THE EFFECT OF SUBSTITUTED BENZOIC ACIDS ON
ADAPTIVE ENZYME FORMATION IN A
MYCOBACTERIUM

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Certain Mycobacterium form adaptive enzymes for the oxidation of benzoic
acid (1). The oxidation proceeds through catechol and protocatechuic
acid (2) and is thus similar to that in Pseudomonas fluorescens (3). Neither
cyclohexanecarboxylic acid nor any substituted benzoic acid is oxidized by
these Mycobacteria, but certain of these compounds can induce the or-
ganism to form adaptive enzymes for benzoic acid and catechol. This is
shown by the following experiments.

EXPERIMENTAL

Mycobacterium tuberculosis BCG 8420 was grown for 3 to 5 days on Long’s
medium. The surface mass was broken up and centrifuged in a Hopkins
tube. The cells were washed twice with distilled water and finally sus-
pended in 0.05 m phosphate buffer, pH 7.8 or 6.7, 0.1 ml. of packed cells
per ml. of buffer. 0.5 ml. of this suspension was used in each Warburg
vessel, which had a final fluid volume of 2.0 ml. The substituted benzoic
acids were incubated with the cells for varying times, then 1.0 mg. of sodium
benzoate or catechol was added from the side arm and the rate of oxygen
uptake measured. These cells have a high rate of autorespiration, and
the addition of the substituted benzoic acids over a wide concentration
range had no appreciable effect upon it. Melting points were taken on
all the compounds used and several were recrystallized. The evidence
indicates that the effects of the substituted benzoic acids were not the
result of their contamination with benzoic acid.

Preincubation of the cells with o-fluoro-, o-chloro-, o-bromo-, o-nitro-, and
o-aminobenzoic acids increased the oxidation rate of benzoic acid; i.e.,
shortened or almost eliminated, depending upon the time of preincubation,
the latent period of 25 to 30 minutes which occurs when benzoic acid is
added to untreated cells, and which represents the time for adaptive en-
zyme formation (Table I). o-Hydroxybenzoic acid was without effect in
any concentration used. p-Fluorobenzoic acid stimulated enzyme forma-
tion, although the other para and meta isomers, which had no effect in
themselves, prevented the stimulation of enzyme formation normally in-
duced by the ortho isomers (Table II). Cyclohexanecarboxylic acid behaved like the ortho isomers (Table I). All the compounds gave the same results when catechol was used instead of benzoic acid (Table III). In other words, although not oxidized, these compounds stimulated the formation of the enzyme for catechol as well as that for benzoic acid. Protocatechuic acid is oxidized by this organism by what is apparently a constitutive enzyme (2) and the substituted benzoic acids had no effect on its oxidation rate.

In these studies, 2 hours were taken as an arbitrary time for preincubation, but, regardless of the concentration of the added compound, maximal

**Table I**

*Effect of Preincubation with Analogues of Benzoic Acid on Subsequent Oxidation of Sodium Benzoate*

The preincubation time was 2 hours at pH 7.8 before the addition of 1.0 mg. of sodium benzoate. The oxygen uptake by control cells (15 to 20 c.mm. per 10 minutes) was subtracted from experimental values to yield the net oxygen uptake.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Amount in pre-incubation mixture</th>
<th>Net oxygen uptake, c.mm.</th>
<th>10 min.</th>
<th>20 min.</th>
<th>40 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>-3</td>
<td>-2</td>
<td>9</td>
</tr>
<tr>
<td>o-Nitrobenzoate</td>
<td>200</td>
<td></td>
<td>5</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
<td>o-Aminobenzoate</td>
<td>200</td>
<td></td>
<td>9</td>
<td>15</td>
<td>32</td>
</tr>
<tr>
<td>o-Fluorobenzoate</td>
<td>20</td>
<td></td>
<td>26</td>
<td>54</td>
<td>139</td>
</tr>
<tr>
<td>o-Chlorobenzoate</td>
<td>100</td>
<td></td>
<td>19</td>
<td>40</td>
<td>109</td>
</tr>
<tr>
<td>o-Bromobenzoate</td>
<td>200</td>
<td></td>
<td>12</td>
<td>28</td>
<td>84</td>
</tr>
<tr>
<td>p-Fluorobenzoate</td>
<td>50</td>
<td></td>
<td>11</td>
<td>23</td>
<td>73</td>
</tr>
<tr>
<td>o-Hydroxybenzoate</td>
<td>200</td>
<td></td>
<td>-4</td>
<td>-2</td>
<td>10</td>
</tr>
<tr>
<td>Cyclohexanecarboxylate</td>
<td>200</td>
<td></td>
<td>21</td>
<td>51</td>
<td>125</td>
</tr>
</tbody>
</table>

