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METABOLIC PATHWAYS OF MALATE ENANTIOMERS

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ABSTRACT

A strain of *Pseudomonas non-fluorescens* has shown to metabolize certain phenolic compounds through a pathway containing D-malic acid. This same bacterium is able to oxidize D-malate slowly when it is used as the sole carbon source, but is unable to grow on it. Possible pathways and explanations of this phenomena are postulated.

In recent years the metabolism of malate enantiomers in various species of *Pseudomonas* has been the subject of considerable investigation. This results from the fact that the microbial metabolism of the dihydroxyphenol, gentisic acid, has been shown to include D-malate in the metabolic chain (Hopper, Chapman, and Dagley, 1968, p. 65). This compound is not the naturally occurring form of malate found in the tricarboxylic acid cycle, but its enantiomer.

In general, the process of splitting the stable benzene ring is initiated by the addition of first one, then two hydroxy-groups to the benzene ring by a series of enzymes known as "monooxygenases." A second series of enzymes known as "dioxygenases" are responsible for the fission of the benzene nucleus, and in the particular case of gentisic acid, the α -keto dicarboxylic acid, maleylpyruvate, is formed (Figure 1) (Dagley, 1967, pp. 287-288).

This ring fission product, maleylpyruvate, exists as a keto-enol tautomer. In some species of bacteria the existence of *cis-trans* isomerase which converts maleylpyruvate to fumarylpyruvate has been shown. This is then hydrolyzed to form fumarate and pyruvate, which are constituents of the tricarboxylic acid cycle. However, in the species of *Pseudomonas* studied (a non-flourescent species designated 2,5X for its isolation on 2,5-xyleneol) no such isomerase has been detected. Instead, the maleylpyruvate is split into maleate and pyruvate. The maleate is then hydrated to yield D-malate (Hopper, Chapman, and Dagley, 1968, p. 65). The problem now arises as to how the D-malate is subsequently degraded for use in the cell.

Several methods for the degradation of L-malate have been recorded in the literature. First, it can be oxidized with NAD by the enzyme L-malate dehydrogenase to give oxaloacetate (Figure 2a). This is the reaction which takes place in the tricarboxylic acid cycle. Oxaloacetate can be formed by an L-malate dehydrogenase which does not require NAD, and is particulate rather than soluble (Figure 2b). Finally, L-malate can be decarboxylated by "malic enzyme" to yield pyruvate, which can either enter the tricarboxylic acid cycle directly, or be recarboxylated by pyruvate carboxylase to form oxaloacetate (Figure 2c) (Francis, Hughes, Kornberg, and Phizackerley, 1963, pp. 430-438).

