THE MALONIC DECARBOXYLASE OF PSEUDOMONAS AERUGINOSA

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Malonate oxidation has long been thought to proceed by direct decarboxylation to acetate. The enzymic processes involved, however, have not been demonstrated. The chief difficulty encountered in attempts to prove decarboxylation to acetic acid has been the strong acetate oxidation characteristic of the species of *Azotobacter* examined (Lineweaver, 1933; Karlsson, 1950) which prevents accumulation of acetate and excludes isolation of the slow initial decarboxylation step.

This report confirms the suspected pathway of malonate oxidation by showing that malonate adapted *Pseudomonas aeruginosa* cells, which no longer oxidize acetate after vacuum or acetone dehydration, can decarboxylate malonic acid with the accumulation of acetic acid. The crude enzyme has been extracted from dried cells, and some properties of a new system, malonic decarboxylase, are described.

MATERIALS AND METHODS

Culture and growth. The HMS strain of *P. aeruginosa* was grown in a fluid medium containing 1.5 per cent tryptic digest of casein (NZ-case), 0.01 per cent $MgSO_4 \cdot 7H_2O$, and M/80 potassium phosphate buffer, pH 6.7. When malonate adapted cells were desired, 0.3 per cent sodium malonate was included in the medium. Large blake bottles were used to provide shallow cultures with a large surface area. Cultures were incubated at 30 C and vigorously shaken twice daily during the 70 hour growth period. Cells were collected by centrifugation and washed twice with an amount of water equal to 20 per cent of the original culture volume. The washed cells were resuspended in three volumes of water to one volume of cell paste.

Dried cell preparations. A petri dish containing a quantity of the thick cell suspension sufficient to cover its surface, circa 5 ml, was placed in a vacuum desiccator over fresh calcium sulfate ("drierite"). After one hour under a vacuum produced by a hyvac pump, the desiccator valve was closed and the apparatus was placed overnight in a refrigerator. The system was opened then, and the dried material was reduced to a uniform powder by grinding lightly in a mortar for 15 seconds.

Acetone powders were prepared by shaking sedimented cells with 15 volumes of cold acetone for 20 seconds. Then the cells were centrifuged immediately and the supernatant discarded. The centrifuge tubes were placed immediately in a vacuum desiccator and processed in the manner previously indicated.

Both types of dry powders were stored in sealed tubes over drierite in the refrigerator. Such preparations were stable for many weeks.

Crude cell free enzyme. Dried cells were suspended in M/50 phosphate buffer, pH 6.5, at a concentration of 50 mg of dried cells per ml. Such suspensions were permitted to stand at room temperature for 3 hours with occasional stirring. Centrifugation at 20,000 × gravity yielded a clear yellowish supernatant.

Manometric and chemical determinations. Respiratory gas exchanges were measured by conventional techniques with a micro-Warburg apparatus (KO_2 = 0.5) at 35 C. Aerobic oxygen consumption and carbon dioxide evolution were determined by the direct method with air as the gas phase. Anaerobic carbon dioxide production was measured in an atmosphere of nitrogen. The dried cell powder was prepared for use by suspending a weighed amount in buffer. Except for large scale experiments designed for the isolation of reaction products, an aliquot of suspension containing 2, 5, or 10 mg was used in a system with a tota

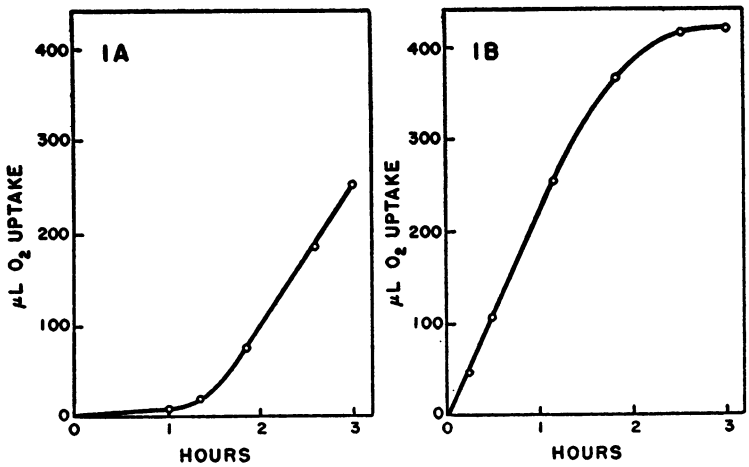

Figure 1. Oxidations of malonate by control (A) and malonate adapted (B) *Pseudomonas aeruginosa.* Substrate: 10 μM malonate in M/40 phosphate, pH 7.0.

fluid volume of 0.8 ml. The reaction system was M/40 with respect to potassium phosphate buffer, pH 6.9. Substrates were tested at a concentration of 10 μM.