enzyme production was not elicited in this time. As shown in Table IV for cyclohexanecarboxylic acid, once a certain concentration is reached, the rate of enzyme formation by the cell in response to the stimulus is the limiting factor, so that, for instance, 200 γ of the compound preincubated with cells for 4 hours elicit more enzyme than 2400 γ incubated for 2 hours. The same results were obtained with all the other compounds. o-Fluorobenzoic acid was by far the most active compound, while o-nitro- and o-aminobenzoic acids were least active (Table I). There seems to be a rough inverse correlation between the size of the substituting atom or group and the effectiveness of the compound with the exception of o-hydroxybenzoic acid. Not only was o-hydroxybenzoic acid inactive, but it in-
hibited the stimulation of enzyme formation by the active compounds. p-Fluorobenzoic acid is another exception since, as shown in Table II, the other para- as well as meta-substituted benzoic acids inhibited enzyme formation. There appears, moreover, to be a mass action effect, for the

**Table II**

**Effect of Some Para- and Meta-Substituted Benzoic Acids on Enzyme Formation in Response to o-Chlorobenzoic Acid**

The preincubation time was 2 hours at pH 7.8. 1.0 mg. of sodium benzoate was then added.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Amount in pre-incubation mixture</th>
<th>10 min.</th>
<th>20 min.</th>
<th>40 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>-1</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>o-Chlorobenzoate</td>
<td>100</td>
<td>11</td>
<td>29</td>
<td>76</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ p-chlorobenzoate</td>
<td>100 + 10</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ m-chlorobenzoate</td>
<td>100 + 10</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ p-bromobenzoate</td>
<td>100 + 10</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ m-bromobenzoate</td>
<td>100 + 10</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>&quot;</td>
<td>25</td>
<td>14</td>
<td>20</td>
<td>69</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ p-chlorobenzoate</td>
<td>25 + 10</td>
<td>-4</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>50</td>
<td>10</td>
<td>22</td>
<td>75</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ p-chlorobenzoate</td>
<td>50 + 10</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>&quot;</td>
<td>100</td>
<td>9</td>
<td>27</td>
<td>79</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ p-chlorobenzoate</td>
<td>100 + 10</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>&quot;</td>
<td>150</td>
<td>3</td>
<td>20</td>
<td>75</td>
</tr>
<tr>
<td>&quot;</td>
<td>+ p-chlorobenzoate</td>
<td>150 + 10</td>
<td>2</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table III**

**Effect of Preincubation with Benzoate or o-Fluorobenzoate on Subsequent Oxidation of Sodium Benzoate and Catechol**

The preincubation time was 2 hours at pH 6.7. 1.0 mg. of sodium benzoate or of catechol was then added.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Amount in preincubation mixture</th>
<th>Sodium benzoate</th>
<th>Catechol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Net oxygen uptake, c.mm.</td>
<td>10 min.</td>
<td>20 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 min.</td>
<td>20 min.</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>-1</td>
<td>6</td>
</tr>
<tr>
<td>Benzoate</td>
<td>5</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>o-Fluorobenzoate</td>
<td>5</td>
<td>13</td>
<td>40</td>
</tr>
</tbody>
</table>
inhibition by a given amount of para-substituted compound depends upon
the concentration of the ortho-substituted compound used. This effect,
illustrated in Table II with \( o- \) and \( p- \) chlorobenzoic acids, was obtained
with all the other ortho compounds as well as with cyclohexanecarboxylic
acid and with benzoic acid itself. If \( p \)-fluorobenzoic acid behaved like
the other \( p \)-substituted compounds, it should inhibit enzyme formation
by the ortho compounds. This it does not do, and it therefore appears
unlikely that the effect of this para compound is the result of its con-
tamination with the ortho isomer.