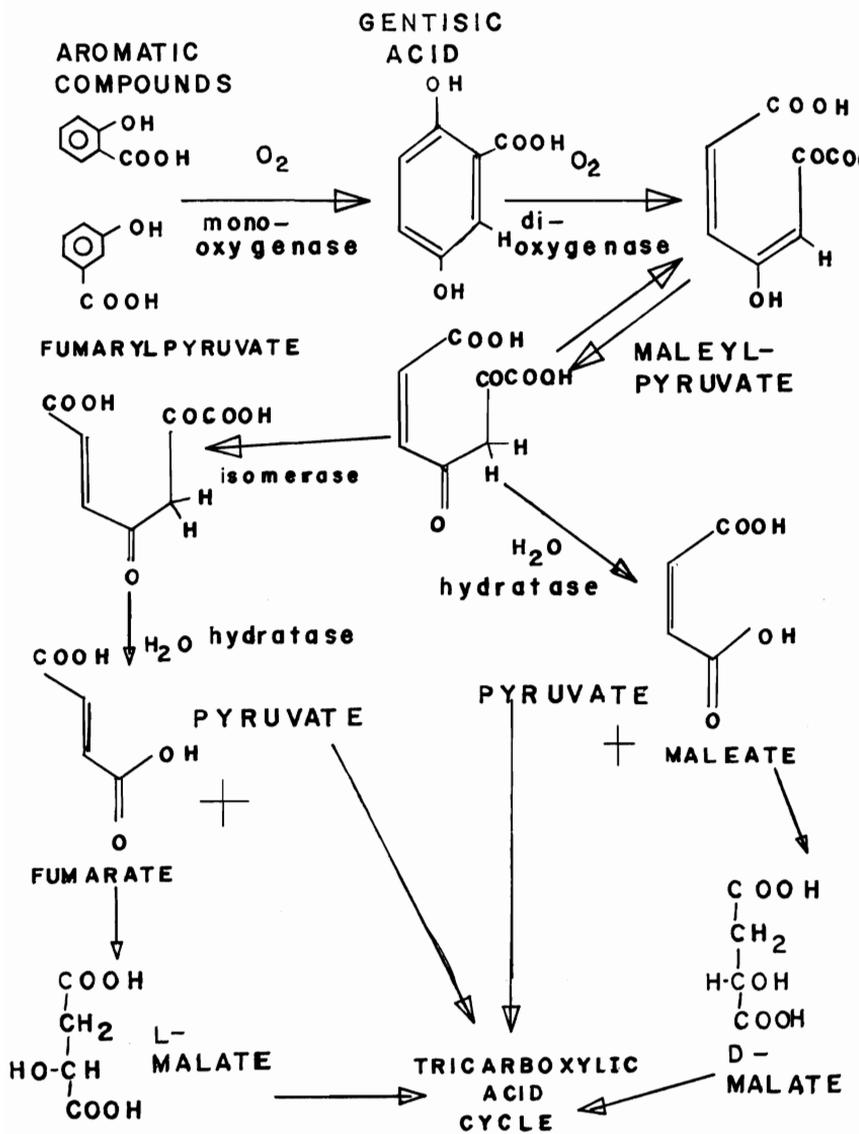


Figure 1
Degradation of Aromatic
Compounds By Soil Microbes

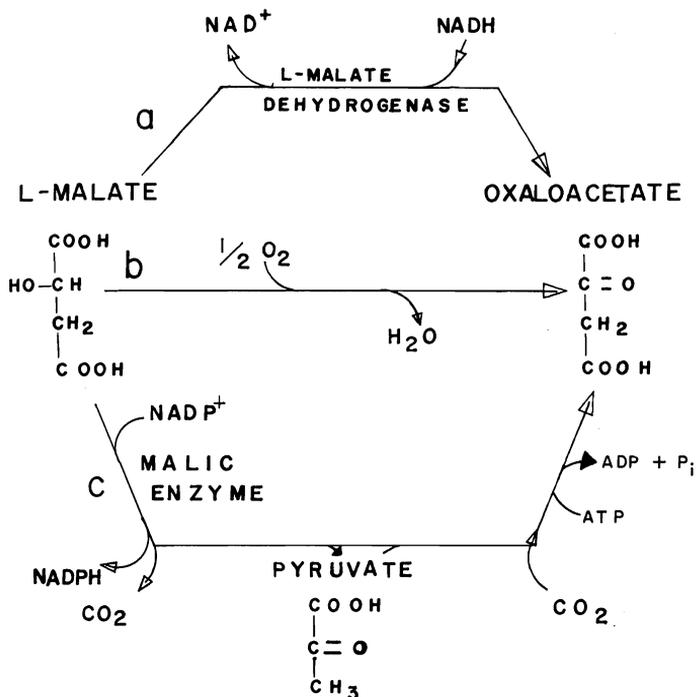


Figure 2
Metabolic Pathways
of L-Malate

The oddity that arises with the *Ps. 2,5X* organism is that it will grow well with L-malate as the sole carbon source; however, it will not grow with D-malate as the sole carbon source, despite the fact that this compound can arise from the metabolism of gentisate. In addition, whole cell suspensions oxidize L-malate rapidly, and oxidize D-malate slowly, no matter what substrate the cells are grown on (Figure 3).

The presence of an NAD-dependent L-malate dehydrogenase and an NAD-independent L-malate dehydrogenase has been shown in the *Ps. 2,5X* organism, but there is no evidence that either an NAD-dependent or an NAD-independent D-malate dehydrogenase is present.

