METABOLISM OF DICARBOXYLIC ACIDS IN ACETOBACTER XYLI NUM

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Received for publication 15 August 1963

ABSTRACT

BENZIMAN, Moshe (The Hebrew University of Jerusalem, Jerusalem, Israel), and A. AbeliovitZ. Metabolism of dicarboxylic acids in Acetobacter xylinum. J. Bacteriol. 87:270–277. 1964.—During the oxidation of fumarate or L-malate by whole cells or extracts of Acetobacter xylinum grown on succinate, a keto acid accumulated in the medium in considerable amounts. This acid was identified as oxaloacetic acid (OAA). No accumulation of OAA was observed when succinate served as substrate. These phenomena could be explained by the kinetics of malate, succinate, and OAA oxidation. OAA did not inhibit malate oxidation, even when present at high concentrations. When cells were incubated with OAA or fumarate in the presence of C\textsuperscript{14}O\textsubscript{2}, only the beta-carboxyl of residual OAA was found to be labeled. Evidence was obtained indicating that nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) are not directly involved in malate oxidation by cell-free extracts. The results suggest that malate oxidation in A. xylinum is irreversible, and is catalyzed by an enzyme which is not NAD- or NADP-linked.

During the incubation of washed succinate-grown Acetobacter xylinum cells with fumarate or L-malate, no cellulose was formed but considerable amounts of a keto acid accumulated in the medium. However, incubating such cells with succinate resulted in a net synthesis of cellulose, without the accumulation of a keto acid (Benzi-
nan and Burger-Rachaminov, 1962). The purpose of this investigation was to study the manner in which these dicarboxylic acids are metabolized in A. xylinum. A preliminary report of this work has appeared (Abeliovitz and Benzi-
nan, 1963).

MATERIALS AND METHODS

Succinate-grown cells of A. xylinum, were grown and harvested as described previously (Benzman and Burger-Rachaminov, 1962). Cell-free extracts were prepared by submitting cell suspensions to disruption in a Raytheon model DF 101 magnetorestrictive oscillator at 200 w and 10 kc per sec for 20 min. The sonic extract was centrifuged at 9,000 × g for 15 min in the cold, and the precipitate was discarded.

Oxidation experiments were carried out in a Warburg apparatus by use of standard manometric techniques (Umbricht, Birris, and Stauffer, 1957). For other experiments, a Dubnoff metabolic shaker was used. All experiments were carried out at 30 C. Endogenous oxidation rates represented less than 5% of total readings. Values reported were corrected for endogenous rates.

Substrates used were in the form of the potassium salts. Oxaloacetic acid (OAA) solutions were freshly prepared before each experiment.

For determination of reaction products, HCl was added to incubation mixtures to a final concentration of 0.2 n (unless otherwise indicated), and mixtures were centrifuged in the cold at 9,000 × g for 15 min. The supernatant fluid was kept in the cold until assayed.

Analytical methods. OAA was determined enzymatically with malic dehydrogenase as follows. Proper samples of deproteinized sample, 10 \textmu moles of tris(hydroxymethyl)aminomethane (tris) base, 50 \textmu moles of tris-HCl buffer (pH 7.5), 0.2 \textmu moles of reduced nicotinamide adenine dinucleotide (NADH), and 0.3 units of malic dehydrogenase were added to a glass cuvette (total volume 1.0 ml), and the optical-density changes at 340 nm were determined spectrophotometrically (E\textsubscript{NADH} = 6.22 × 10\textsuperscript{4}). OAA was also determined manometrically by beta decarboxylation with Al\textsuperscript{3+} according to Krebs and Eggleston (1945), and colorimetrically by the method of Kalnitsky and Tapley (1958).

Total keto acids were determined by the direct method of Friedman and Haugen (1943).

Succinate, isolated on a Celite column by the method of Swick and Wood (1960), was determined with succinic dehydrogenase, by the spectrophotometric method of Rodgers (1961). Acetic acid, separated by steam distillation,
was identified on the basis of mobility on paper, in a solvent system containing butanol-water-diethyl amine (100:15:1, v/v; Jones, Dowling, and Skraba, 1953).

Reduction of 2,6-dichlorophenolindophenol and ferricyanide was determined spectrophotometrically by changes in optical density at 600 and 420 nm, respectively (Green, Mii, and Kohout, 1955; Slater and Bonner, 1952).

All spectrophotometric determinations were carried out in a Zeiss spectrophotometer by use of cuvettes with 1-cm light path.

