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α-Methylaminomalonate-Dependent Reactions of 5-Deoxypyridoxal†

John W. Thanassi

ABSTRACT: The reactions between α-methylaminomalonic acid and the B_6 analog, 5-deoxypyridoxal, have been studied at pH 5.2 (30°). The initial and required decarboxylation step results in a carbanionic Schiff base which partitions into the formation of four ultraviolet-absorbing products, three of which have been identified by their nuclear magentic resonance and mass spectra. The reaction pathways leading to these products involve a transaminative decarboxylation and at least two carbanion condensations, one of which leads to a dimer (structure I) incorporating one molecule of 5-deoxypyridoxal and one molecule of 5-deoxypyridoxamine. In addition to these reactions, there is buffer catalysis of the 5-deoxypyridoxal-catalyzed decarboxylation of α-methylaminomalonate; this catalysis is suggested to be general acid in nature. The results of these experiments are discussed in terms of the mechanism of action of B_6-dependent enzymes, with particular reference to Dunathan's suggestions concerning the reaction specificity of such enzymes.

Vitamin B_6 dependent enzymes catalyze a wide variety of extremely important reactions in the intermediary metabolism of amino acids. For example, decarboxylation of histidine and 5-hydroxytryptophan by B_6-requiring decarboxylases give rise to the pharmacologically active substances, histamine and serotonin, respectively. All of the amino acid transaminases also require vitamin B_6. These enzymes fulfill a key role in overall nitrogen balance and serve to connect amino acid and carbohydrate metabolism. Many other types of reactions are catalyzed by pyridoxal phosphate requiring enzymes, and there are a number of general reviews available which discuss these in detail (Braunstein, 1960; Snell, 1958).

In an attempt to elucidate the role of this vitamin in the molecular mechanisms of an apparently large variety of enzyme-catalyzed reactions, model systems have been extensively examined (Metzler *et al.*, 1954; Auld and Bruice, 1967; Bruice and Benkovic, 1966). Although most of the enzymatic reactions have been reproduced in such studies, they generally require elevated temperatures and/or relatively long reaction times. In contrast, as shown in a previous communication (Thanassi, 1970), the reactions between aminomalonic acid, $NH_2CH(COOH)_2$, and 5-deoxypyridoxal, a B_6 analog, occur very rapidly at 30°. Thus, aminomalonate derivatives appear to be uniquely sensitive to pyridoxal-catalyzed reactions. The experiments reported herein deal with the reactions occurring between 5-deoxypyridoxal and α-methylaminomalonic acid, $NH_2C(CH_3)(COOH)_2$.

Experimental Section

Materials. 5-Deoxypyridoxal was synthesized by the method of Mühlradt and Snell (1967), as described previously (Thanassi, 1970). The preparation of the monoammonium salt of α-methylaminomalonic acid is described elsewhere (Thanassi, 1971). All other chemicals were reagent grade. Water employed in these experiments was house-distilled water, redistilled from all-glass apparatus. D_2O (99.8%) and AG 50W ion-exchange resin were obtained from Bio-Rad.

Reaction between 5-Deoxypyridoxal and α-Methylaminomalonic Acid; Isolation of Fractions I–IV. 5-Deoxypyridoxal (608 mg, 4.0 mmoles) was dissolved in 400 ml of 0.04 N ammonium acetate buffer at pH 5.2. To this solution was added 2.42 g (16 mmoles) of the monoammonium salt of α-methylaminomalonic acid. The reaction was allowed to proceed for 1 hr in the dark in a nitrogen atmosphere at room temperature. After acidification from pH 5.3 to pH 1 with concentrated HCl, the reaction solution was applied to a 1.5 × 100 cm column of AG 50W × 8 (200–400 mesh) in the hydrogen form. The column was washed with 2.0 N HCl and then eluted in a stepwise fashion with increasing concentrations of HCl at a flow rate of 30–40 ml per hour (Figure 1). Fractions were collected at 30-min intervals, and the absorbancies at 295 nm were measured. The tubes containing the individual fractions designated I through IV in Figure 1 were combined, and the separate pooled fractions were concentrated to dryness on a rotary evaporator at a bath temperature not exceeding 40°. Approximately 80% of the absorbancy applied to the column was recovered in the four fractions. The individual fractions were then decolorized with charcoal. Fraction IV was recrystallized from 2-propanol–ether. Fractions I–III were recrystallized from water–2-

