SIMULTANEOUS ADAPTATION: A NEW TECHNIQUE FOR THE
STUDY OF METABOLIC PATHWAYS

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During work on the oxidation of aromatic substances by Pseudomonas fluores-
cens, a useful technique for the elucidation of metabolic pathways by the analysis
of adaptive behavior was discovered. Since it could undoubtedly be applied
to many other microbial dissimilations, a brief account of its principles and
applications seems merited.

METHODS

Adaptation was determined manometrically, by following the oxygen uptake
after addition of the substrate to a cell suspension in the Warburg apparatus.
All experiments were conducted at 30 °C in an atmosphere of air, using 2.0 ml
of cell suspension and 0.2 ml of 0.01 M substrate.

One strain of Pseudomonas fluorescens (str. A. 3.12) was used throughout.
The cells were grown on agar plates at 30 °C and harvested after 20 to 45 hours
by suspension in M/60 phosphate buffer (pH 7.0). After centrifugation they
were resuspended in the same buffer mixture. The mineral media employed
for cultivation of specifically adapted cells had the following composition:
specific carbon source, 0.1 to 0.25 per cent; NH₄NO₃, 0.1 per cent; K₂HPO₄,
0.1 per cent; MgSO₄, 0.05 per cent; and agar, 1.5 per cent; pH 7.0 to 7.2.

The precision and sensitivity of the manometric technique make it ideal for
studying adaptation to nonvolatile compounds, but complications arise when
such substances as benzaldehyde are tested. Even in 0.01 M solution, the vapor
pressure of benzaldehyde is sufficiently high at 30 °C to cause a marked distil-
ation from the side arm into the main compartment of the Warburg vessel,
and adaptation consequently begins before the contents of the side arm are added
to the cell suspension. Even when the period of thermal equilibration is held
to a minimum, the effect is noticeable, showing up as an apparently more
rapid adaptation to benzaldehyde than to nonvolatile substrates. Hence the
results with this substance cannot be strictly compared to those obtained with
the remaining aromatic compounds investigated.

THEORY

If we accept the well-tested Kluverian axiom (Kluver, 1931) that every
dissimilation is the result of a series of simple, chemically intelligible step-re-
actions, it follows that the complete oxidation of even a relatively small organic
molecule will involve the formation of a large number of intermediate compounds.
In the case of microorganisms, the further probability exists that at least some

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of these intermediates will be attacked by adaptive enzymes. On the general theory of enzymatic adaptivity (cf. Karström, 1937), cells adapted to attack the primary substrate should be adapted simultaneously to attack all the intermediates formed during the oxidation of that substrate, but not to attack other substances the dissimilation of which is brought about by adaptive enzymes that fail to participate in the over-all dissimilatory process in question. Thus by growing cells on the primary substrate or on assumed intermediates and then testing for adaptation to a variety of related substances, one should be able to obtain convincing evidence of whether or not assumed intermediates do actually occur, together (in positive instances) with information about their position in the reaction chain. The argument can be summarized in the following three postulates:

1. If the dissimilation of a given substance A proceeds through a series of intermediates B, C, D, E, F, G . . . , and if the individual steps in this chain of reactions are under adaptive enzymatic control, then growth on a medium that contains A will produce cells that are simultaneously adapted to A, B, C, D, E, F, G, . . .

2. If growth on A fails to adapt the cells to a postulated intermediate X, then X cannot be a member of the reaction chain.

3. Growth on E will adapt the cells for F, G, . . . but not necessarily for A, B, C, and D. The probability that growth on E will adapt the cells to precursors decreases with the number of intervening steps; i.e., adaptation to D is more probable than adaptation to A.

