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Spectral Properties of Schiff Bases of Amino Acid Esters with Pyridoxal and Pyridoxal *N*-Methochloride in Ethanol*

LaVerne Schirch and R. Arden Slotter

ABSTRACT: The reaction of amino acid esters with pyridoxal and pyridoxal *N*-methochloride in ethanol has been studied. The spectra of the imines formed with glycine butyl ester were found to be similar to those formed with amino acids in aqueous solutions. However, a compound was formed between diethyl aminomalonate and pyridoxal *N*-methochloride that did not behave as a typical imine.

Some of the differences which were observed are noted as follows. The compound exhibited an absorption peak at 480 mμ with an ε of at least 40,000. Upon the addition of an excess of a strong base the 480-mμ peak did not shift to a shorter wavelength. The compound disappeared in a few hours at room temperature. Sodium methoxide accelerated both the formation and disappearance of the compound. The possible relation of the data to the mechanism of pyridoxal catalysis was discussed.

The study of the interaction of pyridoxal with amino acids in nonenzymatic systems has been helpful in understanding the role of this coenzyme in enzymatic reactions. However, there is at least one notable difference between the enzymatic and nonenzymatic systems. Several B_6 enzymes have been found to form enzyme–substrate complexes which absorb near 495 mμ (Jenkins, 1961a,b; Schirch and Mason, 1963; Marino and Snell, 1965). Compounds formed between pyridoxal and amino acids in nonenzymatic systems have not been found to absorb at wavelengths in this region of the spectrum. The evidence available from the enzymatic studies indicates that the enzyme–substrate complexes which absorb near 495 mμ are due to a compound in which the α carbon of the amino acid has lost a proton (Schirch and Jenkins, 1964). This structure was postulated by Metzler *et al.* (1954) to be the key intermediate in nonenzymatic transamination. Since the intermediate is a carbanion, one would expect to observe its existence in nonenzymatic systems only in very weakly acidic solvents. Little work, however, has been done with nonenzymatic systems in solvents other than water. Matsuo (1957) reported the spectrum of several Schiff bases of pyridoxal and amino acids in ethanol and showed that transamination occurred at room temperature and in the absence of metal ions. The lack of additional data in nonpolar solvents suggests that this is a fruitful area for further investigation. The purpose of this paper is to study the spectral properties of Schiff bases of amino acid esters with pyridoxal and pyridoxal *N*-methochloride in absolute ethanol. A compound with several unique spectral properties was found to be formed between the amino acid ester diethyl aminomalonate and pyridoxal *N*-methochloride. The possible significance of this compound is discussed.

Experimental Section

Materials. Pyridoxal hydrochloride was purchased from the Sigma Chemical Co. Diethyl aminomalonate was synthesized by the method of Hartung *et al.* (1960) and purchased from the Aldrich Chemical Co. Glycine butyl ester hydrochloride was obtained from Nutritional Biochemicals Corp. and glycine ethyl ester

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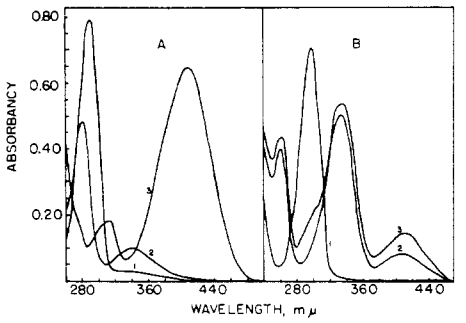


FIGURE 1: Spectra of pyridoxal and pyridoxal *N*-methochloride in acidic, neutral, and basic solutions of absolute alcohol. (A) Spectra of 1×10^{-4} M pyridoxal hydrochloride with the following concentrations of sodium methoxide: curve 1, 0; curve 2, 1×10^{-4} M; curve 3, 1×10^{-3} M. (B) Spectra of 1×10^{-4} M pyridoxal *N*-methochloride with the following concentrations of sodium methoxide: curve 1, 0; curve 2, 1×10^{-4} M; curve 3, 1×10^{-3} M.

