THE BACTERIAL OXIDATION OF AROMATIC COMPOUNDS

II. THE PREPARATION OF ENZYMATICALLY ACTIVE DRIED CELLS AND THE INFLUENCE THEREON OF PRIOR PATTERNS OF ADAPTATION

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Our evidence concerning the pathways for the oxidation of aromatic compounds by Pseudomonas fluorescens (Stanier, 1947, 1948; Sleeper and Stanier, 1950) has been accumulated almost entirely by use of the technique of simultaneous adaptation. Although we believe that the logical basis of this method is sound, the technique is necessarily an indirect one. Consequently it appeared desirable to confirm the reactions postulated by isolation and study of the various enzymes involved. The present paper is concerned with preliminary phases of this work: the techniques for preparing dried cells and the effect of pre-established adaptive patterns on enzymatic activity in vitro.

METHODS

Cultures of P. fluorescens for making dried cell preparations were grown in media with a mineral base, containing a single aromatic compound as the energy source (for the formulae for these media, see Stanier, 1947). When small batches of dried cells were needed, the cultures were grown either on agar plates or in flasks on a shaking machine. Large batches of dried cells (approximately 10 grams) were prepared from cultures grown in a glass tank of 30-liter capacity, equipped with a mechanical agitator and a system for forced aeration. The tank could not be sterilized; however, the selective nature of the media, coupled with the use of a large inoculum (10 per cent of the volume of the medium) and the short period of incubation (7 to 8 hours), rendered contamination a negligible factor. All cultures were incubated at 30 C.

Following growth, the cells were harvested by centrifugation, washed once with phosphate buffer (m/30, pH 7.0) and a second time with distilled water. The paste of packed cells thus obtained was spread out in a thin layer in a beaker and slowly dried in vacuo at room temperature. The resulting glassy residue was ground to a fine powder and stored in air in the freezing compartment of a refrigerator. The viable count is greatly reduced by this method of drying, and the subsequent storage of the preparations in air results in the destruction of most remaining viable cells after a few days. Storage of the preparations for 4 to 5 days before use is desirable, since immediately after drying the endogenous respiration is undesirably high, and it drops during storage to a negligible value.

1 This work was done in part under a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.
The only difficulties that we have encountered with this very simple technique have arisen from too rapid drying, a particular danger with small batches; when this occurs, the preparations behave metabolically on subsequent suspension in buffer in a manner similar to fresh cell suspensions.

The enzymatic activity of the whole dried cells was determined manometrically by oxygen uptake after resuspension in phosphate buffer (M/30, pH 7.0). At first, methylene blue was added to provide an extraneous hydrogen carrier, but this precaution turned out to be needless; the oxidations catalyzed by the dried cells proceeded equally rapidly in its absence, and consequently methylene blue was omitted in most of the experiments to be reported.

### TABLE 1
Patterns of adaptation to aromatic compounds in *P. fluorescens*

<table>
<thead>
<tr>
<th>CELLS GROWN ON</th>
<th>CELLS ADAPTED TO</th>
</tr>
</thead>
<tbody>
<tr>
<td>MANDELATE</td>
<td>MANDELATE</td>
</tr>
<tr>
<td>BENZOATE</td>
<td>BENZOATE</td>
</tr>
<tr>
<td>PHENOL</td>
<td>PHENOL</td>
</tr>
<tr>
<td>P-HYDROXYBENZOATE</td>
<td>P-HYDROXYBENZOATE</td>
</tr>
</tbody>
</table>

* Adaptation to protocatechuate extremely rapid.

### TABLE 2
Patterns of enzymatic activity against aromatic compounds in dried cells of *P. fluorescens*

<table>
<thead>
<tr>
<th>CELLS GROWN ON</th>
<th>DRIED CELLS ACTIVE AGAINST</th>
</tr>
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<tbody>
<tr>
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</table>

* Very slight activity (approx. 2 per cent of that in p-hydroxybenzoate-grown cells).

