THE OXIDATION OF BENZOIC ACID BY MYCOBACTERIA

I. Metabolic Pathways in Mycobacterium tuberculosis, Mycobacterium butyricum, and Mycobacterium phlei

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The BCG strain of the tubercle bacillus forms an adaptive enzyme for the oxidation of benzoic acid, but *Mycobacterium phlei* does not do so (Fitzgerald and Bernheim, 1947). According to Stanier (1947) enzymes for the intermediates in the bacterial metabolism of benzoic acid should also be formed adaptively, and Eadie, Bernheim, and Fitzgerald (1948) have studied the kinetics of this process. Thus, simultaneous adaptation can be used as a means of testing for such intermediates. The criterion used is primarily the reduction in the latent period when the compound is added to cells pre-adapted to benzoic acid. Negative results are, however, very difficult to evaluate since permeability effects may delay or prevent the access of the added compound to the enzyme. Intermediates in the oxidation of benzoic acid by mycobacteria thus far have not been identified. The purpose of this work was to determine some of the more stable intermediates formed in the oxidation of benzoic acid by various species of mycobacteria.

MATERIALS AND METHODS

The organisms employed were *Mycobacterium butyricum* and *Mycobacterium tuberculosis*, strain BCG, obtained from the Department of Bacteriology at Duke Hospital, and *M. phlei*, strain 10142, obtained from the American Type Culture Collection. The BCG was grown for 4 days in 250 ml Erlenmeyer flasks at 37 C in a liquid medium containing 2.0 g $(NH_4)_2SO_4$, 2.0 g K_2HPO_4, 4.0 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 10.0 g glucose, and 5.0 g proteose peptone (Difco) per 1,000 ml distilled water. *M. butyricum* and *M. phlei* were grown for 3 days on Long's synthetic medium under the same conditions. The cells were centrifuged first at 2,000 rpm for 15 minutes in 20 by 100 mm test tubes, resuspended in Hopkins tubes in distilled water, centrifuged for 10 minutes, resuspended in buffer (0.2 M KH_2PO_4 with 0.2 M NaOH; pH 6.8), centrifuged again for 10 minutes, decanted, and finally resuspended in buffer so that each ml of suspension contained 0.05 ml packed cells. One ml of this suspension was used in each Warburg vessel, which had a fluid volume of 2.2 ml, except in CO_2 measurements where vessels with double sidearms and fluid volumes of 2.5 ml were employed. All substrates to be tested were dissolved so that the desired amount was contained in 0.5 ml distilled water, this portion being added from the side-

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arm. The main compartment of the vessel contained 0.5 ml distilled water besides the cell suspension. When cells were to be adapted to a given compound, the latter was added to the main compartment of the vessel. Two-tenths ml of 20 per cent NaOH was used to absorb CO_2. The temperature of the bath was 37.5 C, and the shaking rate 100 per minute. The sodium salts of all acids except β-ketoadipic, α-ketoglutaric, and oxaloacetic, and phenol were used. For the Rothera tests, cells were incubated with the appropriate substrate in either a Dubnoff metabolic shaking incubator or in 125 ml Erlenmeyer flasks in the Warburg bath.

RESULTS

Mycobacterium tuberculosis, strain BCG: In confirmation of earlier work (Fitzgerald and Bernheim, 1947) none of the three monohydroxy derivatives of

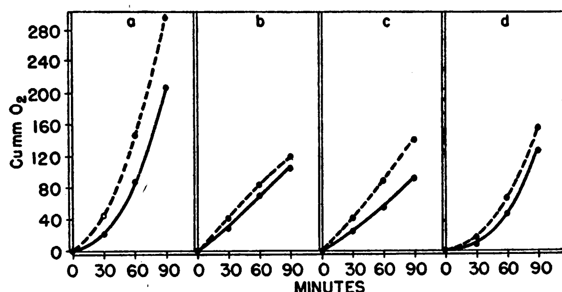

Figure 1. a: The increase in the rate of oxidation of 5 μM of benzoate by washed cells of BCG strain after preincubation for 45 minutes with 20 μg of benzoate. b: The increase in the rate of oxidation of 5 μM of protocatechuate under the same conditions. c: The increase in the rate of oxidation of 5 μM of catechol after preincubation for 60 min with 5 μg of benzoate. d: The increase in the rate of oxidation of 5 μM of benzoate after preincubation for 60 min with 5 μg of catechol.

