Phosphorescence of Pyridoxal*

JOHN E. WAMPLER AND JORGE E. CHURCHICH

From the Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37916

SUMMARY

The effect of pH on the relative distribution of pyridoxal structures in aqueous solution was examined by fluorescence spectroscopy. The hemiacetal structure, which is the predominant species at neutral pH, exhibits a symmetrical emission band at 378 nm and is characterized by a quantum yield of 0.08. Luminescence studies at low temperature (77°K) have permitted the analysis of the phosphorescence properties of pyridoxal. The long lived emission of pyridoxal (phosphorescence lifetime, 1.1 sec) originates in a triplet state (π → π* transition) and shows a structureless phosphorescence band (λmax at 420 nm). An energy level diagram for pyridoxal is proposed, and the possible role of pyridoxal as a sensitizer in photobiological processes is discussed.

The relative distribution of the structures of pyridoxal in aqueous solution has been investigated by spectrophotometric and spectrofluorometric methods in several laboratories (1-5). In spite of these spectroscopic studies, little is known about the luminescence properties of pyridoxal at low temperature (77°), which are important in understanding the nature of the electronic transitions. The work reported herein is part of an extensive experimental study designed to define the luminescence parameters of pyridoxal and to explain the origin of the fluorescence and phosphorescence normally observed at room temperature and liquid nitrogen temperature, respectively. Experimental studies on the luminescence of free and bound pyridoxal 5-phosphate are also presented; the results obtained have direct bearing on the question of events occurring at the catalytic site of the enzyme, aspartate aminotransferase.

EXPERIMENTAL PROCEDURE

Methods Luminescence spectra were recorded in an instrument designed in our laboratory. Radiation from a 150-watt xenon lamp (Hanovia) was passed through a 500-mm Bausch and Lomb monochromator (blazed at 300 nm; dispersion, 3.3 nm per mm) and focused onto the cell compartment. The luminescence was observed at right angles to the exciting beam with the use of a 500-mm Bausch and Lomb monochromator (blazed at 300 nm; dispersion, 3.3 nm per mm) and detected by an EM1 62568 photomultiplier tube. The signal was amplified and fed to the Y axis input of a Moseley X-Y recorder (model 135AM). The X axis of the recorder was coupled to the wavelength drive of the analyzing monochromator. Luminescence measurements at liquid nitrogen temperature were conducted in a Dewar housing assembly adapted for observations in our spectrofluorometer. A rotating shutter (Aminco) was used to examine the phosphorescence properties of the samples. The decay of phosphorescence was recorded on a Tektronix 532 oscilloscope. The standard error in a group of phosphorescence decay measurements was 0.1 sec.

Calibration of the exciting light source and detector system of the spectrofluorometer was carried out as described previously (6). Quantum yields of fluorescence were determined according to the method of Parker and Rees (7), with standards of known quantum yield, such as salicylic acid and fluorescein (8). Quantum yields of phosphorescence were determined by comparing the area of the phosphorescence spectra with that from a solution of benzophenone made up to an equal absorbance at the exciting wavelength. The quantum yield of benzophenone is 0.85 (9). All of the luminescence spectra reported were obtained at a band width of 2 nm. Polarization of fluorescence was measured with a photometer built in our laboratory (10).

Materials—Pyridoxal and pyridoxal 5-phosphate were purchased from Sigma. Deoxypyridoxal was provided by Professor D. A. Metzler of Iowa State University. The reagents used in the experiments were of analytical grade and the solvents (ethanol and glycerol), of spectroscopic grade.

The enzyme, aspartate aminotransferase, was purified from pig heart according to the method of Martinez-Carrion et al. (11). After chromatography through carboxymethyl Sephadex C-50, four cytoplasmic subforms were isolated. The fractions of greatest specific activity, Fractions III and IV, were used throughout the fluorometric experiments described.

