Aminomalonate as an Enzyme Inhibitor

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When *Rhodopseudomonas spheroides*, which had been grown in a biotin-deficient medium, was transferred to a medium containing glycine, α-oxoglutarate and fumarate, and which normally promotes formation of much porphyrin, little or no porphyrin was formed (Lascelles, 1956); but, when glycine and α-oxoglutarate were replaced by δ-aminolaevulic acid, the biotin-deficient organisms were able to form porphyrin. The work of Lascelles was confirmed and extended by Gibson, Neuberger & Tait (1962), who showed that the addition of biotin to the medium enables biotin-deficient organisms to form porphyrin from glycine and α-oxoglutarate. The condensation of these compounds to form δ-aminolaevulate, catalysed by δ-aminolaevulate synthetase, was shown in a cell-free system to be inhibited by commercial preparations of avidin. These findings suggested that biotin might be concerned in the synthesis of δ-aminolaevulate, the first step in the formation of porphyrin. Two possibilities for the role of biotin were considered. One was that biotin was involved in the decarboxylation of α-amino-β-oxoadipate, at the time thought to be the immediate precursor of δ-aminolaevulate. The other possibility was that one of the substrates for aminolaevulate synthetase was not glycine but aminomalonate, i.e. monocarboxylated glycine: this hypothesis was attractive on chemical grounds because the α-carbon atom of aminomalonate is a more reactive nucleophilic centre than that of glycine.

Aminomalonate was therefore synthesized and examined as a substrate for aminolaevulate synthetase. As reported briefly by Neuberger (1961), it was found to be not a substrate but a powerful inhibitor of the enzyme. This work is now reported in more detail, together with the effect of aminomalonate on other enzymes that have glycine as substrate or pyridoxal phosphate as coenzyme. A preliminary communication has been published (Gibson, Matthew, Neuberger & Tait, 1961).

EXPERIMENTAL

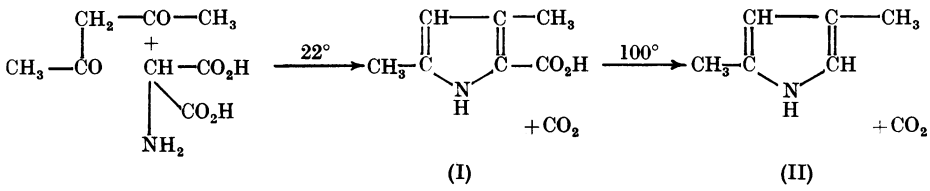
Chemical determinations

Determination of aminomalonate and δ-aminolaevulate. In experiments in which it was desired to measure the effect of aminomalonate on the enzymic formation of δ-aminolaevulate from succinyl-CoA and glycine by aminolaevulate synthetase, it was noticed that aminomalonate, when treated with acetylacetone and Ehrlich's reagent, gave a colour similar to that of 3-acetyl-4-(2-carboxyethyl)-2-methylpyrrole obtained from δ-aminolaevulate. Although the molecular extinction coefficient of the aminomalonate compound at its $\lambda_{max.}$ (533 mμ) was about 10% of that of the compound formed from δ-aminolaevulate ($\lambda_{max.}$ at 553 mμ), the amount of aminomalonate necessarily used in these experiments made it impossible to use the acetylacetone-condensation method of Mauzerall & Granick (1956) for the determination of δ-aminolaevulate in the presence of aminomalonate. However, aminomalonate does not give a chromogen with ethyl acetoacetate, and this difference between the reagents has been exploited to determine δ-aminolaevulate with ethyl acetoacetate in the presence of aminomalonate. Acetylacetone has been used in other experiments to determine aminomalonate alone.

Determination of δ-aminolaevulate in the presence of aminomalonate. To determine δ-aminolaevulate in the presence of aminomalonate or its derivatives the ethyl acetoacetate colour reaction (Shuster, 1956) was modified as follows. To 1 ml. of the solution containing 0–1·0 μmole of δ-aminolaevulate was added ethyl acetoacetate (0·05 ml.) followed by 10N-NaOH (0·3 ml.). The mixture was heated at 100° for 10 min., cooled, and then 5N-HCl (0·6 ml.), Ehrlich's reagent (Mauzerall & Granick, 1956) (0·6 ml.) and ethanol (1·5 ml.) were added in that order. Extinctions were read at 550 mμ after 10 min.

Nature of the reaction between aminomalonate and acetylacetone

Attempts were made to isolate the product of the condensation of aminomalonate and acetylacetone both under the conditions of the colour reaction (100° for 10 min.) and by allowing the compounds to react for several days at 22°. These were not successful because, judging by the poor colour yield, only a small proportion of the theoretical amount of pyrrole had been formed. The product had the same $\lambda_{max.}$ (533 mμ) when allowed to react with the modified Ehrlich's reagent as had an authentic sample of 2,4-dimethylpyrrole (II) (Treibs & Schmidt, 1952). Because of the rapidity with which Ehrlich's reagent reacted with the product, it seemed likely that this was a pyrrole with a free α-position. Aminomalonate and acetylacetone also condensed in the cold to form a compound that reacted with Ehrlich's reagent on warming. The finding that aminomalonate condenses only with a β-dicarbonyl compound and not one where a carbonyl group is in a β-position to a carboxyl group indicates that both carbonyl groups are concerned in the condensation, the first product probably being 2-carboxy-3,5-dimethyl-



Scheme 1. Reaction between aminomalonate and acetylacetone.

pyrrole (I) which, when heated, loses CO_2 to form 2,4-dimethylpyrrole (II) (Scheme 1). This is essentially the same type of reaction as that described by Kleinspehn (1955) in which 2-ethoxycarbonyl-3,5-dimethylpyrrole is formed by the reductive condensation of ethyl oximinomalonate with pentane-2,4-dione (acetylacetone).

A solution of aminomalonate (10–20 μmoles) in water (10 ml.) was adjusted to pH 8 with NaOH. A few drops of acetylacetone were added and the mixture was allowed to stand at 22° for 1·5 hr. The solution was then brought to pH 10 with 5% (w/v) Na_2CO_3 and extracted with ether (extract I). The aqueous layer was acidified to pH 4 with 3N-HCl and again extracted with ether (extract II). Another drop of acetylacetone was then added to the aqueous layer, which was then heated at 100° for 10 min., cooled, and extracted as before (extracts III and IV). Extract II gave a positive reaction, but only when heated, with Ehrlich's reagent; extract III gave a positive reaction in the cold; extracts I and IV gave no positive reaction, indicating that aminomalonate and acetylacetone reacted in the cold to form an acidic pyrrole which lost its carboxyl group(s) on heating.

Chemicals

Adenosine 5′-phosphate, adenosine 5′-triphosphate and (−)-3-phosphoglyceric acid (barium salt) were from Boehringer und Soehne G.m.b.H., Mannheim, Germany. Acid-hydrolysed casein, *p*-chloromercuribenzoic acid, folic acid, nicotinic acid, riboflavin, ribose 5-phosphate (barium salt), thioglycollic acid and uracil were from British Drug Houses Ltd., Poole, Dorset. 2-Mercaptoethanol was from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. CoA (approx. 70% pure), glycinamide hydrochloride and pyridoxal hydrochloride were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. L-Azaserine was from Parke, Davis and Co., Detroit, Mich., U.S.A. The ^{14}C-labelled compounds were from The Radiochemical Centre, Amersham, Bucks. Biotin, calcium pantothenate and pyridoxal phosphate were from Roche Products Ltd., Welwyn Garden City, Herts. δ-Aminolaevulic acid hydrochloride was prepared by Dr W. G. Laver, using the method of Shemin (1957). Tetrahydrofolic acid was prepared from folic acid by the method of Blakley (1957*a*), except that the catalytic hydrogenation was carried out in 8 equiv. of $NaHCO_3$ instead of 2.

