fig. 1 (filled square)). The 1L_a 0-0 band is not identifiable in absorption spectra for either solvent due to spectral overlap between the 1L_a and 1L_b transitions. Instead, the short wavelength absorption peak maximum is chosen as the 1L_a transition marker. Indeed, for many indole derivatives in a variety of solvents, the 1L_a and 1L_b transitions are energetically degenerate. The following indoles did not fluoresce or fluoresced very weakly: 4- and 7-hydroxy and 5-bromoindoles in cyclohexane, and 3-cyano and 7-hydroxyindoles in water and so no point appears on the histogram for these species. Trend lines were fit to the spectral data where linear trends were apparent. This process yielded three near-parallel lines for pyrrole-substituted indole spectral data (Fig. 1, left side): through the 1L_a absorption maxima of aqueous indoles (filled triangle), the 1L_b absorption maxima of indoles in cyclohexane (filled square) and the emission maxima of indoles in cyclohexane (triangle). In general, the energy of these maxima decrease with increased methyl substitution to the pyrrole ring. A trend line is also applied to the emission maxima for pyrrole derivatives in aqueous solution (Fig. 1, left, filled circle). The slope of the trend line to these emission maxima is 1.8 times larger than those fit to the absorption maxima for pyrrole derivatives.

Trend lines were also fit to selected sets of spectral data for benzyl-substituted indoles, shown on the right side of Figure 1. Fits were made to the 1L_a absorption maxima, cyclohexane solvent (diamond), the 1L_b absorption maxima, cyclohexane solvent (filled square), and to the aqueous emission maxima (filled circle). These trend lines reveal a different energy relationship between the absorption and emission transitions for benzyl-substituted indoles. The energy separation between the 1L_a and 1L_b absorption maxima is roughly 2 times greater for the benzyl-substituted indoles than the pyrrole-substituted ones, placing the 1L_a absorption maxima for benzyl substituted indoles at higher energy. The trend line fit to the aqueous emission maxima for the benzyl-substituted indoles is anti-parallel to the corresponding absorption trend lines. For 5-methoxy and 5-hydroxyindole at the right edge of the plot, the energy separation between the aqueous and cyclohexane emission maxima is less than the Stokes shift between the 1L_b absorption maximum and the emission maximum in cyclohexane solvent. Another noteworthy feature of the plotted results is the lack of Stokes shift for 6-hydroxyindole.

Resolution of the 1L_a and 1L_b transitions

The absorption and fluorescence excitation spectra for 5- and 6-hydroxyindole in cyclohexane are given in Figure 2a and b, respectively. These indoles are singled out because of the apparent resolution of their 1L_a and 1L_b transitions. The 5-hydroxyindole absorption spectrum (Fig. 2a, solid line) consists of a low energy band with three distinct

peaks at 297 nm, 302 nm and 308 nm and a shoulder at 291 nm. This is the 1L_b transition. A more intense, higher energy band is resolved into two peaks at 263 nm and 270 nm with a shoulder at 281 nm. This is the 1L_a transition. The lower energy band of the corresponding fluorescence excitation spectrum (Fig. 2a, bold line) duplicates the peak position and intensity of the lower energy absorption 1L_b band with little exception. The higher energy band, however, only has the peak at 267 nm of the 1L_a transition, and shoulder at 281 nm with a broad shoulder at 264 nm

A similar pattern of bands and peaks is found for 6-hydroxyindole, shown in Figure 2b. Here, the low energy absorption band (solid line), the 1L_b transition, is the more intense. Peaks are blue-shifted 6-7 nm relative to those in the absorption spectrum for 5-hydroxyindole. The higher energy 1L_a absorption band for 6-hydroxyindole also has two peaks, here centered at 260 nm and 267 nm. The low energy band of the excitation spectrum for 6-hydroxyindole (Fig. 2b, bold line) also recapitulates the 1L_b transition of the absorption spectrum. A high energy peak at 267 nm corresponds to the absorption peak at the same wavelength.

Figure 2c originates from reference (26), and is included here for comparison with the results of Fig. 2a and b. It illustrates both experimental (dashed line) and calculated (solid line) 1L_a and 1L_b absorption transitions for indole. The experimental spectra were obtained from fluorescence anisotropy measurements for indole in propylene glycol at -58°C (27). The calculated spectra were derived from CIS/4-31G excited state and HF/4-31G ground state geometries (28). Comparison of the resolved transitions from Fig. 2c with the absorption and excitation spectra in Figs. 3a and b reveals a strong resemblance between the lower energy bands of the hydroxyindole spectra (Fig. 2a,b) and the calculated 1L_b transition (Fig. 2c, solid line) where more vibronic detail is evident. The higher energy absorption band for the hydroxyindoles (Fig. 2a,b) more strongly resembles the band shape of the experimental 1L_a transition (Fig. 2c, dashed line) where, again, there is resolution of distinct peaks. The high energy peak of the excitation spectra for the hydroxyindoles at 267 – 270 nm (Fig. 2a,b bold line) corresponds to the 1L_a transition peak at 280 nm (Fig. 2c, dashed line).

