INHIBITION OF THE d-AMINO ACID OXIDASE BY BENZOIC ACID

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Benzoic acid has been reported to inhibit the oxidation of succinic acid by muscle (1), the oxidation of butyric acid and crotonic acid by liver slices (2), and the oxidation of acetoacetic acid by kidney slices (3). It has also been reported to inhibit the oxygen uptake of liver and kidney slices, diaphragm, minced liver, kidney, and brain (4).

In the present work it was found that benzoic acid had a slight inhibitory effect on the oxygen uptake of broken cell preparations of rat liver and kidney. It had no effect on the oxidation of succinic acid, lactic acid, hypoxanthine, xanthine, uric acid, choline, tyramine, l(-)-tyrosine, l(-)-proline, l(-)-phenylalanine, sarcosine, or ethyl alcohol by the liver preparation. The oxidation of d-amino acids by various preparations of the d-amino acid oxidase was markedly inhibited by benzoic acid.

EXPERIMENTAL

Effect of Benzoic Acid on Oxidation of d-Amino Acids—The oxidation of d-amino acids by rat liver and kidney slices and by the preparations of the d-amino acid oxidase described below and the effect of benzoic acid on the oxidation were tested by measuring oxygen uptakes manometrically. The measurements were made with the usual Warburg apparatus at 37.5°.

The extra oxygen uptake of liver slices in the presence of 0.0075 M d(-)-alanine was 30 microliters per 30 minutes per 100 mg. of wet tissue. This extra oxygen uptake was completely inhibited by 0.001 M benzoic acid. The presence of glycine did not affect the inhibitory effect of benzoic acid. The benzoic acid did not
inhibit the oxygen uptake of the liver slices in the absence of the amino acid. The extra oxygen uptake of kidney slices in the presence of 0.0075 M d(-)-alanine was 53 microliters per 30 minutes per 100 mg. of wet tissue. 0.001 M benzoic acid inhibited the rate 50 per cent. The oxygen uptake of the kidney slices in the absence of the substrate was inhibited 6 per cent by benzoic acid.

The oxidation of 1 mg. of d(-)-alanine, d(+)-phenylalanine, and the d isomer of dl-proline by broken cell preparations (5) of rat liver and kidney and the washed proteins (6) of rat liver was completely inhibited by 0.01 M benzoic acid at pH 7.0, 7.5, and 8.0. With 0.001 M benzoic acid the rate of oxidation of these amino acids was inhibited about 90 per cent. In most cases the oxygen uptake of the broken cell preparations alone was inhibited about 10 per cent at pH 6.6 and 5 per cent at pH 8.1. The inhibition usually decreased as the pH approached 7.4, and then increased up to pH 8.1. In a few experiments the inhibition was negligible at pH 6.6 to 8.1.

Benzoic acid inhibited the activity of two purified preparations of the d-amino acid oxidase. One preparation was made by extracting 1 gm. of dry pig kidney (7) with 10 ml. of water, and then centrifuging. 1 ml. of the supernatant liquid plus 1 ml. of 0.1 M pyrophosphate buffer, pH 8.3, was used to oxidize the amino acids. The other preparation was a reconstructed oxidase composed of 3 to 6 mg. of a preparation of the protein fraction of the oxidase plus 2.4 to 4.8 γ of flavin-adenine dinucleotide. The protein preparation was made from the dry pig kidney and the flavin from yeast (7). Neither preparation of the oxidase could oxidize l-amino acids.

Data illustrating the effect of benzoic acid on the oxidation of d-amino acids by the reconstructed oxidase are given in Fig. 1. The data show that 10⁻⁴ M benzoic acid inhibits the activity 79, 52, and 50 per cent in the case of alanine, methionine, and proline respectively. The extent of inhibition is calculated from the oxygen uptakes at 10 minutes. In addition, the rate of oxidation of 1 mg. of d(+)-phenylalanine, d(+)-leucine, and d(+)-tyrosine was found to be inhibited 57, 28, and 64 per cent by 10⁻⁴ M benzoic acid. The data for methionine in Fig. 1 show that the extent of oxidation was not affected by benzoic acid. This was
also true in the case of the other amino acids when the experiments were of longer duration. Similar data were obtained with the kidney extract. The benzoic acid also inhibits the rate of reduction of methylene blue by mixtures of the oxidase preparations and amino acids.

