THE TRIPLET STATE OF PYRIDOXAL*

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The absorption spectra, as well as the relative distribution of the structures of pyridoxal in aqueous solution, have been investigated in several laboratories (Williams and Neilands, 1954; Metzler and Snell, 1955; Anderson and Martell, 1964). In spite of these spectrophotometric studies, little is known about the luminescence properties of pyridoxal at low temperature (77° K), such as fluorescence and phosphorescence. It is the purpose of this note to show that the phosphorescence of pyridoxal originates in a triplet state which is also detected by esr spectroscopy. Evidence is presented that electronic excitation energy is transferred from the triplet state of pyridoxal to the singlet state of L-kynurenine.

Methods.

Luminescence measurements at 77° K were conducted in a Dewar Housing assembly adopted for observations in our spectrofluorometer (Churchich, 1967). A rotating shutter (Aminco) was used when it was desired to examine the phosphorescence properties of the samples. The decay of phosphorescence was recorded on a Tektronix 532 oscilloscope and the most probable error in a group of phosphorescence decay measurements was 0.1 second. Benzophenone was used as a standard for quantum yield calculations (Giltmore *et al.*, 1955).

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The esr measurements were made with a Varian V 4502-12 spectrometer operating at 9.235 GHz. Illumination was provided by 500 watt high pressure mercury arc (Philips) and the wavelengths selected by a 500 mm Bausch and Lomb monochromator. Further details on this apparatus are given by Ten Bosch *et al.*, 1968. Low temperature absorption spectra were obtained on a Perkin-Elmer Model 450 spectrophotometer.

Table I

Luminescence characteristics of pyridoxal in the mixed solvent system ethanol-water (95:5, v:v) at 293° K and 77° K. T is the absolute temperature, λexcit. is the exciting wavelength, λ_f is the maximum of fluorescence emission, Q_f is the quantum yield of fluorescence, λ_p is the maximum of phosphorescence emission, Q_p is the quantum yield of phosphorescence, and τ_p is the phosphorescence lifetime.

Conc. of Pyridoxal	T	λexcit. (mμ)	λf(mμ)	Q_f	λp(mμ)	Q_p	τ_p
5×10^{-5} *M*	293	290	330	0.03			
5×10^{-5}	77	290	315	0.40	420	0.43	1.2
10^{-3}	77	290	315		420		1.2

Results and Discussion

Fluorescence studies of pyridoxal in the mixed solvent system ethanol-water (95:5, v:v) were conducted at 293° K and 77° K. Table I shows the fluorescence characteristics of pyridoxal at the temperatures examined. At 293° K, the emission spectrum shows a maximum at 330 mμ which corresponds to the absorption maximum at 290 mμ. At 70° K, the band position of the emission spectrum is shifted towards shorter wavelengths and the fluorescence yield is ten-fold increased. When the phosphorescence spectrum of pyridoxal was examined (Fig. 1), it was found that the long lived emission band is devoid of vibrational structure and exhibits a maximum at 420 mμ. The observed phosphorescence decay is an

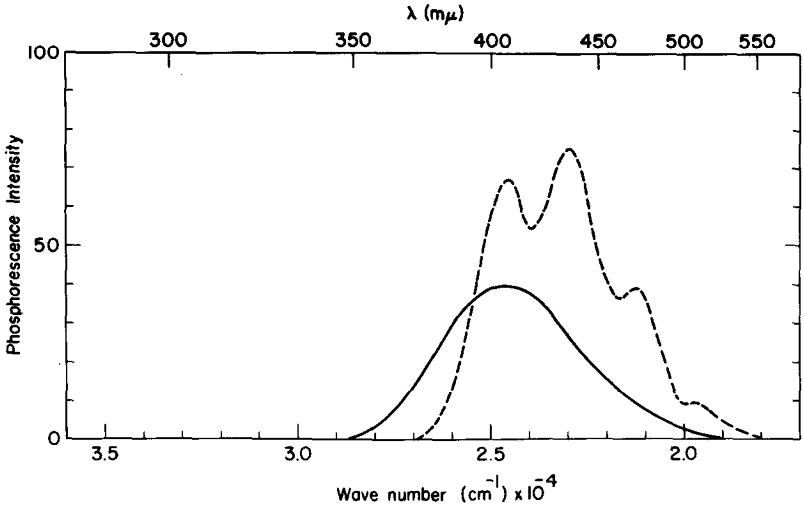

Fig. 1. Phosphorescence spectra of pyridoxal, ————, and benzophenone, – – – – –, in ethanol-water glass (95:5, v:v) at 77° K. Both samples had an absorbancy of 0.10 in 1 cm. cuvettes at the exciting wavelength (290 mμ).

exponential process that can be described by the expression:

$$I = I_o e^{-kt}$$

where I is the phosphorescence intensity at time t and k is the rate constant for phosphorescence deactivation.