Absorption and fluorescence excitation spectra for indole vapor at 75°C are given in Figure 2d. These results are included because individual vibronic bands are particularly well-resolved in the vapor phase. The resolved 1L_a and 1L_b transitions for the 5- and 6-hydroxyindoles now make possible assignment of peaks where the transitions overlap. There is a one-to-one correspondence between the 1L_b peaks in the 6-hydroxyindole absorption spectrum (Fig. 2b) and the absorption and fluorescence excitation spectra peaks of indole vapor (269 – 284 nm). Likewise, the 253 nm and 259 nm peaks in the absorption spectrum of the indole vapor match the 1L_a transition peaks at 263 nm and 270 nm (Fig. 2a). The indole vapor excitation spectrum shows only the 259 nm peak of the 1L_a transition.

Comparison of absorption and excitation spectra for indoles can be extended to tryptophan containing proteins where vibronic bands are mostly obscured. The absorption and fluorescence excitation spectra for the single tryptophan in *Pseudomonas aeruginosa* azurin are shown in Fig. 2e. The broad, relatively featureless peaks are typical of spectral results for tryptophans in proteins, even for those buried in an hydrophobic environment (2). While there is a weak vibronic peak at 291 nm that indicates the 1L_b origin, the remainder of each spectrum is without detail. The absorption and excitation peaks at 281 nm and 283 nm (Fig. 2e), respectively, are similar to the featureless calculated 1L_a band in Fig. 2c, but also suggest a fair degree of overlap for the 1L_a and 1L_b transitions for tryptophan in azurin.

Fluorescence anisotropy reveals the relative orientation of the 1L_a and 1L_b transitions

Fluorescence excitation anisotropy is a spectroscopic technique that reveals the orientation of the excitation transition dipole relative to the emission dipole. In the case of indole derivatives, where there are two absorption transition dipoles, 1L_a and 1L_b , of variable energetic overlap, calculated orthogonality (29, 30) (Scheme 1, bottom), and highly variability in the energy of the 1L_a transition, anisotropy measurements have the potential to resolve the relative contribution of these transitions to fluorescence emission. The requirement of a static population of oriented dipoles for anisotropy measurements requires a medium that can be frozen to low temperature and yet maintain optical transparency. A glycol glass is one such medium. However, it is not as apolar as cyclohexane and so transition resolution matching that obtained in cyclohexane (Fig. 2a,b) is not expected due to solvent-induced inhomogeneous broadening.

Absorption and fluorescence excitation spectra for 5-hydroxyindole and 6-hydroxyindole in poly(ethylene glycol)/ethylene glycol solution are given in Fig. 3a, b (dashed and solid line, respectively); the absorption results were acquired at room temperature while the excitation results were obtained at 77K. These spectra reveal the position of each transition in the more hydrophilic glycol solvent environment, and therefore aid in the interpretation of the anisotropy measurements. As the glycol solvent is more hydrophilic than cyclohexane, vibronic detail is lacking in both hydroxyindole absorption and excitation spectra (Fig. 3a, b dashed and solid line, respectively). The solvent-sensitive 1L_a transition maximum for 5-hydroxyindole appears at 275 nm in the excitation spectrum (Fig. 3a, solid line), and at 273 nm in the absorption spectrum (Fig. 3a, dashed line). The 1L_b origin appears at 309 nm in the excitation spectrum (Fig. 3a, solid line) and at 310 nm in the absorption spectrum (Fig. 3a, dashed line). Relative $^1L_a / ^1L_b$ absorption and excitation band intensity for 5-hydroxyindole in glycol solution (Fig. 3a, dashed and solid line, resp.) follows that for cyclohexane (Fig. 2a, solid line and bold line, resp.).

Much vibronic detail is also absent in the absorption and excitation spectra of 6-hydroxyindole in glycol solution (Fig. 3b, dashed and solid lines, respectively). Peaks are located at 295 nm (1L_b) and 273 nm (1L_a) in both absorption and excitation spectra. The relative intensity of the 1L_a and 1L_b absorption and excitation bands for 6-hydroxyindole in glycol solution mirror those for cyclohexane solution (Fig. 2b, solid and bold lines, resp.).

Fluorescence anisotropy measurements for 5-hydroxyindole and 6-hydroxyindole in the glycol glass at 77K are also given in Figure 3, a and b (---■---). Anisotropy values, r_0 , follow the relationship (24, 27):

$$r_0 = 0.4 \frac{(3\cos^2\alpha - 1)}{2} \quad (3)$$

Where α is the angle between the excitation and emission dipoles. The values of r_0 range from 0.4, corresponding to collinear excitation and emission dipoles ($\alpha = 0^\circ$) to -0.2, where the excitation and emission dipoles are orthogonal. At wavelengths shorter than 250 nm, overlap with the higher energy 1B_b transition is possible (31), and so the anisotropy above 250 nm will not be considered.

