# Electronic Absorption and Fluorescence Spectra of 5-Hydroxytryptamine (Serotonin). Protonation in the Excited State

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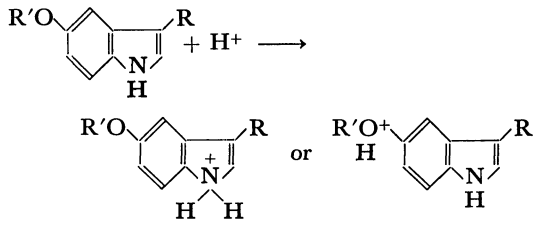
The fluorescence spectra of 5-hydroxytryptamine (serotonin) in acidic media have been studied by means of fluorescence polarization, temperature dependence of fluorescence, and kinetics of the protonation in $H_2O$ and $D_2O$. The red-shifted emission appearing by protonation in the excited state is ascribed to the protonated form of serotonin at $C_4$. In order to confirm the assignment of electronic spectra, the absorption spectra of the single crystal of 5-methoxyindole-3-acetic acid have been measured by means of reflection technique, the directions of transition moments being determined by polarization analysis. Calculation was carried out on the energy levels of the excited state for serotonin and its protonated forms, and the directions of transition moments were compared with the experimental results.

5-Hydroxytryptamine (serotonin) found in many mammal tissues has several unknown physiological effects, such as on nervous impulse transmission and mental activity.[1] The fluorescence assay of serotonin is important in biomedical analysis. Udenfriend *et al.*[2] found that the fluorescence of serotonin in a strongly acidic solution appears at 18000 $cm^{-1}$ in the visible region, and at 30000 $cm^{-1}$ in a neutral solution. Bridges and Williams,[3] and Chen[4] studied the mechanism of this anomalous fluorescence and postulated that the emitting species is the protonated excited state form of serotonin. However, no detail of the protonated structure was established, the postulate remaining unsettled.




We have attempted to clarify the structure of the protonated form on the basis of (1) polarization measurement of the fluorescence, (2) temperature dependence of the fluorescence in the acidic media, (3) lifetime measurement of the fluorescence of both unprotonated and protonated serotonin in the acidic media, (4) polarization measurement of the reflection spectra of a crystal of serotonin derivative, and (5) theoretical calculation on serotonin and its protonated forms.

The electronic states and structures of serotonin and its protonated form were clarified from the results. The results are discussed in connection with the electronic spectra of the parent molecule, indole.

## Experimental

*Material.* Serotonin creatinine sulfate (Nakarai Chemical Co.) was purified by repeated recrystallization from water. Ethylene glycol–water (7 : 3) (EGW) with 1.2 M HCl was used as a solvent for the fluorescence polarization measurement. The fluorescence lifetime were measured with solutions of $2 \times 10^{-5}$ M in $H_2O$ or $D_2O$ by adding appropriate amounts of HCl or DCl. 5-Methoxyindole-3-acetic acid (pfs grade, Sigma Chemical Co.) was recrystallized from methanol–water. The compound was supplied by Prof. K. Tomita of Osaka University.

*Methods.* The temperature dependence of the fluorescence was measured in a thermostated cell with a Carl Zeiss spectrofluorometer in the temperature range −56—6 °C. A HTV-R446UR photomultiplier tube was used, no correction being made for the spectral responce of the detecting system. The polarization of the fluorescence was measured with the same apparatus combined with a calcite polarizer for excitation and a polacoat polarizer for analysis at −52 °C. The polarization characteristic of the polacoat polarizer was checked before the measurement, the stray light being less than 1%. The instrumental depolarization effect was corrected following the procedure of Azumi and McGlynn.[5] Fluorescence lifetimes were measured with an Ortec 9200 photon counting system. An Ortec nanosecond light pulser was used as an excitation source through a Toshiba DV-25 filter, with 20% $NiSO_4$ solution and 0.2% $K_2CrO_4$ solution of each 1 cm path. The fluorescence was detected through filters of a Toshiba UV-D1B and a Nd glass for the 29600 $cm^{-1}$ band and a Fuji Color SC-52 for the 17800 $cm^{-1}$ band. The reflection spectra were recorded by the microscopic reflecting system constructed in this laboratory.