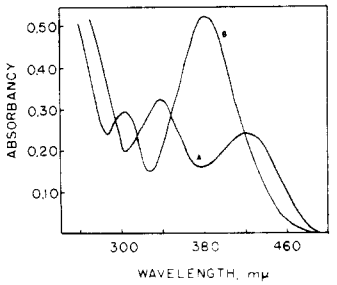


FIGURE 2: Spectra of ethanol solutions of 1×10^{-4} M pyridoxal hydrochloride, 3.3×10^{-3} M glycine butyl ester hydrochloride, and sodium methoxide (curve A, 4×10^{-3} M; curve B, 6.6×10^{-3} M).

hydrochloride from the Aldrich Chemical Co. Absolute alcohol was purchased from United States Industrial Chemical Co. Sodium methoxide was obtained from the Mathieson Chemical Corp. Pyridoxal *N*-methochloride was a gift from Dr. David Metzler.

Methods. Spectrophotometric measurements were made with a Beckman DB spectrophotometer connected to a Sargent SRL recorder. Constant temperature, 30 ± 0.2°, was maintained by a circulating water bath. All spectra were obtained with solutions in glass-stoppered cuvets with a 1-cm path length.

Reactions in which the disappearance of pyridoxal or pyridoxal *N*-methochloride was to be determined were carried out in stoppered test tubes under a nitrogen atmosphere and in the dark. Stock solutions of diethyl aminomalonate, pyridoxal hydrochloride, pyridoxal *N*-methochloride, and sodium methoxide were prepared fresh in oxygen-free absolute alcohol or water prior to each experiment. The reactions were usually started

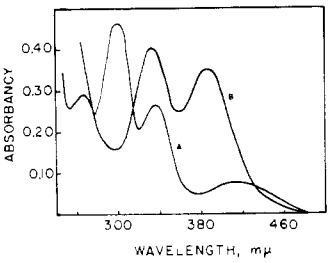


FIGURE 3: Spectra of ethanol solutions of 1×10^{-4} M pyridoxal *N*-methochloride, 3.3×10^{-3} M glycine butyl ester hydrochloride, and sodium methoxide (curve A, 4×10^{-3} M; curve B, 6.6×10^{-3} M).

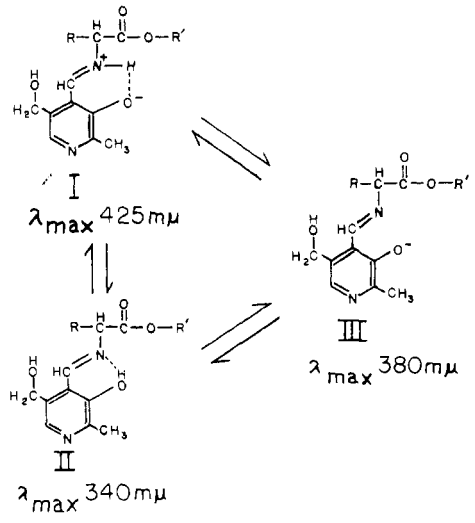
by the addition of sodium methoxide. At various time intervals 0.5-ml aliquots were removed and added to 2.5 ml of the phenylhydrazine reagent described by Wada and Snell (1961). The phenylhydrazones were formed by heating in a water bath at 60° for 20 min. The solutions were allowed to cool to room temperature before determining their absorbance at 410 mμ.

Diethyl aminomalonate was found to form a compound in absolute alcohol which reacted with phenylhydrazine to form another compound which exhibited an absorption maximum at 355 mμ. In alkaline solutions this compound was formed in such large amounts that it masked the 410-mμ peaks of the phenylhydrazones of pyridoxal and pyridoxal *N*-methochloride.