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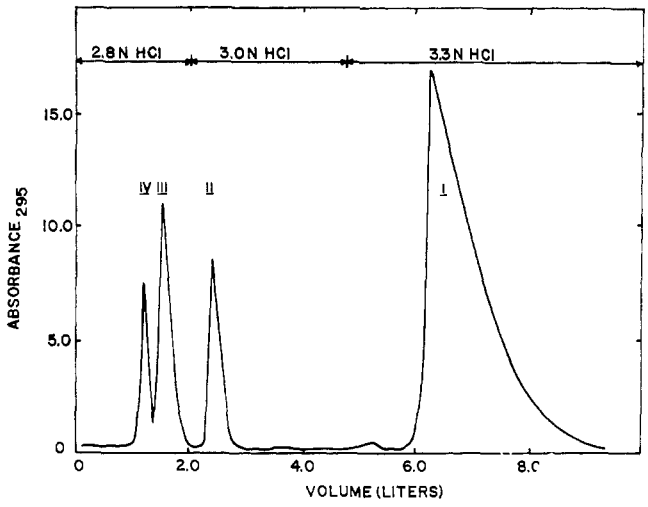


FIGURE 1: Elution diagram of products obtained from a reaction mixture containing α-methylaminomalonic acid and 5-deoxypyridoxal (pH 5.2, 30°).

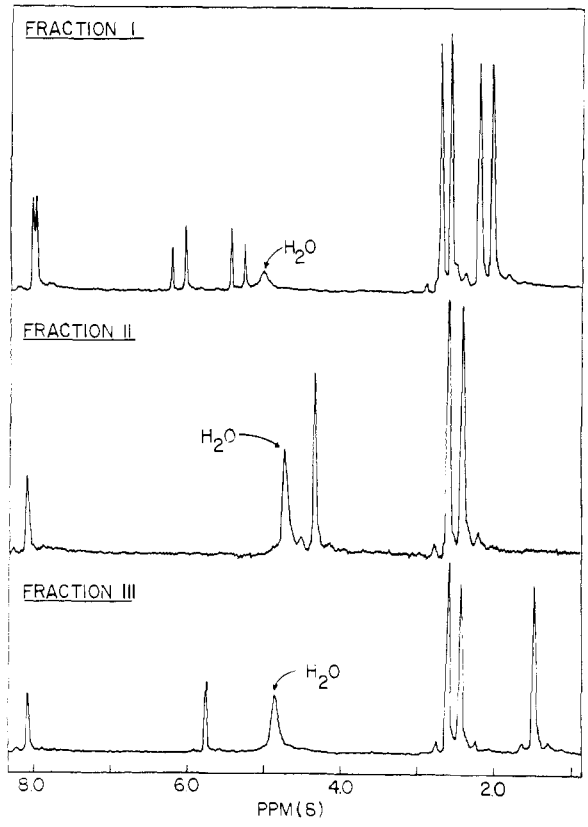


FIGURE 2: Nmr spectra of fractions I, II, and III; spectra were taken in D_2O.

propanol. The nuclear magnetic resonance (nmr) spectra of fractions I–III are found in Figures 2-I, 2-II, and 2-III, respectively. The electron impact mass spectrum of fraction II and the chemical ionization mass spectrum of fraction I are shown in Figures 3-II and 3-I, respectively.[1]

The ultraviolet spectra of all four fractions are extremely similar. In 0.1 N KOH, fractions II–IV all have maxima at

[1] For a discussion of chemical ionization mass spectra, reference is made to Milne *et al.* (1970), and references therein, and Field (1968).

