# PROBLEMS OF BACTERIAL OXIDATIVE METABOLISM[1]

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At the present time, we possess a satisfactory conceptual scheme for the most important fermentative processes, which takes into account the stepwise transformations undergone by the substrate molecules, the mechanism of hydrogen transfer, and the manner in which part of the energy released by fermentation is made available for the driving of other cellular reactions. Of course, not every biochemical detail of every fermentation is understood, but at least the main outlines have been established with as much finality as is ever possible in science, and it is the peripheral problems which still await solution. If we now turn our attention to microbial oxidative metabolism, we find a very different state of affairs. Here we stand today in a position roughly comparable to that occupied forty years ago by Harden and Neuberg vis-à-vis alcoholic fermentation; that is to say, we possess a number of interesting and suggestive facts in isolation, but the connecting links needed to fit these facts together into a coherent conceptual scheme are lacking. Hence an over-all picture of bacterial oxidative metabolism, if one is hardy enough to attempt its construction, strongly resembles a seventeenth century geographer's map of North America. The shape of the continent is roughly outlined, some small local areas already penetrated by the pioneers are correctly shown, but vast regions are either simply left blank or filled in as fancy dictates.

Before we can elaborate a satisfactory conceptual scheme for oxidative metabolism, we must have information about three closely interrelated aspects of the oxidative process, which are shown in seventeenth century fashion in figure 1. The first of these is the nature of the stepwise degradations undergone by the carbon skeletons of the various classes of substrate molecules during their transformation into the two main end-products of normal oxidative metabolism, carbon dioxide and synthesized cell material, which, for want of more detailed knowledge, is conventionally designated as ($CH_2O$). The study of this aspect also confronts us with the important question, still highly controversial, of whether bacteria possess one or possibly more terminal respiratory cycles similar in function to the tricarboxylic acid cycle of higher animals. The fact that synthesized cell material, "($CH_2O$)", is a major end-product of so-called complete microbial oxidations must eventually lead us also to the biochemical investigation of the connection between respiration and synthesis. The second aspect that requires elucidation is the nature of the carrier systems responsible for hydrogen transport in the functioning intact cell, and of the enzymes responsible for re-

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duction of the final hydrogen acceptor. We already have a good idea of the nature of these terminal enzymes in systems for which molecular oxygen is the final hydrogen acceptor, but virtually nothing is yet known about terminal enzymes in bacterial oxidations that function anaerobically with nitrates, sulfates or carbonates as final hydrogen acceptors. Thirdly, we need to know the reactions in substrate degradation and hydrogen transfer through which inorganic phosphate is mobilized and energy-rich phosphate generated. It goes without saying that this program in its entirety is a formidable one, and the work of our group discussed below has been centered so far on certain phases of the first-mentioned aspect—namely, the fate of the substrate carbon skeleton.

This particular aspect of bacterial oxidative metabolism has been studied most intensively in the past by analysis of the "incomplete" oxidations, or *blocked oxidations*, as they might be more logically designated, which are catalyzed by such organisms as the acetic acid bacteria. The blocked oxidations, which are

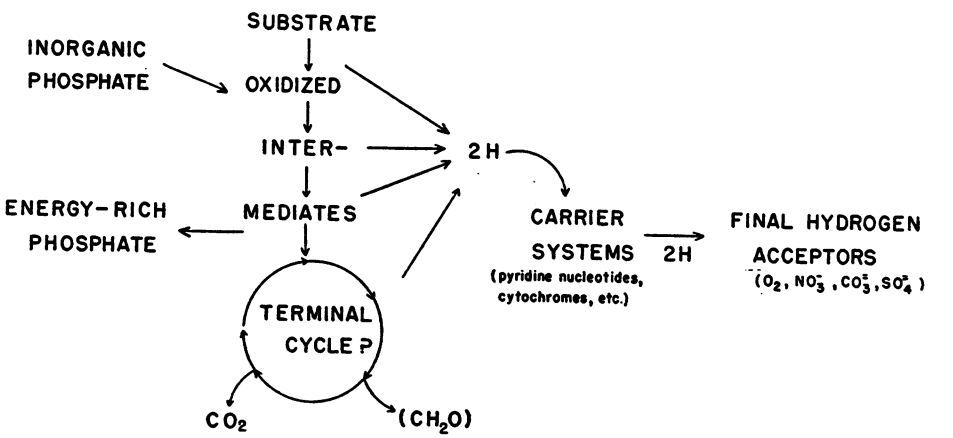



FIG. 1. A highly schematic diagram of oxidative metabolism.