## Results and Discussion

The fluorescence spectra of indole in polar solvents exhibit a conspicuous red shift.[6-11] It is considered that the second excited level is stabilized and the fluorescence occurs from this state. The absorption and fluorescence spectra of serotonin are shown in Fig. 1 together with the polarization values for the excitation and emission. There are more than three absorption bands in the 31000—47000 $cm^{-1}$ region. The first band appears in the 31000—34000 $cm^{-1}$ range, the second band overlapping with it at 33000 $cm^{-1}$ and extending to the 37000 $cm^{-1}$ region, and the third band appearing with a maximum at 45300 $cm^{-1}$. Although the first and the second bands overlap in the 33000 $cm^{-1}$ range, their maxima appear at different energies, differing from the case of indole where they overlap strongly.

The fluorescence spectra measured at −52 °C in an acidic media are shown in Fig. 1. The emission band with a maximum at 29800 $cm^{-1}$ is ascribed to a neutral molecule and the band appearing at 17800 $cm^{-1}$

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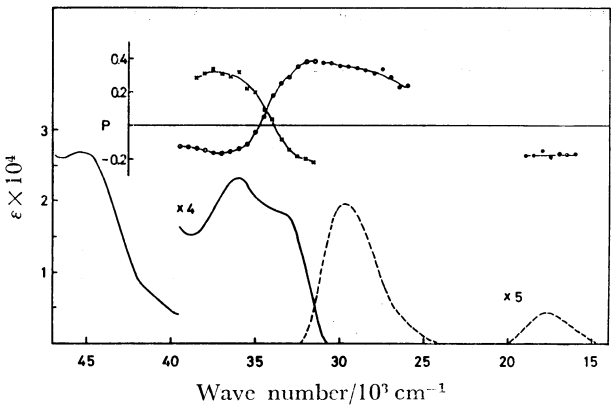



Fig. 1. The absorption (——), emission (---), and polarization spectra of serotonin in EGW with 1.2M HCl at −52 °C.
The fluorescence polarization (FP) spectra (-●-●-) were measured by exciting at 32500 cm$^{-1}$. The fluorescence excitation polarization (APF) spectra were recorded by monitoring the fluorescence at 30000 cm$^{-1}$ (-O-O-) and at 18000 cm$^{-1}$ (-×-×-).

is assigned to the protonated form. The maximum of this emission shifted to 17600 cm$^{-1}$ when correction was made on the response of the photomultiplier tube.

*Polarization Measurement of Fluorescence.* The fluorescence excitation polarization (APF) spectra for the neutral molecule were measured by setting the detecting monochromator at 30000 cm$^{-1}$. It was found that the first absorption band shows $p$-values of 0.4—0.2. This indicates that the fluorescence originates from the first excited state. The second absorption band showed negative $p$-values of −0.12——0.17. Thus the direction of the transition moment is considered to be nearly perpendicular to the first band.

The fluorescence polarization (FP) of 29800 cm$^{-1}$ emission band was measured by exciting at 32500 cm$^{-1}$, the $p$-values being always positive (0.4—0.25) in the range 31000—26000 cm$^{-1}$. This shows that the emission band consists of a unique origin, namely the fluorescence occurs from the lowest excited state.

The emission bands at 17800 cm$^{-1}$ appearing in a strongly acidic solution showed a quite different polarization character as compared to the 29800 cm$^{-1}$ band, its FP spectra measured by exciting at 32500 cm$^{-1}$ showing a constant negative $p$-value of −0.18. In line with this results, the APF spectra in the first absorption region showed negative $p$-values of −0.2——0.1, while the second absorption band showed positive $p$-values of 0.2—0.3 in the 35000—39000 cm$^{-1}$ range.

The 17800 cm$^{-1}$ band is considered to be due to the protonated form of serotonin.[4] The present results show that the rotational motion of serotonin molecule in the excited state is frozen at −52 °C in EGW to give definite $p$-values, while the proton transfer occurs under these conditions and the protonated serotonin is formed consequently. The long wavelength fluorescence shows a different polarization character as compared to the parent molecule. The absorption spectra of serotonin in 1M HCl solution was almost the same as those in neutral solution. It was confirmed that the protonation occurs after the excitation of the neutral molecule, since a close correlation was found between the absorption and polarization spectra of the parent molecule and the protonated form. The direction of the transition moment of the protonated emitting species was shown to be nearly perpendicular to the first absorption band and parallel to the second absorption band.