RESULTS

Fluorescence of Pyridoxal—The hemiacetal form of pyridoxal (dipolar ion) shows a symmetrical emission band (378 nm) when excited at the strongest absorption band (318 nm) (Fig. 1). The shape and the band position of the emission spectrum are not affected by a decrease in the pH of the solution to pH 1, whereas the absorption band is shifted towards shorter wave lengths as
Phosphorescence of Pyridoxal

Vol. 244, No. 6

1478

Fig. 1. Absorption (---) and emission (- - - -) spectra of pyridoxal (hemiacetal form) at 10^{-4} and 10^{-5} M, respectively. The exciting wave length for the emission spectrum was 317 mp. Experiments were conducted in aqueous solution (pH 7) at 20°C.

TABLE I

<table>
<thead>
<tr>
<th>Fluorescence properties of pyridoxal</th>
</tr>
</thead>
<tbody>
<tr>
<td>solvent</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>pH 1</td>
</tr>
<tr>
<td>pH 7</td>
</tr>
<tr>
<td>pH 9</td>
</tr>
<tr>
<td>Ethanol-water, 95:5</td>
</tr>
<tr>
<td>Glycerol-buffer, pH 7</td>
</tr>
<tr>
<td>50:50</td>
</tr>
<tr>
<td>70:30</td>
</tr>
<tr>
<td>90:10</td>
</tr>
</tbody>
</table>

shown in Table I. This behavior is characteristic of several pyridoxal derivatives, and seems to indicate that excited state dissociation of the phenolic group occurs within the fluorescence lifetime of the excited state (12). At pH values higher than 9, the dissociation of the proton from the heterocyclic nitrogen atom yields several species in solution, some of which are distinguished by spectrophotometric methods (4). Fluorescence measurements, on the other hand, showed the presence of two distinctly different emission bands. The major emission band (360 mp), which was also detected by Morozov et al. (5), corresponds to the hemiacetal form of pyridoxal (anion). A less intense fluorescence band, amounting to only 5% of the 360 mp band, was observed in the spectral region, 450 to 540 mp. This minor fluorescence band, obtained by excitation at 400 mp, may be tentatively assigned to the free aldehyde species in solution.

Rotational Relaxation Time of Pyridoxal—The polarization of fluorescence measurements designed to evaluate the rotational relaxation time of pyridoxal were conducted in the mixed solvent system, glycerol-phosphate buffer, pH 7, at 25°C. This solvent system is appropriate for depolarization measurements for the following reasons: (a) it makes available an almost 500-fold variation in the viscosity of the solution, and (b) the relative distribution of the species of pyridoxal in the mixed solvent system is similar to that observed in aqueous solution at pH 7, as shown by a comparison of the excitation spectra, emission spectra, and fluorescence yield values (Table I).

In his theory of depolarization of fluorescence of solutions, Perrin proposed that the rotational motion of fluorescence molecules is governed by the Brownian laws of rotation for spherical particles suspended in the solvent. Accordingly, the degree of polarization of fluorescence (P) is a function of the fluorescence lifetime (τ) and the rotational relaxation time (τR) of the fluorophor in solution (Equation 1)

\[
\frac{1}{P} - \frac{1}{3} = \left( \frac{1}{P_0} - \frac{1}{3} \right) \left( 1 + \frac{3\tau}{\tau_R} \right)
\]

(1)

for a spherical particle

\[\rho_k = \frac{3V\eta}{RT}\]

where η represents viscosity; T, absolute temperature; V, the volume of the fluorophor; and R, the gas constant.

Fig. 2. Polarization of fluorescence (at 25°C) of 10^{-4} M pyridoxal in glycerol-buffer (pH 7) solutions of different compositions. 1/P - 1/3 is plotted against T/τ for solutions excited at 320 mp. The emitted light was passed through a Corning CS3-74 glass filter.

