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Aminomalonic Decarboxylase*

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A study has been made of the aminomalonic decarboxylase of rat liver, and evidence has been obtained for the role of pyridoxal phosphate as a co-factor. Under the test conditions employed, the enzyme appears to be specific for aminomalonic acid, and is inhibited by several structural analogues (*e.g.* α-methylaminomalonic acid, L-serine) which are not substrates. The nonenzymic decarboxylation of aminomalonic acid and α-methylaminomalonic acid at *p*H 6 is promoted by pyridoxal phosphate or pyridoxal (10^{-4} M and higher), and diethyl aminomalonate is decomposed at this *p*H in the presence of 10^{-5} M pyridoxal phosphate.

The existence of an enzyme that catalyzes the decarboxylation of aminomalonic acid was reported in a brief note by Shimura *et al.* (1956), who found it in the silk-gland tissue of silkworms and in rat-liver homogenates. In their studies of the silk-gland enzyme, these authors demonstrated that glycine and CO_2 were the products of the reaction, that the *p*H optimum was near *p*H 6, and that the enzymic activity was inhibited by cyanide and hydroxylamine. A careful search of the biochemical literature has failed to reveal any subsequent paper on this subject by the above authors. A later note from their laboratory (Nagayama *et al.*, 1958) reported the finding, in the silk-gland of silkworms and in rat-liver homogenates, of a transaminase that catalyzes the amination of ketomalonic acid (mesoxalic acid) by L-alanine and several other amino acids. The possibility was raised that a route of glycine synthesis in the silk-gland may be: hydroxymalonic acid (tartronic acid) to ketomalonic acid to aminomalonic acid to glycine. The question of the role of aminomalonic acid as a glycine precursor had been discussed several times previously in the biochemical literature (Knoop, 1914; Haas, 1916; Knoop and Oesterlin, 1927); in particular, Shemin (1946) excluded this possibility in the conversion of L-serine to glycine in the intact rat because the COOH-C^{13}/N^{15} ratio in the glycine (isolated as urinary hippuric acid) produced was the same as in the administered labeled serine. In his well-known note, Ogston (1948) commented on this conclusion by calling attention to the possibility that aminomalonic acid, although a symmetrical compound, may be handled asymmetrically by an enzyme so as to cause the selective loss, by decarboxylation, of only one of the apparently equivalent carboxyl groups. Later work on the mechanism of the serine-glycine interconversion has led to the recognition of the role of the tetrahydrofolic acid compounds as co-factors (for a review, see Huennekens and Osborn, 1959), and the possible intermediate role of aminomalonic acid in amino acid metabolism has received little attention recently.

Because of our interest in the possibility that aminomalonic acid may be a naturally occurring amino acid, it appeared desirable to examine more closely the enzyme-catalyzed decarboxylation reported by Shimura *et al.* (1956). In the present communication we describe experiments with a preparation from rat liver, and offer evidence in favor of a pyridoxal phosphate-dependent enzymic process. Observations are also reported on the catalysis of the nonenzymic decarboxylation of aminomalonic acid by pyridoxal phosphate. During the course of these studies with model systems, we noted that Neuberger (1961) had already observed the pyridoxal phosphate-promoted decarboxylation of aminomalonic acid.

A number of syntheses of aminomalonic acid have been described since its first preparation by Baeyer (1864) by the reduction of oximinomalonic acid (obtained from isonitrosobarbituric acid) with sodium amalgam. Later methods based on this approach have involved the preparation of diethyl isonitrosomalonate by the treatment of diethylmalonate with $NaNO_2$ and acetic acid (Cerchez, 1930; Zambito and Howe, 1960), followed by reduction with aluminum amalgam (Piloty and Neresheimer, 1906; Putochin, 1923; Cerchez, 1930), with hydrogen sulfide (Johnson and Nicolet, 1914), with Pt or Pd and H_2 (Putochin, 1923; Schneider, 1937; Snyder and Smith,

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1944; Hartung *et al.*, 1960), or with Zn and acetic acid or formic acid (Galat, 1947; Hellmann, 1949; Hellmann and Lingens, 1954; Meek *et al.*, 1959). The last reduction procedure has been performed with both the diethyl and dimethyl esters and leads to the formation of the acetamido- or formamidomalonates. Other synthetic routes to aminomalonic acid have been the reduction of nitromalonamide with sodium amalgam, followed by alkaline hydrolysis (Ruhemann and Orton, 1895); the treatment of chloromalonic acid with ammonia in methanol (Lutz, 1902); the treatment of uramil (5-aminobarbituric acid) with hot concentrated alkali (Piloty and Finckh, 1904); and the catalytic hydrogenation of carbobenzoxyaminomalonic acid (Beaujon and Hartung, 1952, 1953).