benzoic acid was oxidized either by adapted or unadapted cells. This excludes them as possible intermediates. This was also true of phenol. Muconate was not oxidized. Protocatechuate was oxidized, the rate increasing when the cells were preincubated with benzoate (figure 1). The oxygen uptake was 1.5 mole per mole and the CO_2 release 1.5 mole per mole. Catechol was rapidly oxidized, and the latent period is markedly reduced with cells adapted to benzoate (figure 1). Moreover, cells adapted to catechol showed a reduced lag phase when benzoate was oxidized (figure 1). This may be considered an example of backward adaptation. Under the conditions of these experiments 1 mole of benzoate took up 3.5 moles of O_2 and produced 3 moles of CO_2. Catechol took up 2.5 moles of O_2 and produced 2 moles of CO_2 per mole. Since the same O_2 uptake would be expected with both protocatechuate and catechol, this would infer that more than one pathway exists for the oxidation of benzoate by this species.

β-Ketoadipic acid is formed in the oxidation of catechol by *Vibrio*, strain 0/1 (Kilby, 1948). Catechol- or benzoate-adapted cells or unadapted cells of BCG are unable to oxidize it. In order to eliminate possible permeability effects, the

adapted cells were subjected to ultrasonic vibrations and were ground with "hyflo supercel". These preparations still oxidized catechol but had no effect on β-ketoadipic acid. Sodium dodecyl sulfate was added as a possible means of altering the surface permeability. This detergent, in a concentration of 1.0 mg per ml cell suspension, inhibited the formation of the adaptive enzyme for benzoate entirely, and partially inhibited the oxidation in pre-adapted cells (figure 2). β-Ketoadipic acid was not oxidized, however, in the presence of this concentration of the detergent or in smaller concentrations which inhibited the oxidation of benzoate to a lesser degree. Finally, Bloch (1950) has shown that petroleum ether-extracted tubercle bacilli are still viable despite the loss of a fatty substance

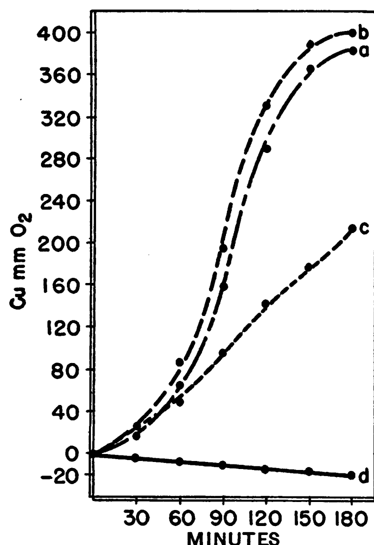

Figure 2. The inhibition of the formation of the adaptive enzyme for benzoate by BCG strain in the presence of sodium dodecyl sulfate (SDS). Cells were adapted by preincubation with 5 μg of benzoate for 60 min. Concentration of SDS = 1.0 mg per ml cell suspension. *a:* unadapted cells; *b:* adapted cells; *c:* cells adapted before the addition of SDS with substrate; *d:* cells adapted in the presence of SDS.

from their surface. This procedure was used on the BCG strain. The extracted cells oxidized benzoate and catechol and formed adaptive enzymes approximately 50 per cent as rapidly as the unextracted controls, but β-ketoadipic acid was again not oxidized. Moreover, the Rothera test for ketone bodies was still negative after 5 hours when catechol was used as substrate.

Mycobacterium butyricum. This species oxidized benzoate with an O_2 uptake of 2 moles per mole and gave off 1.5 moles CO_2 per mole. None of the monohydroxy derivatives of benzoate was adaptively attacked, but they did cause an increase in the rate of autorespiration in the order: salicylate < *m*-hydroxybenzoate < *p*-hydroxybenzoate. A slight increase in the rate of O_2 uptake was also noted when protocatechuate was added, but this was apparently only an increase in the rate of autorespiration, the magnitude being only about 50 mm^3

for a 3 hour period. Cells adapted to benzoate did not show a corresponding increase in the rate of O_2 uptake when either protocatechuate or *p*-hydroxybenzoate was added. Muconate was not oxidized. Catechol was oxidized, the gaseous exchange being 1 mole O_2 consumed and 0.5 mole CO_2 released per mole of catechol. Cells adapted to benzoate were simultaneously adapted to catechol (figure 3). β-Ketoadipic acid was oxidized with an O_2 uptake of 0.5 mole per mole, but cells adapted to either benzoate or catechol were not simultaneously adapted to this compound.