Determination of protein

Simultaneous determination of nucleic acid and protein during the course of enzyme purification was carried out by reading extinctions at 280 and 260 mμ (Warburg & Christian, 1942) and then using a nomogram (California Corp. for Biochemical Research). Large quantities of protein were determined by the biuret method (Layne, 1957), and small quantities by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin as a standard.

Extinctions

Extinctions were generally read on Unicam SP. 500 or SP. 600 spectrophotometers. Spectra were plotted and values for $\lambda_{max.}$ determined on the Unicam SP. 700 spectrophotometer.

Electrophoresis of amino acids and related compounds

Electrophoresis was generally carried out on Whatman 3MM paper in 1·5N-formic acid–0·2N-acetic acid buffer, pH 1·85, in a water-cooled high-tension electrophoresis apparatus at a potential gradient of approx. 60 v/cm. Under these conditions aminomalonate stayed at the origin and glycine moved about 10 cm. towards the cathode in 15 min. The compounds were located by dipping the dried paper in 0·2% (w/v) ninhydrin in acetone containing 5% (v/v) of pyridine.

Preparation of enzymes and determination of enzyme activities

Aminolaevulate synthetase. A culture of *R. spheroides* was a gift from Dr J. Lascelles, Oxford. It was maintained in stab culture and grown semi-anaerobically in 'medium S' of Lascelles (1956). A crude enzyme extract was prepared from it as described by Gibson *et al.* (1962) and used for all experiments except when another preparation is mentioned in the text. The assay system contained, in a volume of 0·725 ml.: potassium phosphate buffer, pH 7·4, 72·5 μmoles; glycine, 40 μmoles; succinyl-CoA (Simon & Shemin, 1953), approx. 0·2 μmole; pyridoxal phosphate, 0·05 μmole; enzyme, protein content corresponding to the activity of about 0·3 mg. of protein in the unpurified *R. spheroides* extract. Incubations were carried out at 37° for 45 min. Then 0·1M-iodoacetamide (0·025 ml.) was added, followed by 20% (w/v) trichloroacetic acid (0·25 ml.). The precipitates were removed by centrifuging and δ-aminolaevulate was determined in the supernatants. The enzyme was purified approximately threefold for determination of the affinity constants by fractionating with $(NH_4)_2SO_4$ between 30 and 50% saturation. The enzyme was also prepared from chicken erythrocyte particles (Gibson, Laver & Neuberger, 1958); about 1·5 mg. of unpurified protein was used in the assay system.

Aspartate aminotransferase (EC 2.6.1.1). Aspartate aminotransferase, specific activity 1500 units/ml., was a gift from Dr C. A. Vernon, University College London. The preparation of the enzyme and the conditions of assay ('method I') are described by Banks & Vernon (1961).

Tyrosine decarboxylase (EC 4.1.1.25). A culture of *Streptococcus faecalis* R, which was a gift from Dr J. Lascelles, Oxford, was grown for 12 hr. in a medium similar to that of Sloane-Stanley (1949), but containing tyrosine (2·2 mM). A crude enzyme extract was prepared by incubating an acetone powder with 22 mM-potassium phosphate buffer, pH 5·5, for 21 hr.; this was assayed as described by Epps (1944), crude coenzyme being replaced in the reaction by 0·4 μmole of pyridoxal phosphate.

Glycine acyltransferase (EC 2.3.1.13). Partially purified enzyme [precipitated by $(NH_4)_2SO_4$ between 35% and 50% saturation] was prepared from pig kidney cortex (Schachter & Taggart, 1953); it was assayed by a method which was essentially the same as that of Schachter & Taggart (1954).

Phosphoribosylglycinamide synthetase (EC 6.3.1.3). An enzyme preparation capable of forming glycinamide ribotide from ribose 5-phosphate, glutamine and glycine was prepared and assayed by the method of Goldthwait & Greenberg (1955). Radioactivity was measured with a Geiger end-window counter.

Serine hydroxymethyltransferase (EC 2.1.2.1). The enzyme was purified as described by Blakley (1955) as far as the step involving precipitation by $(NH_4)_2SO_4$ between 1·47 M and 2·06 M. Because aminomalonate and formaldehyde condense non-enzymically to form serine, serine hydroxymethyltransferase could not be assayed by measuring the serine formed by it from glycine and formaldehyde. Instead, [U-^{14}C]glycine was incubated with formaldehyde in the serine-hydroxymethyltransferase system described below. The serine formed was isolated by paper electrophoresis and its total radioactivity was counted. By this method enzymic activity could be determined even when aminomalonate was present in the reaction in quantity, since it gave rise only to unlabelled serine. At slightly alkaline pH aminomalonate, in the presence of pyridoxal phosphate and formaldehyde, forms primarily serine and not glycine, so that the radioactive substrate would be little diluted with inactive material. There was a linear relationship between the amount and the radioactivity of glycine standards put through the whole procedure of electrophoresis, ninhydrin-staining and counting. It therefore appears that the loss of radioactivity that might be caused by volatility of the reaction products was so slight as to be insignificant.

The assay system contained, unless otherwise stated, in a volume of 1·0 ml.: $NaHCO_3$, 9 μmoles; uniformly ^{14}C-labelled glycine, [U-^{14}C]glycine, 100 μmoles; formaldehyde, 10 μmoles; tetrahydrofolic acid, 2·4 μmoles; pyridoxal phosphate, 0·4 μmole; enzyme, approx. 1·5 mg. of protein. The glycine had in different experiments an activity of 1×10^3–2×10^4 counts/sec./μmole. The tetrahydrofolic acid was kept in an evacuated Thunberg tube, dissolved in 0·16 M-2-mercaptoethanol, giving this a final concentration of 0·01 M in the reaction mixture. Incubation was for 1 hr. at 37°; protein was precipitated by the addition of dialysed iron (0·25 ml.) and removed by centrifuging. Dialysed iron was from Hopkin and Williams Ltd.; it is a colloidal solution of $Fe(OH)_3$ containing 3–4% (w/v) of iron. The supernatants were frozen and dried in a desiccator at 0·2 mm. The residues were dissolved in water (0·1 ml.) and samples of 0·02 ml. were subjected to paper electrophoresis as described above. The serine spots were cut out and divided into smaller pieces, which were placed in counter pots with 0·2 ml. of water and 3·8 ml. of a dioxan-based scintillator (Bray, 1960). Radioactivity was measured in an I.D.L. scintillation counter with the upper-gate-discriminator bias set at 35 v, giving about 30% efficiency. The samples were counted after a minimum of 5 hr., by which time most of the quenching, due to the coloured ninhydrin complex, had died down. A quenching correction was made by adding 0·02 ml. of a standard solution of known and fairly high radioactivity to each sample after counting, recounting and calculating an appropriate correction from the difference reading. Preliminary experiments indicated that efficiency of counting was little affected by the presence of a fairly wide variation in the sizes and quantity of pieces of paper present, and that there was a linear relationship between the amounts of radioactive compounds placed on paper and the counts recorded.