The anisotropy measurements for both hydroxyindoles follow a general pattern of decreasing value with decreasing wavelength. For 5-hydroxyindole (Fig. 3a, ---■---), the anisotropy at 321 nm is at a maximum value of 0.21, corresponding to a 34° angle between the emission and excitation dipole. At 309 nm, the location of the 1L_b origin, the anisotropy drops to 0.15. The anisotropy continues to decrease with wavelength, 'plateauing' twice

more at 305 nm and 291 nm. The anisotropy for 5-hydroxyindole finally reaches a minimum of -0.03 at 275 nm near the 1L_a transition absorption maximum.

The anisotropy for 6-hydroxyindole (3b, $-\blacksquare--$) at the short wavelength edge of the excitation band is 0.37, a limiting anisotropy that is close to the theoretical limit of 0.4 (27). This anisotropy value corresponds to a 13° angle between the excitation and emission dipoles; that is, they are nearly collinear. At 300 nm, the apparent 1L_b origin, $r_0 = 0.13$. Near the 1L_a maximum at 272 nm, a minimum value in anisotropy is found: $r_0 = 0.034$, yielding $\alpha = 51^\circ$. An angle of 54.7° , the so-called 'magic angle,' indicates a random orientation of dipoles.

Fluorescence emission spectra for some indoles

The fluorescence emission spectra for 5- and 6-hydroxyindole in cyclohexane, indole vapor and *Pseudomonas aeruginosa* azurin are shown in Figure 4. The hydroxyindole spectra (Figs. 4a, b) are included to illustrate the very small Stokes shift for the emission of 6-hydroxyindole in cyclohexane versus the near identical Stokes shift for the emission of 5-hydroxyindole in cyclohexane and water (Fig. 1). The emission spectrum of indole vapor (Fig. 4c) is included because of the sharpness of the vibronic bands. The *P. aeruginosa* azurin emission spectrum (Fig. 4d) represents the case where tryptophan is buried in a hydrophobic protein environment and emission shows vibronic structure. The source of emission for such a buried tryptophan--- 1L_a or 1L_b transition---has been discussed at length in the literature (2, 26, 32).

The fluorescence emission spectrum for 5-hydroxyindole shows a maximum at 325 nm. The blue-edge 1L_b origin emission at 313 nm is Stokes-shifted 5 nm from the corresponding excitation band (Fig. 2a). 1L_b origin absorption wavelengths and emission maxima for other indoles, given in Fig. 1, show that this is not an atypical Stokes shift for indoles in cyclohexane. The fluorescence emission spectrum for 6-hydroxyindole in cyclohexane (Fig. 4b) exhibits the vibronic detail and blue-shifted emission maximum (304 nm) characteristic of indole in a hydrophobic environment. However, the 293 nm emission peak corresponds to the 301 nm excitation peak (Fig. 2b), which means that there is no Stokes shift for 6-hydroxyindole in cyclohexane. The excitation wavelength for the 6-hydroxyindole emission shown in Fig. 4b was 284 nm in order to collect the full emission band. Excitation at 299 nm shifted the emission maximum to 319 nm with a secondary vibronic band at 326 nm but identical bandwidth (Data not shown). The lack of Stokes shift for the emission of 6-hydroxyindole in cyclohexane will be discussed below.

The fluorescence emission spectrum of indole vapor (Fig. 4c) is narrow, extremely blue-shifted with an emission maximum at 295 nm, and shows vibronic structure. The high energy vibronic detail at 290 nm is Stokes-shifted 14 nm from the corresponding excitation peak (Fig. 2d). The band shape, although very narrow, is similar in profile to that of 6-hydroxyindole.

The emission spectrum for the *P. aeruginosa* protein, azurin (Fig. 4d), shares the features of the other blue-shifted emission spectra just described. The emission peak is at 305 nm--- close to that of 6-hydroxyindole---and the short wavelength vibronic peak appears at 295 nm. At the long wavelength edge, a shoulder is apparent, which is also defined in the indole vapor emission spectrum (Fig. 4c).

Fluorescence lifetime measurements for 5- and 6-hydroxyindoles

Fluorescence lifetime measurements were made for 5- and 6-hydroxyindoles in water and cyclohexane. All measurements were carried out at two excitation wavelengths, 281 nm and

293 nm. The results are given in Table 2 along with lifetime measurements from the literature for 5-hydroxyindole in water, cyclohexane and in a helium jet expansion (1, 21, 23) and for 6-hydroxyindole in helium jet expansion (22). Fluorescence lifetime measurements on 6-hydroxyindole in solution have not been found in the literature. The low fluorescence intensity of 6-hydroxyindole, resulting from excited state formation of a keto tautomer (22), a nonradiative process, discourages lifetime measurement. Single lifetimes were obtained for all but 6-hydroxyindole in cyclohexane, where two lifetimes were obtained. A ~10 ps lifetime attributed to scattering was necessary for acceptable fits ($\chi^2 \sim 1$) to the 6-hydroxyindole in cyclohexane data. A lifetime for 6-hydroxyindole in water with 293 nm excitation could not be obtained because the fluorescence decay was of the same timescale as the instrument response function. The lifetimes obtained for each hydroxyindole did not vary significantly with the excitation wavelengths of 281 nm and 293 nm.