Results

The spectra of pyridoxal and pyridoxal *N*-methochloride in acidic, neutral, and basic ethanol solutions are shown in Figure 1. Comparing these with those previously reported for aqueous solutions (Johnston *et al.*, 1963; Metzler and Snell, 1955), one observes a 5–10-mμ shift of the absorption peaks to a longer wavelength and a greater absorption at 400 mμ in the neutral and basic solutions. The latter difference indicates that in alcohol the pyridoxal and pyridoxal *N*-methochloride exist as the free aldehyde to a greater extent than in aqueous solutions.

In Figures 2 and 3 are recorded the spectra of solutions of glycine butyl ester with pyridoxal and pyridoxal-*N*-methochloride. The spectra are similar to those described by Martell (1963) for methanolic solutions of *N*-(3-hydroxy-4-pyridylmethylene)valine and the aqueous solutions of pyridoxal *N*-methochloride and valine reported by Johnston *et al.* (1963). These authors provide evidence for the structures of the imines which exhibit the absorption maxima recorded in Figures 2 and 3. The neutral solutions of the imines are characterized by having absorption peaks at 420 and 336 mμ. With pyridoxal these two peaks are attributed to the existence of structures I and II in Scheme I. For the solutions containing pyridoxal *N*-methochloride the peaks would be attributed to the analogous structures IV and V in Scheme II. Free pyridoxal *N*-methochloride



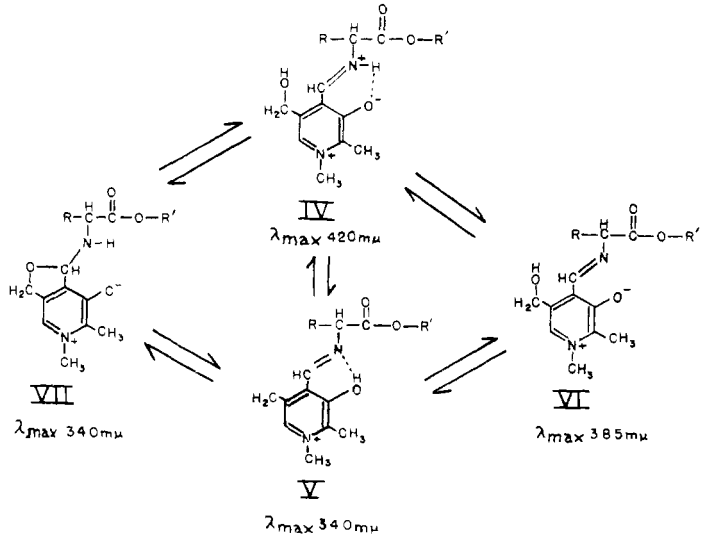
SCHEME I

and structure VII of Scheme II would also be expected to contribute to the 340-mμ peak. Upon making the solutions basic with sodium methoxide the 420-mμ peaks disappear with the formation of a new peak at about 380 mμ. This transition can be interpreted as being due to the formation of structures III and VI, respectively. The formation of the imines required about 30 min to reach equilibrium after which no spectral changes occurred for a period of 4 hr.

