gave the same CarAc pattern on electrofocusing (H. L. White and J. C. Wu, unpublished).

CarAc has been proposed as one of several possible enzyme systems which facilitate transport of the acetyl moiety from within mitochondria to the cytosol of nerve cells where ACh synthesis occurs (Tučcek, 1970). According to this theory, acetylcarnitine formed by intramitochondrial CarAc passes through mitochondrial membranes and is converted to acetyl-CoA by extramitochondrial CarAc. Acetyl-CoA then is able to stimulate ACh formation by cytoplasmic ChAc. The *in vitro* interactions of choline and ACh with CarAc may therefore have relevance with respect to *in vivo* regulation of ACh synthesis.

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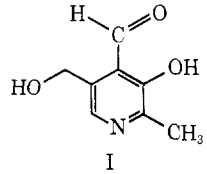
The Isolation and Reactions of a 1,4-Dihydropyridine General Intermediate for Vitamin B-6 Catalysis†

Edwin H. Abbott* and Michael A. Bobrik

ABSTRACT: Evidence is presented that in cold concentrated aqueous solution, the Schiff base formed from pyridoxal (vitamin B-6) and diethyl aminomalonate exists principally in the form of its 1,4-dihydropyridine tautomer. The dihydropyridine may be precipitated from solution, representing the first isolation of what is postulated to be the general intermediate for vitamin B-6 catalysis. The corresponding methyl-substituted amine, diethyl aminomethylmalonate forms only normal Schiff bases with pyridoxal. Nuclear magnetic resonance (nmr) data show that in these systems pyridoxal catalyzes the rapid cleavage of the carboxyethyl group to form ethyl alcohol and ethyl alanate. The mechanism of this reaction is suggested to be analogous to vitamin B-6 catalyzed decarboxylations but to proceed *via* prior saponification. These data are used to discuss the factors controlling various products which are produced in vitamin B-6 catalyzed reactions.

The heterocyclic aldehyde pyridoxal, I, and its 5-phosphate ester are active forms of vitamin B-6. This vitamin is an essential cofactor to a host of enzymes which catalyze amino acid reactions. Of particular interest to chemists is the fact that many of these reactions proceed slowly by pyridoxal catalysis even in the absence of the enzymes. This initially gave rise to the suggestion that the reactions go through Schiff base formation and subsequent tautomerization to the dihydropyridine II → III (Metzler *et al.*, 1954).



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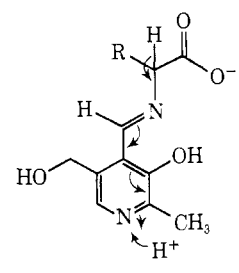

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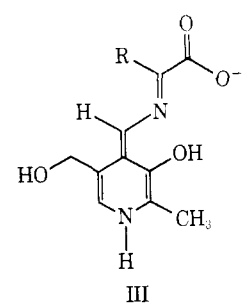

III

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The presence of the Schiff base on the reaction pathway has been demonstrated by kinetic and equilibria studies (Thanassi *et al.*, 1965; Auld and Bruice, 1967a–c). Evidence for the dihydropyridine is more tenuous. Isotope effects have been observed with a C–H bond breaking being rate determining in II → III (Blake *et al.*, 1963; Auld and Bruice, 1967c). Also, absorptions have been detected at or above 460 nm in several enzyme-free (Thanassi and Fruton, 1962; Schirch and Slotter, 1966; Maley and Bruice, 1968; Matsumoto and Matsushima, 1972) as well as enzyme-containing systems (Jenkins, 1961; Morino and Snell, 1967; Jenkins and Taylor, 1969). Since the Schiff bases absorb at about 415 nm this means that a longer π system is present, as expected in III. In one of the nonenzymic studies, the long-wavelength absorption was correlated with the rate of transamination of alanine (Maley and Bruice, 1969).

Experimental Section

Pyridoxal hydrochloride, diethyl aminomalonate hydrochloride, alanine, and ethyl alanate were obtained from Mann Laboratories and used without further purification. D_2O, NaOD, and DCl were supplied by Bio-Rad. Other reagents were of the highest purity readily obtainable. Diethyl aminomethylmalonate was prepared by the method of Thannassi and Fruton (1962).