For the present work, the method of synthesis found to be the most convenient involved the cleavage of the readily available diethyl formamidomalonate with HCl in ethanol to yield diethyl aminomalonate hydrochloride, which was carefully saponified. For the study of the specificity of aminomalonic decarboxylase, α-methylaminomalonic acid was prepared by methylation of diethyl formamidomalonate, and subsequent treatment of the α-methyl derivative in the manner outlined above for the preparation of aminomalonic acid.

In agreement with the report of Shimura *et al.* (1956), homogenates and aqueous extracts of rat liver were found to contain aminomalonic decarboxylase activity. No appreciable activity was found in extracts of calf kidney; saline extracts of calf liver exhibited some activity, but this was markedly reduced upon dialysis against water. For the characterization of the rat-liver enzyme, a preparation was obtained in the following manner: From the supernatant fluid (fraction I) from a homogenate prepared with 0.15 M KCl, an acetone powder was obtained. The dialyzed aqueous extract (fraction II) of this powder was fractionated with ammonium sulfate, and the material that precipitated between 40% and 55% saturation was collected and dialyzed (fraction III). The details of a representative preparation are given in the experimental section, and the data on the specific activity of the fractions are presented in Table I.

TABLE I
PREPARATION OF RAT LIVER AMINOMALONIC DECARBOXYLASE[a]

Fraction[b]	Enzyme Units[c] Total	%	Protein Total (g)	%	Specific Activity
I[d]	68,640		33		2.08
II	67,400	98	30.6	93	2.20
III	24,200	35	5.8	18	4.15

[a] Based on a preparation from 620 g of rat liver (for details, see experimental section). [b] The designation of the fractions is given in the text. [c] Enzyme assays conducted with 2 mg of protein per 2.5 ml incubation mixture, which also contained 10^{-5} M pyridoxal phosphate. [d] Before assay, a 5-ml sample was dialyzed for 4 hours against 20 liters of water.

TABLE II
EFFECT OF PYRIDOXAL PHOSPHATE ON ENZYMIC DECARBOXYLATION OF AMINOMALONIC ACID
Standard conditions of enzyme assay (see experimental section), except as noted.

Pyridoxal Phosphate Added per ml (mμmoles)	CO_2 Evolution in 10 Minutes: Enzyme Present (μl)	Enzyme Absent (μl)
None	27	0
0.01	28	
0.1	32	
1.0	41	
10	59[a]	7
100		25
1000		108

[a] With 0.1 ml of 10% KOH in the center well of the Warburg vessel, no CO_2 evolution was observed. Also, after the enzyme preparation had been heated at 100° for 2 minutes, no CO_2 evolution was noted in a test assay.

It is evident that only slight concentration of enzymic activity was attained by this procedure, and efforts were made to effect further purification. Although encouraging results were obtained by the further treatment of fraction III with calcium phosphate gel and by column chromatography on DEAE-cellulose, these purification steps led to preparations that were unstable upon storage. For this reason, attempts to obtain a more highly purified enzyme preparation were suspended in the hope that better knowledge of the properties of the crude preparation might make future efforts in this direction more successful.

As might have been expected from the nature of the chemical reaction catalyzed by the enzyme preparation, it is promoted by the addition of pyridoxal phosphate (Table II). The study of the effect of increasing concentrations of this co-factor was limited by the fact that, at *p*H 6 and 37.5°, pyridoxal phosphate (at 10^{-4} M or higher) catalyzes appreciable decarboxylation of the substrate in the absence of the enzyme preparation. For this reason, the enzymic experiments were conducted in the presence of 10^{-5} M pyridoxal phosphate. The nonenzymic decarboxylation of aminomalonic acid and related compounds will be discussed later in this communication. The fact that some CO_2 evolution is observed in the enzyme assay without the addition of pyridoxal phosphate (Table II) suggests that this co-factor is present in the enzyme preparation, as in the case of other crude preparations of amino acid decarboxylases from animal tissues (see, for example, Schales and Schales, 1949).

The *p*H dependence of aminomalonic decarboxylase activity was studied in the presence of

the following buffers (all at 0.1 M and as sodium salts): succinate (*p*H 4.0–5.8), citrate (*p*H 4.8–5.8), cacodylate (*p*H 5.3–6.6), phosphate (*p*H 5.8–6.8). The *p*H dependence curve showed maximal activity with a plateau in the region *p*H 5.5–6.5, and therefore *p*H 6.0 was selected for the standard assay of the enzyme. The *p*H-dependence curve shows a marked drop at *p*H values more acid than 5.5; this is attributable to the instability of the enzyme, since incubation of the preparation for 30 minutes at 37.5° and *p*H values below 5 before testing causes complete loss of enzyme activity (tested at *p*H 6 in the usual manner). The enzyme preparation appears to be stable over the *p*H range 5.5–8.0.