Non-enzymic reaction of aminomalonate and its derivatives with aldehydes

The standard mixture for non-enzymic reactions contained, in a volume of 0·2 ml.: $NaHCO_3$, 2·8 μmoles; aminomalonate or a related compound, 10 μmoles; aldehyde, 10 μmoles; pyridoxal phosphate, 0·3 μmole, or pyridoxal hydrochloride, 1·5 μmoles. Incubations were usually carried out at 37° for 1 hr. Esters of aminomalonate and glycine were converted into glycine by hydrolysis with an equal volume of conc. HCl for 2 hr. at 100°. The hydrolysates were dried in a vacuum oven at 60° overnight and the residues dissolved in 0·2 ml. of water. Portions of 0·02 ml. were subjected to high-tension electrophoresis as described above, samples containing aminomalonate being applied to the paper without heating. In most cases the relative amounts of compounds thus separated were estimated visually by comparison with standards run with them. Semi-quantitative estimation of hydroxyamino acids after their separation by electrophoresis was carried out by a modification of the method of Visakorpi & Puranen (1958). The papers were stained with ninhydrin, the spots of hydroxyamino acid cut out with equal areas of 'blank' paper, and each spot further cut up and eluted by standing in $CuSO_4$ reagent (4 ml.) for 30 min. The reagent is made by dissolving 0·2 g. of $CuSO_4,5H_2O$ in 60 ml. of water and adding 60 ml. of ethanol. The extinctions were read against the appropriate blank at 520 mμ for serine and threonine and 470 mμ for phenylserine, and compared with standards, which had also been subjected to paper electrophoresis, containing 0·01–0·5 μmole of the appropriate amino acid.

Preparation of compounds

Aminomalonic acid had been prepared by aminating bromomalonic acid (Lutz, 1902); isolation was facilitated by the use of an ion-exchange resin.

In our work it soon became clear that solutions of aminomalonic acid, its monoesters and monoamide lose CO_2 to a significant extent if they are allowed to stand at room temperature or even at 0° for more than a few hours (cf. Baeyer, 1864). Decarboxylation is rapid in acid solution. Preparations of aminomalonate are thus liable to be contaminated with small amounts of glycine that in the early work on aminomalonate could not have been easily recognized. We have used high-tension electrophoresis as a rapid and convenient method for testing preparations of aminomalonate for the presence of glycine. Since the present studies are mainly concerned with the influence of amino-

malonate on enzymes for which glycine is a substrate, it was particularly important that this compound should be absent from the aminomalonate preparations. Solutions of aminomalonate neutralized to pH 7 appear to be stable. To minimize the risk of decarboxylation it is important that the final step in the preparation of aminomalonate and its monoesters and monoamide should be carried out at a low temperature and as quickly as possible. We worked in the cold room (4°) where this was practicable, and used large amounts of catalyst to remove benzyloxycarbonyl groups.

It appears that determination of m.p. is of little use for the characterization of aminomalonate. Lutz (1902) gives the m.p. of the monohydrate as 108–109° (decomp.), whereas Haas (1916) gives the m.p. as 140° (decomp.). We have consistently found a m.p. of about 120° (decomp.), which is almost independent of the rate of heating for both the monohydrate and anhydrous compound.

Transesterification of the benzyloxycarbonyl derivative of ethyl aminomalonate to the corresponding methyl ester was achieved in fairly good yield by using toluene-*p*-sulphonic acid as catalyst and allowing the reaction to proceed slowly at a comparatively low temperature.

The first ester group of ethyl or methyl benzyloxycarbonylaminomalonate is readily saponified, but the charge effect of the resulting carboxylate is such that the second ester grouping is far less susceptible to nucleophilic attack. Free half esters were obtained from ethyl or methyl benzyloxycarbonylaminomalonate by treating these compounds with just over 1 equiv. of alkali, then acidifying the reaction mixture and extracting the half ester into ether.

Synthetic experiments

All melting points are uncorrected.

Analyses for C and H were performed by Weiler and Strauss, Oxford. Analysis for N was carried out by the Kjeldahl method in this Laboratory.

Aminomalonic acid. Ammonium aminomalonate was prepared in 50% overall yield by bromination of malonic acid to yield monobromomalonate and treatment of this with methanolic ammonia. Monobromomalonic acid crystallized readily, provided that the bulk of acetic acid was removed from the reaction mixture by distillation. On treating monobromomalonic acid with approx. 30 equiv. of methanolic ammonia (saturated at 0°), the first product that separated was ammonium bromomalonate, but this dissolved on the addition of 0·11 vol. of water. Ammonium aminomalonate, which is insoluble in 90% (v/v) methanol, was precipitated when the mixture was allowed to stand for several days at room temperature.

The free acid was obtained in 95% yield by passing the ammonium salt through a Zeo-Karb 225 (H^+ form) column which was run in the cold room (4°). The product was freeze-dried immediately. One mol.prop. of water of crystallization was lost very slowly in a desiccator containing H_2SO_4 (Piloty & Finckh, 1904; Ruheman & Orton, 1895). The equivalent weights of the hydrated and anhydrous compounds were determined by electrometric titration to an appropriate pH.

The potassium salt was prepared by neutralizing the acid to pH 7 with KOH (glass electrode) and removing water with an oil pump at 35°. It was recrystallized from water–ethanol (1:2, v/v) and had m.p. 227–230° (decomp.). The yield was 78%. The sodium salt, m.p. 227–228·5° (decomp.), was prepared similarly.

Monoethyl aminomalonate. Ethyl aminomalonate hydrochloride was prepared by the method of Redeman & Dunn (1939) (see also Witkop & Foltz, 1957). Catalytic hydrogenation of ethyl isonitrosomalonate was carried out at atmospheric pressure with 'palladized charcoal' (10% of Pd) in 1·1 equiv. of ethanolic HCl. Ethyl *N*-benzyloxycarbonylaminomalonate was prepared essentially by the method of Frankel, Harnik & Levin (1952), but the method of Schneider (1937) for partial saponification was modified to give an improved product and yield.

Ethyl *N*-benzyloxycarbonylaminomalonate (11·904 g.; 1 equiv.) was dissolved in 70% (v/v) ethanol (90 ml.). To the solution, stirred with a stream of N_2, was added, over 2·5 hr., 3·635N-KOH (10·6 ml.; 1 equiv.) in 70% (v/v) ethanol. The vessel containing the reaction mixture was sealed for 16 hr. and then a further 0·98 ml. of KOH (0·093 equiv.) was added during 2·25 hr. The pH was adjusted (glass electrode) to 7·5 with 2N-HCl; water (400 ml.) was added and the cloudy solution was extracted with ethyl acetate (150 ml.) to remove unchanged diester. The aqueous layer was brought to pH 1–2 (long-range paper); the white oil which separated was extracted with ether (3 × 150 ml.) and washed with water (1 × 150 ml.). The product was crystallized from CCl_4 in 77% yield and had m.p. 65–67°.

A solution of monoethyl *N*-benzyloxycarbonylaminomalonate dissolved in 70% (v/v) ethanol containing 1 equiv. of acetic acid was hydrogenated at atmospheric pressure for 3·5 hr. by using 'palladized charcoal' (10% of Pd; 50 mg./m-mole). The hydrogenation flask was shaken in an ice bath and the catalyst filtered off at 4°. Solvent was removed by distillation by using an oil pump at 30°. A white crystalline product was obtained on treating the oily residue with a few drops of water followed by ethanol and anhydrous ether. The yield after reprecipitation was 71%.