2,4-Dinitrophenylhydrazones of keto acids were prepared, isolated, and chromatographed on paper, according to El Hawary and Thompson (1953), in solvent systems containing n-butanol–ethanol–0.5 N NH₃ (7:1:2, v/v) or 0.1 M glycine, pH 7.8 (Virtanen, Meittinen, and Kuntu, 1953). Separation of 2,4-dinitrophenylhydrazones on silica gel columns was done according to Aronoff (1956), with a chloroform–n-butanol (15:88, v/v) mixture as solvent. Radioactivity of keto acids was determined as follows. To a sample to be assayed, containing 30 to 40 μmoles of keto acid, 6 ml of 0.5% solution of 2,4-dinitrophenylhydrazine were added. After incubation at 30 °C for 30 min, the mixture was allowed to stand in the cold for a few hours, and the precipitate, collected on Fiberglas discs, was washed twice with 2 N HCl and petroleum ether and dried in the cold over P₂O₅. Radioactivity was measured in a gas-flow counter. Values were corrected for self-absorption. Recovery of the 2,4-dinitrophenylhydrazones in the precipitate was 80% of the theoretical, and values were corrected accordingly.

Spots of 2,4-dinitrophenylhydrazones, on paper chromatograms, were eluted according to El Hawary and Thompson (1953), and the radioactivity of the separated keto acid derivatives was determined after drying the ethyl acetate extract.

Radioactivity in the beta-carboxyl of OAA was determined by decarboxylation with Al₄⁺. The liberated CO₂ was trapped in KOH, precipitated as BaCO₃, collected on Fiberglas discs, and counted.

Chemicals. Succinic acid and 2,6-dichlorophenolindophenol were purchased from the British Drug Houses; L-malic acid from Nutritional Biochemicals Corp., Cleveland, Ohio; fumaric acid from Fluka AG Chemisch Fabric, Bucks SG, Switzerland; OAA from Mann Research Laboratories; nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), NADH, and reduced NADP (NADPH) from Sigma Chemical Co., St. Louis, Mo.; fluoracetate (sodium salt) from Delta Chemical Works Inc., New York, N.Y.; and Na₂C₄O₄ from the Radiochemical Center, Ambersham, England. 2,4-Dinitrophenylhydrazine, obtained from the British Drug Houses, was recrystallized twice from 2 N HCl before use. Malic dehydrogenase (pig heart; specific activity, 36) was obtained from C. F. Boehringer & Soehne GmbH, Mannheim-Waldhof, Germany.

Results

Identification of the keto acid formed during malate or fumarate oxidation. Cell suspensions or cell-free extracts of A. xylinum were incubated with L-malate or fumarate. Treatment of the reaction mixtures with 2,4-dinitrophenylhydrazine, followed by paper chromatography in two different solvent systems (see Materials and Methods), revealed OAA as the only keto acid present in considerable amounts in the reaction mixtures. In all chromatograms, small amounts of pyruvic dinitrophenylhydrazones could also be detected.

That OAA is the keto acid accumulating in the reaction mixture, can also be concluded from the results shown in Table 1. The values obtained for the amount of total keto acid are in good agreement with those obtained by the OAA-specific methods of determination and by the beta-decarboxylation method. Pyruvic acid was the only keto acid identified by paper chromatography, after beta-decarboxylation treatment of the reaction mixtures.

Oxidation of malate and fumarate. The time curves for OAA accumulation during oxidation of malate by whole cells and cell-free extracts of A. xylinum are shown in Fig. 1 and 2. At the early stages of the reaction, OAA accumulated. Under the experimental conditions used, the maximal amount of OAA accumulating was equivalent to about 40 and 25%, respectively, of the total malate added to whole cells and cell-free extracts. In extracts, acetate was identified chromatographically as the principal product of malate oxidation beyond OAA. The observed RQ values (corrected for accumulation of OAA) were consistent with oxidation of malate to acetate.
TABLE 1. Determination of the keto acid formed from malate or fumarate by Acetobacter xylinum

<table>
<thead>
<tr>
<th>System</th>
<th>Beta-decarboxylation</th>
<th>Kalnitsky and Tapley</th>
<th>Enzymatic</th>
<th>Total keto acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumarate +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>14.5†</td>
<td>14.2</td>
<td>14.3</td>
<td>15.1</td>
</tr>
<tr>
<td>fumarate +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extract</td>
<td>11.8</td>
<td>12.1</td>
<td>12.2</td>
<td>12.7</td>
</tr>
<tr>
<td>L-Malate +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>15.2</td>
<td>14.9</td>
<td>15.3</td>
<td>16.1</td>
</tr>
<tr>
<td>L-Malate +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extract</td>
<td>12.2</td>
<td>12.4</td>
<td>12.7</td>
<td>13.2</td>
</tr>
</tbody>
</table>

* Cells or cell-free extract [equivalent to 25 mg (dry weight) of cells] was incubated with 0.01 M substrate in 5.0 ml of 0.05 M phosphate buffer (pH 6.0) for 30 min. Determinations as described in Materials and Methods.
† Decarboxylation with aniline citrate (Gre-ville, 1939), which decomposes all keto acids, gave similar results.
‡ Keto acid formed is expressed as micromoles of OAA.

