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Vitamin B_6-Catalyzed Reactions of α-Amino- and α-Amino-α-methyldiethylmalonate

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The reactions of pyridoxal phosphate with α-amino- and α-amino-α-methyldiethylmalonate have been investigated at pH 6.25 (30°C, N_2 atmosphere). The data indicate that the parent α-amino compound is converted to glycine ethyl ester by a reaction sequence involving rate-limiting, B_6-independent formation of the half-ester of α-aminomalonic acid, and then B_6-dependent decarboxylation. The reaction of α-amino-α-methyldiethylmalonate with pyridoxal phosphate follows a similar pathway except that, in contrast to the parent compound, the vitamin enhances the rate of formation of the half-ester from the α-methyl derivative. Likely mechanisms for these reactions are discussed.

The reported (*1*) pyridoxal phosphate-catalyzed release of carbon dioxide from α-aminodiethylmalonate, $NH_2CH(COOEt)_2$, is a reaction of considerable interest with respect to the mechanism of vitamin B_6 catalysis, and for a number of years there has been speculation regarding the mechanism of this reaction and the role of the vitamin therein. For example, a hydrolytic mechanism involving formation of a half-ester sensitive to decarboxylation has been advanced (*1*, *2*). Another possible pathway involves pyridoxal-promoted carbon–carbon bond cleavage with the transient formation of ethyl carbonate (*1*).[1] Still another mechanism invokes pyridoxal-assisted ketene formation and hydration to the half-ester, followed by decarboxylation (*3*). Finally, neighboring group participation by a carbinolamine adduct of pyridoxal and α-aminodiethylmalonate, in a hydrolytic pathway leading to the half-ester, has been suggested (*3*).

We have reexamined this reaction and have determined that the release of carbon dioxide proceeds by way of an initial, pyridoxal phosphate-*independent* reaction, leading to a half-ester. The subsequent decarboxylation of the half-ester then occurs extremely rapidly in the presence of pyridoxal phosphate:

$$NH_2CH(COOEt)_2 \xrightarrow[k_1\ (B_6\text{-independent})]{-EtOH\ (slow)} NH_2CH(COOH)COOEt \xrightarrow[k_2\ (B_6\text{-dependent})]{-CO_2\ (fast)} NH_2CH_2COOEt. \quad (1)$$

[1] This mechanism has also been considered for the α-methyl derivative of α-aminodiethylmalonate (*2*).

In Fig. 1a are shown the effects of increasing time of incubation at pH 6.25 of α-aminodiethylmalonate prior to the addition of pyridoxal phosphate. It can be seen that there is a B_6-independent reaction that occurs during the incubation time leading to the formation of a decarboxylation-sensitive product, and that the addition of the vitamin causes a very rapid release of carbon dioxide, the amount of which is solely dependent on, and proportional to, the incubation time in buffer alone.[2] There is no CO_2 evolution observed until the pyridoxal phosphate is tipped into the main compartment, the time of addition of the vitamin being indicated by the arrows in Fig. 1.

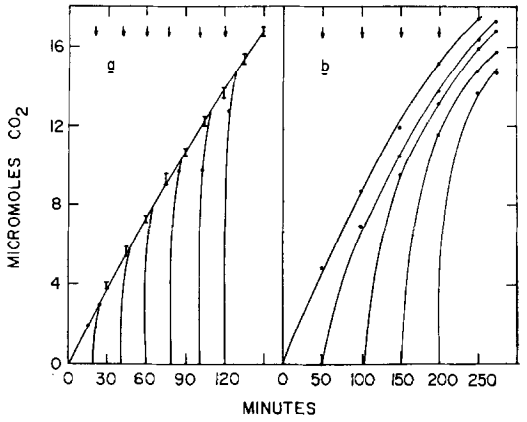

FIG. 1. a. CO_2 evolution was followed in a nitrogen atmosphere at 30°C in a Warburg apparatus. Double sidearm flasks were used. The contents of every flask were identical. The main compartment contained 2.2 ml of 0.6 *M* potassium phosphate buffer at pH 6.25 ($\mu = 1.0$ with KCl) and an amount of 2 *N* KOH sufficient to neutralize the aqueous α-aminodiethylmalonate hydrochloride solution in sidearm 1 (71 μmoles in 100 μl). Sidearm 2 contained 12.5 μmoles of pyridoxal phosphate in 200 μl of buffer plus 2 *N* KOH to neutralize the vitamin. After gassing and temperature equilibration, sidearm 1 of all setups were tipped in (zero time). At the desired times, indicated by arrows in the figure, sidearm 2 was tipped in. The bars represent the limits of the experimental data. b. Identical to a except that α-amino-α-methyldiethylmalonate hydrochloride was used.

The intermediacy of the half-ester of aminomalonic acid was demonstrated by thin-layer chromatography after incubation of a reaction mixture at pH 6.25 containing the diester and buffer alone, as shown in Fig. 2 in the tracks marked AMDE. It can be seen that in the absence of B_6 a spot corresponding to the monoester, AMME, is evident. (The streaking of this compound is most likely a result of decarboxylation of this reactive compound on the acidic tlc sheet.) In the presence of B_6, the half-ester has disappeared and a spot corresponding to glycine ethyl ester has appeared. Similarly, if authentic half-ester of aminomalonic acid[3] is incubated in the presence of B_6, glycine ethyl ester is formed (tracks marked AMME). Reaction mixtures containing

[2] The amount of CO_2 released at pH 6.25 depends on the concentration of the buffer. Hence the k_1 step in Eq. (1) is subject to general buffer catalysis.