Postulate (3) perhaps requires a few additional words of explanation. In a complex dissimilation, it is conceivable that an enzyme will act at more than one stage in the dissimilatory process. Hence when two intermediates, say D and E, are separated by one enzymatic step, the possibility exists that the enzyme catalyzing that particular step (D→E) may also function later on in the oxidation of E, and that growth on E will also adapt the cells completely for the attack on D. However, if two intermediates, say B and E, are separated by several intervening steps (B→C→D→E), the probability that all three enzymes involved also take part in subsequent reactions is small, and thus growth on E is not likely to produce cells completely adapted for the oxidation of B.

**ANALYSIS OF A SPECIFIC BIOCHEMICAL PROBLEM BY MEANS OF SIMULTANEOUS ADAPTATION**

The application of the postulates may be illustrated with a relatively simple system, consisting of the following five compounds:

- **CH₂COOH**: phenylacetic acid
- **CHOHCOOH**: dl-mandelic acid
- **CHO**: benzaldehyde
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Each of them is readily utilized (in the case of the acids, as the sodium or potassium salt) by a strain of Pseudomonas fluorescens as the sole source of energy for aerobic growth in an otherwise mineral medium.\(^2\) Washed cell suspensions prepared from yeast extract agar are unadapted for the oxidation of these aromatic compounds: the oxygen uptake remains at the autorespiratory rate for the first 40 to 70 minutes following the addition of the substrate, and then increases exponentially to a steady maximum rate which is maintained to the point of substrate exhaustion. Cells grown in the presence of any one of the five substances show complete adaptation to that particular substance when tested in the same manner. These points are illustrated for benzoate in figure 1.

It can be seen that the system is excellently suited for analysis along the lines of the postulates enunciated above, since it consists of five closely related compounds the oxidation of which by the biological agent employed is in all cases under primary adaptive control. Inspection of the structural formulæ would suggest as a provisional hypothesis that these compounds comprise five successive members of an oxidative reaction chain:

\[\begin{align*}
\text{benzoic} & \quad \text{OH} \\
\text{acid} & \quad \text{para-hydroxybenzoic acid}
\end{align*}\]

\(^1\)Both isomers of mandelic acid are attacked at the same rate, and a racemic mixture has been used throughout the experiments herein reported.
Analysis by simultaneous adaptation has provided conclusive evidence that this is not the case, and that in reality three separate primary oxidations are involved. The evidence for this is presented in figures 2, 3, 4, and 5. Cells were grown on four mineral agar preparations containing, respectively, benzate, para-hydroxybenzoate, mandelate, and phenylacetate and then tested manometrically for adaptation to the four acids and to benzaldehyde. Only the data for the four acids are shown on the graphs.

Figure 2 demonstrates that para-hydroxybenzoate is not an intermediate in
the oxidation of benzoate, since benzoate-grown cells are unadapted for its oxidation. The immediate attack at maximum rate on para-hydroxybenzoate by cells grown in its presence (figure 3) shows that the initial lag in its oxidation by benzoate-grown cells cannot be ascribed to permeability effects.

Figure 4 shows that benzoate is oxidized at the same rate as mandelate by cells grown on the latter substrate, suggesting that benzoate is an intermediate in mandelate oxidation. As might be expected if this were the case, mandelate-grown cells are unadapted to para-hydroxybenzoate.

The results presented in figure 5 for cells grown on phenylacetate are perhaps the most interesting of all. In the first place, the typically adaptive curve for the oxidation of benzoate proves that phenylacetate cannot be oxidized along this pathway. The curve for mandelate shows a new feature: it has a double break, the initial rapid rise in oxygen uptake being followed (after a brief re-
turn to the autorespiratory rate) by an exponential rise that parallels with reasonable closeness, but at the higher absolute level initially established, the strictly adaptive curve for benzoate. The first break comes at a point that corresponds approximately to an oxygen uptake of one mole per mole of substrate. The only likely interpretation of such a curve is that growth on phenyl-

\[
\text{CHOHCOOH} + \text{H}_2\text{O} \rightarrow \text{COOH} + \text{CO}_2 + 4\text{H}
\]

acetate has activated the dehydrogenases involved in the initial oxidation of mandelate to benzoate—
The peculiar action of phenylacetate-grown cells on mandelate made possible a further experiment in substantiation of the hypothesis that benzoate really is an intermediate in the oxidation of mandelate. Adaptation to either benzoate or phenylacetate singly fails to bring about complete adaptation to mandelate (figures 2 and 5), but if the deductions drawn from the experiments above are correct, cells adapted to both of these substances should also be adapted, by a process of complementary activation, to mandelate. As shown in figure 6, this expectation is realized.

