FACTORS WHICH AFFECT THE OXIDATION OF BENZOIC ACID BY A STRAIN OF PSEUDOMONAS AERUGINOSA

FREDERICK BERNHEIM AND WILLIAM E. DE TURK

Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina

Received for publication June 2, 1952

It has been shown that Pseudomonas fluorescens (Stanier, 1947) and various species of mycobacteria (Fitzgerald et al., 1948) are able to form adaptive enzymes for the oxidation of benzoic acid and related compounds. The conditions for the formation of these enzymes which occur in suspensions of washed resting cells have not been studied extensively although it is known that certain drugs in low concentrations inhibit the formation (Fitzgerald et al., 1949) and that an energy source is probably a requisite. In the following report, a strain of Pseudomonas aeruginosa has been used which oxidizes benzoic acid after a long latent period, and \( p \)-hydroxybenzoic acid after a somewhat shorter one. The various factors which affect the latent period have been studied in some detail.

MATERIALS AND METHODS

A strain of P. aeruginosa originally supplied by Dr. Koppers was used. It was grown as previously described (Bernheim and DeTurk, 1951). The washed cells were suspended in 0.05 m Na-K-phosphate buffer pH 7.8 and placed in Warburg vessels in a final fluid volume of 2.0 ml. When the substrate was catechol or protocatechuic acid, a similar buffer of pH 6.0 was used. The oxygen uptake was measured in the usual way and ammonia determinations made by the method of Speck (1949). The Theis and Benedict method (1924) was used to estimate the presence of aromatic hydroxy compounds.

RESULTS

When \( p \)-hydroxybenzoic acid was added, the test showed a progressive disappearance of the aromatic hydroxy group as soon as oxidation started. When benzoic acid was added, a positive test was obtained which indicates a hydroxylation of the ring. Calculated as \( p \)-hydroxybenzoic acid, the concentration reached a maximum of 6.0 \( \mu g \) when the oxygen uptake of benzoic acid had reached approximately half the final value and 0.5 mg of benzoic acid had been added. Both acids took up 9 atoms of \( O_2 \) per molecule and produced 4 molecules of \( CO_2 \) per molecule. The ring therefore was broken. Since both catechol and protocatechuic acid were oxidized by the organisms, and more rapidly in benzoic acid adapted cells, it is probable that the metabolic pathways are similar to those outlined by Stanier (1947) and Sleeper and Stanier (1950). Meta and ortho-hydroxybenzoic acids, \( 2,4 \)-dihydroxybenzoic acid, and \( p \)-aminobenzoic acid were not attacked.

The autorespiration of the washed cell suspensions was small, i.e., 15 to 20 mm² of \( O_2 \) taken up per hour, and 5 to 10 \( \mu g \) of ammonia was produced per hour. When benzoic acid was added, the ammonia values were unchanged during the latent period, but after the oxidation was complete little or no endogenous ammonia could be recovered. This suggested that ammonia was assimilated during the oxidation of benzoic acid and might be a limiting factor in the latent period. Accordingly, ammonia as \( (NH_4)_2SO_4 \) was added. It had no effect on the autorespiration but decreased the latent period from 120 to 90 min and increased the oxidation rate (figure 1). Another factor prolonging the latent period might have been the low rate of autorespiration since energy might be necessary for enzyme synthesis. Succinic acid therefore was added to the organisms with and without \( (NH_4)_2SO_4 \). It has been shown previously that ammonia is assimilated during the oxidation of succinic acid (Bernheim and DeTurk, 1951). When the oxidation was complete, benzoic acid was added, and as shown in figure 1 the latent period was shortened by the succinic acid and more so by the succinic acid and ammonia. Other acids of the tricarboxylic acid cycle produced similar effects. Despite the fact that ammonia had been assimilated during the oxidation of succinic acid, more was assimilated during the oxidation of benzoic acid. The amount, 41.0 \( \mu g \) NH\( _4 \)-N/mg
of benzoic acid, was the same as the amount assimilated by benzoic acid without previous incubation with succinic acid. Thus ammonia assimilation is an obligatory accompaniment of the oxidation of benzoic acid and this may explain why addition of ammonia shortens the latent period. On the other hand, addition of ammonia has no effect on the latent period of catechol and protocatechuic acid.

**Figure 1.** The effect of various compounds on the latent period in the oxidation of 0.5 mg sodium benzoate added when the oxidation of succinic acid was complete. (A) benzoate; (B) + 0.5 mg (NH₄)₂SO₄; (C) + 0.5 mg succinic acid; (D) + (NH₄)₂SO₄ and succinic acid; (E and F) + (NH₄)₂SO₄, succinic acid, and 10 µg and 30 µg benzoate, respectively, added at the beginning of succinic acid oxidation. The control oxygen uptakes have been subtracted in this and all subsequent figures. All experiments were done at pH 7.8 and 37 C.