Total volatile acids were determined by steam distillation and micro titration. Acetate was identified by the ether water partition method of Osburn, Wood, and Werkman (1936) and by Virtanen and Pulkki (1928) semidistillation values.

RESULTS

Activity of cells grown under various conditions. The HMS strain of *P. aeruginosa* grew so very slowly on a synthetic medium with malonate or acetate as a sole carbon source that a tryptic digest of casein medium was employed. Nonadapted cells oxidize malonate adaptively in the Warburg apparatus. Adaptation occurs very slowly; two hours elapse before the maximum oxidation rate is achieved (figure 1A). When the culture medium was supplemented with malonate, adapted cells were obtained which oxidized malonate immediately and more than 90 per

cent of the way to completion (figure 1B). Malonate adapted cells oxidize acetate more rapidly than control cells.

Comparative activities of dried and untreated malonate adapted cells. Dried cell preparations were deficient in many of the activities demonstrated for ordinary water washed resting cells (table 1). The endogenous metabolism was reduced and negligible activity remained for pyruvate, *alpha*-ketoglutarate, propionate, and ethyl alcohol. Succinate, fumarate, and malate were oxidized to the oxalacetate level, and the latter was then decarboxylated. Oxidative activity toward these dicarboxylic acids was lower in vacuum dried cells than the control cells and was further reduced in acetone dried cells and in cell free extracts. The loss in ability to oxidize acetate was complete. A similar loss in activity against ace-

TABLE 1

Comparative activities of preparations of malonate adapted Pseudomonas aeruginosa

WASHED CELLS		SUBSTRATE	DRIED CELLS	
O_2 uptake	CO_2 production		O_2 uptake	CO_2 production
+	++	Malonate	−	+++
+	++	Oxalacetate	−	+++
+	+	Pyruvate	−	−
++	++	Acetate	−	−
−	−	Citrate	±	−
+	+	*alpha*-Ketoglutarate	−	−
++	++	Succinate	+	+
++	++	Malate	+	+
++	++	Fumarate	+	+
+	+	Propionate	−	−
+	+	Formate	−	−
+	±	Ethyl alcohol	−	−

Q_{O_2} or Q_{CO_2} − less than 3
± 3–15
\+ 15–75
++ 75–350
+++ over 350.

tate was also recently reported for dried cells of another strain of *P. aeruginosa* by Campbell and Stokes (1951). No attempt was made to restore activity to these preparations by adding catalytic agents such as diphosphopyridine nucleotide, flavinadenine dinucleotide, adenosinetriphosphate, or cytochrome C, since the primary objective at this stage was to segregate malonate decarboxylation from as many other activities as possible.

As was expected, the loss in ability to oxidize acetate was accompanied by a loss in capacity to attack malonate oxidatively. Fortunately, the ability to decarboxylate malonate remained. Malonate decarboxylase activity was demonstrated by dried and cell free preparations under both aerobic and anaerobic conditions. The only other conventional compound which was directly decarboxylated by these preparations was oxalacetate (table 2). Oxalacetate decar-

boxylase is present in both control and malonate adapted cells. It is present also in the cell free enzyme from both types of cells, but malonic decarboxylase is obtained cell free only from adapted cells.

Optimum conditions for malonate decarboxylation. There is no sharp pH optimum for malonate decarboxylation. Decarboxylation occurs in the pH range 6.0 to 8.0. Activity is difficult to gauge accurately in the neutral and alkaline ranges because corrections for CO_2 binding are largely due to the protein, buffer, and upward creep in pH due to the formation of a monocarboxylic from a dicarboxylic acid. Activity appears greatest at pH 6.9 to 7.4.

None of the preparations requires Mg, Mn, cocarboxylase, or biotin for maximum activity. Perhaps further purification will show one or more of these to be necessary.

The activity of the cell free enzyme is linear with respect to enzyme concentration when an excess of malonate is present (figure 2). The rate is the same dur-

TABLE 2

Decarboxylase activity of dried Pseudomonas aeruginosa cells

SUBSTRATE: 10 μM IN M/40 PO_4, pH 6.9 IN AIR	μL CO_2 EVOLVED/30 μG N/15 MIN	
	Malonate adapted	Not adapted
Malonate	40	0
Oxalacetate	40	45
Pyruvate	0*	0
alpha-Ketoglutarate	0	0
Succinate	0	0
Glutarate	0	0

* 0 means less than 1.0.

ing the first 7.5 minutes as the last. The assay system, therefore, is valid. Boiling destroys the activity of both dried and cell free preparations.