Since β-ketoadipic acid is formed in the oxidation of catechol by *Vibrio*, strain 0/1, more evidence was desired before this compound could be eliminated as a possible intermediate. The Rothera test for ketone bodies became positive after approximately 2 hours when either catechol or benzoate was employed. The color disappeared entirely after 4 hours with benzoate and somewhat more than 3 hours with catechol. Therefore, if β-ketoadipic acid were a metabolic in-

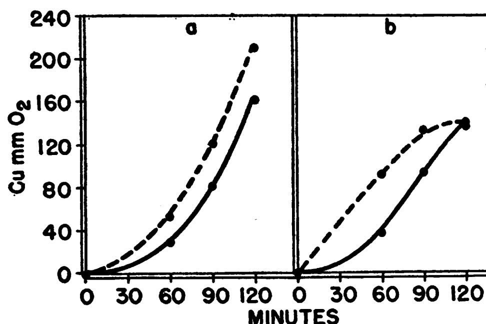

Figure 3. The increase in the rates of oxidation of (a) benzoate and (b) catechol by washed cells of *Mycobacterium butyricum* after preincubation with 20 μg of benzoate for 45 min.

termediate, the test should become negative after a certain period of incubation with this compound. This was not found to be the case. The test was still strongly positive after 6 hours. It must be concluded, therefore, that although β-ketoadipic acid is oxidized, it is not an intermediate in the breakdown of either benzoate or catechol, and its end product is itself a ketone body. The three keto acids in the Kreb's cycle, pyruvate, α-ketoglutarate, and oxaloacetate, were oxidized, but cells adapted to benzoate did not display an increased rate of O_2 uptake when these acids were added as substrate.

Mycobacterium phlei. This organism did not oxidize any of the previously mentioned compounds. Benzoate, catechol, β-ketoadipic acid, *p*-hydroxy- and *m*-hydroxybenzoate produced slight increases in the rate of autorespiration, but in no case did oxidation occur.

DISCUSSION

Surface permeability in mycobacteria is a subject which deserves serious consideration in studying the utilization of various compounds by these organisms. The lipid layer surrounding the cell would presumably act as a barrier to cer-

tain compounds; the degree of blocking would be the resultant of several factors. Among these are molecular size, structure, and polarity. It is, perhaps, the latter which enters into this case. Stanier (1947) obtained a much more rapid rate of oxidation of benzoate and some of its derivatives in adapted cells of *Pseudomonas* than is apparent in this work. An organism such as *Pseudomonas* without the lipid layer which is characteristic of mycobacteria would allow a more rapid passage of certain substances into the cell. This is a factor to be considered when comparing adaptation curves of mycobacteria with those of other organisms.

In most of his work, Stanier adapted the cells to certain substances by growing them in the presence of the compounds. His procedure is not applicable in all cases, however, since catechol, when added to the broth used for the BCG strain, autooxidized in 2 to 3 days as indicated by the dark brown color formed. Hence, a method of adaptation in a shorter period of time was a necessity in this case.

It is obvious that either permeability or differences in the methods used for adaptation could markedly alter the rates of oxidation. The first one mentioned would perhaps exert its greatest effect at the beginning of the reaction when the substrate is initially passing through the cell wall, and this may partially explain the persistence of a certain degree of lag when the substrate is added after the cells have been pre-adapted with a small amount of the same compound.

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SUMMARY

The evidence obtained by the method of simultaneous adaptation indicates that catechol is formed in the oxidation of benzoic acid by the BCG strain of *Mycobacterium tuberculosis* and *Mycobacterium butyricum*. The steps between these two compounds are probably the same as judged by the amounts of oxygen taken up. However, by this same criterion, each organism apparently oxidizes catechol by a different pathway.

The fact that benzoate-adapted cells of the BCG strain show some degree of adaptation to protocatechuate indicates that benzoate can be transformed to this compound. The amount of O_2 taken up, however, excludes its being an intermediate between benzoate and catechol. This would seem to infer at least two metabolic pathways for the oxidation of benzoate by this organism.

M. butyricum forms a ketone from benzoate which is further broken down so as to no longer give the Rothera test. This ketone is neither β-ketoadipic acid nor any of the keto acids in the Kreb's cycle. The failure of the BCG strain to give this test is further evidence that the two organisms oxidize catechol via different pathways.

M. phlei did not oxidize any of the compounds tested.

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