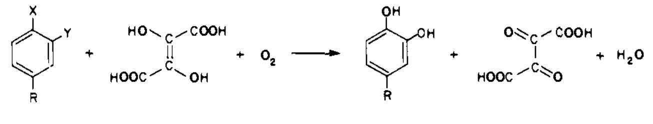
Scheme I. Peroxidase-Catalyzed Hydroxylation of L-Tyrosine (I), D-(−)-*p*-Hydroxyphenylglycine (II), and L-Phenylephrine (III) in the Presence of Oxygen and Dihydroxyfumaric Acid



I: R = $CH_2CH(NH_2)COOH$, X = OH, Y = H

II: R = $CH(NH_2)COOH$, X = OH, Y = H

III: R = $CH(OH)CH_2NHCH_3$, X = H, Y = OH

hydroxyphenylglycine (II), and L-epinephrine (adrenaline)[9] from L-(−)-phenylephrine (III).

An Erlenmeyer flask was filled with 1 L of a 2 mM solution of I, II, or III in 60 mM of acetate buffer (pH 5.0). This system was cooled to 0 °C and then 4 mmol of dihydroxyfumaric acid and 0.5 mg of horse radish peroxidase were added. Oxygen was bubbled through the solution and the reaction was carried out at 0 °C with vigorous stirring. Four millimoles of dihydroxyfumaric acid were added after 1 h and again after another hour; 3 h after beginning the process the reaction was terminated by acidification (in the case of III, dihydroxyfumaric acid was added every 30 min and the reaction was terminated after 1.5 h). Both consumption of I, II, or III and production of their dihydroxylated successors were measured simultaneously during the syntheses by using specific colorimetric assays.[10] After 3 h, 1.4–1.5 mmol of L-DOPA and D-(−)-3,4-dihydroxyphenylglycine were produced and 0.5–0.6 mmoles of I and II, respectively, remained. In the case of epinephrine, 0.8–1.0 mmol were produced after 1.5 h and 1.0–1.2 mmoles of unreacted III remained.

In each of the syntheses, the sum of the amounts of the substrate and the product was, within experimental error, equal to the initial amount of the substrate. This implies that no byproducts were formed. In agreement with this conclusion, no other aromatic products were detected by paper chromatography for the first two syntheses and by silica gel chromatography for the third one.

The resulting mixtures of the substrate, product, and other components in each of the three syntheses were separated by chromatography on a Dowex-50-4X (200–400 mesh) H^+ form column, followed by elution with 0.7 N HCl. The L-DOPA, D-(−)-3,4-dihydroxyphenylglycine and L-epinephrine produced were positively identified by comparison of their ultraviolet spectra, R_f values in paper and silica gel chromatography,[11] and retention times in ion-exchange chromatography with those of the authentic samples. The specific optical rotations of the compounds in 0.7 N HCl were −11, −99, and −47° as compared with −11 and −50° for the authentic samples of the first and third compounds, respectively (for the second one it is not known). That is, no racemization has occurred during the enzymatic hydroxylation.

It is critical that the enzymatic synthesis be carried out at 0 °C since at room temperature in the case of I some other hydroxylated tyrosines in addition to L-DOPA are formed. We have found that at 0 °C in the absence of peroxidase no hydroxylation of tyrosine takes place. However, at room temperature this is not the case: dihydroxyfumaric acid and O_2 hydroxylate tyrosine with a rate which is as high as 30–50% of that in the presence of the enzyme. It is conceivable that this nonenzymatic (and therefore probably nonspecific) hydroxylation may result in the formation of several hydroxylated species.

Using the peroxidase-catalyzed hydroxylation of L-tyrosine we have found that (i) replacement of O_2 with air results in slowing down of the reaction, (ii) an increase in the reaction time (beyond 3 h) or the concentration of the enzyme or dihydroxyfumaric acid does not appreciably increase the rate of the enzymatic process, (iii) a reduction in the dihydroxyfumaric acid concentration results in a decreased rate of L-DOPA formation, and (iv) the pH optimum of the enzymatic reaction is at pH 5.

We have discovered that two other peroxidases, lactoperoxidase from cow milk and cytochrome *c* peroxidase from yeast,[12] are also capable of hydroxylating L-tyrosine to L-DOPA in accordance with Scheme I.

It is hoped that the simple and efficient enzymatic hyroxylation described herein could be useful for preparative transformations of various pharmaceuticals and fine chemicals.

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[9] For various clinical uses of epinephrine, see: McLean, J. R. In "Encyclopedia of Chemical Technology", 3rd ed.; Wiley: New York, 1980; Vol. 9, pp 241–250.

[10] Arnow, L. E. *J. Biol. Chem.* **1937**, *118*, 531–537. Waite, J. H.; Tanzer, M. L. *Anal. Biochem.* **1981**, *111*, 131–136. The assay for the dihydroxylated compounds is not sensitive to the corresponding monohydroxylated compounds and *vice versa*. Neither assay has been found to be capable of detecting a trihydroxylated compound, e.g., 3,4,6-trihydroxyphenylalanine (obtained from Sigma).