The dihydropyridine was prepared from pyridoxal and diethyl aminomalonate as follows. Pyridoxal hydrochloride (0.20 g) and diethyl aminomalonate (0.20 g) were dissolved in 10 ml of water and the solution was cooled in an ice bath, 10 ml of a 0.1 N NaOH solution was added with stirring, and the intensely yellow solution was left to stand in the ice bath. After a few minutes, a bright red precipitate began to form. The solution was left to stand in the dark for 2 hr. The dihydropyridine was filtered from the solution as an intensely red solid. It was washed with anhydrous ethyl alcohol followed by ether and then was dried at 70° *in vacuo* for 2 hr. *Anal.* Calcd for $C_{15}H_{20}N_2O_6$: C, 55.55; H, 6.18; N, 8.65. Found: C, 55.38; H, 6.42; N, 8.79. The yield was 0.096 g, 32% based on total pyridoxal; however, it should be noted that only half the amount of the base required for complete formation of the dihydropyridine was used. This was done so that the solution would be self-buffered at a pH low enough to prevent side reactions.

Nuclear magnetic resonance (nmr) spectra were obtained on a Varian A-60-A. Samples were prepared as described previously (Abbott and Martell, 1970). Infrared spectra were recorded from Nujol mulls using a Perkin-Elmer 521 spectrophotometer.

In this paper, pD is the negative logarithm of deuterium ion activity and was measured by adding 0.41 to the reading of pH meter standardized against buffers in H_2O (Covington *et al.*, 1968).

Results

Formation of a Pyridoxal Dihydropyridine. When pyridoxal hydrochloride and diethyl aminomalonate hydrochloride are mixed in dilute DCl they give a solution with a pH of about 2 and an nmr spectrum identical with that of the components. If the pH is raised to 4 by the addition of NaOH, an intense yellow color appears, indicative of Schiff base formation. Upon standing for 1 min, the color darkens and then a flocculent red precipitate begins to form and is deposited slowly over the course of 1 hr at room temperature. A variety of data indicated that the red precipitate has the 1,4-dihydropyridine structure, IV.

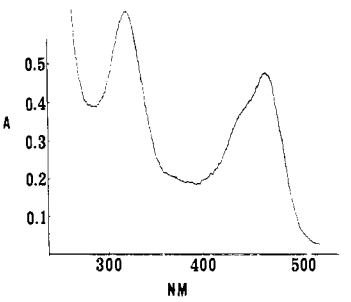
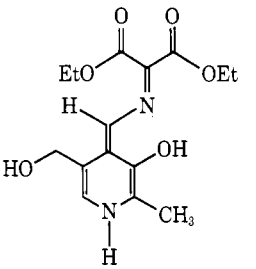


FIGURE 1: Absorption spectrum of a solution initially 0.1 M in diethyl aminomalonate and 1.0×10^{-4} M in pyridoxal after about 45 min at pH 7.15.



IV

Compound IV is sufficiently soluble in neutral aqueous media that its visible absorption spectrum may be observed together with those of its components, with which it is in equilibrium. In solutions of the diethyl aminomalonate (0.1 M) and pyridoxal (1.0×10^{-4} M) between pH 4 and 9, the appearance and increase of intensity of an absorption maximum at 465 nm may be observed during the first few minutes after mixing (Figure 1). Schiff bases derived from pyridoxal have an absorption maximum near 415 nm (Metzler, 1957) and so the 465-nm absorption must arise from a species with a longer π-electron system than is present in the Schiff bases. The dihydropyridine has such a π-electron systems. No absorptions at longer wavelengths than 465 nm are observed. The intensity of absorption is not stable, for reasons discussed below, and so extinction coefficients and formation constants have not been determined.

Similar spectroscopic observations have been reported for related systems in both ethanol and water (Thanassi and Fruton, 1962; Schirch and Slotter, 1966; Maley and Bruice, 1968). In the present work, the slow appearance of the 465-nm absorption in dilute solution suggests that it arises from the same compound as the red precipitate, which appears during the same time scale from solutions more concentrated in pyridoxal. At the same time, the slowness of the 465-nm absorption to develop suggests that it cannot be attributed to a Schiff base, as these species are formed with half-lives of the order of tens of seconds under our conditions (French *et al.*, 1965; Auld and Bruice, 1967b).