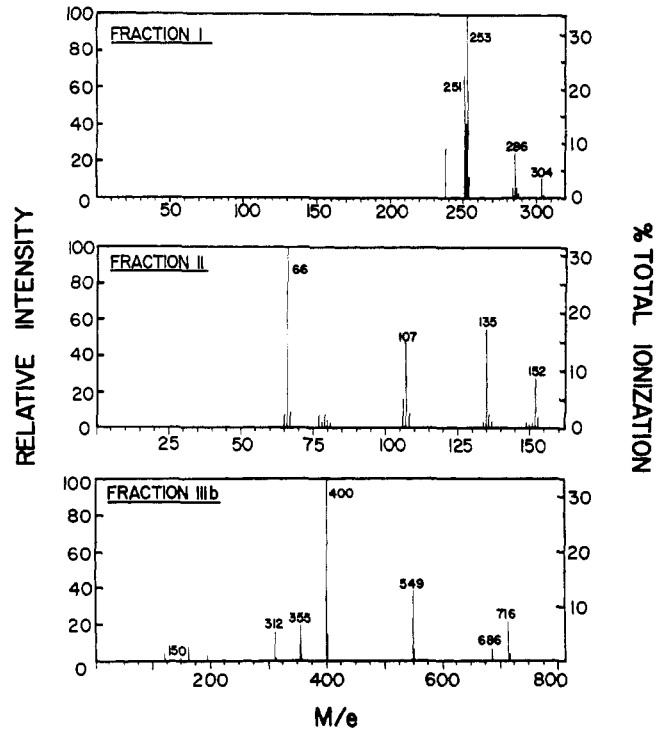


FIGURE 3: Mass spectra of fractions I, II, and IIIb.

305 nm and minima at 270 nm. In the same solvent, fraction I shows maximal absorbance at 310 nm and has a minimum at 270 nm.

Esterification and Tri-p-nitrobenzoylation of Fraction III. Fraction III was esterified by the conventional Fischer procedure employing HCl-saturated anhydrous ethanol. The nmr spectrum revealed that signals corresponding to those of an ethyl group had been added to the spectrum shown in Figure 2-III. The ethyl ester derivative was then reacted with excess *p*-nitrobenzoyl chloride in dry pyridine and worked up according to the procedure described by Viscontini *et al.* (1951). After decolorization with charcoal, the product (IIIb) was recrystallized several times from acetone–methanol. The chemical ionization mass spectrum of this derivative is provided in Figure 3-IIIb.

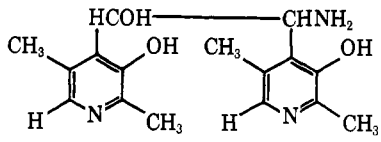
Methods. Ultraviolet, nuclear magnetic resonance, and pH measurements were made as described previously (Thanassi, 1970). CO_2 evolution and aldehyde disappearance were also measured as described previously except that the path length for ultraviolet studies was 2.0 mm and not 0.5 mm as stated earlier (Thanassi, 1970).

Results

Figure 1 indicates that four ultraviolet-absorbing fractions can be isolated by cation-exchange chromatography from a reaction mixture containing 5-deoxypyridoxal and α-methylaminomalonic acid at pH 5.2 (30°). These are designated fractions I–IV. The following paragraphs deal with each of these fractions separately.

Fraction I. The nmr spectrum of fraction I (Figure 2-I) reveals that the compound has four aromatic methyl groups, two coupled CH protons and two aromatic hydrogen atoms. The chemical ionization mass spectrum (Figure 3-I) shows a quasimolecular ion at *m/e* 304 $(M + 1)^+$. Loss of mass 18 (H_2O) and of mass 17 (NH_3) leads to signals at 286 and 287,

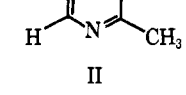
respectively. Further loss of ring substituents results in signals in the *m/e* regions of 251–254. The molecular weight of 303 and the nmr spectrum clearly indicate that a dimer of the starting aldehyde has formed. Synthesis of this information leads to structure I ($C_{16}H_{21}N_3O_3$; 303). This product gives an



I

orange color with ninhydrin which is characteristic of pyridoxamine derivatives. A suggested mechanism for the formation of I is provided in the Discussion section; proposed mechanisms for the formation of products II and III can also be found there.