relatively rare if we consider the bacteria as a whole, are brought about by organisms that are deficient, for either genetic or environmental reasons, in enzymes required for the later steps of substrate breakdown; these oxidations involve, accordingly, a superficial dehydrogenation of the substrate molecule with consequent massive accumulation, either transient or permanent, of slightly oxidized organic products possessing the same carbon skeleton as the substrate molecule. The popularity of the blocked oxidations as objects for research is readily intelligible. Quite apart from their not infrequent technical importance, they are easy to study, since one can readily isolate and characterize the accumulating organic products, an operation that gives the investigator a solid sense of accomplishment largely lacking when he attempts to study "complete" oxidations whose sole detectable end-products are $CO_2$ and unidentified cell material. It should of course be remembered that the blocked oxidations of the acetic acid bacteria, by virtue of their very chemical simplicity, provided the experi-

mental material with which Wieland succeeded in establishing the basic concept of biological oxidations as dehydrogenations (20). However, I have long felt that their current value as material from which to construct our needed conceptual scheme is limited, since we cannot learn from them the nature of the key reactions that give rise to the two normal major end-products of oxidative metabolism, $CO_2$ and $(CH_2O)$.

We have, accordingly, directed our attention to the complete oxidations. As biological material we have used exclusively members of the genus *Pseudomonas*, and for the most part strains of one large and well-known species group, *Pseudomonas fluorescens*.[2] This choice was suggested by several features of the organisms in question which make them well-nigh ideal objects for such work. Firstly, they are obligately oxidative, which eliminates any possible complications which might arise from the simultaneous presence in the cells of a fermentative apparatus, as is the case, for example, in *Escherichia coli* and *Bacillus subtilis*. Secondly, they can oxidize a wider range of simple organic compounds than any other known bacteria, a fact first established through the classical studies of den Dooren de Jong (1). This permits one to study many different oxidative pathways without a shift in biological material. Thirdly, the varied oxidations catalyzed by *P. fluorescens* are nearly all complete ones, aldoses (6, 7, 17) and ethanol (12) being the only substrates known to undergo substantially incomplete oxidation. Lastly, *P. fluorescens* is devoid of accessory growth factor requirements, developing rapidly and abundantly in simple media with a mineral base and any one of the many potentially oxidizable organic substrates.

The compounds whose oxidation we have so far principally studied are simple substituted aromatic substances, such as benzoic acid, phenol and phenylacetic acid. At the time when our work was begun very little attention had been paid to the oxidation of ring compounds; this made it possible to approach the problem with an absence of preconceived notions, or perhaps one should say a naïveté, which would have been difficult to achieve if we had ventured onto one of the better-trodden biochemical paths, such as the oxidation of carbohydrates or fatty acids. Preliminary experiments showed that, provided the initial substrate concentration was kept low to avoid toxic effects, many aromatic substrates were rapidly oxidized by *P. fluorescens* to $CO_2$, with substantial accompanying oxidative assimilation. In addition, it was ascertained that the attack on these substrates was strictly adaptive, cells grown on a yeast extract or asparagin medium being incapable of immediate attack on any aromatic substrate (13, 14). In order to proceed beyond this point in the analysis of the transformations in question we urgently needed some sort of signpost, and at first sight the problem seemed as bare of signposts as the terrain of a novel by Franz Kafka. However, the strictly adaptive nature of attack on aromatic substrates suggested to us

[2] This name is used here to designate any strain conforming to the morphological definition of the genus and further characterized by its ability to produce a water-soluble, yellow-green fluorescent pigment without accessory phenazine pigments. It is possible that specific sub-divisions of the *P. fluorescens* species-group should be made, but these can only be based on a detailed comparative systematic study, which nobody has yet undertaken.