The elemental analyses reported in the Experimental Section indicate that the red compound is constituted as a 1:1 ratio of pyridoxal and diethyl aminomalonate. The nuclear magnetic resonance of the solution resulting from acid hydrolysis confirms this. The red compound is too insoluble for nmr itself, however, when shaken with 5% DCl, it is rapidly dissolved with loss of the red color. An nmr spectrum of such a

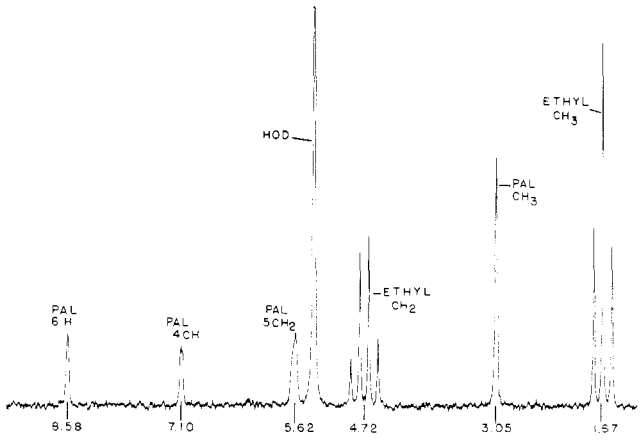


FIGURE 2: 60-MHz nmr spectrum of the solution resulting from acid hydrolysis of the 1,4-dihydropyridine formed from pyridoxal and diethyl aminomalonate. Pal = pyridoxal, shifts are in parts per million from hexamethyldisiloxane.

solution appears in Figure 2. Integrals of the spectrum show that pyridoxal and diethyl aminomalonate are present in a 1:1 ratio and that the functional groups of the malonate are still present.

Figure 2 also shows that the α-H of the diethyl aminomalonate has been replaced by deuterium in the preparation or hydrolysis of the red compound. This is as expected if the structure is IV. The argument may, however, be raised that the α-H is adjacent to two carboxyethyl groups and is so acidic that it exchanges before the nmr measurement can be made. Thus the red compound could be the Schiff base instead of the dihydropyridine and could be exchanging its α-H very rapidly and exchange could occur after hydrolysis to the aminomalonate as well. In 5% DCl, however, the α-H of diethyl aminomalonate is readily observed (Figure 3) and the intensity of its resonance decreases with a half-life of about 10 min at room temperature. This excludes exchange of aminomalonate α-H. The Schiff base between aminomalonate and pyridoxal differs from the aminomalonate itself in that it has an immonium instead of an ammonium nitrogen bound to the α carbon. Though an argument may be made that the Schiff base would be more acidic, the fact that in strong acid it is completely hydrolyzed in a matter of seconds

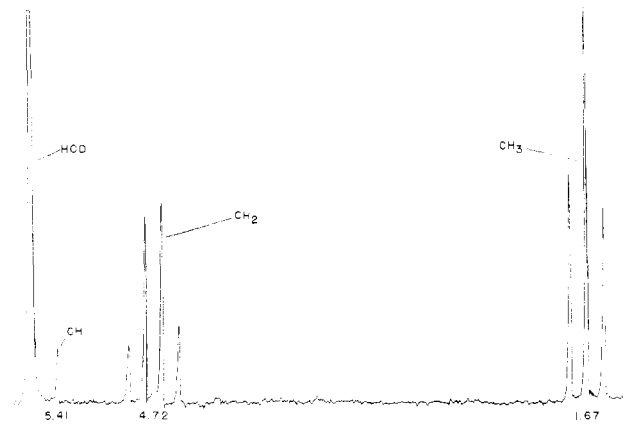


FIGURE 3: 60-MHz spectrum of diethyl aminomalonate dissolved in a 5% solution of DCl. Shifts are in parts per million from hexamethyldisiloxane.

TABLE I: Principal Infrared Bands in the Dihydropyridine and Its Partially Deuterated Derivatives.