A second organism, *Ps. 3,5X*, was found to grow well on L-malate and slowly on D-malate as the sole carbon source. However, whole cell suspensions of the *Ps. 3,5X* organism oxidized both D- and L-malate almost equally well (Figure 4). Preliminary evidence indicates that this organism

utilizes the same metabolic pathways in the degradation of gentisic acid as the *Ps. 2,5X* organism.

Therefore, an experiment was run to determine whether the enzymes used in the breakdown of D-malate are constitutive enzymes; that is enzymes produced by cells regardless of the composition of the medium on which they grew, or whether they were induced enzymes, produced by cells in response to the presence of a particular substrate.

A Warburg experiment was set up using cells of *Ps. 3,5X* grown on either succinate or *m*-hydroxybenzoate (which is metabolized in this organism via the gentisic acid pathway) as the sole carbon source. Each experiment was subdivided into groups, one to which chloramphenicol was added, and a control group without chloramphenicol. Chloramphenicol is an antibiotic which inhibits the production of new protein, and would thus inhibit the production of any enzymes not already present.

Succinate grown cells oxidized D-malate and L-malate at similar rates. When chloramphenicol was added, little change was observed in the rates of oxidation (Figures 5 and 6).

Using cells grown on *m*-hydroxybenzoate, however, the D-malate showed a significantly slower rate than the L-malate. When chloramphenicol was added, the oxidation of D-malate and DL-malate showed markedly slower rates (Figure 7 and 8). This slow rate would indicate a basal metabolism of D-malate as a result of the small amounts of enzyme that are present in the cell at all times.

Since the D-malate was oxidized rapidly by the succinate grown cells of *Ps. 3,5X*, even in the presence of chloramphenicol, the enzymes concerned must be constitutive, or at least produced along with the succinate enzymes. D-malate oxidation was inhibited somewhat with the *m*-hydroxybenzoate grown cells, while showing only the basal rate with chloramphenicol added; therefore, the enzymes concerned with the metabolism of D-malate in these cells must be induced. In other words, the same enzymes are not used in the metabolism of D-malate derived from *m*-hydroxybenzoate as are used in the metabolism of D-malate.

Next, a search was made for either an NAD-dependent or an NAD-independent D-malate dehydrogenase in the cell free extract of DL-malate grown *Ps. 3,5X* cells, using the methods of Wolfe and Neilands (Wolfe and Neilands, 1956, p. 61) and Britten (Britten, 1968, p. 330). The cell free extract was prepared by freezing freshly harvested cells, and forcing them through a fine space between two metal plates (Hughes Press Method). The NAD-dependent L-malate dehydrogenase was detected in both the particulate and supernatant fractions of the cell free extract. In addition, an NADP-dependent L-malate dehydrogenase and an NAD-independent L-malate dehydrogenase were detected in both the supernatant and particulate fractions. Neither an NAD-independent D-malate dehydrogenase nor an

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FIGURES 3, 4, 5, 6
OXIDATION OF VARIOUS SUBSTRATES BY *Ps.* sp.