Upon paper chromatography (Whatman No. 1 paper) of an incubation mixture with *n*-propanol–ammonia–water (6:3:1 v/v), ninhydrin-positive components corresponding to aminomalonic acid (R_F 0.30) and to glycine (R_F 0.43) were observed, in agreement with the report of Shimura *et al.* (1956).

In the enzyme-catalyzed decarboxylation of aminomalonic acid, 10^{-5} M pyridoxal phosphate cannot be replaced by an equal concentration of either pyridoxal or pyridoxamine phosphate. As will be seen from Table III, pyridoxal did not promote the enzymic reaction at concentrations as high as 10^{-2} M, the CO_2 evolution at this concentration being entirely attributable to the action of pyridoxal in catalyzing the nonenzymic decarboxylation of aminomalonic acid. At a concentration of 10^{-2} M pyridoxamine phosphate, the rate of CO_2 evolution was about 80% of that observed in the standard control assay; it appears possible that the crude enzyme preparation caused the formation of sufficient pyridoxal phosphate to promote the decarboxylation reaction. Pyridoxine (pyridoxol) had no stimulatory or inhibitory effects at concentrations as high as 10^{-2} M.

The conclusion that aminomalonic decarboxylase is a pyridoxal phosphate–dependent enzyme

TABLE III
EFFECT OF PYRIDOXAL ON THE DECARBOXYLATION OF AMINOMALONIC ACID

Standard conditions of enzyme assay (see experimental section), except that pyridoxal was used in place of 10^{-5} M pyridoxal phosphate.

Pyridoxal Added per ml (μmoles)	CO_2 Evolution in 10 Minutes: Enzyme Present (1) (μl)	Enzyme Absent (2) (μl)	(1) − (2) (μl)
0.01[a]	22	5	17
0.1	23	10	13
1.0	36	24	12
10	138	119	19
None	23	0	23

[a] A control experiment with 10^{-5} M added pyridoxal phosphate gave a CO_2 evolution of 60 μl during the first 10 minutes.

TABLE IV
EFFECT OF ALDEHYDE REAGENTS ON AMINOMALONIC DECARBOXYLASE

Standard conditions of enzyme assay (see experimental section).

Reagent	% Inhibition of Enzyme Activity: 10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
Hydroxylamine	55	92		
Semicarbazide	5	15	79	100
Phenylhydrazine	2	23	62	88
Sodium cyanide	1	17	60	85
Sodium bisulfite	0	2	16	51

is supported by the inhibitory effect of aldehyde reagents. As shown in Table IV, hydroxylamine, semicarbazide, phenylhydrazine, cyanide, and bisulfite (in the concentration range 10^{-5}–10^{-2} M) are inhibitors, with hydroxylamine the most effective one of those tested.

During the course of the attempts to purify the enzyme by salt fractionation, it was observed that the enzymic activity was inhibited by KCl at concentrations higher than 0.1 N. Further examination of this phenomenon showed that the addition of other salts showed a similar inhibitory effect. With KCl, NaCl, and imidazolium chloride, the decrease in rate was proportional to salt concentration over the range 0–0.7 N. The concentrations of the salts causing 50% decrease in enzymic activity (under the standard conditions of assay) were: KCl, 0.55 N; NaCl, 0.4 N; imidazolium chloride, 0.45 N. It was of interest to find that 0.4 N NaI caused almost complete inhibition of enzyme activity, with a 50% decrease at about 0.2 N, whereas 0.7 N Na_2SO_4 inhibited the activity only about 40%. Clearly, these effects cannot be explained solely on the basis of the influence of the ionic strength of the medium on enzymic activity. It may be added that the inhibitory effect of 0.7 N NaCl is reversible, since the activity of a salt-treated sample dialyzed against water was the same as that of a control solution dialyzed in the same manner. The possibility exists that the inhibition of enzymic activity by high concentrations of salts may be in part a consequence of the dissociation of the pyridoxal phosphate–enzyme complex.

The enzymic activity (in the presence of 10^{-5} M pyridoxal phosphate and 0.1 M cacodylate buffer, *p*H 6) was completely inhibited by 10^{-3} M $CuCl_2$ and by 10^{-4} M $HgCl_2$; it was only slightly inhibited (10–20%) by the presence of 10^{-3} M concentrations of the following salts: $MgSO_4$, $MnCl_2$, $SnCl_2$, $CoCl_2$, $ZnCl_2$, $NiCl_2$, $FeSO_4$, $FeCl_3$, and $Al(SO_4)_3$. No inhibition was observed with 10^{-2} M ethylene diaminetetraacetate or β-mercaptoethanol. On the other hand, 10^{-2} M β-mercaptoethylamine and L-cysteine were strongly inhibitory (54% and 71% respectively). This action of aminothiols may be attributed to their

reaction with pyridoxal phosphate to form thiazolidines (Heyl *et al.*, 1948; Buell and Hansen, 1960).