Whole cells, however, oxidize malate and fumarate to CO₂ and water. Conversion of malate to acetate was also observed by Gromet-Elhanan (1960) when glucose-grown cells of A. xylinum oxidized malate in the presence of fluoracetate. It seems likely that, under the conditions employed, the extracts are unable to oxidize acetate (see Neeman and Benziman, 1962).

The time curves of OAA accumulation during oxidation of fumarate by whole cells and cell-free extracts were similar to those obtained when malate was the substrate. This is to be expected in view of the high fumarate activity present in the cells (Benziman and Burger-Rachamimov, 1962).
Effect of OAA on malate oxidation. When extracts preincubated with ethylenediaminetetraacetic acid (EDTA) were allowed to oxidize malate, OAA accumulated at a constant rate (Fig. 3), indicating that OAA does not inhibit malate oxidation.

Nonparticipation of exogenous NAD and NADP in malate oxidation. Addition of NAD or NADP had no effect on malate oxidation, even when a dialyzed extract was used (24 hr, against 0.04 M phosphate buffer, pH 6.0). When the standard spectrophotometric assay method for mammalian NAD-malic dehydrogenase (Mehler et al., 1948) was applied to extracts of A. xylinum, no oxidation of NADH or NADPH could be observed upon addition of OAA.

Stoichiometry of malate oxidation. Malate oxidation by extracts of A. xylinum could be coupled with the reduction of artificial electron acceptors. The stoichiometry of the reaction with oxygen, ferricyanide, or dichlorophenolindophenol as oxidants is shown in Table 2. The results demonstrate that 1 atom of oxygen or 2 moles of ferricyanide were consumed, or 1 mole of dichlorophenolindophenol was reduced, per mole of OAA formed. The rates of malate oxidation in the presence of the different electron acceptors were of the same order of magnitude, with ferricyanide giving the highest rate of oxidation. On the other hand, the supernatant fluid obtained by high-speed centrifugation of extracts (100,000 × g, 30 min in a Spinco model L ultracentrifuge) showed low malate-oxidizing activity with oxygen, while it still possessed more than 50% of the original oxidizing activity with ferricyanide.

Kinetics of OAA accumulation. Whereas OAA accumulated during malate oxidation by extracts of A. xylinum, under the same experimental conditions exogenous OAA was rapidly utilized by these extracts (Fig. 4). To establish whether OAA accumulation is due to the low rate of OAA utilization, under the conditions prevailing during its formation from malate, experiments were carried out to study the rate of malate and OAA utilization by extract as a function of substrate concentration (Fig. 5). From these curves, we calculated the velocity of malate and OAA utilization under conditions where the rates of both reactions were linearly proportional to substrate concentration. The values obtained per ml of reaction mixture were, for malate and OAA, respectively, 0.39 and 0.50 μmoles of substrate oxidized per mg cells per hr per μmole of initial substrate concentration. The amounts of OAA which accumulated during malate oxidation (Fig. 2) were found to be in close agreement with those expected from calculations based on these velocity values.

Oxidation of succinate. No accumulation of OAA was observed when extracts of A. xylinum were incubated with succinate, under the same conditions as those described for malate oxidation.

### Table 2. Stoichiometry of malate oxidation by extracts of Acetobacter xylinum

<table>
<thead>
<tr>
<th>Expt</th>
<th>Acceptor*</th>
<th>OAA formed†</th>
<th>Acceptor reduced‡</th>
<th>Rate of oxidation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O2</td>
<td>2.4</td>
<td>1.3</td>
<td>1.45</td>
</tr>
<tr>
<td>2</td>
<td>Ferricyanide</td>
<td>3.0</td>
<td>6.1</td>
<td>1.80</td>
</tr>
<tr>
<td>3</td>
<td>Dichlorophenol indophenol</td>
<td>2.4</td>
<td>2.3</td>
<td>1.45</td>
</tr>
</tbody>
</table>

* Extract (from 10 mg of cells) was incubated 15 min with 0.05 M EDTA in 0.1 M phosphate buffer (pH 6.0), prior to addition of acceptor (ferricyanide, 0.02 M; dichlorophenol indophenol, 0.006 M) and malate (0.05 M). Final volume, 1.0 ml. Experiments 2 and 3 under N₂. In experiment 3, sodium azide (5 × 10⁻³ M) was added.