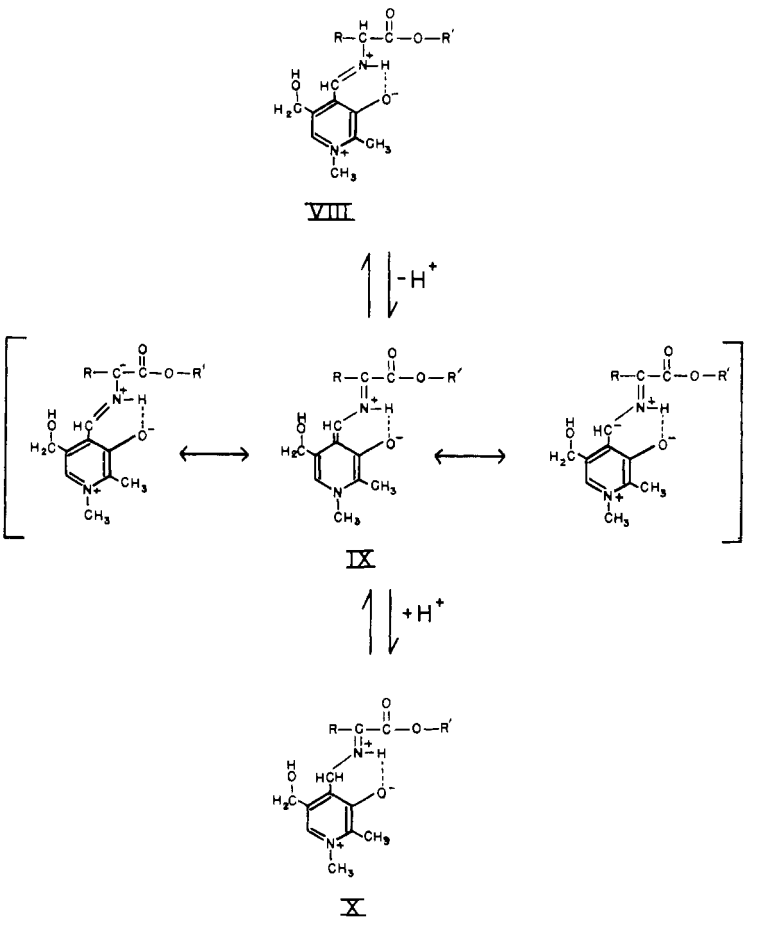
In contrast to the readily interpretable spectra of the imines of glycine butyl ester are those of diethyl aminomalonate with pyridoxal and pyridoxal *N*-methochloride. Curve 1 in Figure 4 shows the spectrum of solutions of 1×10^{-2} M diethyl aminomalonate and 7×10^{-5} M pyridoxal hydrochloride in ethanol. The curve is similar to the one shown for glycine butyl ester and pyridoxal (curve 1, Figure 2). The 440- and 340-mμ peaks can be attributed to structures I and II of Scheme I. The addition of sodium methoxide to yield a concentration of 1×10^{-2} M results in a gradual shift of the 440-mμ peak to 470 mμ (curve 2, Figure 4). A small shift of the 340-mμ peak to 343 mμ is also noted. The small shoulder at 300 mμ indicates that a small amount of free pyridoxal is present. The addition of sodium methoxide (1.8×10^{-4} M) to the solution used to obtain curve 2 in Figure 4 is followed by the rapid formation of products characterized by an absorption peak at 403 mμ with shoulders at 450 and 375 mμ (curve 3, Figure 4). Continued incubation of this solution resulted in the slow disappearance of the peaks at 403 and 450 mμ with the one at 375 mμ remaining. The peak at 403 mμ is probably attributable to free pyridoxal since at lower amino acid concentrations it was slightly higher and the shoulders at 375 and 450 mμ less pronounced.

The reaction of 1×10^{-2} M diethyl aminomalonate hydrochloride with pyridoxal *N*-methochloride gives a solution with absorption maxima at 340 and 450 mμ. Under these conditions the spectrum is similar to that observed with solutions of pyridoxal as shown in curve 1 of Figure 4. The addition of sodium methoxide to 1×10^{-2} M is followed by a shift of the 450-mμ peak to 480 mμ with a severalfold increase in height. The peak at 480 mμ reaches its maximum height in about 12 min (curve 1, Figure 5). The rapid increase in absorption at 480 mμ is accompanied by the formation of a peak at 263 mμ. Figure 5 shows that this band also disappears with the disappearance of the 480-mμ band.