It can be shown from the data in Fig. 1 that the oxidation of the amino acids in the presence and absence of benzoic acid is a first order reaction; i.e., a straight line is obtained when the logarithm of the concentration of unoxidized amino acid is plotted as a function of time. This indicates that the enzyme is not pro-

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Effect of benzoic acid on the activity of a reconstructed d-amino acid oxidase. The continuous lines represent oxidation of amino acids in the absence of benzoic acid; broken lines, oxidation in the presence of $10^{-4}$ M benzoic acid. The oxidase consisted of 3 ml. of the protein preparation and 2.4 $\gamma$ of flavin-adenine dinucleotide in 2 ml. of 0.05 M pyrophosphate buffer, pH 8.3. Curves 1, 0.0038 M dl-proline; Curves 2, 0.0056 M dl-alanine; Curves 3, 0.0034 M dl-methionine.

gressively inhibited by benzoic acid. This is also indicated by the fact that incubation of the enzyme and benzoic acid for 30 minutes before the addition of the amino acid does not affect the extent of inhibition.

A comparison of the effect of benzoic acid and that of other active acids is given in Table I. Under the same conditions isophthalic, hippuric, nicotinic, quinoline-$\beta$-carboxylic, and pyrazinecarboxylic acids were slightly active (0.08 or less), while p-aminobenzoic, phthalic, phenylacetic, picolinic, quinolinic, dinicotinic, 6-hydroxynicotinic, pyrimidine-4-carboxylic, acetic,
iodoacetic, stearic, and malonic acids, and benzamide, aniline, phenol, toluene, benzene, nicotinamide, trigonelline, pyridine, and glycine were inactive. These data show that compounds containing the benzene ring are more effective than those containing other rings, that the carboxyl group is necessary for inhibition, and that further substitution in the ring, or between the ring and the carboxyl group, depresses the effectiveness of the compound. The following observations indicate that the inhibition produced by benzoic acid is reversible. (a) The inhibitory effect disappeared after dialysis. (b) Preparations of the protein component of the oxidase made from kidney extract to which benzoic acid was added had the same activity as preparations made from untreated extracts. (c) The protein component can be recovered without loss of activity from solutions containing benzoic acid by treatment (7) with acid and ammonium sulfate. (d) Flavin-dinucleotide precipitated as the silver salt (7) from solutions containing benzoic acid had the same activity, after regeneration with hydrogen sulfide, as that precipitated from pure aqueous solution.

Assuming the formation of a reversibly dissociating substrate-enzyme complex and inhibitor-enzyme complex as suggested by

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**TABLE I**

Comparison of Inhibitory Effect of Benzoic Acid on d-Amino Acid Oxidase with Effect of Other Compounds

The activity of the kidney extract was tested in the presence of 2 mg. of dl-alanine. The decrease in activity produced by each substance was calculated from the oxygen uptakes at 10 minutes. The measure of inhibition given in the table is the ratio of the decrease in activity produced by the substance in 10^{-4} M concentration to that produced by 10^{-4} M benzoic acid. This concentration of benzoic acid decreased the activity 60 per cent.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Inhibition, 10^{-4} M benzoic acid = 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Aminobenzoic acid</td>
<td>0.25</td>
</tr>
<tr>
<td>m-Aminobenzoic &quot;</td>
<td>0.33</td>
</tr>
<tr>
<td>o-Hydroxybenzoic acid</td>
<td>0.32</td>
</tr>
<tr>
<td>m-Hydroxybenzoic &quot;</td>
<td>0.21</td>
</tr>
<tr>
<td>Terephthalic acid</td>
<td>0.19</td>
</tr>
<tr>
<td>2,4,5-Triiodobenzoic acid</td>
<td>0.13</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid</td>
<td>0.80</td>
</tr>
</tbody>
</table>
Michaelis and Menten (8), the relation between the concentrations of the components of an oxidase solution containing amino acid and benzoic acid would be