DISCUSSION

Substituent sensitivity of 1L_b transition

The solvent sensitivity of the 1L_a transition is well-documented (14, 26, 33-35), and is the source of the large Stokes shift observed for indoles in a polar environment, as illustrated by the emission maximum data points for pyrrole-substituted indoles in aqueous solution on the left side of Fig. 1. The effect of solvent relaxation is to lower the energy of 1L_a , allowing it to become the emitting dipole. The trend lines to the pyrrole-substituted indole spectral data show that methylation changes the energy of 1L_a and 1L_b absorption maxima in tandem with the emission maxima in both solvents.

Substitution on the benzyl ring with electron withdrawing groups, however, simultaneously raises the energy of the 1L_a absorption and lowers that of the 1L_b absorption maxima as indicated by the nonparallel trend lines fitted to the absorption maxima for 1L_a and the 1L_b origin. For 5-methoxy and 5-hydroxyindole at the right hand edge of Fig. 1, the energetic difference between the 1L_a and 1L_b absorption transitions is sufficient to obviate any large polar solvent-induced relaxation of the solvent-sensitive 1L_a transition. Thus, the aqueous fluorescent emission maxima for these two indoles are close to their emission maxima in cyclohexane. The insensitivity of 5-hydroxyindole to solvent polarity has also been documented by two other groups (23, 35).

The energy separation between the 1L_a and 1L_b transitions for both 5- and 6-hydroxyindole presents the unique opportunity to study the resolved 1L_a and 1L_b transition bands without resorting to any spectral manipulation. A comparison of the absorption spectra for 5- and 6-hydroxyindole reveals that the 1L_a transition is more intense for 5-hydroxyindole, and there are peak intensity differences between the two absorption spectra, e.g., the 270 nm peak of 5-hydroxyindole is of equal intensity to the 263 nm peak while the corresponding peak intensities differ in the 6-hydroxyindole spectrum. Such differences are to be expected as the difference in hydroxyl substitution site effects the ring charge distribution. Remarkably, the 1L_a peak profiles for 5- and 6-hydroxyindole both recapitulate features of the anisotropy-resolved 1L_a band shape for indole in Fig. 2c. The 1L_b band for these hydroxyindoles exhibit vibronic detail that is not resolved in the anisotropy-derived 1L_b transition but appears in the calculated 1L_b band. In fact, the calculated 1L_b transition and those of the hydroxyl indole absorption and excitation spectra differ only in the resolution between the two lowest energy peaks and the relative intensity of the higher energy peak for 6-hydroxyindole. As the 1L_a and 1L_b absorption bands for 5- and 6-hydroxyindole show a small overlap, a curve fit to each has been carried out, and these are shown in Figures S2 and S3, respectively (see Supplementary Materials). The small overlap of the red edge of 1L_a with the blue edge of 1L_b for both hydroxyindoles shows that excitation of 1L_b only is possible.

Because vibronic peaks can now unambiguously be assigned, the extent of 1L_a and 1L_b overlap for other indole spectra can be determined. In the case of indole vapor (Fig. 2d), several vibronic peaks are apparent. While there is still significant overlap of the 1L_a and 1L_b transitions, it is clear that peaks at 253 nm and 259 nm belong to the 1L_a transition while those at 273 nm, 278 nm and 284 nm belong to 1L_b . The peak at 268 nm may well be a superposition of the low energy shoulder from 1L_a and the high energy shoulder from 1L_b . The lack of vibronic detail in spectra for azurin (Fig. 2e) ---only the 1L_b peak at 291 nm is resolved---and the compressed bandwidth in the absorption (Fig. 2e, solid line) and excitation (Fig. 2e, bold line) spectra suggest that the 1L_a and 1L_b transition energies are degenerate.

Excitation spectra clarify the $^1L_a/^1L_b$ contribution to blue-shifted emission spectra

The spectral overlap of the 1L_a and 1L_b transitions for pyrrole-substituted indoles, including aqueous tryptophan, and the solvent sensitivity of the 1L_a transition, which leads to a featureless emission envelope due to solvent relaxation, obscures determination of the emitting dipole.

The separation of the 1L_a and 1L_b transitions for 5- and 6-hydroxyindoles in cyclohexane clarifies the source of emission in an hydrophobic environment. The fluorescence excitation spectra for 5- and 6-hydroxyindole in cyclohexane (Fig. 2a,b) make plain the contribution of both the 1L_a and 1L_b transitions. While the primary source of fluorescence emission is the 1L_b transition, there is undeniably a contribution from 1L_a as the high energy peaks at 267 nm (Fig. 2b) and 270 nm (Fig. 2a) in the excitation spectra align directly with an 1L_a absorption band. The same reasoning can be applied to the fluorescence excitation spectrum of indole vapor (Fig. 2d).