Figure 3. *Ps.* 2,5X
grown on L-malate

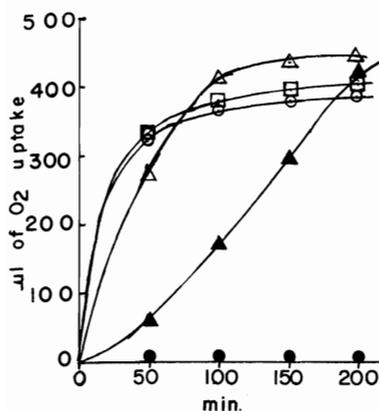


Figure 4. *Ps.* 3,5X
grown on DL-malate

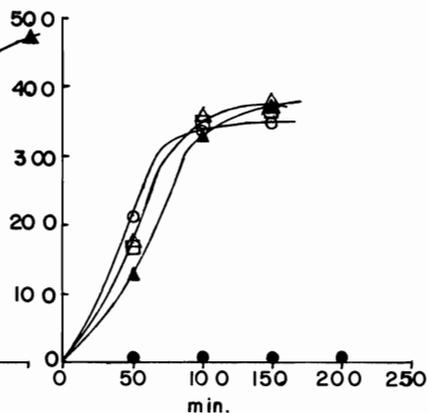


Figure 5.
Ps. 3,5X grown on
succinate

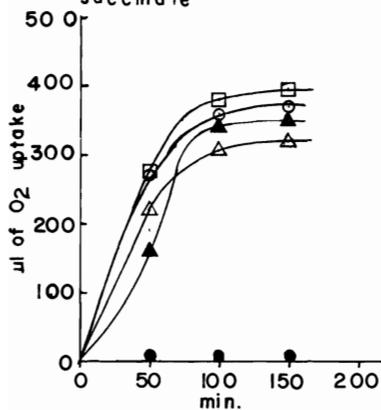
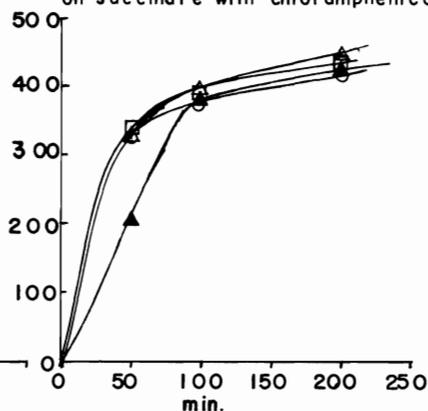


Figure 6. *Ps.* 3,5X grown
on succinate with chloramphenicol



- L-malate
- Fumarate
- △ DL-malate
- ▲ D-malate
- Maleate

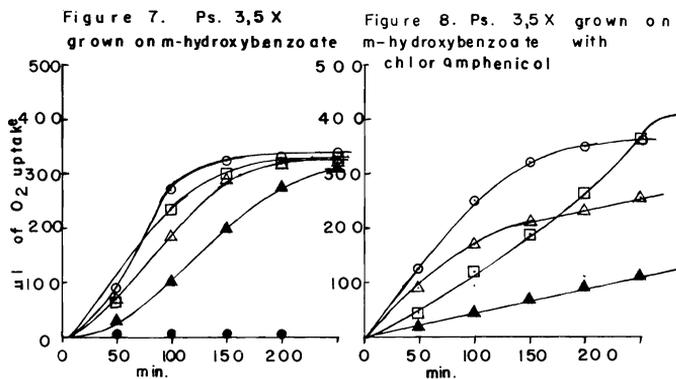
Each flask contained 3-7mg. of cells, 10μmole substrate, .20ml 5N NaOH (in center well), made up to a total volume of 3.0 ml. with .05 M pH 7.0 Phosphate buffer.

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NAD-dependent D-malate dehydrogenase were detected in either fraction. Later work has shown that an NAD-dependent D-malate decarboxylase is present in *Ps.* 3,5X cells grown on D-malate, but not in cells grown on other substrates.

An experiment was also run to determine the presence of a maleate hydratase in the cell free extract of either DL-malate or *m*-hydroxybenzoate grown *Ps.* 3,5X. This experiment involved following an enzymatic hydration of unsaturated dicarboxylic acids spectrophotometrically at 250 nm. The extract was prepared by the ultrasonic disruption of the bacterial cells. In the DL-malate grown cells, only the fumarate hydratase was present, but in the *m*-hydroxybenzoate grown cells, in addition to the fumarate hydratase, maleate and citramaleate hydratases were also present. These results would indicate that malate (D- or L-) is not dehydrated to give maleate and that maleate occurs specifically in the degradation of gentisate, or compounds that follow the gentisate pathway.

From the results presented here, the following conclusions may be drawn. First, the observed slow oxidation of D-malate, relative to L-malate, by *m*-hydroxybenzoate grown *Ps.* 3,5X (Figure 7 and 8) indicates that D-malate as such probably does not occur in the degradation of *m*-hydroxybenzoate. Second, the absence of a D-malate dehydrogenase and maleate hydratase in cells grown on DL-malate would suggest that D-malate is not metabolized by conversion to oxaloacetate or maleate. If this is also true for the metabolism of D-malate derived from *m*-hydroxybenzoate, then the following speculative hypothesis to account for D-malate metabolism may be proposed.