A further shortening of the latent period occurred when 10 to 30 µg of benzoic acid was added with the ammonia and succinic acid to direct the enzyme formation. When 0.5 mg benzoic acid was added at the end of the succinic acid oxidation, its oxidation proceeded after a latent period of only 10 to 15 minutes. This remaining latency probably represents the time necessary for the molecules to diffuse into the cell because if benzoic acid were oxidized to completion under any of the experimental conditions the further addition of benzoic acid did not result immediately in a resumption of the oxygen uptake but showed a similar latent period of 10 to 15 minutes. All these results could be duplicated with p-hydroxybenzoic acid which assimilated 50.0 µg NH₄—N/mg.

When the organisms were grown in the medium to which 0.4 per cent sodium benzoate was added, the adaptive enzyme presumably was formed because washed suspensions of such organisms oxidized added benzoic acid with the minimum latent period (figure 2) and, as was to be expected, ammonia was without effect. The latent periods for p-hydroxybenzoic acid, catechol, and protocatechuic acid were also reduced. Conversely, organisms grown in p-hydroxybenzoic acid showed a reduced latent period for the oxidation of benzoic acid. This is not, however, unequivocal evidence for a crossed effect since benzoate adapted cells also oxidized pyruvic and succinic acids somewhat more rapidly than the controls although the same volume of cells was used in each case. If the washed resting cells were primed with 10 µg of p-hydroxybenzoic acid with or without ammonia and succinic acid, the latent period for the oxidation of benzoic acid was
somewhat reduced (figure 3) and the reverse was also true, but this also may be a nonspecific effect.

A number of antibiotics in low concentrations inhibit ammonia assimilation in this organism (Bernheim and DeTurk, 1952). Figure 4 shows that they inhibited the oxidation of benzoic acid. As little as 0.25 \( \mu g \) per ml gave a demonstrable effect. The reactions inhibited are apparently those necessary for the initiation of the oxidation which include the formation of the enzymes because these drugs were completely without effect if added after the oxidation had started. Their action thus resembles that of streptomycin on the inhibition of adaptive enzyme formation in certain mycobacteria (Fitzgerald et al., 1948). Addition of 1.0 mg per ml of sulfanilamide had no effect on the oxidation of benzoic acid whether it was added before or during the oxidation.

Since ammonia assimilation is associated with oxidation, the possibility exists that ammonia might be oxidized to hydroxylamine. To test this, hydroxylamine was substituted for ammonia and added with ammonia to both \( p \)-hydroxybenzoic acid and succinic acid. As little as 10.0 \( \mu g \) per ml of hydroxylamine HCl inhibited the oxidation of succinic and \( p \)-hydroxybenzoic acids in the presence or absence of added ammonia. This seems to rule out hydroxylamine as a possible intermediate and indicates that it may act as an inhibitor of the assimilation process. Methylamine, ethanolamine, and ethylene diamine could not substitute for ammonia in the oxidation of either succinic or \( p \)-hydroxybenzoic acids. Thus it is possible that the assimilated ammonia is directly incorporated into compounds of greater complexity. These might be aspartic or glutamic acids or amides, but it has been shown previously (Bernheim and DeTurk, 1951) that these compounds are rapidly deaminated when added to these organisms.

**DISCUSSION**

The long latent period which occurs when benzoic acid or \( p \)-hydroxybenzoic acid is added to this strain of *P. aeruginosa* before these compounds are oxidized and the low rate of autorespiration of the washed cells have been
useful for determining the factors which may influence the latent period. These factors may be divided into two classes, nonspecific and specific. Since the oxidation of these acids is coupled in an obligatory way with ammonia assimilation, the latent period can be shortened by supplying ammonia. Since ammonia assimilation requires energy which must be supplied by the oxidation of the substrate (Bernheim and DeTurk, 1951), the latent period can be shortened further if the energy is supplied by the previous oxidation of a substance such as succinic acid which is rapidly oxidized by a constitutive enzyme. Finally, since adaptive enzymes must be formed presumably for the oxidation of the benzoic acids, the addition of a priming amount of benzoic acid, the specific factor, along with ammonia and succinic acid, the nonspecific factors, almost completely eliminates the latent period. That which remains can be accounted for by the time necessary for diffusion into the cell. All antibiotics tested, but not sulfanilamide, inhibited the oxidation of benzoic acid in very low concentrations. It has been shown previously (Bernheim and DeTurk, 1951) that they can inhibit ammonia assimilation without interfering with the oxidation of substrates, and this suggests that they block the formation or utilization of energy rich compounds involved in assimilation.

SUMMARY

A washed suspension of cells of a strain of Pseudomonas aeruginosa oxidizes benzoic acid after a long latent period and p-hydroxybenzoic acid after a somewhat shorter one. Ammonia is assimilated during the oxidation. No other aromatic hydroxy or amino acids tested are oxidized.

The latent period can be shortened by addition of ammonia, succinic acid, or other acids of the tricarboxylic acid cycle, by succinic acid plus ammonia, and can be eliminated almost completely by the addition of succinic acid, ammonia, and a priming amount of benzoic acid.

Streptomycin, aureomycin, terramycin, and chloramphenicol, but not sulfanilamide, inhibit the oxidation of benzoic and p-hydroxybenzoic acids when added in the latent period but are without effect once the oxidation is started.

Hydroxylamine also inhibits the oxidation.

REFERENCES