[3] This compound was prepared by a modification of the procedure of Matthew and Neuberger (*4*). Elemental analyses (C, H, and N) were all within 0.25% of theory. All of the other compounds used in these experiments were either commercially available or were synthesized by published procedures.

pH 6.25 buffer, pyridoxal phosphate, and the half-ester of aminomalonic acid, under the conditions described in the legend to Fig. 1, evolved CO_2 at a rate which was too fast to follow by conventional Warburg manometry. Hence, k_2, in Eq. (1) is faster than k_1, which becomes the rate-limiting step.

In Fig. 1b are provided data for CO_2 release from α-amino-α-methyldiethylmalonate. It is important to note that, in contrast to the parent compound, the amount of CO_2 released from the α-methyl derivative shows a dependency on the presence of pyridoxal phosphate, in that the longer the substrate is incubated with the vitamin, the greater is

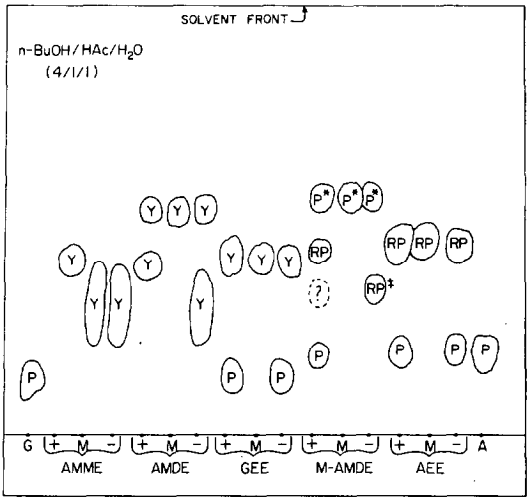

FIG. 2. All solutions were 0.06 *M* in the respective amino acid derivative, 0.2 *M* in potassium phosphate buffer ($\mu = 1.0$; pH 6.25), and, where present, 5×10^{-3} *M* in pyridoxal phosphate. The reaction solutions were kept at room temperature for 24 hr under N_2 in screw-cap vials in the dark. Five microliters of each solution, corresponding to 0.3 μmole of reactant, were then applied to an Eastman No. 6060 chromagram sheet previously activated at 100°C for 30 min. Marker solutions denoted by M were prepared immediately prior to application. The + and − symbols indicate the presence and absence of B_6. Abbreviations: G, glycine; AMME, α-aminomalonate monoethyl ester; AMDE, α-aminomalonate diethyl ester; GEE, glycine ethyl ester; M-AMDE, α-methyl-α-aminomalonate diethyl ester; AEE, alanine ethyl ester; A, alanine. Initial color after spraying with ninhydrin solution: P, purple; Y, yellow; RP, reddish purple. *Color developed only after prolonged standing in the dark. ‡Relative R_f indicates that this compound is the half-ester of α-methyl-α-aminomalonate.

the amount of CO_2 released. Results of thin-layer chromatography with reaction solutions at pH 6.25 containing α-amino-α-methyldiethylmalonate are provided in Fig. 2, in the tracks marked M-AMDE. In the absence of B_6, a compound is present which, from its mobility, is the half-ester of α-amino-α-methylmalonate. In the presence of B_6, this spot virtually completely disappears and a spot corresponding to alanine ethyl ester, AEE, becomes evident.

From these observations, it is clear that α-aminodiethylmalonate is converted to a half-ester in a reaction that is entirely independent of the vitamin, and that CO_2 release occurs subsequently in a B_6-dependent decarboxylation, as shown in Eq. (1). The formation of the half-ester may take place either directly, by a hydrolytic mechanism,

or, alternatively, by a mechanism involving elimination of ethanol and hydration of the resulting ketene to the half-ester.[4]

Since there is no α-hydrogen on α-amino-α-methyldiethylmalonate, CO_2 release from this compound most likely occurs by way of a hydrolytic pathway to the half-ester. The data provided in Fig. 1b reveal that, although hydrolysis occurs in the absence of pyridoxal phosphate, longer incubation with the vitamin enhances the absolute amount of CO_2 production from the α-methyl derivative. This enhancement in rate is expected because Schiff base formation between the vitamin and the α-amino-α-methyl diester would exert an inductive effect on the carbonyl group, rendering it more sensitive to nucleophilic attack by water. Along these lines, electron withdrawal from the bonds about the α-carbon atom of amino acids in Schiff base linkage with pyridoxal phosphate has been for many years a central part of the theory of vitamin B_6 catalysis (*6*).

The fact that pyridoxal phosphate promotes the hydrolysis of α-amino-α-methyldiethylmalonate (Fig. 1b) but has no apparent effect on the parent compound (Fig. 1a) is intriguing and no doubt relates to the remarkable acidity of the α-hydrogen of α-aminodiethylmalonate when it is in Schiff base linkage with B_6 derivatives (*2*).[5]

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[4] Holmquist and Bruice (*5*) have reported that the hydrolysis of α,α-dimethylethyl *o*-nitrophenylmalonate proceeds by general base, water-catalyzed, and hydroxide-catalyzed mechanisms. However, α-methyl ethyl *o*-nitrophenylmalonate is converted to the ethyl half-ester by a mechanism involving ketene-forming elimination of *o*-nitrophenol.

[5] Experiments in this laboratory have shown that, if one reacts increasing amounts of pyridoxal phosphate with a constant amount of α-amino-α-methyldiethylmalonate, all reactants being mixed together at zero time, then the release of CO_2 is proportional to the amount of the vitamin present, as expected. However, if one does the same experiment with the parent compound which contains an α-hydrogen atom, then the amount of CO_2 released *decreases* as the concentration of the pyridoxal phosphate is increased. Hence, the presence of the vitamin converts α-aminodiethylmalonate to a species that is *less* readily transformed to the sensitive half-ester. This species is most likely the 1,4-dihydropyridine tautomer of the Schiff base, described by Abbott and Bobrik (*2*).