*Temperature Dependence of Fluorescence.* The temperature dependence of the fluorescence in acidic media is shown in Fig. 2, the solution being excited at 33500 cm$^{-1}$. At 6 °C the peaks for neutral and protonated species were found at 29600 cm$^{-1}$ and 17800 cm$^{-1}$, respectively. By lowering the temperature the intensity of the fluorescence from the neutral form increased, the peak showed a blue shift of 200 cm$^{-1}$, and the emission from the protonated form diminished. The results are explained by a retardation of the protonation in terms of increase in the viscosity of the solvent caused by fall of temperature. The disappearance of the visible fluorescence at −196 °C was reported by Chen.[4] This provides another evidence that the emission occurs from the protonated species.

*Lifetime of Fluorescence.* The rise and decay of fluorescence of the neutral and protonated forms are shown in Fig. 3. The lifetime of neutral species

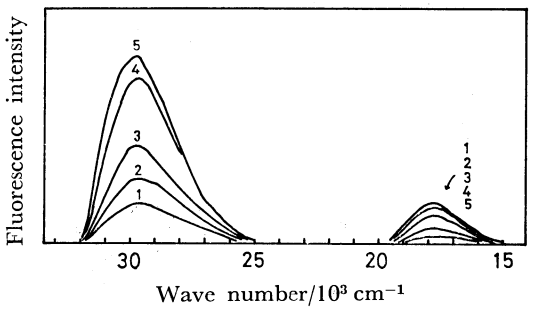



Fig. 2. The temperature dependence of the fluorescence of serotonin in EGW with 1.2 M HCl.
1. 6 °C; 2. −9 °C; 3. −19 °C; 4. −40 °C; 5. −56 °C.

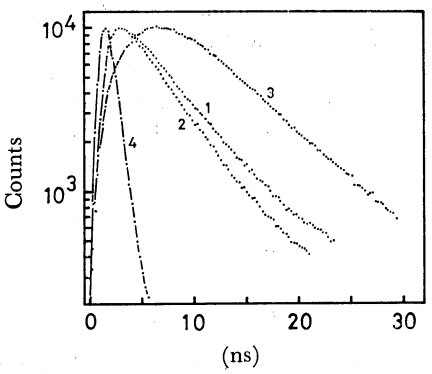



Fig. 3. The rise and decay curves of the fluorescence of the free and protonated serotonin in 0.04 M DCl.
1. Fluorescence intensity of serotonin measured at 30000 cm$^{-1}$, 2. Fluorescence intensity of serotonin with 0.04 M DCl measured at 30000 cm$^{-1}$, 3. Fluorescence intensity of protonated form of serotonin with 0.04 M DCl measured at 18000 cm$^{-1}$, 4. The flash profile.

decreases with increase in acid concentration. The rise and decay curves of the lower energy emission indicate that the excited species were produced after the excitation *via* the protonation of serotonin in the excited state.

Following Chen's[4] scheme, the reaction of the excited species is written as

$$\begin{array}{ccccc} & & S^* + H^+ & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & SH^{+*} \\ & \swarrow^{k_f} \quad \searrow^{k_q} & & \downarrow^{k_f'} \quad \searrow^{k_q'} & \\ S + h\nu & \qquad S & & SH^+ + h\nu' & \qquad SH^+ \end{array} \tag{1}$$

where S* is the excited serotonin, $k_1$ and $k_{-1}$ are the rate constants for the protonation and deprotonation reactions, respectively, $k_f$ and $k_f'$, and $k_q$ and $k_q'$ are the rate constants for radiative and radiationless processes of neutral and protonated species, respectively. If we assume that $k_1[H^+] \gg k_{-1}$, then the lifetime $\tau$ of the excited neutral molecule S* is given by