Fig. 3. Fluorescence (F) and phosphorescence (P) spectra of pyridoxal (---) and pyridoxal 3-phosphate (---) in ethanol-water (95:5, v/v) at 77°C. Each sample had an absorbance of 0.1 in a 1-cm cuvette at the exciting wave length (290 mp). The phosphorescence spectrum of deoxypyridoxal is also shown (---).
Fig. 2 shows the polarization results obtained in the solvent system, glycerol-buffer, when pyridoxal was excited with plane polarized light of 320 mp. The plot of $1/P - \frac{1}{4}$ against $T/\eta$ follows the linearity predicted by Equation 1; therefore the rotational relaxation time ($\rho_0$) was determined from the slope of the straight line when the value of $\tau$ (1.5 nsec) determined by Morozov et al. (5) was included in Equation 1. The rotational relaxation time ($1.2 \times 10^{-10}$ set) obtained for pyridoxal in solution is consistent with the value expected for a rigid particle.

**Table II**

*Phosphorescence yield.*

*Phosphorescence lifetime.*

Fig. 4. Phosphorescence decay of 10⁻⁴ M pyridoxal in ethanol-water (95:5, v/v) at 77° K. Log intensity is plotted against time. The wave length of excitation was 290 mp; the phosphorescence emitted at 420 mp was recorded with a Tektronix oscilloscope.

Fig. 5. Fluorescence spectra of pyridoxal (---), deoxypyridoxal (---), pyridoxal 5-phosphate (-----), and pyridoxal 5-phosphate bound to aspartate aminotransferase (Fraction IV) (-----). Experiments were conducted in 0.05 M phosphate buffer, pH 7, at 25° C with absorbances at the exciting wave length (320 mp) equal to 0.1 for 1-cm cuvettes. The areas beneath the curves are proportional to the fluorescence quantum yields. The fluorescence yield of pyridoxal 5-phosphate is lower than 0.01.

Fig. 6. Energy level diagram for pyridoxal in ethanol-water (95:5, v/v) at 77° K. $S_0$, ground state; $S_1$, singlet excited state; $T_1$, triplet state. $K_F$ and $K_P$ are the rate constants for fluorescence and phosphorescence deactivation, respectively. The rate constant for intersystem crossing between the singlet and triplet state ($K_{IS}$) is proportional to the intersystem crossing ratio, $Q_P/Q_F$.

$$K_{IS} = Q_P/Q_F \times K_F$$

having a volume of approximately 100 cm³ per mole. It should be noted that the Russian authors used a phase fluorometer in the experimental evaluation of $\tau$, and that the value of $\tau$ agrees closely with the fluorescence lifetime calculated by the equations proposed by Forster and by Strickler and Berg (13).

**Luminescence of Pyridoxal at 77° K**—Luminescence studies conducted at 77° K in rigid glasses provided valuable information on the energy levels of the singlet and triplet states of pyridoxal. At 77° K, the band position of the fluorescence spectrum is shifted toward shorter wave lengths (320 mp) when compared to the fluorescence spectrum recorded at 293° K in the mixed solvent, ethanol-water (95:5, v/v) (Table I). In
addition, the quantum yield of fluorescence of pyridoxal is increased 10-fold when the temperature is decreased from 293° K to 77° K. As shown in Fig. 3, the phosphorescence spectrum of pyridoxal peaks at 420 m and is characterized by a phosphorescence yield of 0.4. The observed phosphorescence decay is an exponential process (Fig. 4) that can be described by Equation 2

\[ I = I_0 e^{-kt} \]