Carbon balances. Manometric determinations on dried and cell free preparations from adapted cells showed no oxygen uptake in the presence of malonate. Carbon dioxide production, however, was sufficient to account for the conversion of more than 90 per cent of the malonate to acetate according to the following equation:

$$(1) \qquad HOOC-CH_2-COOH \rightarrow CH_3-COOH + CO_2$$

After measuring carbon dioxide retention, the reaction mixture supernatants were adjusted to pH 1.0 with H_2SO_4, and 10 volumes were steam distilled. Titration of the total volatile fatty acids formed from malonate yielded more than 90 per cent of the theoretical quantity of acid (calculated as acetic acid). Since malonate is decomposed to carbon dioxide and acetate under strongly acidic conditions at the distillation temperature, over acidification of the distillation mixture is to be avoided. If this precaution is not taken some of the residual malonate will appear as acetic acid in the distillate.

While the carbon balances in table 3 are in accordance with equation (1), it

was necessary to further identify the reaction products. Manometric studies made with and without potassium hydroxide in the center well showed that the gas formed from malonate was carbon dioxide which could be completely taken up by the potassium hydroxide; no hydrogen was produced. The distillate from large scale reaction systems (500 mg dry cells, 3 mM malonic acid) provided quantities of acid for analysis. It was shown to consist of pure acetic acid by the ether water partition method of Osburn *et al.* (1936), and by Virtanen and Pulkki (1928) semidistillation values.

Malonate as an inhibitor. Since malonate is a potent inhibitor of succinic dehydrogenase in a wide variety of cell types, it was of interest to study this relationship in pseudomonas cells which oxidize succinate and form an adaptive enzyme for the decarboxylation of malonate. The following types of cell prepara-

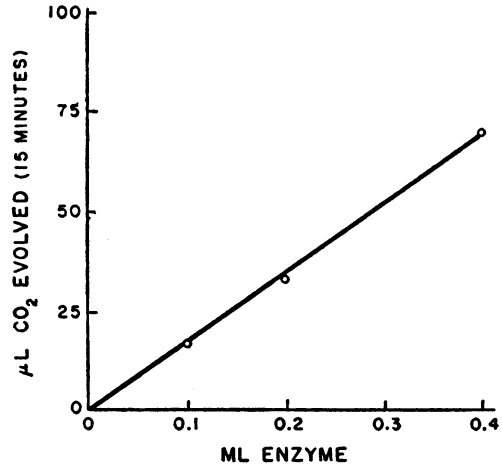

Figure 2. Malonic decarboxylase activity as a function of cell free enzyme concentration. Conditions: an excess of malonate at pH 6.9.

tions were used: (A) unadapted whole cells capable of oxidizing succinate rapidly and forming adaptive enzymes for the oxidation of malonate after a two hour lag; (B) unadapted dry cell preparations capable of oxidizing succinate but not able to form adaptive enzymes for malonate; (C) malonate adapted whole cells which were like (A) but attacked malonate without lag; (D) malonate adapted dry cells which could attack succinate and decarboxylate malonate to the acetate level; and, (E) acetone dried cells (certain preparations only) which were similar to (D) but showed only negligible oxidation of succinate.

Succinate oxidation in (A) was inhibited only 30 per cent by equimolar (0.01 M) concentrations of malonate. This was expected since Quastel and Wheatley (1931) and Randles and Birkeland (1947) showed that malonate partially inhibited succinic dehydrogenase in other strains of the same species. In (B) cells where permeability was increased and adaptive enzyme formation was nil, malonate inhibited 90 per cent. (D) cells, which had lost by drying the property of showing oxygen uptake with malonate characteristic of (C) cells, showed an

80 per cent inhibition of succinic dehydrogenation which was rapidly reduced to 20 per cent as the malonate was decarboxylated. The remaining 20 per cent inhibition was relieved very slowly. It is apparent also that not only the relative concentrations of malonate and succinate, but also the relative concentrations of malonic decarboxylase and succinic dehydrogenase regulate the speed at which both malonate is decarboxylated and succinate is dehydrogenated. The least important factor in this relationship is the concentration of succinate; for succinate in a molar ratio of four molecules to one of malonate in (E) type cells does not inhibit malonic decarboxylase.

Oxalacetate decarboxylase in type (B) cells was not inhibited by equimolar (0.01 M) concentrations of malonate. In this respect *P. aeruginosa* oxalacetate decarboxylase resembles that of *Azotobacter vinelandii* (Plaut and Lardy, 1949) rather than the malonate sensitive system from pigeon liver (Evans *et al.*, 1943). Malonate inhibits tissue succinoxidase by at least two mechanisms (Pardee and Potter, 1949). In addition to inhibiting succinic dehydrogenase, it inhibits oxal-

TABLE 3

Carbon balances

EXPERIMENT	μM MALONATE ADDED	μM CO_2 EVOLVED	μM ACETATE FORMED	PER CENT C RECOVERED
No. 1	25.0	23.4	24.0	95
No. 2	20.0	18.8	19.1	95

acetate decarboxylase. Any succinate leaking through the malonate block is again blocked at the oxalacetate level. The latter, a strong inhibitor of succinic dehydrogenase, reinforces the inhibition. Bacteria, like those described previously which have a malonate insensitive oxalacetate decarboxylase lack this reinforcement mechanism and cannot be expected to be as sensitive to malonate as are tissue preparations.