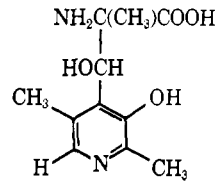
Fraction II. This fraction is readily identified as 5-deoxypyridoxamine (structure II, $C_8H_{12}N_2O$; 152) by the nmr and



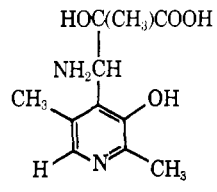
II

mass spectra. The nmr spectrum (Figure 2-II) has in it signals for two aromatic methyl groups, an aromatic hydrogen atom and a singlet integrating for two protons. The electron impact mass spectrum (Figure 3-II) has a molecular ion, M^+ at *m/e* 152, corresponding to the molecular weight of 5-deoxypyridoxamine. Loss of ammonia yields the signal at 135; further loss of CO gives rise to a signal at *m/e* 107. Ring contraction of the pyridine system leads to the base peak at mass 66. Fraction II gives a characteristic orange color with ninhydrin.

Fraction III. The nmr spectrum (Figure 2-III) for this fraction has in it signals for two aromatic methyl groups, an aromatic hydrogen, an alkyl methyl group, and an uncoupled CH proton. It gives a purple color with ninhydrin. The mass spectrum of this fraction as isolated was not unambiguous, probably owing to impurities in the compound. Since there was not much material in hand, this fraction was increased in mass by derivitization to the ethyl ester and subsequently *p*-nitrobenzoylated as described in the Experimental Section. This derivative afforded very nice white crystals after several recrystallizations from acetone–methanol and moved as one spot on thin-layer chromatography (fluorescent silica gel, chloroform solvent). The nmr spectrum of the starting material (Figure 2-III) is consistent with either structure III[1] or its isomer, III[11] ($C_{11}H_{16}N_2O_4$; 240).

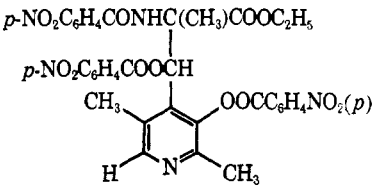


III[1]



III[11]

The chemical ionization mass spectrum of the tri-*p*-nitrobenzoyl ethyl ester (IIIb) of fraction III is shown in Figure



III[1]b

3-IIIb and corresponds to III[1]b (or the tri-*p*-nitrobenzoyl ethyl ester of III[11]). The molecular formula of III[1]b is $C_{34}H_{29}N_5O_{13}$; 715. Figure 3-IIIb reveals that a signal at 716 atomic mass units is present, corresponding to the quasimolecular ion $(M + 1)^+$. Loss of *p*-nitrobenzoic acid gives rise to the peak at mass 549. The signal at 400 is probably due to further protonation of the ion at mass 549 accompanied by loss of *p*-nitrobenzoylacylium ion of mass 150; a signal for an ion of mass 150 is seen in Figure 3-IIIb.