the possibility of a new sort of experimental approach. Preliminary experiments having proved encouraging, this new approach, the technique of simultaneous adaptation, was developed in a systematic way. The experimental basis of simultaneous adaptation is the analysis of adaptive patterns towards a variety of related compounds in cells that have had their enzymatic repertoire pre-conditioned by growth at the expense of a single, specific, adaptively-attacked compound. The ideas underlying simultaneous adaptation had already been touched on by others, notably Marjory Stephenson (18) and Spiegelman (10), and in view of the simplicity of these concepts it is hard to understand why the technique was not introduced into metabolic studies at least ten years ago. At all events, it should not be a matter for surprise that Karlsson (3), Suda, Hayaishi and Oda (19) and the writer (13) developed it independently during the course of work on three entirely different bacterial oxidations.

The complete oxidation of even a relatively simple organic molecule must necessarily involve the formation of many intermediate substances, each successive step being under specific enzymatic control. In order to grasp the idea of simultaneous adaptation it is only necessary to combine this concept with another one: namely, that in the case of adaptive oxidations, not only the initial enzyme $E_A$ acting on the primary substrate A but also the enzymes $E_B$, $E_C$, $E_D$, etc., acting upon the series of intermediates B, C, D, etc., produced successively from A, may well be adaptive. In such an event, the provision to the cells of an adaptively-attacked primary substrate A will act as a trigger mechanism not merely for the synthesis of the single enzyme $E_A$, but for the synthesis of a whole series of enzymes acting in succession on the intermediates formed from A. In other words, by providing a new adaptively attacked substrate we redirect large segments of the enzymatic constitution of the cell into new patterns. From this it follows that if a particular adaptively-attacked compound C is an intermediate in the breakdown of A, cells fully adapted to oxidize A will *also* be fully adapted to oxidize C. Consider, however, the case of a compound X, on purely chemical grounds a feasible intermediate in the oxidation of A, and likewise potentially attackable by an adaptive enzyme $E_x$. If X is not *actually produced* during the breakdown of A, the necessary stimulation for the synthesis of $E_x$ by the cell will not have been provided through exposure to A, and hence cells fully adapted to oxidized A will not be adapted to oxidize X. Thus, by a careful analysis of the adaptive patterns of cells adapted to any given primary substrate A, we should be able to find evidence either supporting or opposing the actual intermediate role of a variety of chemically-feasible postulated intermediate compounds. The technique of simultaneous adaptation is essentially an extension and refinement of the customary kinetic analysis of metabolic problems. Figure 2 illustrates the technique by means of a specific example (8). In this particular case, we were concerned with the problem of intermediates in the metabolic pathways leading from benzoic acid and *p*-hydroxybenzoic acid, which we already knew to be distinct. The work of Evans (2) had suggested that catechol and protocatechuic acid were somehow involved in these oxidations, but

the exact relationship between the four compounds was unclear. From figure 2 it can be seen that the oxidation of both catechol and protocatechuic acid by asparagin-grown cells is adaptive; and further, that benzoate-grown cells are simultaneously adapted to catechol, but not to protocatechuic acid, whereas *p*-hydroxybenzoate-grown cells behave in a complementary manner, being simultaneously adapted to protocatechuic acid, but not to catechol. From this very simple experiment, one may draw the tentative conclusion that catechol is an intermediate in the oxidation of benzoic acid and that protocatechuic acid is an intermediate in the oxidation of *p*-hydroxybenzoic acid, the two reaction chains being distinct, at least to the stage of these intermediates.