Table III shows that \( o \)-fluorobenzoic acid stimulated production of the
enzymes required for oxidation of both catechol and benzoic acid, and that

**TABLE IV**

*Effect of Cyclohexanecarboxylic Acid on Enzyme Formation*

The preincubation times were 2 and 4 hours at pH 7.8. 1.0 mg. of sodium ben-
zoate was then added.

<table>
<thead>
<tr>
<th>Amount of cyclohexane-carboxylic acid in preincubation mixture</th>
<th>Time of preincubation</th>
<th>Net oxygen uptake, c.mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
<td>20 min.</td>
</tr>
<tr>
<td>( \gamma )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

it is more effective than benzoic acid itself. The reason for this is shown
in Table V. When the preincubation period was extended, the enzyme
elicted by benzoic acid began to disappear because, as the enzyme was
formed, the small amount of benzoic acid was completely oxidized, the
stimulus was thus removed, and the enzyme decayed. On the other hand,
\( o \)-fluorobenzoic acid was not oxidized and remained as a constant stimulus
throughout the experiment and elicited almost maximal enzyme production.
The short lag period which still remained can probably be accounted for
by the time necessary for the penetration of the benzoic acid into the cell.
This difference between benzoic and \( o \)-fluorobenzoic acids, together with
the fact that at the beginning they elicit the enzyme at the same rate
(Table V), indicates that the ortho-substituted compounds are not changed
to benzoic acid by the cell and that they contain no significant amounts of
benzoic acid as a contaminant. Furthermore, higher concentrations of these compounds elicit progressively less enzyme, which also indicates that no benzoic acid is present.

There remains the possibility that the ortho-substituted compounds and cyclohexanecarboxylic acid altered some physical characteristic of the cell so that the benzoate subsequently added could produce the enzyme much more rapidly. This appears unlikely since streptomycin, which inhibits enzyme production by benzoic acid (1), also inhibited production by the other compounds. With 0.1 mg. per ml. of streptomycin, enzyme production by 10 γ of sodium benzoate was inhibited 89 per cent, by 5 γ of o-fluorobenzoic acid 90 per cent, by 25 γ of o-chlorobenzoic acid 82 per cent, and by 25 γ of cyclohexanecarboxylic acid 85 per cent.

5 γ of o-fluorobenzoic acid per ml. added to the medium slightly inhibited growth. After 4 days incubation, the washed cells contained the enzymes for benzoate and catechol but not the maximal amount. Thus after 40 minutes in the Warburg vessels, benzoate and catechol had taken up 53 and 56 c.mm. of O₂ respectively, whereas with the controls only 15 and 24 c.mm. were utilized. After 4 days growth with 5 γ per ml. of benzoate, the cells no longer contained the adaptive enzymes. Cells grown with o-fluorobenzoic acid were unable to oxidize it.

DISCUSSION

Apparently the stimulus for the production of the enzymes involved in the oxidation of benzoic acid and catechol is not as specific as the en-
zymes themselves. Any substitution of the benzoic acid prevents its oxidation, whereas ortho substitution stimulates and meta and para substitution inhibit enzyme production. Steric effects probably account for the different actions of the isomers but chemical effects may also play a part. The carboxyl group on the ring is essential since the correspondingly substituted toluenes and phenols are inactive.

**SUMMARY**

1. The ortho isomers of amino-, nitro-, fluoro-, chloro-, and bromobenzoic acids stimulate the production of adaptive enzymes for the oxidation of benzoic acid and catechol by *Mycobacterium tuberculosis* BCG 8420. o-Hydroxybenzoic acid is inactive but cyclohexanecarboxylic acid also stimulates enzyme production. None of these compounds is oxidized.

2. p-Fluorobenzoic acid also stimulates production but the para and meta isomers of amino-, chloro-, and bromobenzoic acid inhibit.

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