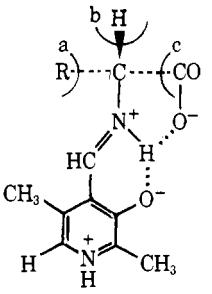
Fraction IV. Fraction IV has an nmr spectrum similar to that of fraction III, showing signals for two aromatic methyl groups, an alkyl methyl group, an aromatic hydrogen atom, and one uncoupled proton. In a Fischer esterification procedure, it forms an ethyl ester indicating the presence of a carboxyl or carboxyl group precursor in the molecule and gives a purple color with ninhydrin. The chemical ionization mass spectrum of the unesterified compound has in it a number of signals in common with the spectrum of impure fraction III (not shown), namely at *m/e* 223 (base peak), 224, 205–207, 195, and 151–154. The last-named are characteristic of the 5-deoxypyridoxine and/or 5-deoxypyridoxamine systems. There is no signal at *m/e* 241, corresponding to the $(M + 1)^+$ ion found in the spectrum for fraction III. Therefore fraction IV cannot be one or the other of the isomers designated III[1] or III[11] above. There are signals in the mass spectrum of IV at *m/e* values higher than 241 but these may be due to contaminants. As can be seen from Figure 1, this particular fraction is the one available in least quantity and sufficient material has not been obtained for proper purification and characterization. Although it appears, therefore, that fraction IV is a close chemical relative of III[1] or III[11] we are unable to definitively assign a structure to it.[2]

It can be seen from inspection of Figure 1 and structures I, II, and III[1] (or III[11]) that the sequence of elution from the strong cation-exchanger, AG 50W, is related to the basicity of the compounds. Thus, in acid, fraction I will carry 3 positive charges and fraction II will bear 2 positive charges. Fraction III has an amino group and a pyridine nitrogen and so will be doubly protonated in acid but, in addition, contains an acidic carboxyl group; fraction IV is the least basic of the four fractions.

Discussion

It is generally accepted that the initial step in all of the reactions catalyzed by B_6-dependent enzymes is the formation of a Schiff base between the amino acid and pyridoxal phosphate (mechanism 1). The subsequent bond-breaking and bond-making steps occurring at a, b, or c in mechanism 1 then determine reaction specificity—*i.e.*, carbon–carbon bond cleavage, transamination, or decarboxylation, respectively.

[2] A referee has suggested that Fraction IV might be an internal lactam of III[11]. If the base peak of *m/e* 223 is in fact the $(M + 1)^+$ ion, then IV would differ in mass from III by 18 mass unit, *i.e.*, a water molecule. This would tend to support this suggestion.



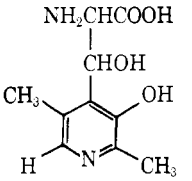
(1)

Most of the enzymatic reactions will also take place in model systems but these do not show the extreme specificity or rapidity observed in the enzyme-catalyzed reactions. Usually a multiple reaction path is observed, and a number of products are formed rather than a single product. The important question, therefore, is how does the enzyme control the course of the reaction so that reaction occurs exclusively at bond a or b or c in mechanism 1?

Dunathan (1966) has proposed that the course of enzyme-controlled, pyridoxal-catalyzed reactions is determined by a steric control mechanism. That is, the sensitive bond must occupy a position perpendicular to that of the extended conjugated system formed by the pyridoxal phosphate–amino acid Schiff base, corresponding to bond b in mechanism 1. A given enzyme, therefore, will interact with its amino acid substrate in such a fashion that the sensitive bond finds itself in the position of bond b. There are theoretical arguments in favor of such a mechanism, and experimental evidence in support of this theory has been forthcoming (Dunathan *et al.*, 1968; Ayling *et al.*, 1968; Bailey *et al.*, 1970).[3]

Perhaps the least studied reaction in model systems has been the decarboxylation reaction, mainly because this type of reaction is not readily observed in such studies. Kalyankar and Snell (1962) have shown that such reactions will take place at elevated temperatures if amino acids lacking in α-hydrogen atom are used, *e.g.*, α-aminoisobutyric acid. These decarboxylation reactions were inhibited by metal ions, in contrast to most other model pyridoxal-catalyzed reactions. From Dunathan's suggestions, this observation is explained by proposing that the carboxyl group is more or less restricted to position c in mechanism 1 by hydrogen bonding of the type shown. Substitution of the bonded hydrogen atom in mechanism 1 by a metal ion would further restrict rotation about the N–C bond of the amino acid. In fact, this is a key point proposed by Metzler *et al.* (1954) in their postulation of a general theory for B_6-catalyzed reactions. Thus it would appear that decarboxylation in model systems is not commonly observed because it is unlikely that the carboxyl group will occupy the reactive position owing to unfavorable spacial constraints.

