THE ENZYMATIC CONVERSION OF MANDELIC ACID TO BENOIC ACID

I. GROSS FRACTIONATION OF THE SYSTEM INTO SOLUBLE AND PARTICULATE COMPONENTS

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Mandelic acid can serve as sole source of carbon and energy for the growth of certain strains of Pseudomonas fluorescens. Analysis of the enzymatic patterns of cells grown on various substrates (Stanier, 1947, 1948; Sleeper and Stanier, 1950) has led to formulation of the following sequence for the initial steps in its dissimilation: Mandelic acid → benzoylformic acid → benzaldehyde → benzoic acid → catechol. All the relevant enzymes are substrate induced. Living cells consume approximately 11 atoms of oxygen per mole of mandelate oxidized with an R.Q. of slightly less than one. After slow vacuum drying, mandelate grown cells yield preparations which consume only two atoms of oxygen per mole of mandelate—sufficient for oxidation to benzoic acid (Sleeper, Tsuchida, and Stanier, 1950). Cells harvested from a medium which contains sodium DL-mandelate, or dried cells prepared from them, oxidize both isomers at equal rate.

In this work the series of enzymes which convert DL-mandelic acid to benzoic acid has been separated and some of their properties determined. In this paper the properties of the overall system, together with the primary separation of the relevant enzymes into particulate and soluble fractions, are reported. The properties of the particle fractions are described in paper two of the series (Stanier et al., 1953), and the further fractionation and characterization of the soluble enzymes, in paper three (Gunsalus et al., 1953).

METHODS

Pseudomonas fluorescens, strain A.3.12, was used throughout this work. Cells containing the enzymes of the mandelate-benzoate reaction

sequence were prepared by growth at the expense of mandelic acid as previously described (Sleeper et al., 1950). Early in the work, slow vacuum-dried or lyophilized cells were used as a source of enzymes. Subsequently, freshly harvested cells (5 g wet weight per 50 ml phosphate buffer, 0.02 M, pH 7.0) were treated for 10 to 15 minutes in a Raytheon 10 kc, 200 watt sonic oscillator. After treatment, the extract was centrifuged in the cold at 7,000 G for 15 minutes to remove unbroken cells. Dried cells, cell-free extracts, and enzymatic preparations were stored at -20 C. Some batches of freshly harvested cells also were frozen and stored at -20 C for several weeks prior to sonic treatment, without apparent effect on their enzymatic activity.

Manometric measurements were made in the customary way (Umbreit et al., 1949) with the Warburg apparatus. Unless otherwise specified, manometric experiments were conducted at 30 C in an atmosphere of air. Studies on the pyridine nucleotide-linked dehydrogenases were conducted with a Beckman model DU ultraviolet spectrophotometer at room temperature.

The protein content of crude extracts and of the first fractions derived from them was estimated colorimetrically using the Folin-Ciocalteu reagent (Hawk et al., 1949). Protein estimations on purified enzymatic fractions which contained little nucleic acid were made spectrophotometrically by the method of Warburg and Christian (1941).

RESULTS

Experiments with whole dried cells. Dried cells of P. fluorescens resuspended in phosphate buffer usually oxidize sodium DL-mandelate with the uptake of two atoms of oxygen and formation of one mole of carbon dioxide per mole of substrate. With many preparations (especially those prepared by slow vacuum drying) the rate of oxygen uptake declines sharply after one atom has been consumed (figure 1, left). The addition
of diphosphopyridine nucleotide (DPN) to such preparations maintains the initial rate of oxidation until the second atom has been consumed. Cocarboxylase (DPT) and Mg++ slightly increase the initial rate of oxidation but do not affect the rate of uptake of the second atom of oxygen. Simultaneous addition of diphosphopyridine nucleotide, cocarboxylase, and Mg++ gives buffer (pH 7.0) with 1 mM of sodium DL-mandelate and incubated at 30°C for 60 minutes with mechanical agitation to provide the necessary aeration. The mixture then was deproteinized with sulfuric acid, clarified by centrifugation, and extracted with ether. The ether was evaporated, and the residue sublimed in vacuo to yield 70 mg of a white crystalline solid. After one recrystal-

![Graph](plot)

**Figure 1 (left).** Influence of cofactors on oxidation of DL-mandelic acid by dried cells of *Pseudomonas fluorescens*. Each vessel contained: 10 μM sodium DL-mandelate, 5 mg dried cells, 2 ml M/10 phosphate buffer, pH 7.0. Where added diphosphopyridine nucleotide (DPN), 200 μg; cocarboxylase (DPT), 100 μg; 10 μM Mg++.  

**Figure 1 (right).** Influence of cofactors on benzoic acid oxidation. Each vessel contained 10 μM benzoic acid, 10 mg dialyzed dried *Pseudomonas fluorescens*, 2 ml M/10 phosphate buffer, pH 7.0.

the same result as addition of diphosphopyridine nucleotide alone. In the light of earlier work (Stanier, 1947, 1948) the slow uptake of the second atom of oxygen (figure 1, left) should reflect a low rate of oxidation of benzoic acid, capable of being increased by addition of diphosphopyridine nucleotide; the data in figure 1 (right) show this prediction to be correct.