The data with benzaldehyde indicate that this substance is probably an intermediate in the oxidation of mandelate to benzoate, although for the reasons mentioned earlier the results are not so clear-cut as those with the aromatic acids. Mandelate-grown cells are completely adapted to benzaldehyde, and phenylacetate-grown cells show "semiadaptation" of the same sort as that discussed above for mandelate, with the difference that the first break in the curve for
benzaldehyde oxidation comes at a level of about 0.5 moles of oxygen per mole of substrate, in accordance with the equation:

\[
\text{CHO} + \text{H}_2\text{O} \rightarrow \text{COOH} + 2\text{H}
\]

Interestingly enough, benzoate-grown cells show complete adaptation for benzaldehyde, suggesting that the benzaldehyde dehydrogenase also functions in the later stages of benzoate oxidation. The adaptation of benzoate-grown cells to benzaldehyde but not to mandelate is a good illustration of the third postulate.

The net result of these experiments has been to show the existence in *P. fluorescens* of three separate oxidative mechanisms involving aromatic substances:
This information could not have been obtained from data on utilization, or even from data on absolute rates of oxidation, which are quite similar for all five compounds.

The fact that growth on phenylacetate activates the dehydrogenases involved in the oxidation of mandelate and benzoic acid to benzoate probably indicates that these two enzymes are nonspecific, and also function at some stage in the oxidation of phenylacetate. Lack of enzymatic specificity is, of course, a limitation to the validity of the technique, and necessitates judicious evaluation of positive findings. It seems most improbable, however, that exactly the same set of enzymes would be involved in two different complex oxidative processes, so that even if growth on one substance activates nonspecifically the first step or steps in the oxidation of another substance, lack of adaptation at some later point in the chain of events will temporarily halt the attack, resulting in a "semiadapted" curve for oxygen uptake. Indeed, the occurrence of such behavior should in itself provide valuable information as to the course of the reaction. It is difficult to see how clear lack of adaptation to a postulated intermediate can be regarded as anything but conclusive negative evidence, provided that permeability effects have been ruled out by a demonstration that cells adapted to the substance in question can oxidize it immediately at the maximum rate.

One further point, at present highly speculative, deserves brief mention. It does not seem excluded that relative rates of adaptation may also provide indications of biochemical interrelationships. A case in point is the relatively rapid adaptation of cells grown either on benzoate or on phenylacetate to para-hydroxybenzoate (figures 2 and 5). A possible interpretation of this behavior is that all three substances have a common intermediate, from which para-hydroxybenzoate is separated by fewer steps than either of the other two, with the consequence that cells grown on benzoate or phenylacetate need to produce fewer adaptive enzymes for the attack on para-hydroxybenzoate than for the attack on one another.
The systematic use of simultaneous adaptation, coupled with the other kinds of data obtainable from manometric experiments, should be particularly valuable in the study of those dissimilatory processes that have so far proved least amenable to analysis—namely, rapid and complete oxidations of relatively complex substances. The only prerequisite is that the enzymatic repertoire of the biological agent employed should be largely adaptive.

SUMMARY

The theory of simultaneous adaptation as a method for the analysis of metabolic pathways is described, and its application is illustrated by a specific example: the oxidation of five aromatic compounds by a strain of *Pseudomonas fluorescens*.

REFERENCES