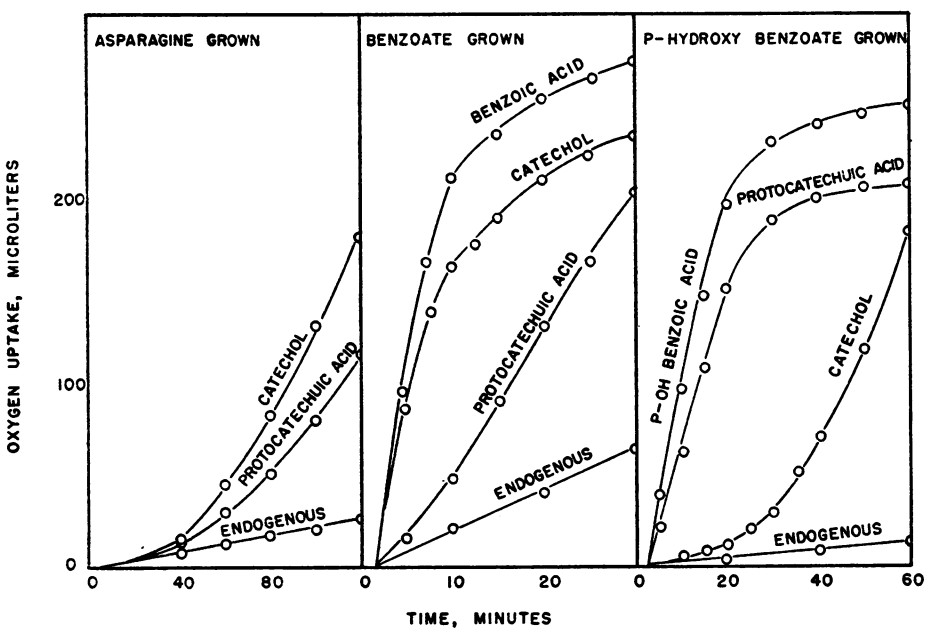



FIG. 2. The effect of prior conditions of cultivation on the adaptive patterns of *Pseudomonas fluorescens* with respect to catechol and protocatechuic acid. Amounts of substrates used: 2 micromoles.

How much dependence should be placed on the results of such experiments *alone?* Underlying the principle of simultaneous adaptation are two assumptions whose validity is open to question. The first is the assumption of a high degree of specificity in the adaptive response, only the normal substrate for a given enzyme being assumed to be capable of evoking maximal synthesis of that enzyme by the cell. Insofar as the adaptive responses of *P. fluorescens* to aromatic substrates are concerned, we have encountered only one example of a complete, nonspecific adaptive response. After exposure to phenylacetic acid, cells possess in maximal concentration the enzymes required for the first three steps in the degradation of mandelic acid, although evidence suggests that the latter com-

pound is not involved in the degradation of the former (13). The mandelate-oxidizing enzymes activated by exposure to phenylacetate carry the oxidation of mandelate only to the benzoate stage, and thereafter the usual adaptive lag ensues while the cells synthesize the enzymes necessary for the degradation of benzoate. In such a case, false positive inferences from the study of adaptive patterns are easily avoided. However, while our own experience to date suggests that nonspecificity of adaptive response is not a major drawback to the technique of simultaneous adaptation, one should be wary of taking specificity of adaptive response too much for granted.

The technique of simultaneous adaptation rests secondly on the assumption that the cell is permeable to all substrates tested. Even if we disregard the hypothesis, now scarcely tenable any longer, that adaptation *an sich* consists of a selective change in the permeability of the cell membrane that permits access of the substrate to pre-existing active enzymes within the cell, the objection may always be raised that certain intermediates are incapable of penetrating the cell *under any circumstances* when externally supplied. If this were so, false negative inferences could easily be drawn from experiments on adaptive patterns. The same objections can be raised whenever one attempts to work with intact cells, and are not uniquely pertinent to the study of adaptive patterns. Since problems of permeability are often lightly dismissed by students of bacterial metabolism, it may be worth while to mention one instance that has occurred in our own work. The compound β-ketoadipic acid was first implicated as a possible intermediate in the oxidations of aromatic substrates when Kilby (4) identified it as an excretion product in cultures of a *Vibrio* sp. growing at the expense of phenol. Subsequently, we showed that specific enzymes capable of oxidizing either catechol or protocatechuic acid to β-ketoadipic acid can be isolated from dried cells adapted to aromatic precursors, but not from unadapted dried cells (16). There seemed, accordingly, good reason to postulate this aliphatic acid as a key intermediate in the oxidation of many aromatic substrates. However, when the oxidation of β-ketoadipic acid by living cells was investigated, it was found that the rate of oxygen uptake was always much less than that with the assumed aromatic precursors. Despite the low rate of its oxidation, β-ketoadipic acid fulfills the postulates of simultaneous adaptation insofar as its assumed intermediate role is concerned; it is attacked adaptively by asparagin-grown cells, but is always oxidized at a steady maximum rate from the moment of addition by cells grown on any of the assumed aromatic precursors (fig. 3). The low rate of oxidation of β-ketoadipic acid by *P. fluorescens* is thus most reasonably explained as a consequence of the slow penetration of this highly polar compound through the cell membrane.