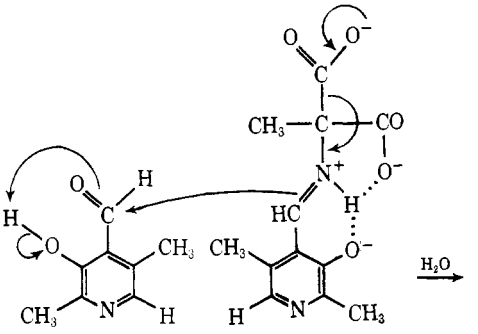
In a previous communication from this laboratory (Thanassi, 1970), it was reported that aminomalonic acid, $NH_2CH(COOH)_2$, showed greatly enhanced decarboxylation in the presence of 5-deoxypyridoxal and buffers, and it was suggested that amino acid decarboxylation in this chemical system took place with general acid catalysis. The powerful electron-withdrawing effect of the extended, protonated, pyridoxylidene–aminomalonate system thus becomes evident. In addition, using Dunathan's suggestions, it would seem that the *pathway* of the reaction of aminomalonic acid in this system results from the fact that it has two equivalent carboxyl groups and so the reactive position b can be filled by either the α-hydrogen atom or a carboxyl group, depending on which of the two apparently equivalent carboxyl groups fills position c in mechanism 1. In agreement with this, it was found that, in addition to a buffer-catalyzed decarboxylation reaction, there occurred a condensation reaction which resulted from the 5-deoxypyridoxal-promoted labilization of the α-hydrogen of aminomalonate and a nucleophilic attack of the resulting carbanion on a second molecule of 5-deoxypyridoxal. The final product of the reaction, designated β-5-deoxypyridoxylserine (V), appeared to be formed quantitatively at zero buffer concentration. Thus, aminomalonate undergoes both a buffer-catalyzed decarboxylation reaction and a reaction involving labilization of the α-hydrogen atom in the presence of 5-deoxypyridoxal.



V

The present experiments are concerned with the reactivity of α-methylaminomalonic acid in the presence of 5-deoxypyridoxal. It is apparent that replacement of the α-hydrogen of aminomalonic acid by a methyl group precludes the possibility of transamination-dependent reactions and that decarboxylation *must* occur as a first step in any reaction sequence. The results show that the reaction which takes place is not a simple decarboxylation reaction yielding only DL-alanine and regenerating 5-deoxypyridoxal, but rather that a fairly complicated reaction sequence ensues, giving rise to one major and three minor ultraviolet-absorbing products. Reaction sequences leading to the identified products are proposed in the discussion which follows.

Fraction I. This is the main product isolated from the reaction mixture, and its formation is suggested to occur by mechanism 2. Intramolecular general acid catalysis by the 3-OH group as shown in mechanism 2 comes from the experiments of Bruice and coworkers (Thanassi *et al.*, 1965; Auld and Bruice, 1967). Although the reaction shown in mechanism 2 is written as a concerted one, a consecutive reaction is also possible. This can be found in Scheme I,

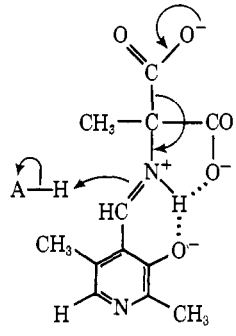


I + CO_2 + $CH_3COCOOH$ (2)

[3] The Schiff base in mechanism 1 is usually drawn with a metal ion instead of the bonded imine proton. However, proton magnetic resonance studies by Abbott and Martell (1970) have led these investigators to propose the existence, in aqueous solution, of the hydrogen-bonded structure shown in mechanism 1. Hence, there is precedent in the literature for a structure of this type (Abbott and Martell, 1970, 1971).

where all of the products are shown to be formed from the partitioning of a common, resonance stabilized, carbanionic intermediate. The 5-deoxypyridoxal ring in Scheme I is denoted by PYR.