Frequency (cm^{-1})	Intensity	Assignment
3500	Broad, weak	O–H stretch (phenolic and primary alcohol)
3200	Weak	N–H stretch
2500	Broad, weak	O–D stretch (phenolic and primary alcohol)
2250	Weak	N–D stretch
1685	Strong	C=O stretch
1640	Medium	C=N stretch
1610	Medium	C=C stretch
1410	Medium	O–H bend (phenolic)
1325	Broad, strong	C–O stretch (phenolic)
1265	Medium	C–N(–D) stretch (conjugated heterocycle)
1250	Medium	C–N(–H) stretch (conjugated heterocycle)
1150	Strong	C–O stretch (ester)
1030	Medium	C–O stretch (primary alcohol)

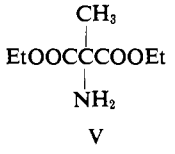
argues against exchange *via* that species. The rate of α-H exchange would have to be several hundred times as great as for the aminomalonate, and this is unlikely.

Infrared spectra of the red compound and its partially deuterated derivatives strongly support the dihydropyridine structure, IV. The data are summarized in Table I. A weak, broad band from 3500 to 3000 cm^{-1} was assigned to the phenolic and aliphatic O–H stretching modes. The shape of the band is characteristic of strong intramolecular hydrogen bonding (Bellamy, 1954, p 86). Separate bending frequencies are observed for the phenolic and aliphatic O–H bending modes. A weak band at 3200 cm^{-1} is assigned to the N–H stretch, its low frequency is acceptable since the spectra were taken in the solid phase (Bellamy, 1954, p 251). Both stretching frequencies were shifted as expected when deuterium was substituted for the exchangeable protons. The possibility that the dihydropyridine exists in the zwitterionic phenolate–immonium form may be further excluded by the absence of the characteristic C=N^+–H bands at 2500–2325 cm^{-1} (Bellamy, 1954, p 260). A peak at 1250 cm^{-1}, shifted to 1265 cm^{-1} in the deuterated derivative, due to vibronic coupling, is typical of C–N stretches of aromatic =C–N–H ring systems (Bellamy, 1954, p 258).

Perhaps the strongest infrared evidence for the dihydropyridine structure comes from the carbonyl stretching frequencies. These are at remarkably low frequency (1685 cm^{-1}) compared to the corresponding band in the diethyl aminomalonate itself (1750 cm^{-1}). Such a shift together with the characteristic ester CO single bond stretch at 1150 cm^{-1} is strong evidence that in the red compound we have an ester in direct conjugation with an extended π system (Bellamy, 1954, p 179). It is interesting to note that the phenolic C–O stretch is shifted about 50 cm^{-1} to higher energy than expected. This can be explained by the presence of substantial double-bond character arising from the weakening of the OH band through hydrogen bonding to the imine nitrogen.

Further chemical evidence that the red compound has the

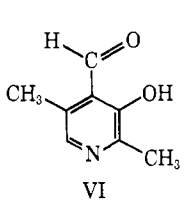
structure shown as IV comes from experiments with diethyl aminomethylmalonate (V). With V, dihydropyridine forma-



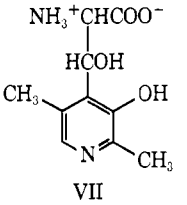
V

tion is much more difficult since it involves cleavage of a carbon–carbon bond rather than a bond to an active hydrogen. Solutions of V with pyridoxal behave precisely as do all other amino acids without reactive functional groups. In solutions 0.2 M in V and 2.0×10^{-4} M in pyridoxal, an absorption maximum is observed at 415 nm indicating Schiff base formation. This could well be due to formation of the Schiff base from V with pyridoxal; however, as discussed below, V reacts to form other amines which could also give Schiff bases. Also solutions like those used to prepare the red compound but with V instead of diethyl aminomalonate do not develop a red color nor does a precipitate form.

Under conditions similar to those we have employed to prepare the dihydropyridine, Thanassi reports that 5-deoxypyridoxal (VI) reacts with aminomalonic acid to form β-(2,5-



VI



VII

dimethyl-3-hydroxypyridyl-4-)serine (VII) (Thanassi, 1970). The striking difference in product when the aminomalonic acid is used instead of the ester may be the result of the greater ease with which the acid is decarboxylated. In any event, the physical properties of VII are quite different from those of the red compound we have prepared.