$$1/\tau = k_f + k_q + k_1[H^+]. \tag{2}$$

The change of the fluorescence lifetime at 20 °C with increase in the hydrogen ion concentration is given in Table 1. The fluorescence quenching by the counter ion $Cl^-$ was examined. It was found that the effect is negligible in the concentration range of $Cl^-$ less than 0.1 M. The plot of $1/\tau$ against $[H^+]$ or $[D^+]$ gives the rate constant, $k_1(H)$ and $k_1(D)$. The results are

$$k_1(H) = 2.9\times10^9\ s^{-1}\ M^{-1},$$
$$k_1(D) = 1.6\times10^9\ s^{-1}\ M^{-1},$$

and

$$k_1(H)/k_1(D) = 1.8.$$

The linear relation between $1/\tau$ and $[H^+]$ or $[D^+]$ supports Scheme 1 for the reaction of the excited species. The kinetic isotope effect found in these reactions can be correlated with the motion of the hydrogen ion in an aqueous solution, since the reaction seems to be a diffusion controlled process. The ratio of the rate constants $k_1(H)/k_1(D)$ shows a normal isotope effect.[12] It is interesting that the value is close to the ratio of the mobilities of proton and deuteron, $\mu(H^+)/\mu(D^+)$ (1.44) at 25 °C.[13] It might imply that the proton transfer occurs in the charge-separated excited species through the Grotthuss mechanism.[14]

Table 1. Fluorescence lifetime of serotonin in the acidic media

| $[H^+]$ | In $H_2O$ | In $D_2O$ |
|---|---|---|
| 0 mM | 4.65 ns | 5.57 ns |
| 40 | 3.72 | 4.81 |
| 60 | 3.30 | 4.43 |
| 80 | 2.99 | 4.13 |

*Theoretical Calculation of Electronic State.* The electronic energy levels of serotonin and its protonated forms were calculated by the Pariser-Parr-Pople method[15,16] by using the Nishimoto-Mataga potential[17] and taking into account the configuration interaction. The calculated transition energy, size and direction of the transition moments are given in Table 2 and Fig. 4. The directions of $^1L_b$ and $^1L_a$ transitions

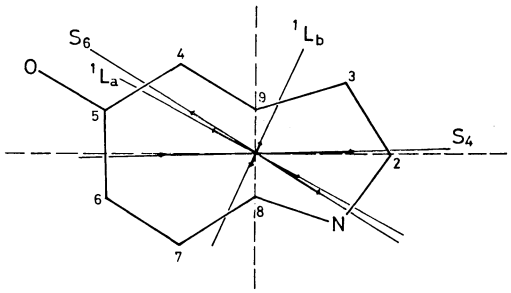



Fig. 4. The theoretical prediction of the direction of transition moments of the excited singlet states ($^1L_a$ and $^1L_b$) of serotonin and its protonated forms. $S_4$ or $S_6$ are is direction of the transition moment of the lowest singlet state of the protonated forms by adding proton at $C_4$ or $C_6$ positions, respectively.

Table 2. Theoretical calculations of the singlet excited states of serotonin and its protonated forms

| | State symbols | State energy $cm^{-1}$ | Transition-moment length $R_x$ | Transition-moment length $R_y$ | Oscillator strength $f$ | Observed values $cm^{-1}$ ($f$) |
|---|---|---|---|---|---|---|
| Serotonin | $^1L_b$ | 32940 | 0.079 | 0.174 | 0.013 | 33000 (0.054) |
| | $^1L_a$ | 38500 | −0.694 | 0.395 | 0.267 | 36000 (0.098) |
| | $^1B_b$ | 43840 | 1.136 | 0.571 | 0.769 | 45300 (0.56) |
| | $^1B_a$ | 46390 | −0.682 | 0.589 | 0.409 | |
| Protonated at $C_4$ | | 22750 | 1.553 | 0.028 | 0.596 | 20000 |
| | | 28720 | 0.161 | −0.059 | 0.009 | |
| | | 39020 | −0.028 | 0.464 | 0.091 | |
| | | 43690 | 0.268 | 0.552 | 0.178 | |
| Protonated at $C_6$ | | 21890 | −1.045 | 0.656 | 0.362 | |
| | | 28730 | 0.662 | 0.576 | 0.240 | |
| | | 37840 | −0.143 | −0.194 | 0.024 | |
| | | 43130 | 0.947 | 0.155 | 0.431 | |