Overlap of excitation and emission spectra for 6-hydroxyindole reveals band symmetry

The fluorescence emission spectra in Figure 4 suggest a contribution of both the 1L_a and 1L_b transitions to blue-shifted emission is possible. While the fluorescence emission spectral envelope can exactly mirror that of the excitation spectrum for some molecules, that is generally not the case for indole derivatives. As shown by the emission spectra for indoles in Figure 4, even such blue-shifted emissions---while featuring vibronic details at the short wavelength edge and suggesting 1L_b as the source---are featureless at the long wavelength edge, obscuring the transition involved. The emission spectrum for 6-hydroxyindole in cyclohexane, however, does not exhibit a Stokes shift and so presents a unique opportunity to compare the excitation and emission spectra. This phenomenon indicates that the molecule is not subject to vibrational relaxation induced by either solvent or collision (24). Absence of Stokes shift has been observed in excitation and emission results for anthracene confined and isolated within cyclodextrin cavities (36).

The excitation and emission spectra for 6-hydroxyindole are shown superimposed in Figure 5. The spectral overlap extends from 290 nm to 305 nm. Clearly, this emission originates from the 1L_b transition. The band profiles of the two spectra also possess an atypical mirror symmetry in the 1L_b vibronic details at 293 nm and 301 nm, and the peak maxima. The spectral overlap also exposes differences: the full-width at half-maximum for the excitation spectrum is 25 nm while that for the emission spectrum is 29 nm. The inset in Fig. 5, where the excitation spectrum has been flipped vertically, shows the difference in peak width more clearly. The excitation spectrum also has a second peak at 267 nm, which lines up with the low energy peak of the 1L_a transition. This peak is not observed in the emission spectrum, but most likely contributes to the increased emission bandwidth. The evidence then points to 1L_b emission with an energetic contribution from 1L_a for 6-hydroxyindole in cyclohexane.

While the emission spectra for 5-hydroxyindole in cyclohexane and indole vapor (Fig. 4a,d) are Stokes-shifted with respect to their excitation spectra, their band shapes and vibronic details bear a similar relationship to their excitation spectra as discussed above. Their excitation spectra feature peaks that align with both 1L_a and 1L_b absorption peaks. These features imply emission from the 1L_b transition with energetic contribution from 1L_a for both 5-hydroxyindole and indole vapor.

Fluorescence excitation anisotropy for 5- and 6-hydroxyindoles under hydrophilic conditions

While a solution of poly(ethylene glycol) and ethylene glycol forms a low temperature glass, it does not create the desired hydrophobic environment necessary for optimal resolution of the 1L_a and 1L_b transitions. A more hydrophobic solution of diethyl ether/2-methyl butane/ethanol, 5/5/2 by volume, has also been reported as forming a transparent glass at low temperature (37). Monitoring the 240 – 350 nm absorbance of 5-hydroxyindole in this hydrophobic solution, however, showed a shift of peaks to 297 nm and 310 nm for the 1L_a and 1L_b absorption bands, respectively, over the time span of a few minutes (Data not shown). This shift to lower energy of the absorption bands suggests dimerization. The reaction, nevertheless, eliminates the possibility of anisotropy measurements in this hydrophobic solution.

The fluorescence excitation anisotropy for 5-hydroxyindole (Fig 4a, --■--) is similar in curve shape to that reported by Wong and Eftink for 5-hydroxyindole in a 50% glycerol-phosphate buffer solution at 77K (38) although the extreme values are greater. Their reported 5-hydroxyindole anisotropy has a high value of 0.25 at 315 nm and diminishes linearly to a minimum of -0.5 at 270 nm. The hydroxyindole anisotropy spectra reported here and elsewhere (38) differ from those reported for indole and tryptophan. Large swings (increases and decreases) in anisotropy value over the 280 – 295 nm wavelength range are seen in the anisotropy spectra for indole (27) and tryptophan (2) where the 1L_a and 1L_b absorption bands overlap. The absence of this feature in the anisotropy spectra of 5- and 6-hydroxyindole may be taken as a signature of at least partial 1L_a and 1L_b absorption band resolution.

The fluorescence excitation anisotropy results for 6-hydroxyindole (Fig. 3b, --■--), reach a high limiting anisotropy value of 0.37 at 321 nm, close to the theoretical limit of 0.4. The highest anisotropy value reached for the anisotropy results of 5-hydroxyindole is only 0.21 so the results for 6-hydroxyindole are used calculate 1L_a and 1L_b bands according to the equation (24, 27):

$$r_0(\lambda) = fa(\lambda)r_{0a}(\lambda) + fb(\lambda)r_{0b}(\lambda) \quad (4)$$

and

$$fa(\lambda) + fb(\lambda) = 1 \quad (5)$$

to the total anisotropy, $r_0(\lambda)$, by each transition at a given wavelength, λ , and $fx(\lambda)$ is the transition band shape at the wavelength, λ . The individual transition contributions to the excitation band shape are calculated from

$$fa(\lambda) = \frac{r_0(\lambda) - r_{0b}(\lambda)}{r_{0a}(\lambda) - r_{0b}(\lambda)} \quad (6)$$

$$fb(\lambda) = \frac{r_{0a}(\lambda) - r_0(\lambda)}{r_{0a}(\lambda) - r_{0b}(\lambda)} \quad (7)$$