Figure 6 records the effect of increasing concentrations of sodium methoxide on the rate of formation and destruction of the compound absorbing at 480 mμ. Increasing concentrations of sodium methoxide are found to increase both the rate of appearance and disappearance of absorbancy at 480 mμ. The maximum absorbance at 480 mμ is also higher at the greater concentrations of sodium methoxide. Imidazole can re-



SCHEME II



SCHEME III

place sodium methoxide as the base in these studies and offers the advantage of being more soluble in alcohol. By using 0.05 M diethyl aminomalonate and 0.2 M imidazole the absorbance at 480 mμ reaches its maximum in 2 min. From this study we have calculated a molar absorptivity constant of 40,000 for the compound absorbing at 480 mμ.

The reactions of pyridoxal phosphate and pyridoxal *N*-methochloride with diethyl aminomalonate in water were also investigated. The conditions were otherwise identical with those recorded in Figures 4 and 5. Pyridoxal 5-phosphate and diethyl aminomalonate hydrochloride react to form a broad absorption band with peaks of equal size at 420 and 440 mμ. The addition of sodium methoxide, to a concentration equal to that of the amino acid hydrochloride, resulted in a shift of the spectrum to one with a peak at 460 mμ. The molar absorptivity constant of this compound has been reported by Thanassi and Fruton (1962) to be 15,000. The addition of more sodium methoxide to this solution gave no further changes in the spectrum.

Pyridoxal *N*-methochloride and diethyl aminomalonate hydrochloride in water gave a peak at 460 mμ upon the addition of sodium methoxide. When the solution was made more basic the 460-mμ band slowly disappeared.

One way of measuring the reactivity of pyridoxal in nonenzymatic systems is to follow the rate of disappearance of the aldehyde group. This is conveniently measured by the phenylhydrazine method of Wada and Snell (1961). It is usually assumed that the loss of reactivity of pyridoxal to phenylhydrazine is due to the tautomerization of structure VIII to structure X as shown in Scheme III. Figure 7 records the rate of disappearance of pyridoxal and pyridoxal *N*-methochloride in solutions of 1×10^{-2} M diethyl aminomalonate and 1×10^{-2} M sodium methoxide. Curves 1 and 3 show the effect of solvent. The disappearance of pyridoxal *N*-methochloride in ethanol is an order of magnitude faster than in water. This difference in rate cannot be attributed to the rate of imine formation. A comparison of curves 2 and 3 shows that, in ethanol, pyridoxal *N*-methochloride disappears twice as fast as pyridoxal. The reactions comparing pyridoxal and pyridoxal *N*-methochloride were also performed at several lower concentrations of sodium methoxide. Although the rate of disappearance of the aldehyde group was slower under these conditions, the rate for pyridoxal *N*-methochloride was about twice that of pyridoxal in each experiment.

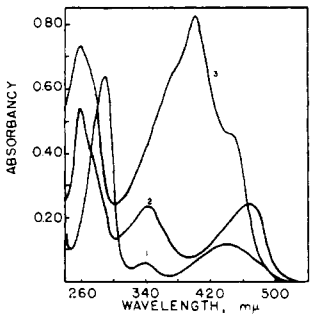


FIGURE 4: Spectra of 7×10^{-5} M pyridoxal hydrochloride and 1×10^{-2} M diethyl aminomalonate hydrochloride in ethanol. Curve 1 was recorded 50 min after mixing the pyridoxal and diethyl aminomalonate. Curve 2 was recorded 70 min after the addition of sodium methoxide (1×10^{-2} M) to the solution used to obtain curve 1. Curve 3 was recorded 5 min after additional sodium methoxide (1.8×10^{-2} M) was added to the solution used to obtain curve 2.