[11] Kirchner, J. G. "Thin-Layer Chromatography", 2nd ed.; Wiley: New York, 1978.

[12] A sample of yeast cytochrome *c* peroxidase, purified to homogeneity and crystallized, was kindly provided by Dr. Thomas Poulos from Professor Joseph Kraut's laboratory at the University of California, San Diego.

Anomalous Dipole Moments of Photoreactive Triplet States

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The photoreduction of carbonyl compounds is among the most extensively studied photoreactions.[1] These reactions are believed to proceed primarily via a $\pi^* \leftarrow n$ excitation whereupon the electron density at the carbonyl oxygen is depleted.[2] In aromatic carbonyls the rates of intersystem crossing and internal conversion are fast compared to the rate of reaction; consequently, the reactive state is the lowest triplet state.[2,3] The reactivity of systems having a lowest triplet state, T_1, which is $\pi\pi^*$ in character has been attributed to the presence of a triplet $n\pi^*$ state, T_2, at a slightly higher energy.[4] 2,4,5-Trimethylbenzaldehyde (TMBA) is thought to belong to this class of molecules.[5]

Many spectroscopic studies aimed at locating and characterizing the triplet states of photochemically active aromatic carbonyls have been of solid-phase samples at low temperature, while the reactions of these molecules have been studied in liquid solution at room temperature. TMBA dissolved in durene crystals (D), however, can be photoreduced[6] under conditions similar to those required for high-resolution spectroscopic investigation. In order to gain more detailed information about the triplet states of aromatic carbonyl compounds, we have obtained the laser-induced phosphorescence excitation spectrum of TMBA isolated in a durene single crystal (TMBA/D) at 25 K and have measured the effect of an electric field (Stark splitting) on several of the bands with the sample at 4.2 K. We have found, as have others for TMBA and similar compounds,[7] that the relative intensities of the bands in the excitation spectrum indicate the presence of a triplet $n\pi^*$ state near T_1, but the results of the electric field

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[1] Turro, N. J. "Modern Molecular Photochemistry", Benjamin Cummings: Menlo Park, CA, 1978; pp 362–409.

[2] Walling, C.; Gibian, M. J. *J. Am. Chem. Soc.* **1965**, *87*, 3361.

[3] Wilkinson, F. "Organic Molecular Photophysics"; Birks, J. B., Ed.; Wiley: New York, 1975; Vol. 2; pp 144–145.

[4] (a) Wagner, P. J. *Acc. Chem. Res.* **1971**, *4*, 168. (b) Mao, S. W.; Hirota, N. *Mol. Phys.* **1974**, *27*, 327.

[5] Migirdicyan, E. *Chem. Phys. Lett.* **1972**, *12*, 473 and cited references.

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[7] (a) Chakrabarti, A.; Sheng, S. J., unpublished results. (b) Hayashi, H.; Nagakura, S. *Mol. Phys.* **1974**, *27*, 969.

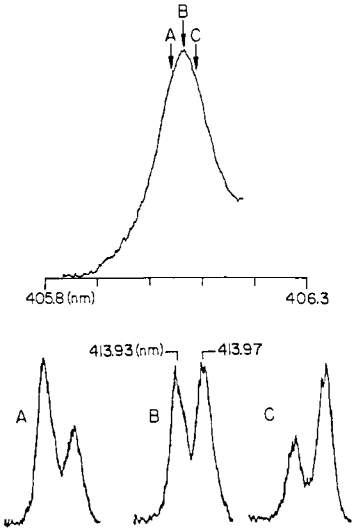

Figure 1. The upper band is the most intense feature in the region of the laser-induced phosphorescence excitation spectrum thought to contain the second triplet state of 2,4,5-trimethylbenzaldehyde isolated in a durene host crystal. The background is not shown. Spectra A, B, and C at the bottom are scans of the Stark split origin of the $T_1(\pi\pi^*)$ phosphorescence. The wavelength region for all three spectra are as indicated for spectrum B. These doublets are obtained when the laser is tuned to the corresponding wavelengths indicated on the excitation spectrum. The ratio of the peak intensities of the Stark components, measured for each doublet, are fitted to a model of the Stark splitting of the excitation band as described in the text. For the example shown, a field of 71 kV/cm was applied along the *b* axis of the crystal. Note that when the laser excites the long wavelength side (C) of the excitation band, the long wavelength component of the phosphorescence doublet is more intense, whereas the short wavelength component is more intense when the laser excites at the shorter wavelength (A). This observation demonstrates that the sign of the $\Delta\mu$ vectors associated with the excitation and phosphorescent transitions are the same along the *b* axis of the crystal.[8] Measurements, with the same result, were made along the other two orthogonal crystal axes as well.

measurements are quite different from those expected[8] for this state.