Replacing the theoretical maximum anisotropy, 0.4, in eqn. 3 above, with the limiting anisotropy of 0.37, and assuming orthogonality of 1L_a and 1L_b ($\alpha = 90^\circ$), the limiting anisotropy of 1L_a , $r_{0a}(\lambda)$, is -0.185 (24, 27). The resulting calculated 1L_a and 1L_b bandshapes, $fa(\lambda)$ and $fb(\lambda)$ (Data not shown), have equal amplitude from 250 nm to 310 nm, and diverge only at the long wavelength edge, where the 1L_b transition reaches a maximum while the 1L_a transition falls to zero. These results are not in agreement with band shapes observed experimentally (Fig. 2a,b) or calculated (Fig. 2c). Band shapes were recalculated using a limiting anisotropy of 0.034 for $r_{0a}(\lambda)$, obtained at 276 nm, for the measured 6-hydroxyindole anisotropy, and $r_{0b}(\lambda) = 0.37$. The band shapes recalculated with these limiting anisotropies, given in Figure S4, show a 1L_a transition that dominates from 250 nm to 303 nm with a gradual drop in intensity from 312 nm to 320 nm. The 1L_b transition has little intensity from 250 nm to 309 nm, but peaks at 321 nm. These anisotropy-derived band shapes are in general agreement with the experimental band shapes given in Fig. 2a,b and the calculated band shapes in Fig. 2c. The 1L_a transition overlaps with 1L_b in the longer wavelength region but is apparently the only contributing transition at shorter wavelengths. At 321 nm, the 1L_a transition still contributes. The 334 nm emission maximum for 6-hydroxyindole in the hydrophilic, glycol glass at 77K (Data not shown) is consistent with emission from the solvent-sensitive 1L_a transition.

Charge acceptors on benzyl ring enhance charge transfer and nonradiative decay

In this work, single fluorescence lifetimes were obtained for 5-hydroxyindole in water and cyclohexane while two lifetimes were obtained for 6-hydroxyindole in cyclohexane, and essentially a single lifetime was obtained for this indole in water (Table 2). The lifetimes obtained for 5-hydroxyindole in water (3.0 ns at two different excitation wavelengths) are in close agreement with the single 3.2 ns lifetime obtained by Sengupta et al. (23). Wong et al. (38), exciting at 305 nm, obtained two lifetimes for 5-hydroxyindole in water. They report an amplitude-weighted average lifetime of 3.55 ns. Using 285 nm excitation and cyclohexane as solvent, Arnold and coworkers (1) obtained a 50% longer lifetime (6.0 ns) for 5-hydroxyindole than that reported here. In He jet-cooled gas expansion, a longer lifetime of 11.1 ns. is obtained for 5-hydroxyindole. In general, longer lifetimes are expected at lower temperatures.

Published fluorescence lifetime studies of weakly fluorescent 6-hydroxyindole are scarce, hampering discussion. One study, by Sulkes and coworkers (22), reports a short lifetime of 2.8 ns for 6-hydroxyindole in a He jet expansion. Lifetimes measured here for 6-hydroxyindole are 1.8 ns and 0.90 – 0.98 ns in cyclohexane and 0.4 ns (~100%) in water (Table 2). In their jet-cooled fluorescence lifetime studies of substituted indoles, Sulkes and coworkers (1, 21, 22) found that 4- and 6-hydroxyindoles display anomalously short lifetimes due to excited state formation of a keto tautomer. The effect is greatest for 4-hydroxyindole, which displays a subnanosecond lifetime in expanded He jet medium (0.23 ns), and somewhat less for 6-hydroxyindole (Table 2). Excited state tautomerism for 5-hydroxyindole is nil in an He-cooled expanded jet, as the 11.1 ns lifetime included in Table 2 suggests (21). Our very short lifetime of 0.4 ns for 6-hydroxyindole in water and 1.8 ns lifetime for this species in cyclohexane suggest that the nonradiative process of excited state tautomerism is operative at room temperature as well. For this and other indoles substituted with charge acceptors on the pyrrole or benzyl ring, charge transfer to the substituent increases the nonradiative decay rate, $k(\text{nrad})$, (1). Charge donating groups, such as CH_3 , placed on the pyrrole ring, enhance charge transfer to the benzyl ring, increasing $k(\text{nrad})$, while donating groups on the benzyl ring diminish charge transfer and decrease $k(\text{nrad})$.

Regarding solvent effects, cyclohexane was found to stabilize charge through polarizability (1).

It is instructive to compare this relationship of charge acceptors and donors to nonradiative decay (Sulkes effect) with the Stark effect, discussed above. Recall that the Stark effect predicts that a negative charge near the benzyl ring or a positive charge near the pyrrole ring results in a blue shift (higher energy) of the fluorescence emission maximum. Converse charge placement yields a red shift in emission maximum. From the perspective of Sulkes, hydroxyl, carboxylates and halogens are charge accepting groups. From the perspective of the Stark effect, these are electron rich groups. Their summary effect is to promote charge transfer, increase the nonradiative rate (Sulkes effect) (1) and increase the fluorescence emission energy (emission blue shift) when placed on the benzyl ring, as shown in Fig. 1.