The preceding remarks imply that the use of simultaneous adaptation alone cannot provide unequivocal evidence for the existence of a metabolic pathway, but should be bolstered wherever possible by other lines of evidence—chemical detection of intermediates, studies on isolated enzyme systems *in vitro*, tracer experiments, etc.—before we can consider a postulated metabolic sequence to be

a reasonably accurate expression of the course of biochemical events. As Kluyver so rightly pointed out 20 years ago (5), no single approach, or even a combination of approaches, to the analysis of the activities of the living cell can ever justify the belief that our conceptual schemes bear a one-to-one correspondence to the real course of events. I do not wish this discussion of the possible shortcomings of simultaneous adaptation to be construed as implying that the technique is without value. Quite to the contrary, it provides us with an extremely simple device, perhaps the best one available, for roughly mapping out a previously unexplored metabolic pathway. I believe that it will also assume a

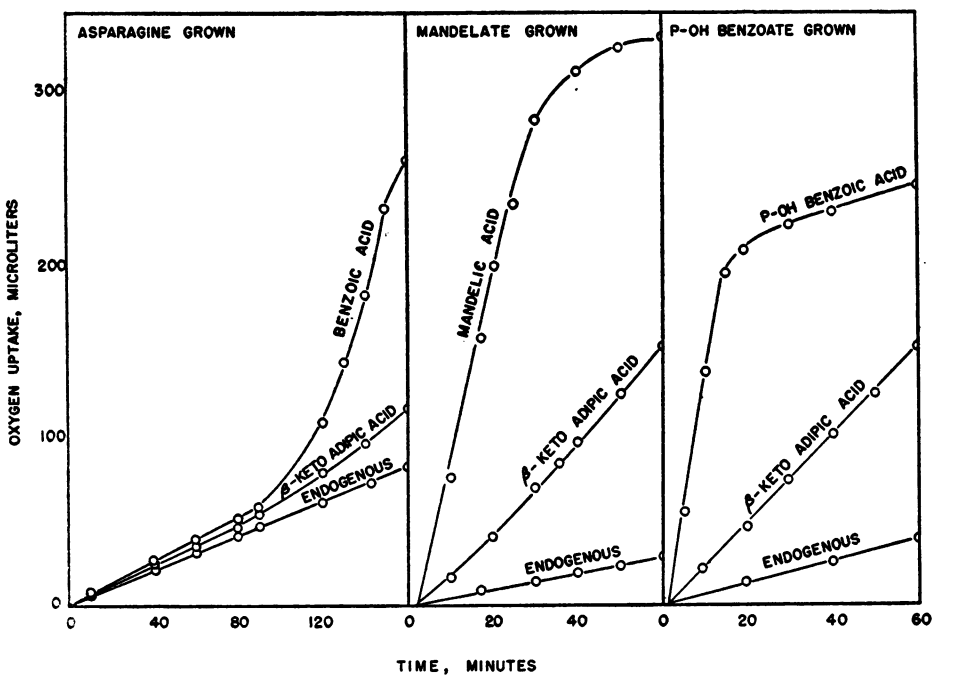



FIG. 3. The effect of prior conditions of cultivation on the oxidation of β-ketoadipic acid by *Pseudomonas fluorescens*. Amounts of substrates employed: 2 micromoles.

valuable place in the later stages of study of an adaptively-controlled metabolic process, when we return once more to the intact cell after analyzing the behavior of isolated enzyme systems *in vitro*. For every intermediate proposed on the basis of enzymatic studies, the postulates of simultaneous adaptation should be fulfilled, except where it can be shown that a permeability barrier exists.

Figure 4, which presents the latest version of our conceptual scheme for the oxidation of aromatic compounds by *P. fluorescens*, shows what has been gleaned by applying the concept of simultaneous adaptation to a single specific problem. All the primary substrates and intermediates shown are adaptively attacked, and the indicated interrelationships could have been postulated from the analysis of

adaptive patterns alone. In addition, we now possess a good many supporting facts obtained by other methods; since these data provide the best evidence for the validity of simultaneous adaptation, a brief survey of them seems appropriate.