The details of sample preparation and the method for obtaining the phosphorescence excitation spectrum are discussed elsewhere.[9] Because the widths of the most intense bands in the excitation spectrum, those assigned to T_2, were large compared to their Stark splittings, a selective excitation method was developed to obtain the unresolved Stark splitting. With the electric field applied to the sample, the laser was tuned to various wavelengths along the broad line. At each excitation wavelength, the emission from the sharp phosphorescence origin was recorded. The Stark doublets thus obtained had varying degrees of asymmetry depending on the Stark splitting of the absorption band and the laser wavelength, as shown in Figure 1. In the absence of energy transfer and electric field effects on relaxation rates, the relative intensities of the Stark components seen in emission should be equal to the ratio of the absorption coefficients of the two unresolved Stark components at the laser wavelength. Ratios of absorption coefficients obtained with the Stark splitting as an adjustable parameter were fit to the experimentally determined phosphorescence intensity ratios. The dipole moment difference vector, $\Delta\mu = \mu$ (excited) $- \mu$ (ground), then was obtained from the relation $\Delta S = 2\Delta\mu\cdot\mathbf{F}$ where $\mathbf{F}$ is the electric field at the molecule. This field was taken to be the anisotropic Lorentz field.[10]

The zero-field phosphorescence excitation spectrum contains four relatively weak bands to the blue of the weak $T_1(\pi\pi^*)$ origin (413.96 nm) followed by an intense band at 406.30 nm and a more intense band at 406.07 nm. Although the appearance of the spectrum is what one might predict for an aromatic carbonyl having a $T_1(\pi\pi^*)$ state near a higher lying T_2 $(n\pi^*)$ state,[5] the electric field measurements described above reveal that the dipole moment difference vector associated with the most intense band (at 406.07 nm) in the region previously assigned[5] to the T_2 $(n\pi^*)$state, is the same in sign and magnitude, within the measurement uncertainty ($\sim$10%), as that for the $T_1(\pi\pi^*)$ origin band (+ 1.1 D). The $\Delta\mu$ vector for a spectroscopic transition reflects the magnitude and direction of the charge redistribution upon excitation. One therefore expects the $\Delta\mu$ vector for a $\pi^* \leftarrow n$ transition to be quite different from that for a $\pi^* \leftarrow \pi$ transition as verified by Udagawa and Hanson.[8]

In the absence of additional information, a possible explanation of this observation is that the intense feature at 406.07 nm is a vibronic level of the $T_1(\pi\pi^*)$ electronic state. The observation that the dipole moment of this level and the origin are the same then means that there are no vibronic coupling effects that affect the dipole moment for this level, but the intensity of this band in the phosphorescence excitation spectrum would seem to be derived from a large vibronic spin–orbit coupling interaction. This explanation, however, does not fit with the extensive correlations[11] of the difference in energy between the $T_1(\pi\pi^*)$ and $T_2(n\pi^*)$ states of several benzaldehye derivatives, including TMBA, with the radiative and nonradiative transition rates and fine structure splittings of the lowest $T_1(\pi\pi^*)$ states of these molecules. In many cases this energy gap was determined from the difference between the T_1 origin and the first intense feature in the phosphorescence excitation spectrum. Changes in this energy difference of 100–600 cm^{-1} from compound to compound are characteristic of a different electronic state rather than a vibrational level. Since TMBA follows the observed energy gap correlations,[11] and since there are no other nearby intense features in the phosphorescence excitation spectrum, one seems forced to conclude that the intense absorption stems from an $n\pi^*$ state in TMBA as well.

We therefore need a description of this state that leads to a dipole moment characteristic of a $\pi\pi^*$ state. Direct electronic coupling between the triplet $n\pi^*$ and $\pi\pi^*$ states fails since this interaction is thought to be small,[11] resulting in mixed states that are primarily $n\pi^*$ (T_2) and $\pi\pi^*$ (T_1). Vibronic coupling of the $n\pi^*$ origin with a single nearby vibronic level of the triplet $\pi\pi^*$ state, as considered by Goodman and Özkan,[12] also is not sufficient. Even an equal mixture of the two results in a dipole moment difference vector pointing in the same direction as that for a pure $n\pi^*$ state, although the magnitude will be quite different.[12,13] On the other hand, if we consider the $n\pi^*$ state to be coupled to a number of closeby vibronic levels of T_1, its character may be sufficiently diluted such that the dipole moment one measures is that of a $\pi\pi^*$ state. It is also possible that a description of the higher excited states of TMBA in terms of a single simple orbital configuration or even a small number of configurations is inadequate.

In summary, we have found, in the laser-induced phosphorescence excitation spectrum of TMBA, intense bands in the region assigned to the photochemically important $T_2(n\pi^*)$ state; however, within the measurement uncertainties, the dipole moment difference vector associated with the most intense band in this region is the same as that for the $T_1(\pi\pi^*)$ origin. Questions remain as to the source of this anomalous result and its relationship to an "electron-deficient oxygen" thought to be needed for the photoreduction reactions of aromatic carbonyls.

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