Each flask here contained 2.3 mg of cells, the rest of contents were the same as before, except for 150 μgr. of chloramphenicol per flask, as indicated.

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Ordinarily, the cell does not have to deal with D-malate as such, but more probably as D-malate attached to some co-factor or as an enzyme bound intermediate. In this state the D-malate is already in the cell, and can be quickly degraded, and enter the cell's metabolic pathways. This attachment of a co-factor may possibly occur before the D-malate has been split from the maleylpyruvate molecule, but in any case after the D-malate is in the cell.

A scheme has been outlined in which itaconate, as its coenzyme A derivative, is converted to citramalyl CoA, which is then split into acetyl-CoA and pyruvate (Figure 9) (Cooper and Kornberg, 1964, pp. 82-91).

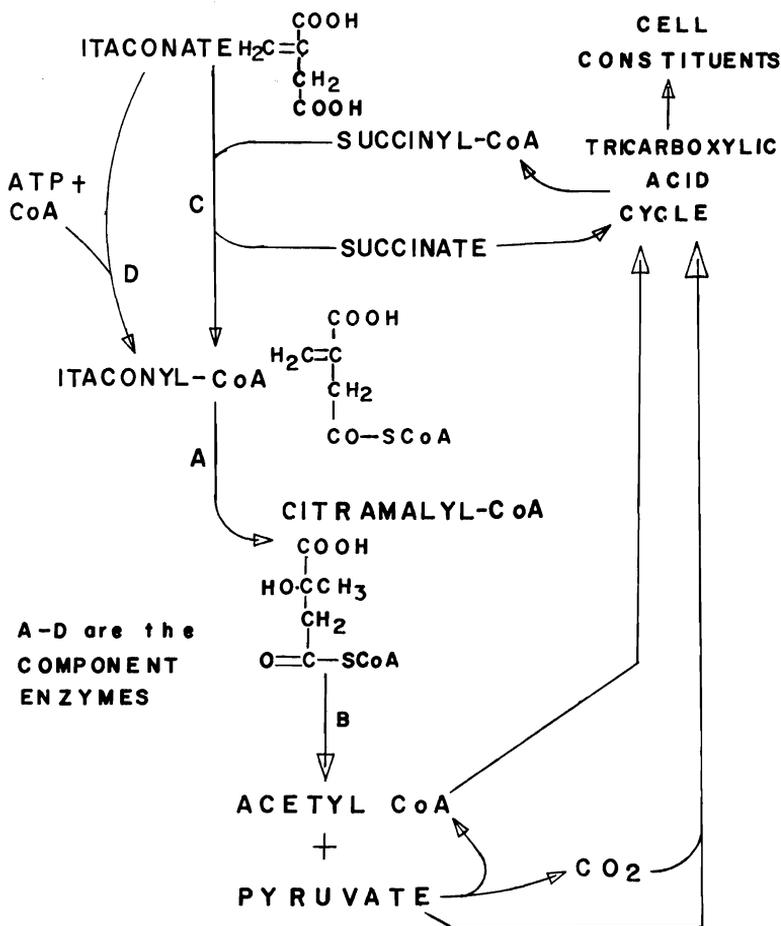


Figure 9
Utilization of Itaconate
By *Pseudomonas* Sp.

rate limiting for D-malate metabolism. Alternatively, high concentrations of D-malate, or D-malyl-CoA might have an inhibitory effect on the D-malyl-CoA complex splitting enzyme. Thus, while still oxidizing D-malate, the rate of energy production would be too slow to allow growth. This might be the case for *Ps. 2,5X* organism. In the *Ps. 3,5X* organism, the decarboxylation reaction of D-malate might be fast enough to make it the prime pathway in the metabolism of D-malate, as the sole carbon source. This reaction is believed to be absent in the *Ps. 2,5X* organism.

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