The formation of benzoic acid as the end product of the oxidation by dried cells was established as follows. One gram of lyophilized cells was suspended in 200 ml of 0.02 M phosphate lization from water, this substance melted at 121.5°C (uncorrected), and the melting point was not depressed by admixture with synthetic benzoic acid. The absorption spectrum between 210 and 300 μm was virtually superimposable on that of pure benzoic acid measured under the same conditions (1 cm cell, pH 7.0, calculated molarity 0.0001 M). Both spectra showed a maximum at 225 μm with molar extinction coefficients of 8,400 (synthetic benzoic acid) and 8,300 (isolated material).

Dried cells, after dialysis overnight at 5°C
TABLE 1

Effect of cofactors on DL-mandelic acid oxidation by a dialyzed dried cell suspension

Per Warburg Cup:

- m/20 phosphate buffer, pH 7
- 5 mg (dry weight) cells
- 10 μM sodium DL-mandelate

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>OXYGEN CONSUMED</th>
<th>CARBON DIOXIDE EVOLVED</th>
<th>R.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM/Mole substrate</td>
<td>μM/Mole substrate</td>
<td></td>
</tr>
<tr>
<td>(1) None</td>
<td>4.2 0.42 0 0 0</td>
<td>9.8 0.98 1.90</td>
<td></td>
</tr>
<tr>
<td>(2) DPT, 50 μg; + Mg++, 10 μM</td>
<td>5.2 0.52 9.8 0.98 1.90</td>
<td>5.7 0.98 1.80</td>
<td></td>
</tr>
<tr>
<td>(3) (2), + DPN, 20 μg</td>
<td>11.0 1.10 10.6 1.06 1.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DIAGRAM 1

Fractionation of sonic extract of mandelate grown cells (5 g wet cells in 80 ml 0.08 m phosphate buffer)

Sonic treated suspension (670 mg protein) centrifuged at 7,000 G for 15 minutes

- Residual intact cells (discarded)
- Pellet resuspended in 0.02 m phosphate buffer (pH 7.0): coarse particle fraction (77 mg protein)
- Supernate extract (455 mg protein): saturated alkaline (NH₄)₂SO₄ added to 0.33 saturation, mixture centrifuged
- 0-0.33 sat. precipitate resuspended in 0.02 m phosphate buffer (pH 7.0): fine particle fraction (54 mg protein)
- Supernatant Saturated alkaline (NH₄)₂SO₄ added to 0.67 saturation, mixture centrifuged
- 0.33-0.67 sat. precipitate resuspended in 0.02 m phosphate buffer (pH 7.0): soluble fraction (225 mg protein)

against 0.1 m phosphate buffer (pH 8.5), consume one atom of oxygen per mole of DL-mandelate with an R.Q. of zero. Addition of cocarboxylase to such a preparation causes formation of one mole of CO₂ without a change in oxygen uptake; addition of diphosphopyridine nucleotide together with cocarboxylase restores the oxygen uptake to its original value of 2 atoms per mole of substrate oxidized. The dialyzed cells do not attack either benzoyleformic acid or benzaldehyde; but addition of cocarboxylase activates the decarboxylation of the former compound, and addition of diphosphopyridine nucleotide activates the oxidation of the latter. Illustrative data are given in Table 1.

These findings confirm the conclusions arrived at on the basis of earlier in vivo studies (Stanier, 1947, 1948) and indicate that dried cells convert mandelic acid to benzoic acid by the following reactions: (a) primary dehydrogenation of mandelic acid to benzoyleformic acid, (b) decarboxylation of benzoyleformic acid to benzaldehyde, requiring cocarboxylase, (c) dehydrogenation of benzaldehyde to benzoic acid, requiring diphosphopyridine nucleotide.

Attempts were then made to extract the enzymes from suspensions of dried cells. Repeated freezing and thawing liberated the benzoyleformic carboxylase and benzaldehyde dehydrogenase, but the mandelate oxidizing system always remained exclusively in the debris. Although the soluble benzaldehyde dehydrogenase no longer connected to molecular oxygen, its activity could be measured spectrophotometrically by diphosphopyridine nucleotide reduction or manometrically with ferricyanide as an electron ac-
enceptor (Haas, 1937). Our inability to solubilize the mandelic dehydrogenase led us to try other methods of breaking the cells, and it was found that concentrated suspensions of living cells could be almost completely disintegrated by brief sonic oscillation to yield a cell-free extract which catalyzed all steps of the mandelate-benzoate reaction sequence. Subsequent experiments were conducted with these preparations.