Consistent with the suggested mechanisms for the formation of I are kinetic data obtained by ultraviolet absorption spectroscopy and Warburg manometry. Reaction solutions (2.5 ml) were prepared which were 0.05 M in α-methylaminomalonate and which contained a total of 2.5 μmoles of 5-deoxypyridoxal. In four separate reaction solutions the buffers (pH 5.12) were 0.25, 0.50, 0.75, and 1.0 M potassium acetate, all at an ionic strength of 1.0 with KCl. The amounts of CO_2 evolved at 30° in 60 min were 1.96, 2.39, 2.85, and 3.12 μmoles, respectively. Spectrophotometric examination of these reaction solutions (path length, 2.0 mm) at 380 nm (aldehyde disappearance) and 320 nm (appearance of products) showed that about half of the absorbancy at 380 nm disappeared in 3–4 min; this decrease was linearly related to the increase at 320 nm. Thus, as is the case with unsubstituted aminomalonic acid, aldehyde disappearance is very rapid. It is apparent, therefore, that at a buffer concentration of 0.25 M, CO_2 evolution (1.96 μmoles) is less than stoichiometric with the amount of 5-deoxypyridoxal present (2.5 μmoles). This is provided for in mechanism 2 because 2 moles of the aldehyde disappear for every mole of CO_2 produced. One also sees the amount of CO_2 increase from a less than to a more than stoichiometric equivalency with 5-deoxypyridoxal as the buffer concentration is increased from 0.25 to 1.0 M, all other conditions being the same. This indicates that there is buffer catalysis of the 5-deoxypyridoxal-catalyzed decarboxylation of α-methylaminomalonic acid. For reasons discussed in detail elsewhere (Thanassi, 1970), this catalysis is suggested to be general acid in nature (mechanism 3).



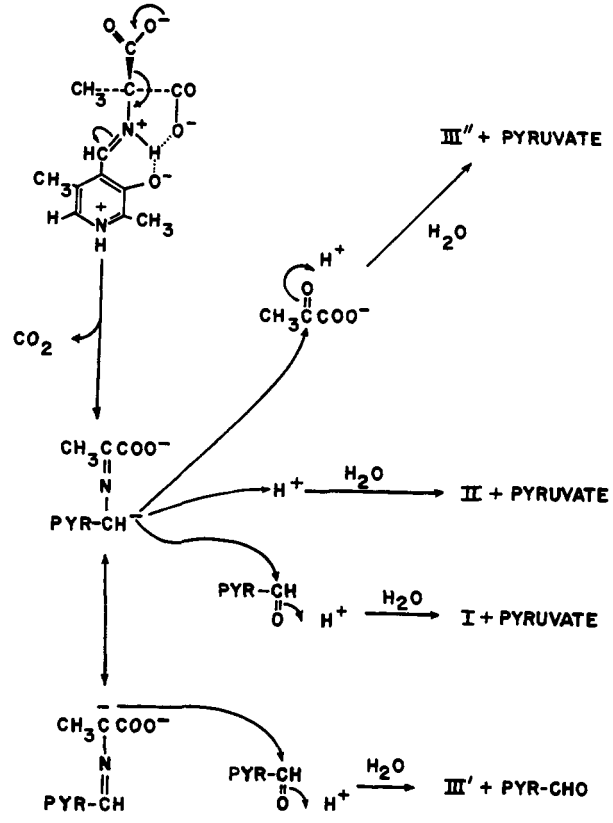
(3)

Fraction II. The mechanism for the formation of 5-deoxypyridoxamine, II, is one of transaminative decarboxylation as originally proposed by Kalyankar and Snell (1962) and is shown in Scheme I. Enzymatic counterparts of this reaction have been found (Bailey and Dempsey, 1967).

Fraction III. Structural evidence indicates that fraction III is either III[1] or III[11]. Suggested mechanisms for the formation of these two possibilities are also shown in Scheme I. According to these mechanisms, III[1] would be formed from a condensation reaction occurring at an amino acid carbanion, and III[11] from a condensation reaction occurring at what is formally the carbanion of a ketimine Schiff base.