It has been shown that CO_2 is rapidly evolved from neutral or basic aqueous solutions of diethyl aminomalonate and pyridoxal (Thanassi and Fruton, 1962). We have confirmed the observation, and we discuss evidence for the possible mechanisms in the next section. However, under the conditions we have described for the preparation of VI, the pyridoxal-catalyzed decomposition of diethyl aminomalonate is quite slow and so it is not a problem in our synthesis. At pD 4, the nmr spectra of a solution 0.2 M in diethyl aminomalonate and 1.0×10^{-3} M in pyridoxal show no ethanol resonances even after several days. At pD 5.5, small concentrations (about 5%) of ethanol are apparent after several hours. In contrast to the acid itself (Matthew and Neuberger, 1963), diethyl aminomalonate is quite stable in weakly acidic solution, no changes in its nmr spectrum being observed in a week at room temperature. Consistent with these observations, IV is stable when exposed to neutral or mildly acidic solution, but decomposes rapidly when exposed to basic solution.

Reactions of Malonates with Pyridoxal. As mentioned above, a number of reactions of the aminomalonate esters proceed under the conditions discussed above. Most obviously, the esters are sensitive to saponification at pH's greater than 7 (Hay *et al.*, 1967). This reaction is most conveniently observed in solutions of V. The nmr spectrum of a solution

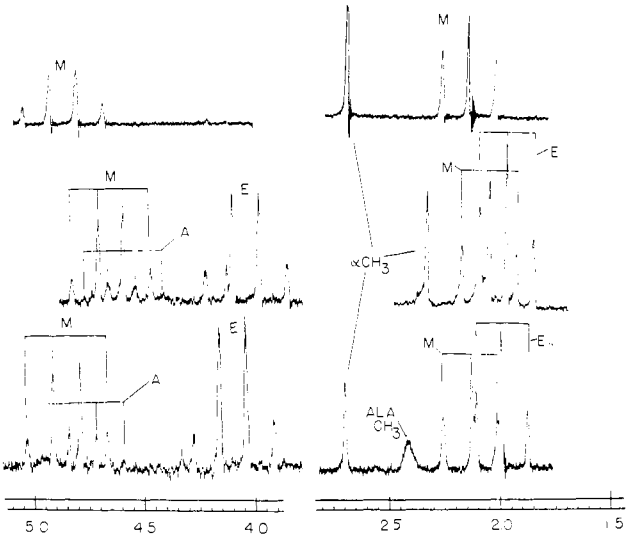


FIGURE 4: 60-MHz nmr spectra showing a solution 0.2 M in diethyl aminomethylmalonate and 0.2 M pyridoxal under different conditions. Upper: solution of the hydrochlorides. Middle: enough base added to raise the pD to 9. Lower: the middle solution with enough DCl added to decrease the pD to about 1.5. Abbreviations: M = diethyl aminomethylmalonate, E = ethanol, A = ethyl alanate, ALA = ethyl alanate + alanine.

of V to which enough base was added to raise the pH to about 9 shows that the saponification to monoethyl aminomethylmalonate anion, and aminomethylmalonate dianion plus ethyl alcohol, proceeds rapidly with a decrease in pH. No resonances assignable to α-deuterioalanine or its ethyl ester are detected, showing that cleavage of the carbon–carbon bonds is negligible.

In the presence of pyridoxal, quite a different reaction is observed. Here when the pD is raised to 9 by addition of NaOD, deuterioalanine and its ethyl ester are produced. Figure 4 shows the changes in the nmr spectrum of such a solution during reaction. Alternate mechanisms for this reaction appear on Plate I. These are the direct cleavage of the carboxyethyl group as in VIII → IX or prior saponification of the ester to the acid or half-ester followed by decarboxylation as in XI → XII (Plate I). The fact that the pH dependence of the decarboxylation reaction is similar to the pH dependence of saponification indicates that decarboxylation probably requires prior saponification. Also, it has been shown that, as expected from the mechanism, an increase in negative charge on the carboxyl group speeds the decarboxylation reaction (Kalyankar and Snell, 1962) by preventing the electron shift away from the carboxylate. The esterification of a carboxylate should likewise decrease the negative charge and inhibit such an electron shift. Presumably this is also why we find that diethyl aminomalonate is stable in solution with pyridoxal at pH values below these where saponification occurs. It is most likely that the evolution of CO_2 from diethyl aminomalonate–pyridoxal solution involves the prior saponification.