(Fig. 4) are given for serotonin; the transition moment to the $^1L_b$ state makes 85° with that of the $^1L_a$, while the observed value estimated from the $p$-values and the relation between the $p$-value and the angle $\theta$ made of transition moments[18,19] given by

$$p = \frac{3\cos^2\theta - 1}{3 + \cos^2\theta} \quad (3)$$

is 90°. The agreement between these two values is satisfactory, since the depolarization and other possible experimental errors might not be completely corrected.

Calculation for the protonated forms was carried out by assuming that the proton attack at $C_4$ or $C_6$ position of the indole ring and positive charge was put on oxygen atom (Table 2). The positions of proton attack were presumed since these protons are known to be easily deuterated in acidic media. The lowest energy transitions were calculated for $C_4$ and $C_6$ adducts at 22750 $cm^{-1}$ and 21890 $cm^{-1}$, respectively. These values are in good agreement with the position of the fluorescence band when we consider a mirror image relationship between the absorption and emission spectra. The directions of transition moments from these levels to the ground state are along $S_4$ or $S_6$ (Fig. 4). A comparison of the calculated directions with the $p$-values suggests that the lowest state of the protonated form has a transition moment along $S_4$. As an example, from the observed $p$-values, the first and second transitions of parent molecule ($^1L_b$ and $^1L_a$) make 69° and 31°, respectively, with the direction of the emission of protonated form, while the calculated values with $S_4$ direction are 65° and 31°, respectively. In contrast, the transition moments to the $^1L_b$ and $^1L_a$ states makes 82° and 2°, respectively, with $S_6$ direction. Thus the position of proton attack may be assigned at $C_4$. However, an alternative choice remains at $C_6$ since a quantitative coincidence of $p$-values may not be easily realized, and the values can be affected by experimental artefact. Chen[4] presumed that the proton attack occurs at $C_6$ position, on the basis of the calculated charge density by DeVoe, who showed that the charge density in the excited state is the largest at this position. In spite of this the predicted $p$-values by the $C_6$ adduct are −0.32 and +0.5 for the first and the second bands, while the observed values are −0.18 and +0.32, respectively. The calculated values by the $C_4$ adduct are −0.15 and +0.33, respectively, the $C_4$ protonated form thus being more plausible than the $C_6$ adduct. We have calculated the charge density in the lowest excited state, the result of which is shown in Fig. 5. We see that the proton attack at $C_4$ is a reasonable process in view of charge distribution. The NMR spectra of serotonin in acidic media have been studied by Daly and Witkop[20] who found that the $C_4$ proton is most labile in an acidic media. This is in line with our conclusion that the proton attacks at $C_4$ position in the excited state of 5-hydroxyindole derivatives in an acidic media.

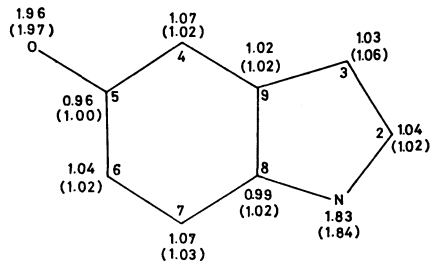
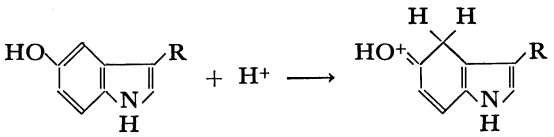



Fig. 5. The charge density of serotonin in the lowest excited state.
The charge density in the ground state was also shown in parenthesis.