## RESULTS

Influence of prior patterns of adaptation on the enzymatic activity of dried cells. Dried cells were prepared from cultures grown on four different media, in which the respective energy sources were mandelic acid, benzoic acid, p-hydroxybenzoic acid, and phenol. Each batch of dried cells was then tested for activity against the four above-mentioned compounds, as well as against protocatechuic acid and catechol. High levels of enzymatic activity against mandelic acid, protocatechuic acid, and catechol were found, but only when the substrate previously employed for growth was one known to cause complete adaptation to the compound in question. In other words, the previous establishment of adaptation to a substrate in vivo is an essential prerequisite for the appearance of enzymatic activity.
in the subsequent dried cell preparation. This cardinal point is clearly evidenced by a comparison of tables 1 and 2, in which are listed the patterns of adaptation in living cells, and of enzymatic activity of dried cells, after growth on the various substrates employed.

Special mention should be made of the behavior of mandelate-, benzoate-, and phenol-grown dried cells with respect to protocatechuic acid. As shown by Sleeper and Stanier (1950), living cells grown on these substrates, though not completely adapted to protocatechuic acid, are nevertheless capable of adapting to it with extreme rapidity. The equivalent dried cells show a very slight, but significant, oxygen uptake with protocatechuic acid, the rate of oxidation being
approximately 2 per cent of that shown by the same cells with catechol (see figure 1). Since dried cells grown on p-hydroxybenzoate, which is known to cause complete simultaneous adaptation to protocatechuic acid, oxidize protocatechuic acid at a rate comparable to the rate of catechol oxidation by mandelate-, benzoate-, or phenol-grown dried cells (figure 2), this suggests that growth on mandelate, benzoate, or phenol causes the production of about one-fiftieth of

![Graph](http://jb.asm.org/)

*Figure 2.* Oxygen uptake with 5 micromoles of Na-protocatechuate by a suspension of p-hydroxybenzoate-grown dried cells (dry weight of cells 10 mg). There was no oxygen uptake in the absence of substrate.

the potential maximum level of enzymatic activity against protocatechuic acid. This level of activity is, of course, far too low to permit of considering protocatechuic acid as an intermediate in the main dissimilatory pathways for compounds in the mandelate-benzoate reaction chain and for phenol.

None of the dried cell preparations that we have obtained show any oxygen uptake with benzoic acid, p-hydroxybenzoic acid, or phenol, even after growth on the homologous substrate (cf. table 2). We assume that in these cases the
technique of drying employed has resulted in the destruction either of the relevant enzymes or of necessary carrier systems, or both.

Extent of the oxidations catalyzed by dried cells. As might be anticipated from the preceding discussion, the oxidations brought about by dried cells proceed with a greatly diminished oxygen uptake per mole in comparison to the oxygen uptake by living cells acting on the same substrate. The oxygen uptake with catechol and protocatechuic acid is 1 mole per mole of substrate (in contrast to the figure of approximately 4.5 moles per mole with living cells), and the rate remains constant throughout the oxidation. The results with mandelic acid are more variable, and appear to be influenced both by the particular batch of dried cells employed and by their age. Total oxygen uptakes ranging from 0.5 to 1.0 mole per mole of substrate have been obtained, and the rate may be constant throughout or may show a marked inflection during the course of the oxidation. Typical data for all three oxidations are shown in figures 1 and 2. The nature and products of these oxidations will be discussed in later papers.

Extraction of enzymes from the dried cells. When dried cells are resuspended in distilled water or buffer solutions, a substantial part of the cell proteins go into solution and can be separated from the cellular debris by subsequent centrifugation at high speeds. The enzyme system responsible for the oxidation of catechol is completely extracted in this manner, and can thus be obtained very easily in a cell-free state. On the other hand, the enzyme systems responsible for the oxidation of mandelic acid and protocatechuic acid remain with the cellular debris. This makes possible a quantitative separation of the mandelate- and catechol-oxidizing enzymes in mandelate-grown dried cells, which contain both.

SUMMARY

Dried cell preparations of Pseudomonas fluorescens that are capable of oxidizing mandelic acid, catechol, and protocatechuic acid have been obtained.

Dried cells oxidize a particular substrate only when the living cells from which they were prepared were adapted to that substrate.

The oxidations catalyzed by dried cells result in a much smaller oxygen uptake per mole of substrate than the equivalent oxidations by living cells.

The enzyme system responsible for the oxidation of catechol can be extracted from the dried cells with distilled water or neutral phosphate buffer, whereas the enzymes oxidizing mandelic and protocatechuic acid cannot be so extracted.

REFERENCES


* Partial extraction of the protocatechuate-oxidizing enzyme system by distilled water has been achieved with some more recently prepared batches of dried cells.