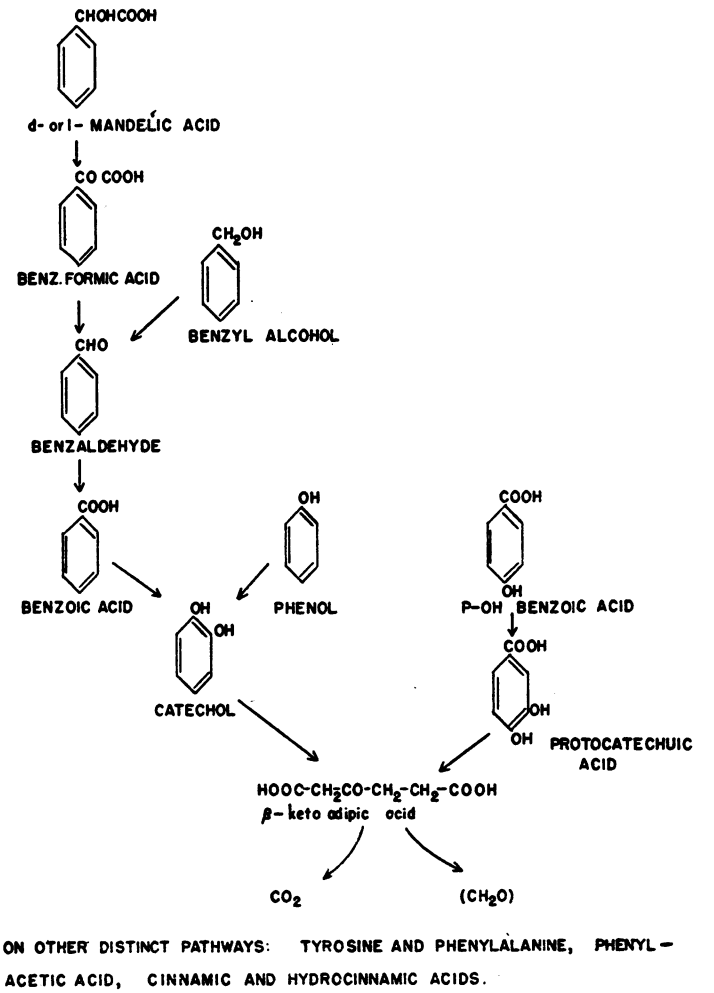



FIG. 4. Present conceptual scheme for the oxidation of aromatic substrates by *Pseudomonas fluorescens*.

Ideally, it could be demanded that a scheme such as that shown in figure 4 be bolstered by the isolation of each enzyme concerned, followed by demonstration *in vitro* of each individual step-reaction. So far, we have not been able to do this, but several large segments of the proposed reaction scheme have been shown to occur with dried cell preparations or cell-free enzyme systems. This phase of our work was inaugurated by Sleeper (9) who first showed that high levels of en-

zymatic activity against certain aromatic substrates were maintained in the cells of *P. fluorescens* following simple vacuum drying. The studies with dried cells were guided by our previous observations on the adaptive patterns of living cells. Since adaptation cannot at present be achieved except with intact living cells, it seemed reasonable to assume that dried cells, if enzymatically active, would show activity only against those substrates to which the living cells had been adapted at the time of drying. Accordingly, a systematic study of the activity of cells dried after the establishment of several different adaptive patterns was undertaken. As shown in table 1, there is a most elegant correlation between the patterns of adaptation in living cells after growth on specific aromatic substrates and the distribution of enzymatic activity in such cells after drying. The

TABLE 1

*Correlations between patterns of adaptation to aromatic compounds by P. fluorescens in vivo and enzymatic activity in vitro*

| CELLS GROWN ON | LIVING CELLS ADAPTED TO | | | | | |
|---|---|---|---|---|---|---|
| | Mandelate | Benzoate | Phenol | *p*-Hydroxybenzoate | Catechol | Protocatechuate |
| Madelate | + | + | − | − | + | −[1] |
| Benzoate | − | + | − | − | + | −[1] |
| Phenol | − | − | + | − | + | 1[1] |
| *p*-Hydroxybenzoate | − | − | − | + | − | + |
| | DRIED CELLS ACTIVE AGAINST | | | | | |
| Mandelate | + | − | − | − | + | −[2] |
| Benzoate | − | − | − | − | + | −[2] |
| Phenol | − | − | − | − | + | −[2] |
| *p*-Hydroxybenzoate | − | − | − | − | − | + |

