THE KINETICS OF THE OXIDATION OF BENZOIC ACID BY CERTAIN MYCOBACTERIA

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Benzoic acid is oxidized by certain Mycobacteria (1). They do not oxidize it completely to carbon dioxide and water; the oxygen uptake usually stops when 5 atoms of oxygen are utilized for each molecule of benzoic acid. The enzymes concerned are adaptive, and are rapidly formed when small amounts of benzoic acid are added to suspensions of the bacteria (2). Benzoic acid may be considered somewhat toxic to the bacteria, because, although small amounts stimulate enzyme formation, larger amounts slow down or inhibit the process, possibly by interfering with reactions providing the energy for the enzyme synthesis. Furthermore, as shown below, the enzyme once formed disappears fairly rapidly in the absence of benzoic acid. Streptomycin prevents the formation of these adaptive enzymes, and thus inhibits oxidation of benzoic acid (2). The rate of oxygen uptake has been carefully measured and the following is an analysis of the kinetics of the reactions involved. Data were obtained from experiments done with Mycobacterium tuberculosis BCG 8240. The technique has already been described (1, 2).

Oxidation of benzoic acid by Mycobacteria proceeds by a number of stages. The substances formed, for the most part, have not been isolated, and will be referred to by letters. Benzoic acid (A) is first hydrated (cf. (3)) to form B. B takes up 1 atom of oxygen and becomes C. C, in turn, takes up a second atom of oxygen to become D, and so on through E, F, and G, until 5 atoms of oxygen have been taken up for each molecule of the original benzoic acid. The time course of each of these reactions, since there is no change in the concentrations of either water or oxygen, will probably be monomolecular. Using small letters to mean "concentration of," we may express these rates as follows:

\[
\frac{da}{dt} = -k_a
\]

\[
\frac{db}{dt} = k_a - k_b
\]

\[
\frac{dc}{dt} = k_b - k_c
\]
Because we do not know how G is further transformed by the bacteria, except that it is not oxidized, it is difficult to write an equation for it; later it will be seen that it is unnecessary to do so. In each case the velocity constant is proportional to the amount of the corresponding enzyme present in any particular experiment; an increase in the constant implies an increase in the amount of enzyme.

Equations similar to these have been integrated by Bateman (4). We may, however, begin by making the simplifying assumption that all the constants, $k_1$, $k_2$, etc., are equal. Integration then leads to a series of equations giving the amounts of each substance present at various times. The general solution giving the amount of the Nth substance is

$$n = e^{-kt} \frac{c_1}{(N-1)!} (kt)^{N-1} + \frac{c_2}{(N-2)!} (kt)^{N-2} + \ldots c_{N+1}$$
The constants of integration, \( c_1, c_2, \) etc., may be evaluated by setting the condition that at zero time there is present in the mixture \( a_0 \) of \( A \), and none of the other substances, \( B, C, \) etc. Then \( c_1 \) becomes \( a_0 \), and the other constants, \( c_2, c_3, \) etc., become zero. Fig. 1 shows the amounts of these substances present at various times when \( a_0 \) is given the value 1, and all the \( k \) values are put equal to 0.1.

To compute the oxygen consumption we proceed as follows. At the time \( t \), each molecule of \( C \) that is present has taken up 1 atom of oxygen, each molecule of \( D \) has taken up 2 atoms, etc. \( G \) presents a slight difficulty since it may have been further transformed; we must therefore say that 5 atoms of oxygen have been taken up by all of the original benzoic acid molecules that have reached this stage or gone beyond it. But all molecules not in the forms \( A, B, C, D, E, \) or \( F \) have reached this stage or gone beyond it, and their concentration must therefore be \( a_0 - (a + b + c + d + e + f) \). The total oxygen taken up, \( X \), will then be given by

\[
X = c + 2d + 3e + 4f + 5(a_0 - (a + b + c + d + e + f))
\]
which simplifies to

$$X = 5a_0 - (5a + 5b + 4c + 3d + 2e + f)$$

When appropriate values for the constants $a_0$ and $k$ are chosen, oxygen consumption at various times may be computed; the smooth curves in Fig. 2 were constructed from such computations. Experimental data, adjusted so that $a_0$ corresponds to exactly 500 c.mm. of oxygen, were plotted on these theoretical curves. It was then seen that the experimental points, although forming a curve of precisely the same shape as the theoretical, all lay a short distance to the right of it. When shifted 10 to 15 minutes to the left, the fit was excellent, as shown in Fig. 2. This shift implies a latent period before any great amount of oxidation occurs; this time interval is presumably occupied by formation of enzyme. It will be discussed further below.