The *monoethyl aminomalonate* had m.p. 106–107° (decomp.) (Found: C, 40·4; H, 6·2; N, 9·5. $C_5H_9NO_4$ requires C, 40·8; H, 6·2; N, 9·5%). The compound gave a purple colour with ninhydrin; when titrated in 5 mM solution (*I* 0·11) at about 20° it had an apparent pK_2 of 7·45 and an equiv. wt. of 147, i.e. theoretical for $C_5H_9NO_4$.

Aminomalonate had an apparent pK_3 of 9·38 when titrated in 0·05M solution (*I* 0·325) at 22°. Esterification of one carboxyl group thus causes a shift of the amino p*K* of the expected direction and magnitude, comparable with the change in pK_2 obtained on esterification of glycine (Neuberger, 1937). Schwarzenbach, Kampitsch & Steiner (1945) titrated aminomalonate in 2·67 mM solution (*I* 0·012) at 20° and found an apparent pK_3 of 9·83.

Methyl N-benzyloxycarbonylaminomalonate. Ethyl *N*-benzyloxycarbonylaminomalonate (dried, but not distilled) prepared from ethyl aminomalonate hydrochloride (23·9 g.) was heated under reflux with anhydrous toluene-*p*-sulphonic acid (19·5 g.; 1 equiv.) and anhydrous methanol (360 ml.; 75 equiv.) at 50° for 13 hr. During this time there was negligible evolution of CO_2. The oil remaining after distillation of methanol under reduced pressure was cooled and diluted with ether (50 ml.), then washed with water (1 × 75 ml., 2 × 50 ml.), saturated $NaHCO_3$ (1 × 25 ml.) and water (4 × 25 ml.), and dried over anhydrous Na_2SO_4. When the solvent was removed by distillation the *methyl*

N-benzyloxycarbonylaminomalonate was obtained in 60% yield (19·0 g.) as rosettes with m.p. 52–54°, which rose to 58–59° after four crystallizations from anhydrous ether (Found: C, 55·0; H, 5·2; N, 5·0. $C_{13}H_{15}NO_6$ requires C, 55·5; H, 5·4; N, 5·0%).

Monomethyl N-benzyloxycarbonylaminomalonate. This compound was prepared in 73% yield by a method exactly analogous to that used for the corresponding ethyl ester. The *monomethyl N-benzyloxycarbonylaminomalonate* was crystallized from CCl_4 and had m.p. 86·5–87°, the equiv. wt. (determined by titration) was 267, i.e. theoretical for $C_{12}H_{13}NO_6$ (Found: C, 53·8; H, 4·9; N, 5·1. $C_{12}H_{13}NO_6$ requires C, 53·9; H, 4·9; N, 5·2%).

Monomethyl aminomalonate. Monomethyl *N*-benzyloxycarbonylaminomalonate (1·353 g.) dissolved in 70% (v/v) methanol was hydrogenated in the presence of palladium black (0·5 g.) and acetic acid (0·285 ml.; 1 equiv.) by the method of Greenstein & Winitz (1961). The amount of CO_2 evolved was determined as described by Muir & Neuberger (1949). In 1·75 hr., 96·5% of the theoretical amount of CO_2 had been collected. Some of the product separated out during the reaction, but was redissolved by the addition of 70% (v/v) methanol (10 ml.) after 1 hr. and water (2 ml.) after 1·75 hr. The catalyst was then filtered off and washed with 70% (v/v) methanol (5 ml.). Anhydrous ether (900 ml.) was added to the combined filtrate and washings to precipitate the desired compound. The *monomethyl aminomalonate* was obtained in 68% yield after reprecipitation and had m.p. 112° (decomp.). It gave a positive ninhydrin reaction and was shown by titration of the amino group, which had an apparent p*K* of 7·55 at 23° (*I* 0·2), to have an equiv. wt. of 133, i.e. theoretical for $C_4H_7NO_4$ (Found: C, 35·2; H, 5·2; N, 10·7. $C_4H_7NO_4$ requires C, 36·1; H, 5·3; N, 10·5%).

Ammonium N-benzyloxycarbonylaminomalonic acid monoamide. This compound was obtained in 77% yield by stirring monomethyl *N*-benzyloxycarbonylaminomalonate with aq. ammonia (sp.gr. 0·88) (50 equiv.) at 22° for 48 hr., then removing the solvent under reduced pressure at 30°. The *ammonium N-benzyloxycarbonylaminomalonic acid monoamide* was recrystallized from methanol–acetone (1:5, v/v) and had m.p. 151·5–153·5° (Found: C, 48·9; H, 5·6; N, 15·5. $C_{11}H_{15}N_3O_5$ requires C, 49·0; H, 5·6; N, 15·6%).

N-Benzyloxycarbonylaminomalonic acid monoamide. Ammonium *N*-benzyloxycarbonylaminomalonic acid monoamide (1·78 g.) was dissolved in water (60 ml.). The solution was filtered and the paper washed with a further 7·5 ml. of water. The filtrate and washings were cooled in ice and agitated in the cold while conc. HCl (2·0 ml.) was added to give an iridescent white precipitate that was quickly filtered off and dried. The yield was 88%. The *N-benzyloxycarbonylaminomalonic acid monoamide* was recrystallized from methanol–anhydrous ether (1:25, v/v) and had m.p. 136–137°, after a very marked shrinking at 118°. (It is possible that the final m.p. may be that of the unknown compound, benzyloxycarbonylglycine amide.) (Found: C, 53·5; H, 4·8; N, 10·8. $C_{11}H_{12}N_2O_5$ requires C, 53·4; H, 4·8; N, 11·1%).

Aminomalonic acid monoamide. N-Benzyloxycarbonylaminomalonic acid monoamide (1·39 g.) was dissolved in 70% (v/v) methanol (50 ml.) and hydrogenated, with palladium black (750 mg.) as catalyst, in the apparatus referred to for the preparation of monomethyl aminomalonate. After 1·75 hr., when just over the theoretical amount of CO_2 had been collected, about 20 ml. of water was added to dissolve the white precipitate that had formed. The catalyst was filtered off and washed with water. Solvent was removed from the combined filtrate and washings by using water and oil pumps at 30°, and a receiver cooled with acetone–solid CO_2. The residual white solid (560 mg.) had m.p. 103–105·5°. Titration of similar material obtained in a pilot run had indicated that the aminomalonic acid monoamide was contaminated with glycinamide to the extent of a molar ratio of approx. 4:1. The following procedure was used to separate the compounds, all solutions being kept at 4°. The amide was dissolved in 1·5 equiv. of 1·0 N-HCl and the solution was quickly brought to pH 5·5 (glass electrode) with 2·25 N-LiOH. Ethanol (1·4 l. approx.) was added to give a flocculent white precipitate of the desired compound which was collected by centrifuging. The yield was 40%. The aminomalonic acid monoamide had m.p. 110–112° (decomp.), gave a positive ninhydrin reaction (first brown, then purple), and was shown by titration of the amino group, which had an apparent p*K* of 8·15 at 23° (*I* 0·12), to have an equiv. wt. of 118, i.e. theoretical for $C_3H_6N_2O_3$. (Found: C, 30·6; H, 5·2; N, 23·4. Calc. for $C_3H_6N_2O_3$: C, 30·5; H, 5·1; N, 23·7%).