The following compounds were tested under the standard assay conditions as substrates (at 0.05 M) in place of aminomalonic acid: carbobenzoxyaminomalonic acid, glycylaminomalonic acid, carbobenzoxyglycylaminomalonic acid, α-methylaminomalonic acid, glycine, L-serine, D-serine, L-threonine, L-cysteine, L-aspartic acid, L-glutamic acid, α-aminoisobutyric acid, α-methylmalonic acid, ketomalonic acid, and hydroxymalonic acid. None of these compounds was decarboxylated during the 30-minute period of the assay. It was of interest to find that CO_2 was liberated upon the incubation of diethyl aminomalonate with the enzyme and pyridoxal phosphate, but control experiments showed that this reaction could be accounted for by a nonenzymic process that is promoted by 10^{-5} M pyridoxal phosphate. It would appear that, of the compounds tested thus far, the only one that serves as a substrate for the enzyme under study is aminomalonic acid itself. Substitution of the amino group, or its replacement by a keto group or hydroxyl group, renders aminomalonic acid resistant to enzymic decarboxylation. Similarly, replacement of the α-hydrogen by a methyl group abolishes the enzyme-catalyzed reaction, although, as will be seen later, α-methylaminomalonic acid is decarboxylated nonenzymically in the presence of 10^{-3} M pyridoxal phosphate.

It should be noted that the failure of the enzyme preparation to catalyze the decarboxylation of ketomalonic acid at *p*H 6 appears to rule out this compound as an intermediate in the enzymic decarboxylation of aminomalonic acid, and makes it unlikely that the enzyme preparation catalyzed a transamination reaction (Braunstein, 1939; Cohen, 1939; Green *et al.*, 1945) with a keto acid present in the incubation mixture, followed by spontaneous decarboxylation of the resulting ketomalonic acid. The ketomalonate–amino acid transaminase found in rat liver by Nagayama *et al.* (1958) is reported to be optimally active at alkaline *p*H values. In this connection, it should be added that Mix (1961) reported a nonenzymic reaction in which pyridine and Cu^{++} promoted the decarboxylation of ketomalonic acid in the presence of α-amino acids (*e.g.*, phenylalanine) at 37° and *p*H 7. This suggested the possibility that aminomalonic acid might react with pyridoxal phosphate to form pyridoxamine phosphate plus ketomalonic acid, which in the presence of an amino acid would undergo decarboxylation. However, the addition of equimolar concentrations of L-glutamic acid, L-aspartic acid, or L-phenylalanine did not promote the rate of decarboxylation of ketomalonic acid at *p*H 6 in the presence of 10^{-5} M pyridoxal phosphate.

Many of the compounds tested as possible substrates were also tested as inhibitors of the enzymic decarboxylation of aminomalonic acid. From Table V it may be seen that the α-amino acids examined were found to be inhibitory in varying degree. Of these, L-cysteine, L-serine, and α-methylaminomalonic acid were the most effective. As noted before, the inhibition by L-cysteine cannot be attributed solely to its sulfhydryl group, since 10^{-2} M β-mercaptoethanol was not an inhibitor, but rather to the fact that L-cysteine is an aminothiol. The inhibition by L-serine, which is more effective than D-serine, suggests that this amino acid reacts with the active region of the catalytic protein; the possibility of oxazolidine formation with pyridoxal phosphate cannot be excluded, although ethanolamine (0.05 M) was not an effective inhibitor (21% inhibition) of aminomalonic decarboxylase. Glycine (a product of the decarboxylation of aminomalonic acid) and L-aspartic acid were inhibitors, whereas L-glutamic acid did not appear to have an appreciable effect. Of special interest is the inhibition by α-methylaminomalonic acid; α-methyl derivatives of aromatic α-amino acids have been found to be inhibitors of an enzyme preparation (from guinea pig kidney) that catalyzes the decarboxylation of 3,4-dihydroxyphenylalanine, 5-hydroxytryptophan, tryptophan, and phenylalanine (Lovenberg *et al.*, 1962).

Under the experimental conditions of the standard assay method, a series of measurements of the initial rate of the decarboxylation of aminomalonic acid as a function of substrate concentration gave values for K_m of 0.013–0.015 M. In these determinations, the slope and intercept were calculated from the data by the method of least squares. Lineweaver-Burk plots for the

TABLE V
INHIBITION OF AMINOMALONIC DECARBOXYLASE BY STRUCTURAL ANALOGUES

Standard conditions of enzyme assay (see experimental section).