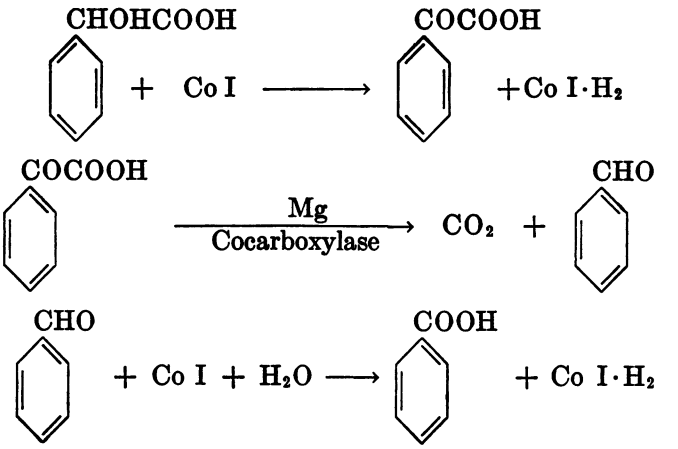
[1] Adaptation to protocatechuate extremely rapid.

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

discovery of such a correlation is by no means original, having been made earlier for other specific adaptive enzymes (e.g., 11); however, I believe our work provides the most comprehensive single illustration of the phenomenon. It is a point that must always be kept in mind by anyone who wishes to work with bacterial enzyme systems.

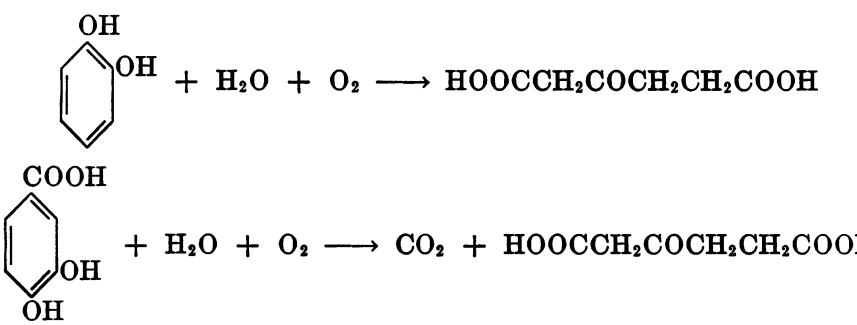
It can be seen from table 1 that certain substrates to which living cells are fully adapted are not oxidized at all by the dried cells. We assume that this is a result of the inactivation of the more labile enzymes of the cell, brought about by the method of drying employed. Whatever the cause, it has a very useful consequence, since those reactions that are still catalyzed by the dried cells are blocked at an early stage, and through their study the nature of a number of intermediates can easily be determined. The oxidation of mandelic acid by dried cells, which has been studied in some detail by Gunsalus (personal communica-

tion), is blocked at the stage of benzoic acid formation. By dialysis and selective addition of cofactors to the dried cells, Gunsalus was able to prove that it consists of the following three steps:




These intermediates are precisely the ones postulated earlier (14) from the analysis of adaptive patterns in living cells.

The two other reactions brought about by dried cells have been studied by our group. These are the oxidation of catechol, which is catalyzed by dried cells grown on mandelate, benzoate or phenol, and the oxidation of protocatechuic acid, which is catalyzed by dried cells grown on *p*-hydroxybenzoate. Both these oxidations give rise quantitatively to an aliphatic product, β-ketoadipic acid, as shown in the following equations:



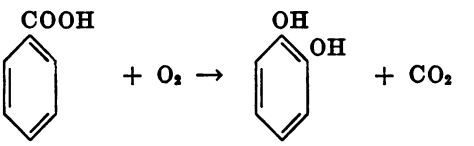

The specific enzymes involved can readily be obtained cell-free. From the overall equations for these oxidations, it is evident that they are complex, probably each involving the action of several enzymes. So far we have not succeeded in showing any of the component steps of catechol oxidation. In the case of protocatechuic acid oxidation, dialysis of the cell-free enzyme system causes inactivation of the decarboxylative step, with accumulation of a 7-carbon intermediate between protocatechuic acid and β-ketoadipic acid. We have good evidence that this compound is an aliphatic, tricarboxylic acid (15), but as yet its constitution is not determined.