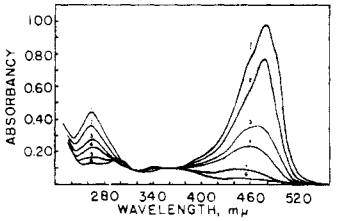


FIGURE 5: Spectral–time study of the reaction of 4.5×10^{-5} M pyridoxal *N*-methochloride, 1×10^{-2} M diethyl aminomalonate hydrochloride, and 1×10^{-2} M sodium methoxide in ethanol. The curves were recorded at the following times after mixing of the solutions: curve 1, 12 min; curve 2, 34 min; curve 3, 65 min; curve 4, 89 min; curve 5, 180 min; curve 6, 312 min. The temperature was 30°.

Under the conditions employed in Figure 7 there was no loss of either pyridoxal or pyridoxal *N*-methochloride when glycine butyl ester was substituted for diethyl aminomalonate. Thanassi and Fruton (1962) have reported that diethyl aminomalonate in aqueous solutions of pyridoxal phosphate is decarboxylated to glycine ethyl ester, carbon dioxide, and ethanol. Using the reaction conditions employed in obtaining the results for Figure 7 we tested for the formation of carbon dioxide and glycine ethyl ester in our system. Our methods, which were sensitive to concentrations of carbon dioxide and glycine ethyl ester of about 1×10^{-4} M, gave no evidence that the decarboxylation of diethyl aminomalonate was occurring.

Because of the great number of functional groups on pyridoxal *N*-methochloride and diethyl aminomalonate, it is possible that the compound absorbing at 480 mμ is not a Schiff base. In an effort to gain confidence that

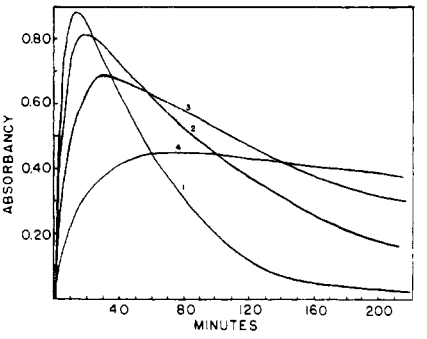


FIGURE 6: Effect of sodium methoxide concentration on the rate of formation and disappearance of the compound absorbing at 480 mμ. The reactions were performed at 30° in glass-stoppered cuvets. Each cuvet contained 4×10^{-5} M pyridoxal *N*-methochloride, 1×10^{-2} M diethyl aminomalonate hydrochloride, and the following concentrations of sodium methoxide: curve 1, 1×10^{-2} M; curve 2, 5×10^{-3} M; curve 3, 2.5×10^{-3} M; curve 4, 1×10^{-3} M. The solvent was absolute ethanol.

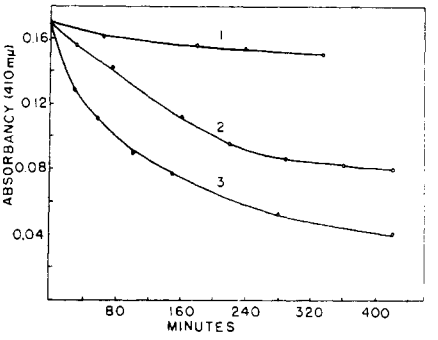


FIGURE 7: Disappearance of pyridoxal and pyridoxal *N*-methochloride as measured by phenylhydrazone formation (see Experimental Section). The reaction mixtures were as follows. Curve 1, 1×10^{-2} M diethyl aminomalonate, 1×10^{-2} M sodium methoxide, and 4.5×10^{-5} M pyridoxal *N*-methochloride; the solvent was water. Curve 2, 1×10^{-2} M diethyl aminomalonate, 1×10^{-2} M sodium methoxide, and 4.5×10^{-5} M pyridoxal; the solvent was absolute ethanol. Curve 3, 1×10^{-2} M diethyl aminomalonate, 1×10^{-2} M sodium methoxide, and 4.5×10^{-5} M pyridoxal *N*-methochloride; the solvent was absolute ethanol.

the compound is a Schiff base the following experiments were performed.