Of five experiments analyzed, three yielded results that fitted such curves; two did not. In these cases the assumption was then made that the velocities of the oxidations of $E$ to $F$ and of $F$ to $G$ were increased to such an extent that these substances, $F$ and $G$, never accumulated in any significant amount. New curves based on this additional assumption were constructed, and experimental results were found to fit these in a satisfactory manner (Fig. 3).
Table I gives values for the $k$ constants and the lengths of the latent periods for one set of experiments. For convenience a set was chosen in which the $k$ values were equal in each experiment. It will be seen that the latent period is longest when no benzoic acid was used for preincubation; it disappears completely after 90 minutes preincubation with 0.2 mg. of benzoic acid. These facts may be explained on the assumption already mentioned, that benzoic acid has a double action. In very low concentrations it stimulates the formation of the oxidizing enzymes, while in high concentrations its toxic action tends to depress the formation of enzymes. Thus when no benzoic acid was added during preincubation no enzymes were formed, the latent period was long, and the total enzyme formed was least, as shown by the value for $k$. With very small amounts, some enzyme was formed, but the addition of the large amount of benzoic acid for the experiment induced some further formation. The latent period was shorter. With larger amounts the maximum amount of enzyme is formed, the $k$ reaches a maximum, and the latent period vanishes. Enzyme already formed is apparently not interfered with.

Disappearance of enzyme was demonstrated in the following way. Bacteria was preincubated with 0.01 mg. of benzoic acid for various lengths

### Table I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Benzoic acid used for preincubation</th>
<th>Without streptomycin</th>
<th>With streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>min.</td>
<td>k</td>
</tr>
<tr>
<td>13-14</td>
<td>None</td>
<td>18</td>
<td>0.028</td>
</tr>
<tr>
<td>16-17</td>
<td>0.05</td>
<td>10</td>
<td>0.050</td>
</tr>
<tr>
<td>21-22</td>
<td>0.10</td>
<td>4</td>
<td>0.050</td>
</tr>
<tr>
<td>24-25</td>
<td>0.20</td>
<td>0</td>
<td>0.050</td>
</tr>
</tbody>
</table>

### Table II

**Demonstration of Disappearance of Enzyme**

In this experiment $k_1$ and $k_5$ were larger than the others; the values given are for $k_1$ to $k_4$ which were equal.

<table>
<thead>
<tr>
<th>Time of preincubation with 10 γ benzoic acid</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.43</td>
</tr>
<tr>
<td>60</td>
<td>0.67</td>
</tr>
<tr>
<td>90</td>
<td>0.67</td>
</tr>
<tr>
<td>120</td>
<td>0.50</td>
</tr>
<tr>
<td>240</td>
<td>0.36</td>
</tr>
</tbody>
</table>
of time. As long as benzoic acid was present the enzymes increased to a maximum; after it was exhausted they gradually decreased. Data are given in Table II.

The effect of streptomycin is to decrease the velocities of all the reactions equally; there is no specific effect on any particular stage. This is true whether all the $k$ values are equal, as in Table I, or whether they are not, as in other experiments.

**SUMMARY**

The kinetics of the oxidation of benzoic acid by *Mycobacterium tuberculosis* BCG 8240 have been analyzed, and six consecutive monomolecular reactions have been postulated. In most cases the velocity constants for these reactions were the same; in other cases those for reactions 5 and 6 were considerably greater, due to a relative increase in the amounts of the adaptive enzymes which catalyze these processes. Oxidation begins with a latent period during which adaptive enzymes are formed. If the bacteria are previously treated with small amounts of benzoic acid, the latent period becomes shorter or vanishes, and the constants become greater up to a maximum, indicating the presence of increased amounts of enzyme. Enzymes gradually disappear in the absence of benzoic acid.

The effect of streptomycin is to depress equally all stages of oxidation.

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**BIBLIOGRAPHY**

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