The kinetics of phosphorescence decay is strictly exponential and the lifetime (τ_p = 1.2 seconds) is concentration independent over the range 5×10^{-5} M to 10^{-3} M, the highest concentration tested. This observation taken together with the finding that the phosphorescence spectrum is structureless suggests that only one metastable state is involved in the long lived emission process. Hence, one can disregard competitive bimolecular processes which are known to be responsible for deviations from exponential kinetics. On the basis of these luminescence studies, the following arrangement of energy levels is proposed for pyridoxal: (1) a lowest singlet excited state (fluorescence) corresponding to an energy of 90 Kcal/mole and (2) a triplet excited state characterized by an energy of 69 Kcal/mole. The phosphorescence originates in this triplet state ($\pi \rightarrow \pi^*$ transition).

The triplet state of pyridoxal was also characterized by electron spin resonance. Thus the triplet state of pyridoxal yields an esr signal corresponding to $\Delta m = \pm 2$ transitions at 1427 gauss in ethanol water (95:5, v:v) at 77° K. The variation of the amplitude of the esr signal for pyridoxal as a function of the excitation wavelength is given in Fig. 2, where it may be seen that the wavelength dependence of the esr signal strongly resembles the absorption spectrum at 77° K (Fig. 2).. The energy level of the triplet state (69 Kcal/mole) as well as the long phosphorescence lifetime (τ_p = 1.2 seconds) suggest that the electronic excitation energy of pyridoxal can be transferred

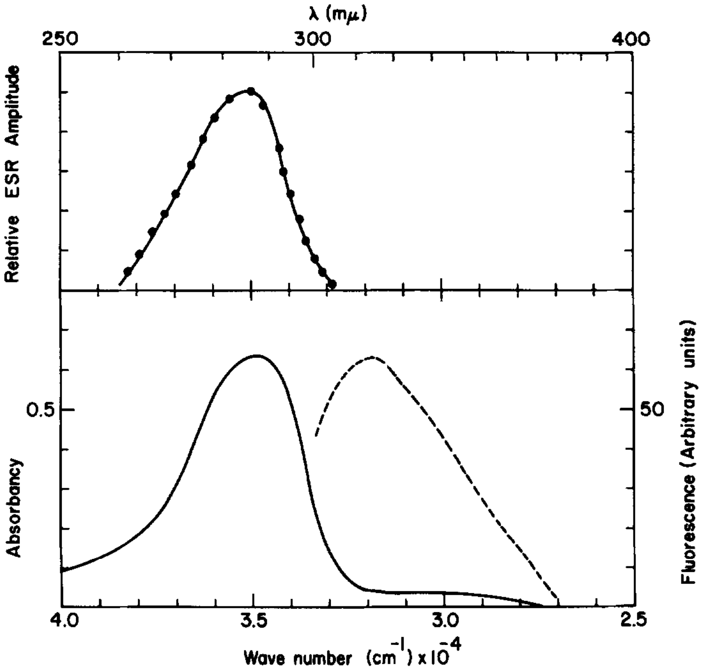

Fig. 2. (Top) Triplet esr signal intensity at 1427 gauss for pyridoxal (10^{-4} M) as a function of excitation wavelength at 77° K.

(Bottom) Absorption spectrum, ————, and fluorescence spectrum (exciting at 290 mμ), — — — — —, of 10^{-4} M pyridoxal in ethanol-water (95:5, v:v) glass at 77° K.

to a suitable acceptor. To test this hypothesis, the mixture pyridoxal-L-kynurenine was chosen for the luminescence studies. L-kynurenine displays an absorption yield of the same order of magnitude as pyridoxal at 77° K, but it has very low absorbancy at 290 mμ. Pyridoxal, on the other hand, shows a maximum of absorption at 290 mμ and a phosphorescence maximum at 420 mμ. When the mixture pyridoxal (2×10^{-4} M), L-kynurenine (5×10^{-4} M) was excited at 290 mμ, and the luminescence recorded over the spectral range 300-550 mμ, it was found that while the phosphorescence of pyridoxal is decreased, a strong emission band was detected in the spectral region coinciding with the fluorescence of L-kynurenine. In addition, the phosphorescence lifetime of pyridoxal was decreased as the concentration of L-kynurenine was increased (Table II).

Table II

Energy transfer from pyridoxal to L-kynurenine
The phosphorescence lifetime of pyridoxal (τ_p)
was measured at 390 mμ with excitations at
290 mμ.

Pyridoxal Concentration	L-Kynurenine Concentration	τ_p (seconds)
2×10^{-4} M	0	1.2
2×10^{-4} M	3×10^{-4} M	1.0
2×10^{-4} M	5×10^{-4} M	0.8
2×10^{-4} M	6×10^{-4} M	0.7

These experiments seem to indicate that a radiationless transfer mechanism (triplet-singlet) is operative, since the extensive overlap between the phosphorescence spectrum of the donor (pyridoxal) and the absorption spectrum of the acceptor (L-kynurenine) meets the requirements of Forster's theory of radiationless energy transfer (Forster, 1959). The possibility that the efficiency of energy transfer is enhanced by complex formation between pyridoxal and L-kynurenine is being investigated in our laboratory.

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