The compound was heated with 0·1 N-HCl (1 equiv.) for 9 hr. at 60° to effect the removal of one carboxyl group. The solution was dried on a rotary evaporator by using a bath at 40°, and the white residue was crystallized from acetic acid as blades with m.p. 203–204° (decomp.). The mixed m.p. of this product with a commercial sample of glycinamide hydrochloride, recrystallized from methanol as blades or needles with m.p. 203–205° (decomp.), was 202–204° (decomp.).

RESULTS

The non-enzymic reaction of aldehydes with aminomalonate and related compounds. During an investigation of the effect of aminomalonate on serine hydroxymethyltransferase it was noted that, in the presence of pyridoxal phosphate, aminomalonate condensed non-enzymically with formaldehyde to form serine. This reaction went best at a slightly alkaline pH, going almost to completion in 20 hr. at 37°. At high pH aminomalonate was stable; at low pH much glycine and little serine was formed. The catalytic role of pyridoxal phosphate was confirmed by an experiment in which the concentration of pyridoxal phosphate was decreased eightfold to 0·25% that of glycine, causing a decrease in the formation of serine of only 50% approx. Pyridoxal phosphate could be replaced as a catalyst by five times the quantity of pyridoxal hydrochloride. Increasing the concentration of formaldehyde gave almost no increase in the formation of serine unless the concentration of catalyst was also increased.

The following experiment indicates that decarboxylation occurs after the condensation and not as a simultaneous reaction. Aminomalonate was incubated with formaldehyde for a period of

Table 1. *Condensations between various aldehydes and compounds related to aminomalonate*

The standard reaction mixture (described in the Experimental section) was modified by replacing aminomalonate with monoethyl aminomalonate, ethyl aminomalonate or glycine ester, and formaldehyde with acetaldehyde or benzaldehyde. The α-amino-β-hydroxy acids formed were estimated semi-quantitatively. Incubations were carried out for 1 hr. with and without pyridoxal hydrochloride.

Compound	Pyridoxal hydrochloride	Conversion of compound (%) Into serine	Into threonine	Into phenylserine
Aminomalonate	+	14	6	38
	−	0	0	0
Monoethyl aminomalonate	+	25	25	18
	−	8	4	35
Ethyl aminomalonate	+	4	0	0
	−	4	0	0
Glycine ester	+	2	2	3
	−	0	0	1

4 hr. and the reaction mixture was then subjected to electrophoresis. A representative part of the strip was cut away and stained. On examination, the aminomalonate spot appeared to consist of two overlapping parts, the more basic staining grey like glycine, and the more acidic blue–grey like serine. These two parts were cut separately from the main strip. The two portions were cut up and agitated for 1 hr. with 1·0 ml. of water; the supernatants were heated for 2 hr. at 100° with equal volumes of conc. hydrochloric acid to effect decarboxylation of aminomalonate or a derivative of this, then dried. The residues were dissolved in water (0·12 ml.), and portions were subjected to electrophoresis. Spots were obtained, derived from the less acidic and more acidic portions of the original aminomalonate spot, that corresponded to those of glycine and serine. These data indicate that formaldehyde condenses with aminomalonate to form first α-amino-α-hydroxymethylmalonic acid. This compound is a slightly stronger acid than aminomalonate and accordingly separates from it on electrophoresis under the conditions used. At neutral pH it loses CO_2 readily to form serine.

Aminomalonate (10 μmoles) was incubated with $NaHCO_3$ (2·8 μmoles), formaldehyde (6 μmoles), pyridoxal phosphate (0·3 μmole), and variously with EDTA (1 and 5 μmoles) or the following metal ions: Al^{3+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+}, or Fe^{2+} ions (0·3 μmole). Serine formation was unaffected by a small quantity of EDTA, but 5 μmoles of the compound (i.e. a substrate quantity) inhibited the reaction by 50% approx. Of the metal ions, Mg^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} ions had no effect on the reaction. Serine formation was increased slightly by Fe^{3+} and Fe^{2+} ions, and approximately doubled by Cu^{2+} ions. The formation of serine was decreased by Al^{3+} ions, but the acidic aminomalonate spot, staining the usual blue–grey as for serine, increased in intensity from being barely detectable to about five times that of the normal serine. It seems likely that Al^{3+} ions stabilize the addition product of formaldehyde and aminomalonate by chelation.

Table 2. *Inhibition of aminolaevulate synthetase by aminomalonate*

Aminomalonate was incubated in the standard assay system (described in the Experimental section) containing 40 μmoles of glycine. Inhibition was measured by comparing the δ-aminolaevulate formed with that formed in a control lacking aminomalonate.

Aminomalonate (μmoles)	Aminomalonate:glycine (molar ratio)	δ-Aminolaevulate formed (μm-moles)	Inhibition (%)
0	—	15·2	0
0·5	0·0125	12·7	16
1	0·025	9·8	36
2	0·05	8·7	43
4	0·1	7·0	54
10	0·25	5·3	65
20	0·5	2·2	86

Aminomalonate also condensed with acetaldehyde and benzaldehyde. Monoethyl aminomalonate, ethyl aminomalonate and glycine ester were also incubated with the three aldehydes, both in the presence and absence of pyridoxal. The extent of the conversion of each amino acid derivative into the appropriate α-amino-β-hydroxy acid was estimated semi-quantitatively (Table 1). The nature of the α-amino-β-hydroxy acid stereoisomer formed was not determined.

This condensation reaction appears to be confined to carbonyl compounds. Neither aminomalonate nor its mono- or di-ethyl esters reacted with acetic anhydride or succinic anhydride or with the CoA derivatives of the corresponding acids.

Inhibition of aminolaevulate synthetase by aminomalonate and some derivatives. When aminomalonate (used as the ammonium or potassium salt) was incubated in the standard assay system for aminolaevulate synthetase, the formation of δ-aminolaevulate was very strongly inhibited, the velocity

of the reaction being halved at a glycine:aminomalonate molar ratio of approx. 10:1 (Table 2). Monoethyl and monomethyl aminomalonate, ethyl aminomalonate, and aminomalonic acid monoamide were also tested in the enzyme system and their inhibitory powers compared with that of aminomalonate (Table 3). The exact degree of inhibition achieved by aminomalonate could not always be reproduced when enzyme prepared from different batches of bacteria was used, but the dicarboxylic amino acid appeared to be an inhibitor about twice as powerful as monoethyl aminomalonate, which in turn was a slightly stronger inhibitor than monomethyl aminomalonate. Ethyl aminomalonate was only a moderately good inhibitor, achieving 50% inhibition when present in a concentration about 1·5 times that of glycine. Aminomalonic acid monoamide was a very weak inhibitor of the enzyme. Aminolaevulate synthetase from avian erythrocyte particles was also strongly inhibited by aminomalonate.

Table 3. *Inhibition of aminolaevulate synthetase by various compounds*

The compounds listed were incubated in the standard aminolaevulate-synthetase assay system (described in the Experimental section) containing 40 μmoles of glycine. Graphs were plotted of the δ-aminolaevulate formed in the presence of different quantities of inhibitor, and from these the amount of inhibitor producing 50% inhibition was read. In the presence of aminomalonate and its derivatives, the ethyl acetoacetate colour reaction was used; in the presence of the other compounds, which in the concentrations used in these experiments did not interfere with the acetylacetone colour reaction, the latter was used to determine δ-aminolaevulate. Enzyme preparation II was obtained from avian erythrocyte particles; the other preparations were crude extracts of *R. spheroides*.