There are certain postulated steps which we have been unable so far to demonstrate *in vitro*, and I would like to conclude by presenting some data on one of the most interesting and puzzling of these; namely, the oxidation of benzoic acid to catechol. The probable occurrence of catechol as an intermediate in benzoate oxidation was first established by Evans (2). Cultures of a *Vibrio* sp. growing on benzoate were shown to accumulate small amounts of catechol at certain stages of development. Subsequently, Evans (personal communication) also established the production of catechol by our strains of *P. fluorescens* when growing

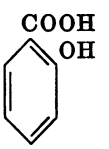
TABLE 2

*Compounds tested as possible intermediates in the oxidation of benzoic acid to catechol*

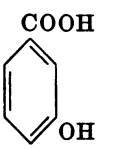
*Over-all reaction:*



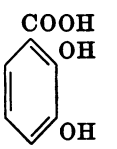

*Compounds excluded by virtue of their unoxidizability:*



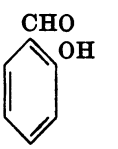

Salicylic acid




*m*-OH benzoic acid

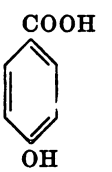



2,3-di-OH benzoic acid



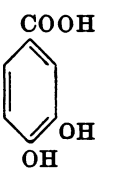

Salicyl-aldehyde

*Potentially oxidizable compounds excluded by the analysis of adaptive patterns:*



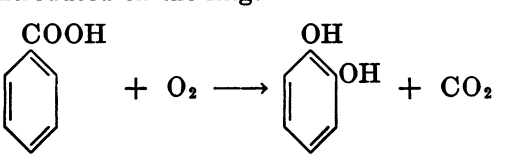

*p*-OH benzoic acid


Phenol




Protocatechuic acid

on benzoate. Our analyses of adaptive patterns (8) are entirely in accord with this finding, since cells grown on benzoate or its precursors are always completely adapted to catechol. These observations accordingly suggest that during the degradation of benzoic acid the carboxyl group is eliminated as $CO_2$ and two hydroxy groups are introduced on the ring:



Since it seemed highly improbable that this would be a single-step reaction, we set about testing possible intermediates by simultaneous adaptation. We have now tested all the likely aromatic intermediates between benzoic acid and catechol (table 2), with completely negative results; either they are unattackable, or they are attacked only after an adaptive lag. Similar negative results have been obtained by Evans *et al.*, using an entirely different technique—paper chromatographic separation and analysis of the intermediate compounds accumulating in culture media (personal communication). Since no method of preparing dried cells which we have yet tried yields preparations capable of oxidizing benzoic acid, we have been forced to study the problem further with living cells by tracer techniques. Preliminary experiments with carboxyl-labelled benzoic acid conducted by Sleeper have shown conclusively the occurrence of the suggested over-all reaction. When carboxyl-labelled benzoic acid is oxidized to completion, all the activity appears as $CO_2$. Furthermore, when such labelled benzoic acid is fed to cells under conditions (low temperature, poor aeration) which permit the accumulation of catechol, the catechol formed is inactive. We have here, then, a reaction in which a carboxyl substituent is apparently split off a benzene ring without the occurrence of ring opening—a novel type of biochemical decarboxylation. In view of the negative outcome of our experiments with possible aromatic intermediates, I am inclined to believe that this decarboxylation is accompanied by a dearomatization of the ring, and that the aromatic character of catechol is secondarily re-established. This hypothesis must, however, await the test of further experiment. The nature of the benzoic acid-catechol transformation is merely one example of the many fascinating problems which still face us in our attempt to contribute to the construction of the hoped-for conceptual scheme of bacterial oxidative metabolism.

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