where \( I \) and \( I_0 \) are the observed phosphorescence intensities at time \( t \) and at zero time, respectively. The rate constant for phosphorescence deactivation \( k \) includes the rate constants \( k_p \) and \( k_s \) corresponding to radiative and nonradiative deactivation, respectively. All the experimental results pertinent to the phosphorescence of pyridoxal are summarized in Table II. From these results it may be seen that the phosphorescence of pyridoxal in three different glasses is strictly exponential and is independent of concentration over the range, \( 5 \times 10^{-4} \) to \( 10^{-3} \) m, the highest concentration tested in these studies. This observation, taken together with the finding that the phosphorescence spectrum is structureless, suggests that only one metastable state (triplet) is involved in the long lived emission process. Similar remarks apply to pyridoxal 5-phosphate and deoxypyridoxal, which displayed phosphorescence properties similar to those of pyridoxal (Table II). Judging from the decay value \( (\tau_p = 1.1 \text{ sec}) \), we believe that the phosphorescence of these compounds originates in a \( \pi \rightarrow \pi \) (triplet) transition. In this context, it is worthy of note that the existence of one triplet state in pyridoxal was recently confirmed by electron spin resonance measurements conducted at 77° K in rigid glasses (14). Thus, the conclusions drawn from phosphorescence measurements are in agreement with the electron spin resonance measurements.

**DISCUSSION**

The present studies have shown that the emission spectra of pyridoxal remains virtually unchanged from about pH 1 to pH 7, indicating that dissociation of the phenolic group occurs within the fluorescence lifetime of the excited state \( (\tau_p, 1.5 \text{ nsec}) \). Thus, the predominant emitting structure in this pH range is the internal hemiacetal form of pyridoxal (dipolar ion). The dissociation of the proton from the heterocyclic nitrogen atom at alkaline pH brings about a change in the absorption spectrum, which is accompanied by a blue shift in the emission spectrum. Although the spectrophotometric analysis clearly shows the existence of several structures in mutual equilibrium, the fluorometric studies revealed that most of the emission detected at alkaline pH (pH 9) is due to the hemiacetal form of pyridoxal (anion).

The fluorescence characteristics of pyridoxal 5-phosphate were examined in aqueous solution at various pH values (Fig. 5). This cofactor shows a broad, structureless emission band characterized by an abnormally low fluorescence yield. Similar behavior was displayed by pyridoxal 5-phosphate linked to the enzyme, aspartate aminotransferase, suggesting that the lifetime of this cofactor is considerably shorter than that of pyridoxal.

If this is indeed the case, then pyridoxal 5-phosphate cannot be used as a chroomophor in experiments designed to evaluate the rotational relaxation time of the macromolecule by means of polarization of fluorescence. In spite of the abnormally low fluorescence yield of pyridoxal 5-phosphate, it has been reported that small changes in the polarization of fluorescence may be related to an increase in the degree of mobility of the cofactor bound to the enzyme, aspartate aminotransferase (15).

The luminescence studies of pyridoxal in rigid glasses at 77° K have shown that the kinetics of phosphorescence decay is strictly exponential. This finding, taken together with the observation that the phosphorescence spectrum is structureless, suggests that only one triplet state is involved in the phosphorescence emission. The existence of one metastable state facilitates the interpretation of the phosphorescence measurements, since one can disregard competitive bimolecular processes which are known to be responsible for deviations from exponential kinetics. On the basis of the luminescence results obtained at low temperature (77° K), the energy level diagram shown in Fig. 6 is proposed for pyridoxal.

The most salient features of this energy level diagram are (a) the singlet and triplet excited states are well separated; the energy splitting is of the order of 21 kcal per mole; (b) the intersystem crossing process is fast enough to complete with the fluorescence emission that originates in the singlet excited state \( (K_{16} = 8 \times 10^{-9} \text{ sec}) \); and (c) the long lived phosphorescence emission originates in a \( \pi \rightarrow \pi \) (triplet) transition, since the decay time is 1.1 sec. Since pyridoxal is characterized by a long phosphorescence decay, it is likely that this compound participates in photochemical reactions of biological interest.

**REFERENCES**

Phosphorescence of Pyridoxal
John E. Wampler and Jorge E. Churchich

J. Biol. Chem. 1969, 244:1477-1480.

Access the most updated version of this article at http://www.jbc.org/content/244/6/1477

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/244/6/1477.full.html#ref-list-1