Enzyme preparation	Compound added	Quantity required to produce 50% inhibition of δ-aminolaevulate formation (μmoles)
I	Aminomalonate	4
II	Aminomalonate	7
	Monoethyl aminomalonate	25
III	Monoethyl aminomalonate	10
IV	Aminomalonate	16
	Monoethyl aminomalonate	30
	Monomethyl aminomalonate	44
	Aminomalonic acid monoamide	>100
V	Ethanolamine	23
	Ethyl aminomalonate	56
	L-Serine	>200
VI	Isobutylamine	26
	L-Valine	154
VII	β-Alanine	44
	L-Threonine	60
	L-Alanine	140
	Sarcosine	>200

Plotting of inhibition data, obtained with partially purified *R. spheroides* enzyme, by the method of Lineweaver & Burk (1934) indicated that aminomalonate and monoethyl aminomalonate were competitive inhibitors of glycine for aminolaevulate synthetase (Fig. 1). The following average values were obtained for K_m for glycine, and K_i for aminomalonate and monoethyl aminomalonate, of 142 μM, 22·5 μM and 31·1 μM respectively.

Inhibition of the enzyme by ethanolamine, L-serine, isobutylamine, L-valine, β-alanine, L-threonine, L-alanine and sarcosine was also studied. When isobutylamine was the inhibitor, a curve was obtained in which $\delta y/\delta x$ increased in magnitude with increasing x (where x is the concentration of inhibitor, y that of the reaction product); with all the other compounds tested, $\delta y/\delta x$ decreased as x increased. Ethanolamine and isobutylamine caused 50% inhibition at a concentration a little over half that of glycine; L-serine and sarcosine were very poor inhibitors; and the remaining compounds were inhibitors of an intermediate order.

Aminoacetone is formed in *R. spheroides*, in a manner analogous to the formation of δ-aminolaevulate, from glycine and acetyl-CoA. The enzyme responsible has been shown by Urata &

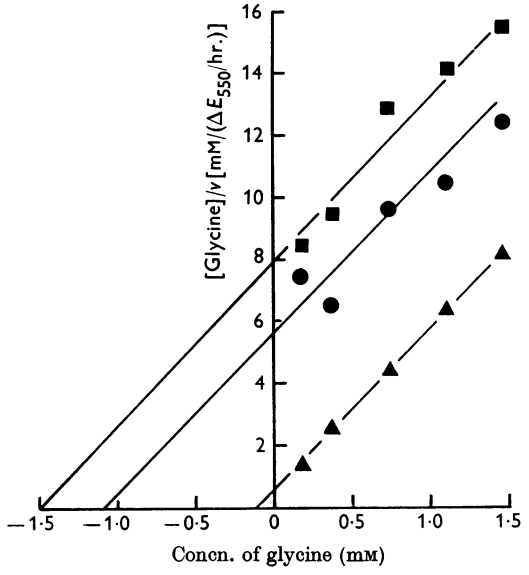


Fig. 1. Competitive inhibition of aminolaevulate synthetase by aminomalonate and monoethyl aminomalonate. The standard assay system (described in the Experimental section) was modified to contain: ▲, 0·184–1·472 mM-glycine; ●, glycine + 0·184 mM-aminomalonate; ■, glycine + 0·276 mM-monoethyl aminomalonate. The initial velocity, v, is represented by ΔE_{550}/hr.

Granick (1963), working with guinea-pig liver mitochondria, to be distinct from aminolaevulate synthetase. However, because aminoacetone synthetase has at most about 10% of the activity of aminolaevulate synthetase in *R. spheroides* preparations, it was not possible to determine whether or not aminoacetone-synthetase activity of crude extracts was inhibited by aminomalonate.

Influence of pyridoxal phosphate concentration on the inhibition by aminomalonate. The coenzyme of aminolaevulate synthetase is pyridoxal phosphate, which is presumed to form a Schiff base with glycine, thus activating the α-carbon atom for condensation with succinyl-CoA (Braunstein, 1960; Neuberger, 1961). The enzyme is not very sensitive to changes in pyridoxal phosphate concentration; the formation of δ-aminolaevulate is increased only from 10 to 27 μm-moles when the concentration of coenzyme is increased more than 30-fold, from 4·3 μM to 138 μM (Table 4). At the latter concentration the enzyme is almost saturated with pyridoxal phosphate. The degree of inhibition of aminolaevulate synthetase by aminomalonate depended to some extent on the concentration of pyridoxal phosphate. The inhibitory effect of aminomalonate was halved when the pyridoxal phosphate concentration was decreased from 100 to 3·1 μm-moles/assay. It was not possible to test higher concentrations of pyridoxal phosphate because they inhibited the colour reaction. Preincubation of pyridoxal phosphate with the enzyme did not protect it from inhibition by aminomalonate.

Comparison of the inhibitory power of amines in the aminolaevulate-synthetase reaction and relevant equilibrium constants measured in the absence of enzyme. The results obtained above indicated that, with crude preparations of aminolaevulate synthetase, the inhibitory power of aminomalonate was most marked when the concentration of pyridoxal phosphate was not less than 0·1 mM. At lower concentrations of coenzyme aminomalonate was not such a powerful inhibitor, suggesting that its inhibitory power at comparatively high concentrations of pyridoxal phosphate might be due to a capacity to react more strongly than glycine with free pyridoxal phosphate, which is loosely bound to the *R. spheroides* enzyme (Gibson, 1958; Kikuchi, Shemin & Bachman, 1958). It was thought that there might be a correlation between the affinity of an amine for free pyridoxal phosphate and its ability to inhibit the aminolaevulate-synthetase reaction. The interaction of pyridoxal phosphate with the inhibitors of aminolaevulate synthetase was accordingly studied at pH 7·4 (the pH at which aminolaevulate synthetase is assayed, and the pH optimum for enzyme activity). The method used was that of Lucas, King & Brown (1962), in which the stability or affinity constant K_d of the imine (Schiff base) formed between pyridoxal phosphate and an amine or amino acid is determined.

$$K_d = \frac{[\text{pyridoxal phosphate}]\,[\text{amine}]}{[\text{imine}]}$$

Thus K_d is analogous to K_m and represents the concentration of amine or amino acid at which half the total pyridoxal phosphate will be present as an aldimine. The affinity constants for pyridoxal phosphate and inhibitors of aminolaevulate syn-

Table 4. *Effect of the pyridoxal phosphate concentration on the inhibition of aminolaevulate synthetase by aminomalonate*

The standard assay system (described in the Experimental section) containing 40 μmoles of glycine was modified in one set of tubes by replacing the normal amount of pyridoxal phosphate (0·05 μmole) by 3·1–100 μm-moles of the coenzyme, giving concentrations in the reaction mixture of 4·3–138 μM. A similar set of tubes contained, in addition, 4 μmoles of aminomalonate. The formation of δ-aminolaevulate was measured, and the percentage inhibition of δ-aminolaevulate formation for each amount of pyridoxal phosphate calculated. The δ-aminolaevulate formed is also expressed as a percentage of that formed with 0·1 μmole of pyridoxal phosphate and no aminomalonate.