Thus the excited-state reaction of 5-hydroxyindole derivatives can be depicted as:




*Reflection Spectra of 5-Methoxyindole-3-acetic Acid.* In order to confirm the assignment of spectra of 5-hydroxyindole derivatives, the polarized reflection spectra were measured with a single crystal of 5-methoxyindole-3-acetic acid on its (100) plane. The crystalline structure was determined by Sakaki, Wakahara, Fujiwara, and Tomita.[21] Projection of the molecule on (100) plane is shown in Fig. 6. It is noted that the short molecular axis is nearly parallel to the crystalline c-axis while the long axis lies nearly perpendicular to this plane. The crystals used for spectral measurement were 0.8×2 mm×*ca.* 0.3 mm. The devel-

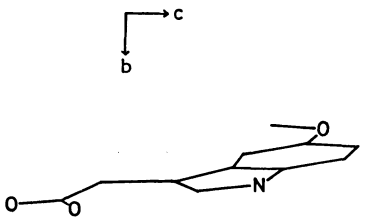



Fig. 6. Projection of 5-methoxyindole-3-acetic acid molecule onto the (100) plane of the crystal.

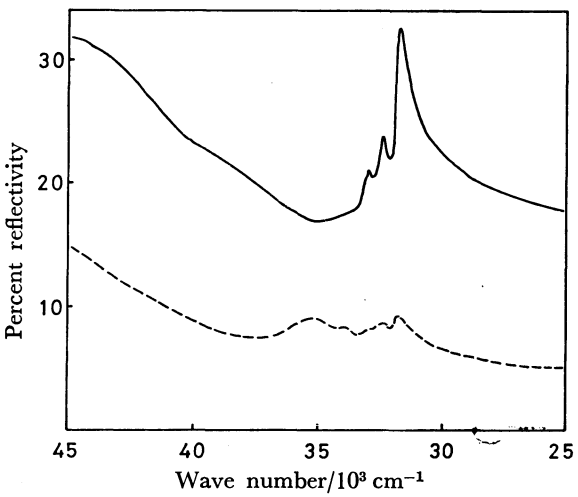



Fig. 7. Reflectivity of 5-methoxyindole-3-acetic acid crystal. Polarization of the light parallel to the b-axis (---) and the c-axis (——).

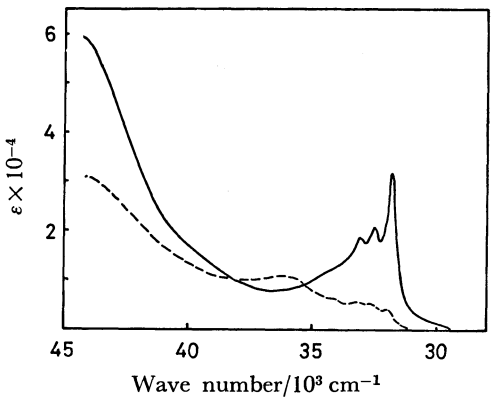



Fig. 8. Absorption spectra of 5-methoxyindole-3-acetic acid crystal obtained from Kramers-Kronig transformation. --- b-axis, —— c-axis.

oped plane was confirmed by X-ray photographs. The reflection spectra calibrated with SiC standard are shown in Fig. 7. The Kramers-Kronig transformation of reflectivity gives the absorption coefficient (Fig. 8).

The first band shows a vibronic structure at 31800, 32500, and 33100 cm$^{-1}$. Appearance of this structure peculiar to the first $^1L_b$ band has also been observed in the spectra of indole in the gaseous states.[22,23] The first absorption band shows a strong dichroism, strong along the c-axis. The direction of the transition moment obtained from the dichroic ratio confirms the calculated one shown in Fig. 4 and the band is thus assigned to the $^1L_b$ state. The second band observed at 36000 cm$^{-1}$ is weak on this crystalline face, its dichroic ratio being $f_b/f_c=2$. The band is assigned to the $^1L_a$ band at 36000 cm$^{-1}$ in solution spectra, its direction being consistent with that of the $^1L_a$ band deduced from theoretical calculation.

In their paper on indole derivatives, Yamamoto and Tanaka[24] estimated the directions of the transition moments of indole for $^1L_b$ and $^1L_a$ bands to make angles of 54 and −38°, respectively, to the long molecular axis. The present result confirms these assignments, *viz.*, although the present molecules have a hydroxyl or methoxyl substituent at $C_5$, the spectral features are very close to those of indole. Actually the calculated result on 5-hydroxyindole differs only by 9° rotation into anti-clockwise way from the result on indole by Yamamoto and Tanaka. Song and Kurtin[9] indicated that the transition moments of $^1L_b$ and $^1L_a$ states of indole are on the first and third quadrant and the second and fourth quadrant, respectively (Fig. 4). This is qualitatively in line with our result, but the directions are not in quantitative agreement with our assignment of indole and serotonin $^1L_b$ and $^1L_a$ bands.