Added Substance	% Inhibition of Enzyme Activity		
	0.01 M	0.03 M	0.05 M
Glycine	12	35	48
L-Alanine	10	15	24
L-Serine	58	71	81
D-Serine	24	40	50
L-Threonine			21
L-Cysteine	71		
L-Aspartic acid	5	21	35
L-Glutamic acid			4
α-Aminoisobutyric acid			11
α-Methylaminomalonic acid	23	70	89
Carbobenzoxyaminomalonic acid			86[a]
Glycylaminomalonic acid			35
Carbobenzoxyglycylaminomalonic acid			81[a]
Malonic acid			6
Methylmalonic acid			19
Ketomalonic acid			4
Hydroxymalonic acid			29

[a] A copious precipitate appeared in the incubation mixture.

enzymic decarboxylation of aminomalonic acid in the presence of α-methylaminomalonic acid (0.02 M) and L-serine (0.005 M) gave data that accorded (within the precision of the method) with the assumption that these compounds are competitive inhibitors; the calculated K_i values were 0.008 M and 0.0015 M respectively.

The observation that L-serine is an effective inhibitor of aminomalonic decarboxylase raised the question of whether the enzyme under study was serine hydroxymethylase, one of whose properties might be to catalyze the decarboxylation of aminomalonic acid. It was found that 0.025 M L-serine at *p*H 6, in the presence of the enzyme preparation and 10^{-5} M pyridoxal phosphate, did not give rise to the formation of measurable quantities of formaldehyde. Furthermore, no formaldehyde disappearance was observed when 0.05 M glycine and 0.006 M formaldehyde were incubated in the presence of the enzyme preparation and 10^{-5} M pyridoxal phosphate. Control experiments showed that this concentration of formaldehyde exerted only a slight inhibitory effect (12%) on the enzymic decarboxylation of aminomalonic acid under the standard conditions of assay.

As noted in Table II, the nonenzymic decarboxylation of aminomalonic acid at *p*H 6 is promoted by pyridoxal phosphate at concentrations higher than 10^{-5} M. Thus, at 10^{-3} M pyridoxal phosphate, and with 0.05 M aminomalonic acid, there is a rapid production of 108 μl of CO_2 (4% of the theory) during the first 10 minutes, with a marked slowing of the rate thereafter. Of special interest is the observation that 10^{-3} M pyridoxal was less effective in the nonenzymic reaction than 10^{-3} M pyridoxal phosphate, the initial rates being in the ratio of about 1:3. This difference may be a consequence of the fact that, at *p*H 6, a large proportion of the pyridoxal is in the form of its hemiacetal (Metzler and Snell, 1955). The addition of glycine (0.05 M) did not inhibit the initial rate of decarboxylation in the presence of either pyridoxal or pyridoxal phosphate (10^{-3} M).

Whereas α-methylaminomalonic acid was not decarboxylated enzymically under the standard conditions of enzyme assay, nonenzymic decarboxylation of this compound at *p*H 6 could be demonstrated in the presence of 10^{-3} M pyridoxal phosphate. Under these conditions, the initial rate of CO_2 liberation was about 50% of that for aminomalonic acid. Paper chromatography (Whatman No. 3 MM paper; phenol-water, 4:1 v/v) of the incubation mixture showed the presence of alanine (R_F 0.60), as well as of α-methylaminomalonic acid (R_F 0.18).

Relatively few examples are known of the nonenzymic catalysis of the decarboxylation of amino acids by pyridoxal or related aldehydes (Snell, 1958). The possibility of the nonenzymic decarboxylation of histidine by pyridoxal (or its phosphate) was suggested by the report of Werle and Koch (1949). More definitive evidence was presented by Kalyankar and Snell (1957, 1962), who showed that α-methyl-α-amino acids, when heated in dilute aqueous solutions with pyridoxal in the absence of metal ions, undergo decarboxylation reactions. The α-methyl compounds were chosen to avoid the complication of competing transamination reactions and the decarboxylation of the keto acids under the experimental conditions employed. The decarboxylation of aminomalonic acid in the presence of pyridoxal or pyridoxal phosphate at *p*H 6 and 37.5° thus represents a reaction of special interest, whose mechanism merits further investigation.

It should be noted that α-methylaminomalonic acid, which undergoes nonenzymic decarboxylation in the presence of 10^{-3} M pyridoxal phosphate, is resistant to the action of the enzyme at a pyridoxal phosphate concentration (10^{-5} M) sufficient to promote rapid decarboxylation of aminomalonic acid. Further studies are needed to establish whether the mechanism of the model reaction is similar to that of the enzyme-catalyzed processes. Experiments with tritiated water and with D_2O may contribute to the elucidation of this question. Mandeles *et al.* (1954) showed that in the decarboxylation of tyrosine (and of other amino acids) by bacterial decarboxylases in the presence of D_2O, only one deuterium atom entered the resulting amine, and Belleau and Burba (1960) demonstrated that the process occurs with retention of configuration about the asymmetric carbon of tyrosine (see Westheimer, 1959).