† Expressed as micromoles per 10 min.
‡ Expressed as micromoles of OAA formed per milligram of cells per hour.

![Fig. 4. Oxidation of OAA by extracts of Acetobacter xylinum. Extract [from 5 mg (dry weight) of cells] was incubated with 10 μmoles of OAA in 1.0 ml of 0.05 M phosphate buffer (pH 6.0).](http://jb.asm.org/)
substrate, 150; the side for OAA Fumarate ............... 65 succinate J. BACTERIOL. of concentrations oxidation Fumarate + ............ 4,350 < 10
[59x648]succinate J. BACTERIOL. of concentrations succinate, up to 10 mm. The value obtained for the velocity of succinate oxidation at concentrations below 10 mm was (per ml of reaction mixture) 0.056 μmoles oxidized per mg of cells per hr per μmole of initial concentration. This value is ten times lower than the respective value obtained for OAA utilization. Thus, assuming succinate oxidation proceeds through malate and OAA, no detectable amounts of OAA could be expected to accumulate during succinate oxidation under our experimental conditions.

Irreversibility of malate oxidation. The irreversibility of malate oxidation by extracts of A. xylinum is suggested by the stoichiometric conversion of malate to OAA. To assess whether this irreversibility exists in the intact cell, advantage was taken of the ability of A. xylinum cells to form radioactive OAA when incubated with C\(^{14}\)O\(_2\) and OAA (unpublished data). If there was reduction of OAA to malate, one would expect, in presence of an active fumarase (Benziman and Burger-Rachamimov, 1962), complete equilibration of the C\(^{14}\) between the alpha and beta carboxyls. [See review by Weinman, Strisower, and Chaikoff (1957).]

Experiments were carried out in which cells were incubated with OAA or with fumarate in the presence of C\(^{14}\)O\(_2\). At the end of the incubation period, the reaction mixture was treated with 2,4-dinitrophenylhydrazine, extracted, and chromatographed on paper. The chromatograms revealed the presence of only OAA and small amounts of pyruvate, of which only the former was found to be radioactive. In similar experiments (Table 3), the distribution of C\(^{14}\) in the OAA remaining in the reaction mixture was determined. The results demonstrate that in all cases there was no detectable randomization of labeling in the OAA. Practically all the labeling

![Graph](https://example.com/graph.png)

**Fig. 5. Effect of substrate concentration on initial rate of malate and OAA oxidation by extracts of Acetobacter xylinum.** Reaction mixtures contained (μmoles/ml): phosphate buffer (pH 6.0), 80; increasing substrate, as indicated; and extract [from 5 mg (dry weight) of cells]. For malate experiment, extract was preincubated 15 min with 0.05 μM EDTA. Activity (V) calculated from amount of OAA accumulated (malate experiment) or utilized (OAA experiment), during first 5-min period after addition of the substrate.

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>OAA remaining in system</th>
<th>Total activity in OAA†</th>
<th>Activity in beta carboxyl of OAA†</th>
<th>Activity in (\text{CH}_2=\text{CO}\text{--COOH}) of OAA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAA</td>
<td>63</td>
<td>6,410</td>
<td>6,530</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Fumarate</td>
<td>65</td>
<td>4,350</td>
<td>4,350</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Fumarate + fluoroacetate (5 × 10^{-4} M)</td>
<td>90</td>
<td>9,400</td>
<td>9,300</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Reaction mixtures (10 ml) in stoppered side-arm flasks contained (in μmoles) in the main chamber: substrate, 150; KH\(_2\)PO\(_4\), 300; phosphate buffer (pH 6.0), 150; and cells, 50 mg (dry weight); and in the side arm: NaHCO\(_3\)O\(_2\) (10,000 counts per min per μmole), 120. Shaken 1 hr at 30 C. The reaction was terminated by sedimenting cells by centrifugation. Supernatant was acidified with HCl and flushed for 5 min with CO\(_2\) and air.
† Expressed as counts per minute.

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TABLE 3. Isotope distribution in OAA after incubation of Acetobacter xylinum with C\(^{14}\)O\(_2\)

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observed in the intact OAA molecule was found in the beta carboxyl, whereas the moiety of OAA containing the alpha carboxyl was unlabeled. The presence of fluoroacetate in the fumarate experiment increased the amount of OAA accumulated but did not effect the distribution of labeling within the OAA.

