THE MECHANISM OF CATECHOL OXIDATION BY MYCOBACTERIUM BUTYRICUM¹

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Bacteria belonging to the *Vibrio* and *Pseudomonas* groups oxidize benzoic acid via catechol, *cis-cis*-muconic acid, and β -ketoadipic acid. Evidence for this sequence has been accumulated by several workers (Evans, 1947; Sleeper and Stanier, 1950; Sleeper, 1951; Evans and Smith, 1951) using a variety of techniques: detection of metabolites in culture filtrates, analysis of induced enzymatic patterns, investigations of reactions catalyzed by cell-free extracts, and tracer studies. Gale (1952a, b) recently examined the oxidation of benzoic acid by several *Mycobacterium* species and concluded that although catechol is an intermediate, β -ketoadipic acid is not. Several different types of evidence were adduced by him to support the nonparticipation of β -ketoadipic acid; we will not analyze them in detail, since for the purpose of our argument it suffices to state that they were all dependent on the metabolic behavior of intact cells in the presence of externally supplied β -ketoadipate.

With pseudomonads, it has been found that intact cells oxidize β -ketoadipate at much lower rates than they oxidize the aromatic precursors (Stanier, 1950); in fact, one strain of *Pseudomonas fluorescens*, shown by enzymatic studies to degrade tryptophan via β -ketoadipic acid (Hayaishi and Stanier, 1951), is wholly incapable of oxidizing externally furnished β -ketoadipate *in vivo* (unpublished data). Such findings may be ascribed reasonably to the partial or complete failure of this highly polar substance to penetrate the cell membrane. If a similar situation were to exist in mycobacteria, it is clear that none of the experiments with intact cells reported by Gale could be expected to furnish valid evidence concerning the role of β -ketoadipic acid in the degradation of benzoic acid and catechol. For this reason, it seemed to us worthwhile to

reexamine the question by enzymatic methods, since cell-free extracts of benzoate adapted mycobacteria should be capable of converting catechol to β -ketoadipic acid, provided that the pathway for benzoate degradation is the one previously established for vibrios and pseudomonads.

The results of the experiments reported below with Gale's strain of *Mycobacterium butyricum* provide good grounds for believing that the dissimilation of catechol by this organism proceeds *exclusively* via β -ketoadipic acid. Hence, the oxidation of aromatic compounds by mycobacteria in all probability involves the biochemical mechanisms previously established with representatives of the *Vibrio* and *Pseudomonas* groups.

MATERIALS AND METHODS

The strain of *M. butyricum* was kindly supplied by Dr. G. Gale. Benzoate adapted cells were grown in a medium consisting of yeast extract (Difco), 0.1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; NH_4NO_3 , 0.1 g; Na benzoate, 0.1 g; 0.02 molar phosphate buffer (pH 6.8), 100 ml. Cultures were incubated aerobically with mechanical agitation for 2 to 3 days at 30 C. Several further additions of sterile Na benzoate were made during the course of growth. By this means the cell crop was increased without exposing the culture to a high concentration of Na benzoate, which is somewhat toxic. Cells were harvested by centrifugation, washed once with phosphate buffer (0.033 molar, pH 6.8) or saline, and disintegrated either by grinding with alumina (McIlwain, 1948) or by treatment of a 10 per cent (wet weight per volume) suspension for twenty minutes in a Raytheon 9 kc sonic oscillator.

The analytical methods used were those described by Stanier *et al.* (1950), with the exception of the method for the manometric determination of β -ketoadipic acid. The customary catalyst for the decarboxylation of β -keto acids (aniline) was replaced by 4-aminoantipyrine,

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which is far more effective (Akamatsu, 1950). The reaction mixture to be analyzed was first acidified to pH 4.2 with acetate buffer, and the decarboxylation was then initiated by the addition of 0.4 ml of 0.1 M 4-aminoantipyrine.

RESULTS

Table 1 presents a summary of manometric data on the oxidation of catechol by a cell-free extract from *M. butyricum*. The results are in complete accord with the requirements of the equation:

The product of the oxidation was isolated from an enzymatic reaction mixture which had been

TABLE 1

Manometric data on the oxidation of catechol by a cell-free extract prepared from benzoate grown cells of Mycobacterium butyricum

	OBSERVED VALUES			THEORETICAL VALUES FOR OXIDATION TO β -KETOADIPIC ACID
	2.0	4.0	6.0	
Catechol furnished, μ moles	2.0	4.0	6.0	
O ₂ uptake, moles per mole of catechol	1.10	1.05	1.05	1.00
CO ₂ evolution, moles per mole of catechol	—	—	0.00	0.00
β -Ketoacid formation, moles per mole of catechol	0.94	0.96	0.96	1.00
Acid formation, equivalents per mole of catechol	1.90	1.92	1.89	2.00

furnished with 220 mg of catechol. Using the procedure described by Stanier *et al.* (1950), 115 mg of a slightly yellowish crystalline product were obtained. This material melted at 120 to 121.5 C (dec) and when mixed with synthetic β -ketoadipic acid at 119 to 121 C (dec). The isolated material and synthetic β -ketoadipic acid were decarboxylated by 4-aminoantipyrine at identical rates.

These experiments clearly show that extracts from *M. butyricum* contain the enzyme system which catalyzes the conversion of catechol to β -ketoadipic acid; however, they do not preclude the possibility that catechol might be dissimilated in another manner by whole cells. This question

was examined by comparing the rates of utilization of catechol by a suspension of intact cells, and by an extract prepared from it. A suspension of cells was prepared in 0.02 M NaCl at a concentration of 10 g (wet weight) per 100 ml. Part of this suspension was disintegrated by sonic oscillation. The rates of oxygen uptake by aliquots (diluted fivefold) of the cellular suspension and the extract then were determined in the presence of excess catechol. The amount of oxygen consumed per mole of substrate decomposed was determined also for each preparation. From these figures, it was possible to calculate relative rates of catechol disappearance (table 2). It is clear that within the limits of experimental error the rates are identical, a fact which strongly suggests

TABLE 2

A comparison of the rates of catechol decomposition by intact cells of Mycobacterium butyricum and by a sonic extract prepared therefrom

	INTACT CELLS	SONIC EXTRACT
1. μ L of O ₂ consumed per μ mole of catechol oxidized	38	22.4
2. O ₂ uptake with excess catechol, μ L/hr/ml of suspension	496, 486	278, 279
3. Rate of catechol disappearance, μ moles/hr/ml of suspension (i.e., (2)/(1))	13, 12.8	12.4, 12.4

that oxidation via β -ketoadipic acid is the only mechanism for the dissimilation of catechol by *M. butyricum*.

SUMMARY

Cell-free extracts prepared from *Mycobacterium butyricum* oxidize catechol to β -ketoadipic acid. Comparison of the rates of catechol disappearance in the presence of intact cells and of extracts indicates that this is the only mechanism for catechol oxidation by *M. butyricum*.

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