Pyridoxal *N*-methochloride was treated with sodium borohydride prior to the addition of diethyl aminomalonate. No absorption at 480 mμ was observed. This indicates that the aldehyde group of pyridoxal *N*-methochloride is necessary for the reaction under study. Evidence that the amino group is necessary is indicated by the failure of diethyl formamidomalonate and

diethyl oxomalonate to alter the spectrum of pyridoxal-*N*-methochloride. That amino acids which arise by the degradation of diethyl aminomalonate are responsible for the 480-mμ peak seems unlikely. During the course of this study many amino acids and amino acid esters were investigated and none of them gave any properties resembling those obtained with diethyl aminomalonate.

Discussion

The key step in pyridoxal catalysis is the prototropic shift as shown by the conversion of structure VIII to structure X in Scheme III. The original mechanism proposed by Metzler and Snell included a carbanion (structure IX in Scheme III) as the key intermediate in all pyridoxal-catalyzed reactions (Metzler *et al.*, 1954). Recent studies by Bruice and Thanassi indicate that in aqueous solutions reactions between pyridoxal and amino acids do not involve the carbanion intermediate but occur by a concerted mechanism involving general acid–general base catalysis (Bruice and Topping, 1963; Thanassi *et al.*, 1965). Cram and Guthrie (1965) have provided evidence that in nonpolar solvents a carbanion intermediate does exist in the prototropic shift.

To demonstrate the existence in a model system of an intermediate similar to structure IX in Scheme III several requirements would have to be met. These requirements would include a pyridinium nitrogen, an amino acid with an acidic α hydrogen, and a very weakly acidic solvent. A nonpolar solvent would also be expected to favor such an intermediate. We felt that the reaction of diethyl aminomalonate with pyridoxal *N*-methochloride in absolute ethanol met these requirements. Although the study does not resolve the question of the mechanism of pyridoxal catalysis, several interesting observations can be made. The most notable is the formation of a compound which absorbs at 480 mμ. Several facts indicate that this spectrum cannot be attributed to an imine similar to structure IV in Scheme II. If it were this imine one would expect a peak at 340 mμ as was found in the experiment with glycine butyl ester (Figure 3). Also, a shift of the 480-mμ band to one around 385 mμ would be expected when the solution was made basic. This would correspond to the conversion of structure IV to structure VI. However, very high concentrations of sodium methoxide did not result in a shift of the 480-mμ peak. The large value for the molar absorptivity constant also indicates a different structure for the compound absorbing at 480 mμ.

The rapid disappearance of the 480-mμ peak may be due to one or several possible reactions. In the presence of pyridoxal phosphate aminomalonic acid has been shown to be a very reactive compound (Thanassi and Fruton, 1962; Matthew and Neuberger, 1963). These investigators found that decarboxylation occurred readily at room temperature and in aqueous solutions. This does not seem to be true in our system. Matthew and Neuberger (1963) also observed the reaction of several aldehydes with aminomalonic acid in the presence of pyridoxal phosphate. Our system is also reactive to aldehydes. The addition of aldehydes to a solution of the compound absorbing at 480 mμ results in a rapid disappearance of the 480-mμ peak. Another possible reaction in our system is the formation of diethyl oxomalonate *via* transamination. We have been unable to test this possibility due to the inability to detect small quantities of diethyl oxomalonate in the presence of large quantities of diethyl aminomalonate.

The difference in the rates of disappearance of pyridoxal *N*-methochloride and pyridoxal in ethanol solution of diethyl aminomalonate (Figure 7) are similar to the results obtained by Johnston *et al.* (1963) in aqueous solution. The large differences in rates of disappearance of pyridoxal *N*-methochloride in water and ethanol indicate a solvent effect which needs further study.