Discussion

In the Results section, we presented a variety of spectroscopic and analytical data which we interpreted as supporting a dihydropyridine structure. Several points deserve further discussion. Experimentally, one of the surprising properties

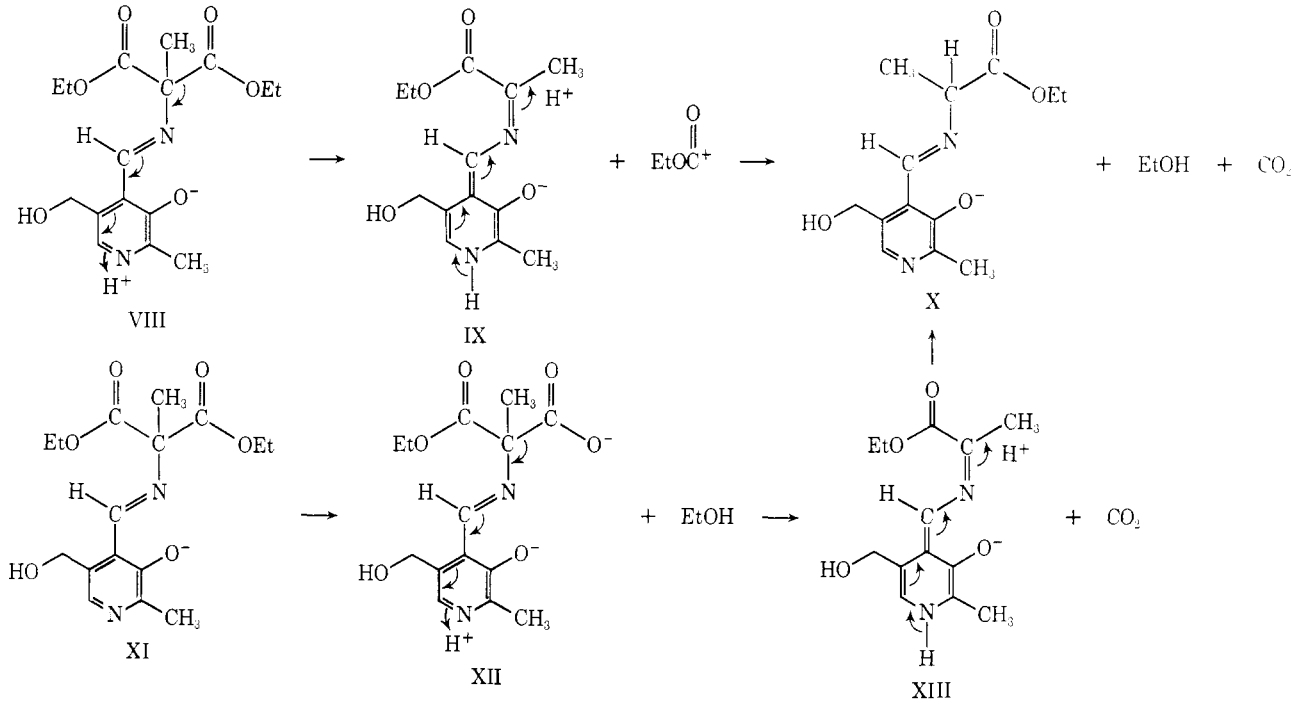


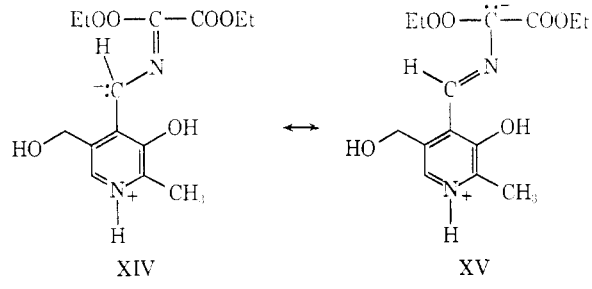
PLATE I: Alternate mechanisms for the decarboxyethylation of diethyl aminomethylmalonate.