Pyridoxal phosphate		Aminomalonate	δ-Aminolaevulate formed		Inhibition of aminolaevulate synthetase
(μm-moles)	(μM)		(μm-moles)	(%)	(%)
3·1	4·3	−	10·0	37	.
6·2	8·6	−	13·7	51	.
12·5	17·2	−	17·4	64	.
25	34·5	−	19·0	70	.
50	69	−	25·6	94	.
100	138	−	27·1	100	.
3·1	4·3	+	7·0	26	30
6·2	8·6	+	8·0	33	37
12·5	17·2	+	8·1	30	53
25	34·5	+	9·2	34	52
50	69	+	11·1	41	57
100	138	+	11·0	41	59

thetase were measured and compared with that for glycine (Table 5). The value found for the K_d for valine (3·3 mM) does not agree with that of 7–10 mM given by Lucas *et al.* (1962), but the difference may be due to slight contamination in the present experiments by ions of heavy metals, since no special precautions were taken to exclude them.

Comparison of the data in Tables 3 and 5 shows that isobutylamine, which forms a very stable imine with pyridoxal phosphate, is a powerful inhibitor of the enzyme, and that aminomalonic acid monoamide, which forms an unstable imine, is a poor inhibitor. Otherwise there is no strict correlation between the K_d for a compound and its inhibitory power. In particular, because aminomalonate does not form a very stable complex with pyridoxal phosphate at pH 7·4, its inhibition of aminolaevulate-synthetase activity cannot be accounted for solely by its reaction with free pyridoxal phosphate. Aminomalonate does not appear to be a general inhibitor of enzymes containing pyridoxal phosphate. At a concentration of 16 mM aminomalonate did not inhibit aspartate aminotransferase, to which pyridoxal phosphate is firmly bound (Meister, Sober & Peterson, 1954); nor, at a concentration of 50 mM, did it inhibit tyrosine decarboxylase, to which the coenzyme is bound loosely (Epps, 1944).

Effect of aminomalonate on other enzymes having glycine as substrate. Another enzyme reaction in which pyridoxal phosphate combines with glycine so that the α-carbon atom is activated for condensation with a second compound is the formation of serine from glycine and formaldehyde in the presence of tetrahydrofolic acid and pyridoxal phosphate. This reaction is carried out by serine hydroxymethyltransferase.

Aminomalonate was a moderately good inhibitor of the enzyme (Table 6), 50% inhibition being achieved when the aminomalonate:glycine ratio was about 1·3:1. Data from another experiment, plotted according to the method of Dixon (1953), indicated that the inhibition was competitive (Fig. 2). Values were found for K_m and K_i of 61 mM and 56 mM respectively. Blakley (1957*b*), using a preparation from rabbit liver purified 14·5-fold from the original phosphate buffer extract, obtained a value for K_m of 28 mM. The degree of inhibition by aminomalonate of serine hydroxymethyltransferase thus resembles that of aminolaevulate synthetase in that, under the conditions of assay, aminomalonate has a greater affinity for the enzyme than has glycine.

Aminomalonate condenses with formaldehyde to form serine in a non-enzymic reaction catalysed by pyridoxal phosphate. It is therefore likely that the inhibition of serine hydroxymethyltransferase by aminomalonate is due in part to this compound's decreasing the concentration of formaldehyde available to the enzyme. Because higher concentrations of formaldehyde are themselves strongly inhibitory towards serine hydroxymethyltransferase (Blakley, 1957*b*), it was not possible to test whether raising the concentration of formaldehyde would overcome the inhibition by aminomalonate.

Glycine participates in two other types of enzymic reaction, in which the carboxyl carbon or the nitrogen are the atoms that condense with the

Table 5. *Affinity constants for pyridoxal phosphate and some amines and amino acids at* pH 7·4

The affinity constants were determined by a method analogous to that of Lineweaver & Burk (1934) for finding K_m by plotting $1/v$ against $1/s$. Amines and amino acids, in concentrations of 0·1 mM to 0·1 M, were equilibrated for 20 min. with 0·1 mM-pyridoxal phosphate in 0·2 M-potassium phosphate buffer, pH 7·4. The positions of $\lambda_{max.}$ for the Schiff bases formed are about 275 mμ, and were determined with a Unicam SP. 700 recording spectrophotometer; $\lambda_{min.}$ for pyridoxal phosphate was 283 mμ. In each case the reciprocal of the increase in extinction ($1/\Delta E$) at λ_{max} was plotted against the reciprocal of the amine or amino acid concentration ($1/s$). The affinity constant, K_d, was found by reading from the graph $-1/K_d$ (the intercept on the abscissa).

Compound	K_d (mM)
Aminomalonic acid monoamide	126
Glycine	89·3
Ethyl aminomalonate	71·4
Aminomalonate	50·3
Monomethyl aminomalonate	44·5
Monoethyl aminomalonate	44·4
L-Alanine	23·2
DL-Serine	19·9
β-Alanine	14·1
Ethanolamine	9·5
DL-Valine	3·3
DL-Threonine	3·1
Isobutylamine	2·7

Table 6. *Inhibition of serine hydroxymethyltransferase by aminomalonate*

Aminomalonate was added to the standard assay system (described in the Experimental section) modified to contain 70 μmoles of [U-^{14}C]glycine. The inhibition was measured by comparing the total radioactivity of the serine formed with that formed in the absence of aminomalonate.

Aminomalonate (μmoles)	Aminomalonate:glycine (molar ratio)	Inhibition (%)
20	0·29	18
40	0·57	27
80	1·14	48
160	2·29	64
240	3·43	71
320	4·57	83
400	5·72	94

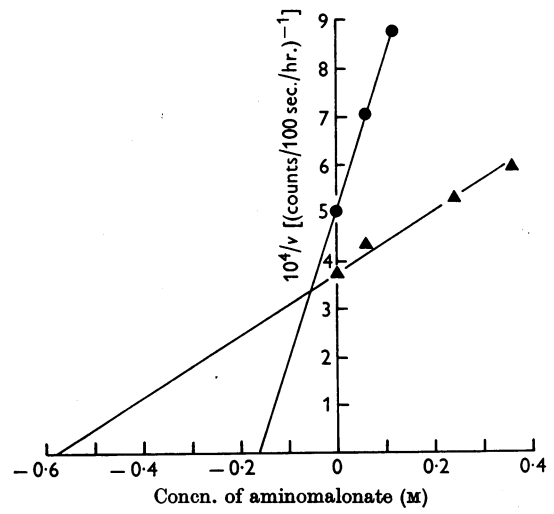


Fig. 2. Competitive inhibition of serine hydroxymethyltransferase by aminomalonate. The standard assay system (described in the Experimental section) was modified by incubating, in a volume of 1·0 ml., aminomalonate (60–360 μmoles) with: ●, 60 μmoles of glycine (s = 60 mM), or ▲, 120 μmoles of glycine (s = 120 mM). The initial velocity, v, is represented by the increase in the total radioactivity of the serine formed (counts/100 sec./hr.).

second substrate. These types are represented by phosphoribosylglycinamide synthetase and glycine acyltransferase. These enzymes were inhibited by aminomalonate only when its concentration exceeded several-fold that of glycine; the inhibition was not very different quantitatively from that produced by several other compounds, e.g. β-alanine, L-alanine, isobutylamine, sarcosine, L-serine and L-valine.