**DISCUSSION**

Malate oxidation in *A. xylinum* seems to be accomplished by a mechanism other than the NAD-linked malic dehydrogenase. This is deduced from the following observations. (i) Malate oxidation by extracts of *A. xylinum* is not affected by addition of NAD or NADP, and in the presence of OAA no oxidation of exogenous NADH or NADPH could be observed. (ii) Malate oxidation by extracts of this organism, was not effected by the presence of even high concentrations of OAA, whereas OAA at even low concentrations strongly inhibits oxidation of malate by the NAD-linked malic dehydrogenase (Ravel and Wolfe, 1963).

In systems containing a NAD-linked malic dehydrogenase, OAA does not accumulate. Moreover, special assumptions were made to account for formation of enough OAA to maintain the high rate of malate oxidation, under physiological conditions, in mitochondria (Johnson, 1960). The accumulation of OAA in the present system is readily explained if it assumed that malate is oxidized irreversibly by a NAD-non-linked enzyme system.

That malate oxidation in whole cells of *A. xylinum* is not readily reversible is strongly suggested from the observed isotope distribution in residual OAA after incubation of cells with OAA or fumarate in the presence of C\(^{14}\)O\(_2\) (Table 3). This distribution points to absence of OAA equilibration with fumarate. Such equilibration should have occurred if malate oxidation in the cells was reversible, considering the high fumarase activity present in the cells. Equilibration of OAA with fumarate catalyzed by NAD-malic dehydrogenase and fumarase is the basis for explaining the observed isotope distribution in glycogen formed from pyruvate-2-C\(^{14}\) or from pyruvate and C\(^{14}\)O\(_2\) (Wood et al., 1945; Topper and Hastings, 1949; Utter, 1959), if pyruvate carboxylation, under physiological conditions, occurs via OAA as postulated by Utter (Utter and Keech, 1960; Utter, 1961). Similarly, the observed distribution of labeling in propionate, formed by propionic bacteria from pyruvate-2-C\(^{14}\) or in the presence of C\(^{14}\)O\(_2\), also demands conversion of OAA to malate. [See review by Wood and Stjernholm (1962); Allen et al. (1963).] The inability of *A. xylinum* to equilibrate OAA with fumarate, explains our earlier observations (Benziman and Burger-Rachamimov, 1962) that exogenous CO\(_2\) failed to serve as a carbon source for cellulose, synthesized by these cells in the presence of pyruvate. This explanation is justified if pyruvate carboxylation to OAA is the first step leading to cellulose from pyruvate. The isotope distribution data of Table 3 excludes the malic enzyme (Ochoa, Mehler, and Kornberg, 1948) as being involved in the OAA-CO\(_2\) exchange reaction in *A. xylinum*.

A malate-oxidizing system, which differed significantly from the NAD-linked malic dehydrogenase, was also reported by Krampitz, Wood, and Werkman (1943) and others (McManus, 1951; Cohn, 1956) to be present in *Micrococcus lysodeikticus*. To explain the accumulation of OAA during malate oxidation by preparations of *M. lysodeikticus*, it was suggested that a "physiological OAA," differing from chemically prepared OAA, is the product of malate oxidation. However, we could explain the OAA accumulation which occurred in our system on the basis of the kinetics of malate and OAA oxidation (Fig. 5). The evidence presented here (Table 1) leaves little doubt that OAA is the product of malate oxidation in *A. xylinum*. However, the possibility that the product is another compound, which is readily equilibrated with OAA, cannot be excluded.

As to the mechanism of a malate oxidation not involving NAD or NADP, Cohn (1958) obtained, during purification of such a system from *M. lysodeikticus*, an unidentified factor capable of stimulating malate oxidation. Kornberg and Phizackerley (1959) reported the existence in a *Pseudomonas*, of a particulate NAD-nonlinked malate-oxidizing system, oxidizing malate with oxygen, ferricyanide, or dichlorophenolindophenol. The oxidation with oxygen was cyanide- and amytal-sensitive, whereas with dichlorophenolindophenol cyanide had no effect and amitil inhibited the reaction. Preliminary experiments with a supernatant fluid obtained by high-speed centrifugation of extracts of *A. xylinum* indicated some similarities between the malate-oxidizing system of *A. xylinum* and that of the *Pseudomonas*, in their reaction towards the above-mentioned reagents. A better understanding as
expected from studies with more purified preparations.

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