The observation, mentioned above, that 10^{-5} M pyridoxal phosphate promotes the liberation of CO_2 from 0.05 M diethyl aminomalonate at *p*H 6 and 37.5° deserves further attention. At 10^{-3} M pyridoxal phosphate, the initial rate of CO_2 evolution is about 4 times that for 0.05 M aminomalonic acid. Since CO_2 is not evolved at a measurable rate from diethyl aminomalonate under the above conditions in the absence of pyridoxal phosphate, it would seem that the decomposition is catalyzed by the intermediate formation of an imine (Metzler, 1957; Matsuo, 1957; Lucas *et al.*, 1962); indeed, when 10^{-3} M pyridoxal phosphate and 0.05 M diester are mixed, a deep yellow color appears in the solution, whose absorption spectrum shows a maximum at 460 mμ. Further work is needed to elucidate the mechanism of the reaction; the decomposition may involve the transient formation of monoethyl carbonate which is rapidly converted in water to CO_2 and ethanol, or may proceed by initial hydrolysis to the monoethyl ester which is decarboxylated.

The fact that the diester is decomposed at *p*H 6 in the presence of 10^{-5} M pyridoxal phosphate, whereas the nonenzymic decarboxylation of aminomalonic acid at this *p*H value requires higher concentrations (*ca.* 10^{-3} M) of the aldehyde may be attributed to the lower pK' value of the NH_3^+ group of the diester. It may be added that, at *p*H 6 and 37.5°, 10^{-3} M pyridoxal phosphate did not promote any CO evolution in the presence of

0.006 M aminomalondiamide (tested at this concentration because of its sparing solubility in water).

The available data suggest that the pyridoxal phosphate–promoted nonenzymic decarboxylation of aminomalonic acid at *p*H 6 involves the formation of an imine, and electron withdrawal from the bond to be cleaved, in the manner postulated for other pyridoxal-catalyzed model reactions (Snell, 1958). In the decarboxylation of aminomalonic acid at acidic *p*H values (in the absence of pyridoxal phosphate), the facilitation of C–C bond cleavage may involve internal hydrogen bonding between a COOH group and a COO^- group, in analogy to the mechanism postulated for the decarboxylation of β-keto acids (Westheimer and Jones, 1941). It is well known that the ionic form $^+NH_3CH(COO^-)_2$ or $NH_2CH(COO^-)_2$ is stable to decarboxylation, and preliminary experiments in this laboratory have shown that the greatest change in the rate of nonenzymic decarboxylation of aminomalonic acid (in the absence of pyridoxal phosphate) occurs in the *p*H range 2 to 6. As noted in the experimental section, the pK_2' of the amino acid is 3.1 (pK_1' has not been determined, but is probably near 1), indicating that the dipolar ion $[^+NH_3CH(COOH)COO^-]$ is the reactive species in the decarboxylation reaction over the *p*H range 1 to 6. The extent to which these suggestions are valid may emerge more clearly from studies, now in progress, of the kinetics of the nonenzymic decarboxylation of aminomalonic acid as function of *p*H in the presence and absence of pyridoxal phosphate and related aldehydes.

Experimental

Preparation of Aminomalonic Acid.—Diethyl formamidomalonate (25 g; Aldrich Chemical Co.) was dissolved in 600 ml of absolute ethanol containing 22 g of HCl and kept at room temperature for 20 hours. The solution was concentrated to dryness under reduced pressure, and the evaporation was repeated three times after the addition of 100-ml portions of absolute ethanol. The residual solid was dissolved in absolute ethanol, and ether was added to induce crystallization. After standing overnight in the refrigerator, the suspension was filtered and the precipitate was washed with ether. Yield, 20 g (77% of the theory); m.p. 159–160°. The following melting points have been reported for diethyl aminomalonate hydrochloride: 162° (Putochin, 1923); 164–165° (Hartung *et al.*, 1960); 170° (Levene and Schormüller, 1934). *Anal.* Calcd. for $C_7H_{14}O_4NCl$ (211.7): N, 6.6. Found, N, 6.5.

A solution of the ester hydrochloride (5 g, 0.0236 mole) in 88 ml of N NaOH was heated on the steam bath for 5 minutes, chilled in ice and adjusted to *p*H 6 with 2 N acetic acid. An aqueous solution (50 ml) of lead acetate trihydrate (12.5 g) was added, and, after the suspension had been kept in the refrigerator overnight, it was filtered. The precipitate was washed with water, suspended in 70 ml of water, and treated with a stream of H_2S for 40 minutes with stirring and cooling. The PbS was removed by filtration, the solution was concentrated to a small volume (bath temperature, 30°), and absolute ethanol was added. After standing in the refrigerator, the suspension was filtered, and the product was washed with ethanol. Yield 2.4 g (85% of the theory): m.p. 140–142° (decomp.) *Anal.* Calcd. for $C_3H_5O_4N$ (119.1): N, 11.8. Found, N, 11.7. Ruhemann and Orton (1895) reported a m.p. of 109° (decomp.) for the monohydrate, which they obtained by hydrolysis of the diamide. Although most of the preparations of aminomalonic acid made by us separated as anhydrous preparations, occasionally the monohydrate was obtained.