of the dihydropyridine is its sparing solubility in neutral water. Pyridoxal Schiff bases are invariably water soluble and as pointed out above, diethyl aminomethylmalonate forms only the typically intense yellow Schiff base solutions even at very high concentration. The reason for the dihydropyridine insolubility in water presumably lies with its having a phenolamine structure, as indicated by the infrared data, rather than the phenolate–immonium tautomeric form which exists extensively in the pyridoxal Schiff bases (Metzler, 1957). The reason that the dihydropyridine's imine nitrogen is less basic than the Schiff base's is probably that its electron pair is better delocalized in the more extensive π system. A polymeric structure for the dihydropyridine would also explain the insolubility. However, since both nmr and ir show all the functional groups to remain intact, and since polymerization normally occurs at the expense of the π system, we exclude this possibility.

As pointed out earlier, long-wavelength absorptions have been observed by other workers, and assigned as arising from pyridoxal dihydropyridine structures. It is important to note the wide range of energies at which these absorptions have been detected. To mention a few, the aminomalonates are close to 465 nm (Thanassi and Fruton, 1962; Schirch and Slotter, 1966), several enzyme dihydropyridine complexes fall near 500 nm (Jenkins, 1961; Jenkins and Taylor, 1969; Morino and Snell, 1967), and the alanine–*N*-methyl-3-hydroxy-4-pyridinecarboxaldehyde dihydropyridine is about 600 nm (Maley and Bruice, 1969). Although the dihydropyridine we have isolated is the only one to have been fully characterized, the evidence presented for the identification of these other dihydropyridine's is quite convincing. Evidently the electronic structure of the dihydropyridine is quite sensitive to small changes in the substituents of both the heterocyclic ring and the amino acid.

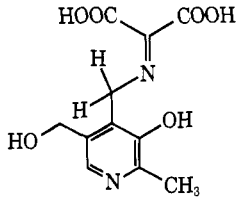
The final and most important point of discussion concerns the acid hydrolysis reaction of the dihydropyridine. Considering the formation of a dihydropyridine starting with pyridoxal and an amino acid, a keto acid may be formed when the ketimine Schiff base resulting from reprotonation α to the ring hydrolyzes. Alternatively the α carbon of the acid could have been reprotonated to give an aldimine Schiff base which may hydrolyze to give back the original amino acid. These two reactions are respectively transamination and racemization, and are both very important in a variety of metabolic processes. Since the rate determining step of both reactions is the removal of the proton on the amino acid α carbon to form the dihydropyridine, detailed study of subsequent reprotonation processes has been impossible prior to our isolation of the dihydropyridine.

From a valence bond point of view, the relative contributions of resonance structures XIV and XV may be invoked



to explain the two different reactions. If for a given dihydropyridine, XIV predominates, transamination will be the result of reprotonation followed by hydrolysis while if XV is the main contributor, a proton will add α to the carboxylate and racemization will be said to have occurred. As we have shown above, in our system acid hydrolysis of the dihydropyridine results almost entirely in racemization type products. This suggests that XV is much more important than XIV so that when the dihydropyridine tautomerizes back to the Schiff base, the proton will bind α to the carboxyl group because

this is the site of greatest negative charge. This is implied by the remarkable acidity of the α-H of the aminomalonates as discussed above; however, verification comes from a preliminary study of the reaction of ketomalonic acid with pyridoxamine. These species react to form a ketimine, XVI,



XVI

which may tautomerize to a dihydropyridine like IV by removing a proton α to the ring and adding one at the pyridine nitrogen. In contrast to the aminomalonate–pyridoxal system, this occurs very slowly because of the very low acidity of the proton α to the ring, a reflection of the high energy of a resonance structure like XIV relative to XV in this system.

Such resonance structure arguments are highly oversimplified and must not be taken too literally, however, they are useful in considering how an enzyme may control the outcome of the reaction. Many factors such as adjacent charged substituents will have a profound effect on whether XIV or XV is favored. Isolation of the dihydropyridines such as that discussed above permits rigorous investigation of these effects. Such work is currently under way in these laboratories both from a theoretical as well as an experimental point of view.

Acknowledgment

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