Although no firm choice can be made between III[1] and III[11] on the basis of the nmr and mass spectra, it is likely that fraction III is, in fact, III[1]. First, as mentioned earlier, an orange ninhydrin color is very characteristic of pyridoxamine derivatives. The fact that fraction III gives a purple color with ninhydrin suggests that the product is the amino acid derivative, III[1], rather than the pyridoxamine derivative III[11]. Second, when the reaction described in the Experimental Section was repeated in the presence of 0.1 M sodium pyruvate, the elution pattern observed on ion-exchange chromatography was very similar to that shown in Figure 1. One would have expected that if the mechanism leading to III[11] in mechanism 5 were operative, more III[11] would be formed at the expense of I in the presence of high concentrations of pyruvate because the formation of I also involves the ketimine carbanion used in the formation of III[11]. A marked decrease in the formation of I and a corresponding increase in III[11] was not observed in the presence of 0.1 M pyruvate.

SCHEME I



The results of these experiments again demonstrate the reactivity of aminomalonates in the presence of pyridoxal derivatives. The replacement of the α-hydrogen of aminomalonate by a methyl group in no way decreases the overall reactivity toward 5-deoxypyridoxal but rather markedly alters the course of the reaction. This must result from the relative stability of the carbanions formed from aminomalonate and α-methylaminomalonate, respectively. In the case of unsubstituted aminomalonate, labilization of the α-hydrogen will yield a carbanion stabilized by the extended Schiff base system and the two carboxyl groups attached to the α-carbon. In the case of α-methylaminomalonate, the carbanion formed can arise *only* from a decarboxylative process. In the experiments with aminomalonate, product analysis shows that the carbanion more resembles an aldimine carbanion since the mechanism leading to V, the exclusive product of the reaction in the absence of buffer, requires carbon–carbon condensation *via* an aldimine carbanion (Thanassi, 1970). However, with α-methylaminomalonate, at least two of the products, I and II, result from ketimine carbanion reactions

(see Scheme I). In enzyme-catalyzed pyridoxal reactions, the protein must therefore determine the nature of the carbanion, presumably by electrostatic and medium effects.

The results with α-methylaminomalonate are supported by Dunathan's suggestions (1966). In the 5-deoxypyridoxal, α-methylaminomalonate Schiff base, reactive position b in mechanism 1 must be occupied by either an unreactive methyl group or one of the two chemically equivalent carboxyl groups. Hence steric constraints to decarboxylation are minimized owing to the fact that a carboxyl group, in the absence of any other factors, has a 50% chance of being in the reactive position. In addition, the rate of disappearance of aldehyde indicates that decarboxylation is not a particularly difficult reaction in model systems if the conditions are carefully chosen. Further studies on the reactions between B_6 derivatives and aminomalonates are being carried out in this laboratory.

Finally, there have been a number of reports of biochemical experiments with aminomalonic acid (Shimura *et al.*, 1956; Thanassi and Fruton, 1963; Matthew and Neuberger, 1963a,b) and these have included speculation regarding a possible biochemical role for this reactive amino acid. Most recently, Meister and coworkers have studied the absolute stereochemistry of the decarboxylation of aminomalonate by aspartate-β-decarboxylase, a multifunctional enzyme from *Alcaligenes faecalis* (Palekar *et al.*, 1970, 1971). It was found that *S*-tritioglycine was formed when the enzyme-catalyzed decarboxylation of aminomalonate was carried out in tritiated water and that the reaction was accompanied by a slower irreversible inactivation of the enzyme. It was also found that radioactivity from ^{14}C-labeled aminomalonate became "tightly bound" to the enzyme. It would appear likely that the inactivation of aspartate-β-decarboxylase is a consequence of the marked reactivity of aminomalonates in the presence of pyridoxal phosphate.

Acknowledgments

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