Preparation and fractionation of sonic extracts. A suspension of living cells is largely disrupted (75 to 90 per cent) by sonic treatment for 15 minutes (Rattheon 10 kc, 200 watt oscillator). After sedimentation of unbroken cells, a reddish-brown extract which shows a marked Tyndall effect is obtained. Centrifugation for one hour at 22,000 G in the high-speed attachment of an International refrigerated centrifuge sediments most of the light scattering material as a translucent, gelatinous amber pellet. Resuspension of the pellet in a small volume of buffer yields an opaque suspension whose brown color is largely masked by intense light scattering (“coarse particle fraction”).

The supernate after high-speed centrifugation still contains some particulate material of relatively large dimensions (“fine particle fraction”) which can be separated from the dissolved proteins by fractionation with alkaline saturated ammonium sulfate (made alkaline by addition of NH₂OH to pH 7.9, as measured with the glass electrode). The particulate material is aggregated completely at 0.33 saturation, whereas most of the dissolved proteins (“soluble protein fraction”) precipitate between 0.33 and 0.70 saturation. The flow sheet for an actual fractionation is shown in diagram 1, and the distribution of some enzymatic activities in the various fractions is presented in table 2.

The data of table 2 reveal that this treatment effects a primary separation of the enzymes of the mandelate-benzoate reaction sequence. The oxido-

<table>
<thead>
<tr>
<th>TABLE 2</th>
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<tbody>
<tr>
<td>Enzymatic activities of sonic extract fractions on mandelate and benzoyl formate (see diagram 1)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>PROTEIN</th>
<th>ENZYMATIC ACTIVITIES*</th>
<th>ACTIVITY/MG PROTEIN</th>
<th>RATIO OF ACTIVITIES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L(+)</td>
<td>p(−)</td>
<td>Mandelate</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>Qos</td>
<td>Qos</td>
</tr>
<tr>
<td>(1) Sonic extract</td>
<td>100†</td>
<td>100†</td>
<td>100†</td>
<td>100†</td>
</tr>
<tr>
<td>(2) Coarse particles</td>
<td>15</td>
<td>72</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>(5) Supernate, after 22,000 G, 60 min</td>
<td>92</td>
<td>22</td>
<td>28</td>
<td>102</td>
</tr>
<tr>
<td>(4) Fine particles</td>
<td>11</td>
<td>18</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>(5) Soluble proteins</td>
<td>62</td>
<td>0</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>(6) Sum of (2), (4), (5)†</td>
<td>88</td>
<td>90</td>
<td>10</td>
<td>103</td>
</tr>
</tbody>
</table>

* Activity with the 2 isomers of Na mandelate measured as rate of oxygen intake at 30°C in air: activity with Na benzoylformate measured as rate of CO₂ evolution at 30°C in nitrogen.
† Expressed as per cent of values for sonic extract (= 100).
‡ Per cent recovery.
particle fraction which, after resuspension in buffer, barely oxidizes the d-isomer. The supernate after high-speed centrifugation, which contains only about 20 per cent of the total mandelate oxidizing capacity of the crude extract, oxidizes the two isomers at equal rates. The fine particle fraction, separated from the supernate by precipitation with ammonium sulfate, carries all the residual mandelate oxidizing capacity. Like the coarse particle fraction, it shows a markedly greater ability to oxidize the L-isomer. The combined recovery of d-mandelate oxidizing activity in the separate fractions is always very low; table 2 shows a recovery of 10 per cent, all in the two particulate fractions, as contrasted with 90 per cent recovery of L-mandelate oxidizing activity. From these findings one can infer that the mandelate oxidizing system associated with the particles is specific for the L-isomer, and that another enzyme, required for the metabolism of the d-isomer and not connected to molecular oxygen, is present in the soluble fraction. As we shall show later (Gunsalus et al., 1953), this enzyme is a racemase.

The enzyme which decarboxylates benzoylformic acid is recovered almost quantitatively (94 per cent) in the soluble protein fraction with negligible activity in the two particle fractions (table 2). The dehydrogenation of benzaldehyde as measured by diphosphopyridine nucleotide reduction proceeds rapidly with crude extracts and with the supernate from high-speed centrifugation, but cannot be quantitated readily because of reoxidation of the reduced diphosphopyridine nucleotide; therefore, data for the distribution of this system have not been included in table 2. It may be stated, however, that the benzaldehyde oxidizing enzyme is located almost entirely in the soluble fraction.

**SUMMARY**

Dried, mandelate grown cells of *Pseudomonas fluorescens* oxidize both isomers of mandelic acid to benzoic acid. Studies with dried cells show that this sequence involves: a mandelate oxidizing enzyme which converts mandelic acid to benzyloformic acid; a benzyloformic carboxylase which requires coenzyme for activity; and a benzaldehyde dehydrogenase, activated by diphosphopyridine nucleotide.

By differential centrifugation and ammonium sulfate fractionation of sonic extracts prepared from living cells, the enzymes of the sequence have been separated into particulate and soluble fractions. The particles contain the L-mandelate oxidizing system, while the soluble fraction contains benzyloformic carboxylase, benzaldehyde dehydrogenase, and an enzyme which is required for the metabolism of the d-isomer of mandelic acid.

**REFERENCES**