The Sulkes effect also predicts that charge acceptors on the pyrrole will increase nonradiative decay. Emission data for indoles containing pyrroles substituted with charge acceptors are missing from Fig. 1, but the aqueous dipeptide zwitterion, tryptophanyl glycine (TrpGly), exhibits a fluorescence emission that is blue shifted relative to the dipeptide anion (39). Molecular dynamics simulation shows that the terminal amine cation is positioned over the pyrrole ring for one dominant conformer (39). From the perspective of the Sulkes effect, the terminal amine cation is a charge acceptor. The average fluorescence lifetime for the TrpGly zwitterion, 1.97 ns, is significantly shorter than that for the TrpGly anion, 6.95 ns (40). This is further evidence of confluence between the Stark and Sulkes effect: the general trend seems to be that electron accepting groups on either indole ring decrease fluorescence lifetime and blue-shift the emission maximum. The results of Fig. 1 suggest that lowering of the 1L_b energy is concomitant with electron accepting group substitution on the benzyl ring.

The Sulkes/Stark effect analogy may be extended to electron donating groups such as methyl groups if the dielectric of the medium is considered. Sulkes found that methyl substitution on the pyrrole ring enhanced the nonradiative rate for measurements made in cyclohexane (1). Emission maxima for indoles where pyrrole rings are methyl-substituted and the solvent is cyclohexane are significantly blue-shifted (Fig. 1, left side). As noted by Sulkes and coworkers, the combined effect of ring substitution with electron donor/acceptor groups and solvent polarizability can work to stabilize intramolecular charge transfer (1). This combined effect is apparently also operable for fluorescence emission maxima shifts.

Other evidence for 1L_a and 1L_b contribution to blue-shifted indole emission

Both theoretical studies and measurements on indoles in van der Waals clusters (8, 18, 19, 41, 42) have found evidence of $^1L_a/^1L_b$ mixing. Gas phase geometrical parameters for the ground and $^1L_a/^1L_b$ excited states of 5-hydroxyindole have been calculated at the CASSCF and TDDFT levels of theory, respectively (43). The 1L_b transition is centered on the benzyl ring in indole, 2,3-dimethyl indole and tryptophan (28, 44), with changes in bond length at the C₄---C₅ (+0.056 Å) and C₈---C₉ (+0.055 Å) bonds (43). The 1L_a transition, however, is delocalized over both indole rings in indole and tryptophan (12, 13), with bond changes at the C₂---C₃ (+0.085 Å), C₄---C₅ (+0.045 Å) and C₆---C₇ (+0.063 Å) bonds (43). As both transitions involve a bond length change at the C₄---C₅ bond and therefore an electronic redistribution along this bond, excitation of the 1L_b transition invariably leads to a change in 1L_a . A change in C₄---C₅ bond length for both the 1L_a and 1L_b transition has also been found by Eisenberg in *ab initio* quantum mechanical calculations for 5-OH indole (45). The excitation spectra for 5- and 6-hydroxyindole in cyclohexane and that of indole vapor (Fig. 2a,b,d) show that 1L_a contributes to emission even when energetically well-separated from 1L_b , which is consistent with mixed $^1L_a/^1L_b$ emission.

CONCLUSION

This spectroscopic study of pyrrole and benzyl substituted indoles under hydrophobic and hydrophilic conditions reveals specific substituent effects on the 1L_a and 1L_b transition energy. For each additional methyl substitution on the pyrrole ring, the energy of both transitions decreases in tandem, with emission maxima following the same trend of energy decrease. Addition of electrophilic or aliphatic groups to the benzyl ring raises the energy of the 1L_a transition while decreasing the energy of the 1L_b transition. The effect on fluorescence emission is to increase the energy of the emission maxima even in aqueous solution. For indoles such as 5-hydroxyindole, the emission maximum in a hydrophobic solvent such as cyclohexane is nearly coincident with the maximum in water. Hydroxyl substitution at either the 5- and 6-ring position of indole reveals experimentally-resolved 1L_a and 1L_b transitions, providing for the first time, the energetic location of the 1L_a origin. Fluorescence excitation spectra for 5- and 6- indole in cyclohexane are also transitionally-resolved, and reveal that the 1L_a transition makes an energetic contribution to fluorescence emission even when emission is from 1L_b . Fluorescence excitation anisotropy measurements have the potential to resolve the relative contribution of overlapping 1L_a and 1L_b transitions to fluorescence emission under favorable solution conditions. For 6-hydroxyindole in a hydrophilic glass, vibrational detail is missing and transition overlap is greater than in cyclohexane solution. Deconvolution of the anisotropy results reveal that the 1L_a is the dominant band under hydrophilic conditions, and while at somewhat higher energy than the 1L_b transition, contributes sufficiently at the excitation spectrum red edge. Lifetimes for 5- and 6-hydroxyindole in aqueous or cyclohexane solution are on the order of 10^0 ns. Combining Stark effects for fluorescence emission maximum shifts and Sulkes effects (1) for charge transfer and the resulting increase in nonradiative decay (lifetime shortening), it is observed that charge acceptor groups on either ring of indole blue shift the emission maximum while increasing charge transfer and the nonradiative decay rate (1). In a hydrophobic environment, charge donor groups on the pyrrole ring blue shift fluorescence emission while promoting charge transfer to the benzyl ring (1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Absorption and fluorescence emission maxima (wavelength, nm) histogram for indole derivatives in cyclohexane and water (25 °C), and for indole vapor (75°C). Results for indoles are ordered to show the effect of substituent ring position on band energy. The indole species acronym key is given in Table 1. Absorption band maxima for: 1La, cyclohexane (diamond); 1La, water (filled triangle); 1Lb band origin, cyclohexane (filled square); Emission: cyclohexane (triangle); water (filled circle). For pyrrole substituted indoles (left side of chart), trend lines are fit to the 1La and 1Lb absorption maxima in cyclohexane and the emission maxima in both solvents. For benzyl substituted indoles (right side of chart), trend lines are fit to the 1La and 1Lb absorption maxima in cyclohexane and the emission maxima in water. Data for eight indole species from Lami (16) are also included.