During the last several years a number of investigators have reported enzyme–substrate complexes of B_6 enzymes absorbing near 495 mμ (Jenkins, 1961a,b; Schirch and Mason, 1963; Marino and Snell, 1965). Schirch and Jenkins studied in some detail one of these complexes which is formed between D-alanine and serine transhydroxymethylase. The complex absorbs at 505 mμ (Schirch and Jenkins, 1964). Several experiments indicated that the formation of this enzyme–substrate complex was accompanied by the loss of a proton from the α carbon of the amino acid. Several similarities between the D-alanine–enzyme complex absorbing at 505 mμ and the compound formed between diethyl aminomalonate and pyridoxal *N*-methochloride absorbing at 480 mμ can be noted. In both studies the rates of formation and disappearance of the compounds absorbing at 480 and 505 mμ were increased by increasing concentrations of base. Both compounds appeared to be formed from ones absorbing at a shorter wavelength. The molar absorptivity constants of the two compounds were considerably higher than those found for other imines of pyridoxal and amino acids. The crucial experiment in determining the similarities of the two systems will be whether the formation of the 480-mμ peak is accompanied by the loss of a proton from the α carbon of diethyl aminomalonate.

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On the Existence of Spectrally Distinct Classes of Flavoprotein Semiquinones. A New Method for the Quantitative Production of Flavoprotein Semiquinones*

Vincent Massey and Graham Palmer†

ABSTRACT: A new method for the conversion of flavoproteins to the semiquinoid form is described. This consists of anaerobic photoirradiation in the presence of EDTA.

A survey of a number of simple flavoproteins with this technique leads to the conclusion that there exist two different classes of flavoprotein free radicals, which are very readily distinguished on the basis of their optical absorption properties. With one enzyme, glucose oxidase, both types of radical are found, the concentrations depending on the pH. These results suggest that the two different spectral species may be due to the neutral and anionic radical forms of the flavin coenzyme prosthetic groups.

Following the original observations of Beinert (1956) it has become generally accepted that the half-reduced or semiquinoid form of flavin coenzymes has an absorption maximum in the region 570–600 mμ. This fact has been amply documented by correlation of optical and epr[1] spectra (Ehrenberg, 1962; Gibson *et al.*, 1962), and quantitative estimates have shown that this absorption band has a considerable extinction coefficient, with values ranging between 3000 and 10000 l. mole^{-1} cm^{-1} (Beinert, 1960; Gibson *et al.*, 1962). On careful anaerobic titration with dithionite a similar absorption band has been observed with glucose oxidase (Massey and Gibson, 1964; Massey *et al.*, 1966) and *Azotobacter* flavoprotein (Beinert, 1965). Again this long wavelength absorption has been found to be well correlated with an epr-detectable free radical, and experimental extinction coefficients in the range 3000–5000 l. mole^{-1} cm^{-1} have been found at 570 mμ. In the case of two other flavoproteins, D-amino acid oxidase (Massey and Gibson, 1964; Massey *et al.*, 1966) and the Old Yellow Enzyme (Nakamura *et al.*, 1965), anaerobic titration with dithionite gave rise to absorption spectra which were very similar to each other, but which were completely different from those seen with glucose oxidase and *Azotobacter* flavoprotein. The D-amino acid oxidase and Old Yellow Enzyme semiquinone spectra are characterized by having very little absorption at 600 mμ, a well-marked peak at 480–490 mμ, and another peak with a very high extinction coefficient around 360–370 mμ. With D-amino acid oxidase a small but well-resolved peak was also observed at 400 mμ. In both cases, 80–100% yields of the free radical species were found by epr spectrometry. We were naturally very interested in determining whether this division of flavoprotein semiquinones into two categories (based on their spectral properties) was valid as a generalization, and

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[1] Abbreviations used: epr, electron paramagnetic resonance; FMN, riboflavin 5′-phosphate; TPNH, reduced triphosphopyridine nucleotide; FAD, FADH, FADH$_2$, oxidized, semiquinoid, and fully reduced flavin-adenine dinucleotide; TPN$^+$, oxidized triphosphopyridine nucleotide.