DISCUSSION

The results show that malonate is enzymatically decarboxylated directly to acetate. The same end products result from chemical decomposition by strong acids, heat, or a combination of these (Christensen and Ross, 1941). In a sense this represents the last stage of a malonic ester synthesis of a fatty acid. The activation of one carboxyl group by another is very strong when they are separated by a single methyl group. From a standpoint of thermodynamics this reaction can proceed more easily than the decarboxylation of succinate to propionate as proposed by Carson *et al.* (1948) and Delwiche (1948) for propionic acid bacteria; for in succinate two methyl groups separate the carboxyl groups. On the other hand, malonate is much more stable than oxalacetate since it undergoes negligible spontaneous decarboxylation in neutral solutions at 35 C.

Lineweaver's (1933) hypothesis that the initial attack by *Azotobacter vinelandii* on malonate is a decarboxylation was based on a shifting respiratory quotient. Karlsson (1950) favored the same hypothesis and supported it with growth

studies and respiration measurements on a mutant of *Azotobacter agilis* which required acetate for growth. Since ethyl alcohol and malonate were the only compounds which could substitute for acetate, he considered them to be oxidized via acetate. Karlsson also showed that there was a net production of carbon dioxide in the early stages of malonate oxidation, but this amounted to only 10 per cent of the theoretical amount necessary for complete conversion to acetate. Since attempts to repress acetate oxidation and obtain only carbon dioxide production from malonate by employing anaerobic conditions did not result in carbon dioxide evolution, he suggested that malonate degradation was coupled with aerobic oxidation processes and that the formation of a phosphorylated intermediate might be required. Since our dried cell preparation and cell free enzyme decarboxylate malonate both aerobically and anaerobically, this is evidently not true for *P. aeruginosa*.

Every since Quastel *et al.* (1928, 1931) made their classical observations on the inhibition of succinic dehydrogenase by malonate, the latter compound has proven a most valuable tool in metabolic studies. It was largely on the basis of malonate inhibition studies that Krebs (1943) was able to formulate his citric acid cycle for tissue cells. While the malonate inhibition technique has been successfully applied to tissue preparations, it has often failed in the case of bacteria.

The suggestion is made frequently that poor malonate inhibition may be explained by the low permeability of bacterial cells for malonate, or that malonate fails to inhibit succinate oxidation because metabolically formed succinate does not pool with added succinate or malonate, but is oxidized *in situ* where it is formed. Permeability difficulties are important, but this may also be complicated by adaptive enzyme formation when fresh whole cells are studied. While there has been no comprehensive survey of bacteria for their ability to metabolize malonate, scattered observations on growth recorded in Bergey's Manual (Breed *et al.*, 1948) and elsewhere suggest that this property is widely distributed in pseudomonas, xanthomonas, azotobacter, and mycobacteria. It is interesting to note that these are highly aerobic genera such as one should like to employ in studies on terminal respiratory processes.

A practical use may be suggested for cell free malonic decarboxylase. There are chemical methods for removing malonate from reaction systems and demonstrating the accumulation of succinate (Krebs and Eggleston, 1940), but there is no effective method of relieving malonate inhibition and keeping the enzyme systems under study in working order. In systems which offer no permeability barrier to the malonic enzyme and when acetate production is not objectionable, the addition of malonic decarboxylase should give the desired result. Thus far we have had partial success using this procedure with water homogenates of rat liver.

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SUMMARY

Dried cell preparations of malonate adapted *Pseudomonas aeruginosa* decarboxylate malonic acid under aerobic and anaerobic conditions with the accumulation of acetic acid. The reaction products were identified and carbon balances show that the reaction is:

$$\text{malonate} \rightarrow \text{acetate} + CO_2$$

The crude enzyme responsible for this reaction has been obtained in a cell free form, and some properties of a new enzyme, malonic decarboxylase, are described.

Dried and cell free preparations decarboxylate only one other conventional substrate, oxalacetate. The oxalacetate decarboxylase is not malonate sensitive.

Malonic decarboxylase and a malonate sensitive succinic dehydrogenase may coexist in the same cell. In such cells malonic decarboxylase gradually relieves malonate inhibition of succinic dehydrogenase.

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