Scheme 1.

Molecular details for the indole molecule. Top: Atomic numbering for ring position.

Bottom: orientation of the 1L_a and 1L_b electronic energy transitions adapted from (26).

Figure 2.

Absorption (solid line) and fluorescence excitation (bold line) spectra for indole species with computed and experimentally-determined 1La and 1Lb transitions for indole reproduced from reference (26) with permission. Absorption spectra have been offset for ease of comparison. a. 5-hydroxyindole in cyclohexane; 325 nm emission wavelength. b. 6-hydroxyindole in cyclohexane; 320 nm emission wavelength. c. 1La and 1Lb transitions resolved from fluorescence excitation anisotropy measurements on indole in propylene glycol at -58°C (dashed lines) from reference (27). Calculated 1La and 1Lb transitions from (28). Reproduced with permission from reference (26). d. indole vapor, 295 nm emission wavelength. e. *Pseudomonas aeruginosa* azurin, 100 mM ammonium acetate, pH 5.3, 320 nm emission wavelength.

Figure 3. Absorption (room temperature, dashed line) and fluorescence excitation (77K, solid line) spectra and fluorescence excitation anisotropy measurements (77K, --■--) in poly(ethylene glycol), 400 MW avg./ethylene glycol (1:2 v/v) for **a.** 5-hydroxyindole; 332 nm excitation for fluorescence measurements. **b.** 6-hydroxyindole; 334 nm excitation for fluorescence measurements.

Figure 4.

Fluorescence emission spectra for indole species. a. 5-hydroxyindole in cyclohexane, 300 nm excitation wavelength. b. 6-hydroxyindole in cyclohexane, 285 nm excitation wavelength. c. indole vapor at 75°C, 285 nm excitation wavelength. d. *Pseudomonas aeruginosa* azurin, 100 mM ammonium acetate, pH 5.3, 275 nm excitation wavelength.

Figure 5. Fluorescence spectra for 6-hydroxyindole in cyclohexane, showing spectral overlap due to the absence of a Stokes shift. Excitation spectrum, 320 nm emission wavelength (dashed line) and emission spectrum, 284 nm excitation wavelength (solid line). These spectra have been superimposed in the inset for band comparison.

TABLE 1

KEY FOR INDOLES IN FIGURE 1 HISTOGRAM

23M 2,3-dimethyl indole	6M 6-methyl indole
235M 2,3,5-trimethyl indole	5F 5-fluoro indole
12M 1,2-dimethyl indole	5M 5-methyl indole
3M 3-methyl indole	5MO2M 5-methoxy-2-methylindole
2M 2-methyl indole	5CN 5-cyano indole
I Indole	6MO 6-methoxy indole
3CN 3-cyano indole	6OH 6-hydroxyindole
I vap indole vapor	5Br 5-bromo indole
7M methyl indole	5Cl 5-chloro indole
4OH 4-hydroxyindole	5MO 5-methoxy indole
7OH 7-hydroxyindole	5OH 5-hydroxyindole
Trp L-tryptophan	

(16)

Table 2

Fluorescence lifetimes for 5- and 6-hydroxyindoles with goodness-of-fit (χ^2)

Molecule	Lifetime (amplitude)/ns			χ^2	
	281 nm	293 nm		281 nm	293 nm
5-OH indole	4.2	4.3	6.0	0.98	1.40
Cyclohexane	3.0	3.0	3.2	1.41(0.35)	1.04
Water				4.74 (0.65)	--
He jet expansion			11.1		
6-OH indole	0.98(0.51)	0.90(0.32)		0.85	1.27
Cyclohexane	1.8 (0.48)	1.8(0.48)			
Water	0.40 (1.0)	--		1.29	--
He jet expansion	4.8 (0.15)	--	2.8		

285 nm excitation(1)

. excitation wavelength not available(23)

305 nm excitation(38)

285 nm excitation (21)

285 nm excitation (22)