$$\frac{[\text{Amino acid}][\text{benzoic acid-oxidase}]}{[\text{Benzoic acid}][\text{amino acid-oxidase}]} = \frac{K_{\text{amino acid}}}{K_{\text{benzoic acid}}}$$  

The parentheses indicate concentration and $K$ the dissociation constants of the complexes.

**Table II**

*Calculation of $K_{\text{amino acid}}/K_{\text{benzoic acid}}$ from Equation I*

The reconstructed oxidase was used in Experiments 1, 2, 3, and 4. 3 mg. of protein preparation plus 2.4 $\mu$ of flavin-dinucleotide were used in Experiments 1 and 4, 3 mg. of protein plus 4.8 $\mu$ of flavin in Experiment 3, and 6 mg. of protein plus 2.4 $\mu$ of flavin in Experiment 2. The kidney extract was used in the other experiments. The oxygen uptakes given in Experiments 2, 3, and 4 are for 7.5 minutes; the others for 10 minutes.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Concentration of d(-)-alanine</th>
<th>Concentration of benzoic acid</th>
<th>Oxygen uptake</th>
<th>$K_{\text{amino acid}}/K_{\text{benzoic acid}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$</td>
<td>$\mu$</td>
<td>Without benzoic acid</td>
<td>With benzoic acid</td>
</tr>
<tr>
<td>1</td>
<td>0.0028</td>
<td>0.0001</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0.0056</td>
<td>0.0001</td>
<td>72</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>0.0056</td>
<td>0.0001</td>
<td>48</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>0.0056</td>
<td>0.0001</td>
<td>44</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>0.0560</td>
<td>0.0001</td>
<td>45</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>0.0280</td>
<td>0.0001</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>0.0056</td>
<td>0.0001</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>0.0028</td>
<td>0.00001</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>0.0028</td>
<td>0.00001</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>0.0028</td>
<td>0.001</td>
<td>21</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mean... ........................................... 115

In Table II are given data which permit the calculation of the ratio indicated in Equation I and the values of the ratio obtained. The rate of oxygen uptake in the absence of benzoic acid was taken as a measure of the concentration of total oxidase. The rate in the presence of benzoic acid was taken as a measure of (amino acid-oxidase). The difference between these rates was used as a measure of (benzoic acid-oxidase). It was assumed that the concentration of bound amino acid and benzoic acid was small com-
pared to the total concentrations, and that the concentration of amino acid was the same in the absence and presence of benzoic acid. For convenience the initial concentration of d-amino acid was used.

Considering the variety of experimental conditions under which the data were obtained, the values for the ratio are in good agreement. Although the agreement cannot be considered as definite proof of the formation of a benzoic acid-oxidase complex, it does make plausible the assumption that the inhibitory effect is due to the formation of such a complex.

The light absorption of solutions of the reconstructed oxidase and of flavin-dinucleotide at wave-lengths of 350 to 500 m\(\mu\) was not affected by the presence of \(10^{-3}\) M benzoic acid. This suggests that benzoic acid does not combine with the flavin or protein component of the oxidase.

**SUMMARY**

1. The activity of the d-amino acid oxidase was decreased by benzoic acid; e.g., the rate of oxidation of 1 mg. of \(d(-)\)-alanine by preparations of the oxidase was decreased about 60 per cent by \(10^{-4}\) M benzoic acid.

2. Data were obtained which suggest that the inhibitory effect of benzoic acid may be due to the formation of a benzoic acid-oxidase complex.

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**BIBLIOGRAPHY**

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