Preparations of aminomalonic acid, upon ascending paper chromatography (Whatman No. 1 paper) with *n*-propanol–ammonia–water (6:3:1 v/v) exhibit an R_F value of 0.30; only slight contamination by glycine (R_F 0.43) was observed.

An aqueous solution (0.01 M) of an analytically pure sample of aminomalonic acid, upon titration at 37° with 0.1 M NaOH in the presence of 0.1 N KCl (Radiometer TTT1 apparatus), gave a value of 3.10 for pK_2' and of 9.00 for pK_3'. The value of pK_2' found here compares favorably with that calculated (3.11) from the electrolytic dissociation constant reported by Lutz (1902).

Preparation of α-Methyl-α-aminomalonic Acid.—Diethyl α-methyl formamidomalonate was prepared by treatment of 10.95 g (0.054 mole) of diethyl formamidomalonate with 100 ml of 0.54 N sodium ethoxide in absolute ethanol and 11.4 g (0.08 mole) of methyl iodide under reflux for 3.5 hours. The reaction mixture was concentrated under reduced pressure to an oil, which was washed with water and then extracted with three 40 ml portions of ether. The combined ether extracts were dried over exsiccated Na_2SO_4, and concentrated under reduced pressure. The product crystallized from an ethanol-petroleum ether mixture. Yield, 8.0 g (68% of the theory); m.p. 49–51°. *Anal.* Calcd. for $C_9H_{15}O_5N$ (217.2): N, 6.45. Found, N, 6.3.

The product (1 g) was deformylated in the manner described above for aminomalonic acid. Crystallization was induced by treatment of the resulting oil with petroleum ether, and the deformylated product was recrystallized from benzene-petroleum ether. Yield, 0.65 g (63% of the theory); m.p. 87–89°. The hygroscopic diethyl α-methyl-α-aminomalonate hydrochloride (0.6 g) was saponified in the manner described above for aminomalonic acid. Yield, 0.28 g (79% of the theory); in contrast to the parent compound, the α-methyl derivative does not decompose below 250°. The product was recrystallized from aqueous ethanol for analysis.

Anal. Calcd. for $C_4H_7O_4N$ (133.1): C, 36.1; H, 5.3; N, 10.5. Found: C, 36.0; H, 5.1; N, 10.4.

Other Compounds.—The following compounds were commercial preparations or were prepared according to the reference cited. Pyridoxal phosphate monohydrate, pyridoxal hydrochloride (Sigma Chemical Co.); pyridoxine hydrochloride (Merck and Co.); pyridoxamine phosphate, β-mercaptoethanol (California Corporation for Biochemical Research); D-serine (Nutritional Biochemical Corp.); L-serine, L-glutamic acid, L-aspartic acid, L-alanine, L-cysteine, α-aminoisobutyric acid, cacodylic acid (Mann Laboratories); glycine, malonic acid, ethanolamine (Matheson, Coleman and Bell); sodium ketomalonate, tartronic acid (Aldrich Chemical Co.); β-mercaptoethylamine hydrochloride (Evans Chemetics); carbobenzoxyaminomalonic acid (m.p. 142° decomp.; Beaujon and Hartung, 1953); carbobenzoxyglycylaminomalonic acid (m.p. 146° decomp.; Schneider, 1937); glycylaminomalonic acid (m.p. 220° decomp.; Schneider, 1937); aminomalondiamide (m.p. 191–192° decomp.; Piloty and Neresheimer, 1906).

Enzyme Preparation.—Except where indicated, all operations were performed in a cold room at 3°. Six hundred twenty g of liver (from 40 rats) was homogenized for 1 minute with 1300 ml of 0.15 M KCl at low speed in a large Waring blendor. Upon centrifugation of the resulting mixture (2.1 liters) for 30 minutes at 9000 rpm in the HR-1 International Centrifuge, 1560 ml of supernatant fluid was obtained; this was centrifuged at 54,000 × *g* for 1 hour in the No. 30 rotor of the Spinco Model L centrifuge. The clear solution (fraction I, 1.2 liters) was dripped rapidly into 10 volumes of acetone kept at −20° with solid CO_2, the precipitate was filtered with suction, and the filter cake was washed five times by resuspending it each time in 3.6 liters of acetone (−20°). The acetone powder was dried overnight *in vacuo* at room temperature and kept at 0°. Yield, 55 g.