DISCUSSION

The non-enzymic reaction between aminomalonate and aldehydes. The formation of serine, threonine, allothreonine and other α-amino-β-hydroxy acids by heating mixtures of glycine, the appropriate aldehyde and pyridoxal hydrochloride has been observed and studied in detail by Metzler, Longenecker & Snell (1954). This reaction proceeds very slowly at room temperature and requires apparently relatively large amounts of pyridoxal hydrochloride and a suitable metal salt. The reaction between aminomalonate and aldehydes described in the present paper proceeds at a reasonable rate at 37° without the addition of metallic salts and requires only small amounts of pyridoxal hydrochloride. It seems reasonable to assume that the reactions of both glycine and aminomalonate with aldehydes are aldol condensations in which the reactivity of the α-carbon atom is increased by aldimine formation, as discussed by Snell (1958) and Braunstein (1960). The greater reactivity of aminomalonate and its monoethyl ester as compared with glycine is almost certainly caused by the fact that the additional carboxyethyl group or the carboxylate group further enhances the nucleophilic character of the α-carbon atom. The monoethyl ester is more reactive than aminomalonate itself; this is probably due to the fact that the removal of one negative charge assists the dissociation of the C–H bond in the α-position. The lack of reactivity of the diester is surprising and suggests that the presence of one negatively charged carboxylate group facilitates in some manner the required electronic shifts in the postulated aldimine structure. The self-catalysed condensation of aldehydes with monoethyl aminomalonate reflects the greater reactivity of this compound, and appears to be similar to the self-catalysed transaminations of glyoxalate and mesoxalate with various amino acids (Metzler, Olivard & Snell, 1954; Mix, 1961). Hellman & Piechota (1960) have exploited the strongly nucleophilic character of the α-carbon atom of monomethyl acetamidomalonate in the addition of aldehydes to this compound under very mild conditions (see also Jaenicke, 1961).

As a preparative method for α-amino-β-hydroxy acids the one described is probably inferior to those of Akabori, Otani, Marshall, Winitz & Greenstein (1959) and Hellman & Piechota (1960).

Inhibition of enzymes by aminomalonate. Enzymes containing pyridoxal phosphate are often inhibited by amino acids, but generally only weakly (Braunstein, 1960). The inhibition of aminolaevulate synthetase by aminomalonate is much more powerful, and is one of the rare examples of competitive inhibition, similar to that of succinate dehydrogenase by malonate (Thorn, 1953), in which K_i is less than K_m. The ability of aminomalonate to inhibit the enzyme by binding at the glycine site is probably not solely due to its structural similarity to glycine, since ethanolamine, L-alanine and L-serine, which are also fairly closely related to glycine, exert differing and much weaker inhibitions.

The aldimine linkage between amino acid and pyridoxal phosphate apparently plays a part in the inhibition by aminomalonate, since this inhibition is somewhat dependent on the concentration of coenzyme. In addition, aminomalonate is a powerful inhibitor of the formation of serine by serine hydroxymethyltransferase, which is also activated by pyridoxal phosphate; but it is only a moderate inhibitor of glycine acyltransferase and phosphoribosylglycinamide synthetase, enzymes that do not require pyridoxal phosphate. The studies on the stability of imines formed *in vitro* between inhibitors of aminolaevulate synthetase and

pyridoxal phosphate are probably more relevant to the inhibition of the enzyme from *R. spheroides* than to that from avian erythrocyte particles, because the coenzyme is bound loosely to the former and firmly to the latter (Gibson, 1958; Gibson *et al.* 1958; Kikuchi *et al.* 1958). The inhibition exerted by isobutylamine is probably due entirely to the stability of the complex that this compound forms with pyridoxal phosphate, since isobutylamine is quite dissimilar in structure from glycine and has an inhibition curve of a shape different from those of the other compounds tested. Apart from the extreme cases of sarcosine (which is unable to form an aldimine), aminomalonic acid monoamide and isobutylamine, ability to inhibit the enzyme does not appear to be related to the value of K_d. As would be expected from consideration of the pK values of the amino groups, the K_d for aminomalonate is higher than those for the monoesters, yet aminomalonate is at least twice as powerful an inhibitor as these compounds.

The comparative inhibitory powers of the compounds tested cannot adequately be accounted for by the criteria of 'fit' and imine formation. On these counts ethanolamine should be a more powerful inhibitor than aminomalonate. The property peculiar to aminomalonate is its double negative charge at pH 7·4, and this appears to be of importance for binding the compound to the active site of the enzyme. When aminomalonate is actually attached to the enzyme, it is probable that the charge is effectively neutralized, thus causing greater activation of the α-carbon atom and increasing its nucleophilic character.

However, it is quite possible that studies of model reactions involving free pyridoxal phosphate are not wholly relevant, since they do not give a clear indication how pyridoxal phosphate combined with protein would react with aminomalonate and related compounds. By analogy with other enzymes containing pyridoxal phosphate (Fischer & Krebs, 1959), it seems that pyridoxal phosphate combined with aminolaevulate synthetase may not possess a free aldehyde group but may be combined as an aldimine with a particular ε-amino group. It thus appears that the degree of inhibition of aminolaevulate synthetase can, for most of the compounds examined, be accounted for by the triple requirements of affinity for pyridoxal phosphate, negative charge and, less importantly, 'fit' for the glycine site on the enzyme.

SUMMARY

1. Aminomalonate combines with acetylacetone, but not with ethyl acetoacetate, to form an Ehrlich-positive compound, which is probably 2-carboxy-3,5-dimethylpyrrole.

2. Monomethyl and monoethyl aminomalonate have been synthesized, and aminomalonic acid monoamide has been prepared by a new route.

3. The non-enzymic condensation of aldehydes with aminomalonate and related compounds under the catalytic influence of pyridoxal has been studied. The aldol condensation occurs under mild conditions and leads ultimately to α-amino-β-hydroxy acids. In acid solution the principal reaction catalysed by pyridoxal is decarboxylation.

4. Aminomalonate was a powerful competitive inhibitor of aminolaevulate synthetase from *Rhodopseudomonas spheroides* and avian erythrocyte particles. The mono- and di-esters of aminomalonate were moderate inhibitors and aminomalonic acid monoamide was a weak inhibitor of the enzyme. Inhibition by aminomalonate was somewhat dependent on the concentration of pyridoxal phosphate.

5. Serine hydroxymethyltransferase was fairly strongly inhibited by aminomalonate. Glycine acyltransferase and phosphoribosylglycinamide synthetase were slightly inhibited by aminomalonate, but tyrosine decarboxylase and aspartate aminotransferase were not inhibited by this compound.

6. The mechanism of inhibition of aminolaevulate synthetase by aminomalonate and other compounds is discussed.

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Comparative Studies of the Metabolism of Strontium and Barium in the Rat

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Although the alkaline-earth metals calcium, strontium and barium follow, in general, similar metabolic pathways, there are important quantitative differences in their metabolic behaviour. In recent years the comparative metabolism of calcium and strontium has been widely studied but relatively little attention has been paid to barium.

Bauer, Carlsson & Lindquist (1956) have studied the comparative metabolism of ^{45}Ca and ^{140}Ba in rats during the first 48 hr. after intraperitoneal injection, and have shown that the clearance of ^{140}Ba from the plasma by skeletal accretion is about twice that of ^{45}Ca, but that the overall retention of ^{140}Ba in the skeleton is less than that of ^{45}Ca. Similar observations have also been made in human subjects (Bauer, Carlsson & Lindquist, 1957). In contrast with bone, however, there is little information available on the turnover of barium in soft tissues.

In the present paper the results of some comparative studies of the metabolism of strontium and barium in the femur, teeth and some soft tissues of young and old rats are reported. To reduce biological variation as far as possible the behaviour of ^{85}Sr and ^{140}Ba has been directly compared in the same animal after the simultaneous administration of ^{85}Sr and ^{140}Ba.