As regards the oscillator strengths of $^1L_b$ and $^1L_a$ bands, whose relative intensity was discussed by Andrews and Forster,[11] the two bands overlap strongly but the whole band can be divided into two parts, $f$-values being estimated as 0.098 and 0.054 for $^1L_a$ and $^1L_b$ bands, respectively. From the crystalline spectra, the $f$-values for the $^1L_b$ band is estimated to be greater than 0.27, which is much greater than the value expected from the solution spectra (0.16), thus giving rise to a batho-chromic effect. The intensity ratio for $^1L_a/^1L_b$ bands is 1.8, whereas Andrews and Forster[11] estimated it to be 3—4 on indole and Yamamoto and Tanaka as 11. The effect of hydroxyl substituent is significant in enhancing the $^1L_b$ band.

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**References**

1) A number of review articles and monographs are available. For example, see H. E. Himwich, "Biochemistry, Schizophrenias, and Affective Illnesses," ed by H. E. Himwich, The Williams and Wilkins Co., Baltimore (1970), Chaps. IV and IX.
2) S. Udenfriend, D. F. Bogdanski, and H. Weissbach, *Science*, **122**, 972 (1955).
3) J. W. Bridges and R. T. Williams, *Biochem. J.*, **107**, 225 (1968).
4) R. F. Chen, *Proc. Natl. Acad. Sci. U.S.A.*, **60**, 598 (1968).
5) T. Azumi and S. P. McGlynn, *J. Chem. Phys.*, **37**, 2413 (1962).
6) H. Zimmermann and N. Joop, *Z. Elektrochem.*, **65**, 61 (1961).
7) N. Mataga, Y. Torihashi, and K. Ezumi, *Theoret. Chim. Acta*, **2**, 158 (1964).
8) J. Eisinger and G. Navon, *J. Chem. Phys.*, **50**, 2069 (1969).
9) P-S. Song and W. E. Kurtin, *J. Am. Chem. Soc.*, **91**, 4892 (1969).
10) C. Cazeau Dubroca, F. Dupuy, M. Martinand, and A. Lopez Campillo, *Chem. Phys. Lett.*, **23**, 397 (1973).
11) L. J. Andrews and L. S. Forster, *Photochem. Photobiol.*, **19**, 353 (1974).
12) R. A. More O'Ferrall, "Proton Transfer Reactions," ed by E. Caldin and V. Gold, Chapman and Hall, London (1975), p. 230.
13) J. O'M. Bockris and A. K. N. Reddy, "Modern Electrochemistry," Plenum Publishing Co., New York (1970), p. 472.
14) C. J. D. von Grotthuss, *Ann. Chim.*, **58**, 54 (1806).
15) R. Pariser and R. G. Parr, *J. Chem. Phys.*, **21**, 466 (1953).
16) J. A. Pople, *Proc. Phys. Soc. Ser., A*, **68**, 81 (1955).
17) K. Nishimoto and N. Mataga, *Z. Phys. Chem. (Frankfurt am Main)*, **13**, 140 (1957).
18) V. L. Levshin, *Z. Phys.*, **32**, 307 (1925).
19) F. Perrin, *Ann. Phys.*, **12**, 169 (1926).
20) J. W. Daly and B. Witkop, *J. Am. Chem. Soc.*, **89**, 1032 (1967).
21) T. Sakaki, A. Wakahara, T. Fujiwara, and K. Tomita, *Bull. Chem. Soc. Jpn.*, **48**, 536 (1975).
22) D. A. Chignell and W. B. Gratzer, *J. Phys. Chem.*, **72**, 2934 (1968).
23) E. M. Strickland, J. Horwitz, and C. Billups, *Biochemistry*, **9**, 4914 (1970).
24) Y. Yamamoto and J. Tanaka, *Bull. Chem. Soc. Jpn.*, **45**, 1362 (1972).