The acetone powder (8 g) was ground with 160 ml of water, the mixture was dialyzed for 4 hours against three 6-liter portions of water, and the insoluble material was removed by centrifugation to yield 164 ml of extract (fraction II). Saturated ammonium sulfate (109 ml) previously adjusted to *p*H 8.3 was added to 40% saturation, the precipitate was removed by centrifugation, and to the supernatant fluid 83 ml of saturated ammonium sulfate was added to 55% saturation. The precipitate was collected by centrifugation and dissolved in 12 ml of water, and the solution was dialyzed against three 6-liter portions of water for 10 hours to yield 27 ml of enzyme solution (fraction III). After dilution to 20 mg protein per ml with water, the solution was dispensed in 2-ml samples which were kept frozen and used as needed. Whereas the acetone powder retains its original enzymic activity for several months, fraction III may lose up to 50% of its activity within a month.

Enzyme Assay.—The decarboxylation of aminomalonic acid was followed manometrically at 37.5°. Except where otherwise stated, the main chamber of the Warburg vessel contained 2 ml of a solution composed of the enzyme preparation, 250 μmoles of sodium phosphate buffer (*p*H 6.0 ± 0.1), and 25 mμmoles of pyridoxal phosphate. The side-arm contained a solution of 125 μmoles of aminomalonic acid in 0.5 ml water (adjusted to *p*H 6 with NaOH). After the solutions had been introduced into the vessels, the manometers were gassed for about 10 minutes with N_2, the stopcocks were closed, and temperature equilibration was allowed to proceed for about 3 minutes. The contents of the side-arm were then tipped into the main chamber, about 45–60 seconds were allowed for temperature equilibration, and manometric readings were taken at 10-minute intervals for 30 minutes. In general, the rate of CO_2 evolution was linear over this time period. In the calculation of the vessel constants, a correction was applied for CO_2 retention at *p*H 6 by the reaction mixture (Umbreit *et al.*, 1957); in experiments to determine the *p*H dependence of enzymic activity, appropriate changes in the correction factor were made for *p*H values more alkaline than *p*H 6. Control experiments showed that the 10-minute equilibration period was sufficient for interaction of pyridoxal phosphate with the enzyme.

An enzyme unit is defined as the amount causing the liberation of 10 μl of CO_2 in 30 minutes, and the specific activity is given as units per mg protein (as determined by the biuret method [Layne, 1957]). Unless otherwise stated, the enzyme concentration was 4 mg of protein per 2.5 ml incubation mixture. In the experiments in which the effect of inhibitors was tested, these compounds were added to the main chamber of the Warburg vessel.

For the determination of the ability of the enzyme preparation to promote the formation of formaldehyde from L-serine or the uptake of formaldehyde in the presence of glycine, the chromotropic acid method of Frisell *et al.* (1954) was employed.

Nonenzymic Decarboxylation Experiments.—Unless otherwise stated, the concentration of the test compounds was 0.05 M in a total volume of 2.5 ml, containing 0.1 M sodium phosphate buffer (*p*H 6). As in the enzyme assay, CO_2 evolution was measured manometrically at 37.5°.

Pyridoxal Phosphate–Catalyzed Decomposition of Diethyl Aminomalonate.– The incubation mixture (5 ml) contained 5 μmoles of pyridoxal phosphate, 250 μmoles of diethyl aminomalonate hydrochloride (adjusted to *p*H 6), and 0.1 M sodium phosphate buffer *p*H 6.0. After being kept at 37.5° for 30 minutes (the *p*H was unchanged), a sample was subjected to paper chromatography (Whatman No. 1 paper; *n*-butanol–acetic acid–water, 4:1:6 v/v). Two major ninhydrin-positive components were found: their R_F values corresponded to those of authentic samples of the diester (R_F 0.81) and of glycine ethyl ester (R_F 0.64) dissolved in 0.1 M phosphate buffer (*p*H 6). In addition, a faint ninhydrin-

positive component of R_F 0.20 (corresponding to that of glycine) was noted in the chromatogram of the incubation mixture. It appears likely that this component arose by the hydrolysis of glycine ethyl ester, since chromatography of an incubation mixture of 0.05 M glycine ethyl ester with 10^{-3} M pyridoxal phosphate, kept at *p*H 6 and 37.5° for 30 minutes, showed the presence of appreciable amounts of the component having R_F 0.20.

When the decomposition of 0.05 M diethyl aminomalonate was followed manometrically at *p*H 6 in the presence of 10^{-3} M pyridoxal phosphate, the CO_2 evolution (in μl) was: 5 minutes, 280; 10 minutes, 370; 20 minutes, 405; 30 minutes, 420. These changes in manometric readings were abolished when 0.1 ml of 10% KOH was present in the center well of the main chamber of the Warburg vessel. In the absence of pyridoxal phosphate, diethyl aminomalonate was not decomposed to a measurable extent under the above experimental conditions.

Spectrophotometric examination of the incubation mixture containing 0.05 M diester and 10^{-3} M pyridoxal phosphate at *p*H 6 showed a strong absorption band at 460 mμ (molecular extinction coefficient, *ca.* 15,000).

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