THE ACTION OF p-AMINOPHENOL ON THE XANTHINE OXIDASE OF LIVER

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The quinimine form of p-aminophenol inhibits the oxygen uptake of tissue suspensions and in very small concentrations inhibits specifically the oxidation of hypoxanthine and xanthine to uric acid by the xanthine oxidase of liver (1). Closely related compounds such as o-aminophenol, p-phenylenediamine, and quinone, although they inhibit to different degrees the oxygen uptake of tissues, have no effect on the xanthine oxidase. Because none of the other common dehydrogenases is affected by the quinimine and because the xanthine oxidase is inhibited by it in concentrations as low as 0.00001 M, it was of interest to study the mechanism of the inhibition more closely.

EXPERIMENTAL

For a quantitative study of an inhibition of this type the use of the purest form of the enzyme is desirable. The xanthine oxidase of milk was therefore prepared according to the method of Dixon and Thurlow (2). Unfortunately, the quinimine had a very variable effect on the milk oxidase. In some preparations a 50 per cent inhibition could be obtained but in most preparations no inhibition occurred under conditions which caused 100 per cent inhibition of the liver oxidase. There were three possible reasons for this discrepancy. Either the liver changed the quinimine, or the quinimine acted on something in the liver which in turn inhibited the oxidase or there was something in milk that prevented the inhibition from taking place. The following experiment indicates that milk contains a substance which inactivates the quinimine. A small amount of milk oxidase was added to liver
and the quinimine inhibition compared to that obtained in the liver alone. The results showed that whereas the oxidation of added xanthine was completely inhibited in the control, the presence of milk oxidase abolished the inhibition and xanthine was oxidized at a rate which showed that both the liver and milk oxidase were catalyzing its oxidation. For the study of the inhibition the xanthine oxidase of rat liver was therefore used. It was necessary to determine first whether the other metabolic activities of the liver had any effect on the xanthine oxidase inhibition. A rat liver was ground with sand in 10 cc. of 0.05 M phosphate buffer at pH 6.7. A part of this was used immediately, a part was put in the ice box for 24 hours, and a part was dialyzed for 24 hours against distilled water. The fresh liver had a large oxygen uptake which after 24 hours had been reduced to a quarter and after dialysis disappeared completely.

### Table I

<table>
<thead>
<tr>
<th>Concentration of quinimine × 10⁻⁴ M</th>
<th>10</th>
<th>5</th>
<th>2.5</th>
<th>1.3</th>
<th>1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of xanthine oxidase</td>
<td>100</td>
<td>94</td>
<td>90</td>
<td>68</td>
<td>45</td>
</tr>
<tr>
<td>&quot; liver</td>
<td>61</td>
<td>45</td>
<td>20</td>
<td>18</td>
<td>16</td>
</tr>
</tbody>
</table>

The xanthine oxidase activity of all three preparations was the same. In each case 0.00005 M quinimine completely inhibited the oxidation of xanthine. The percentage inhibition of the oxygen uptake of fresh liver by different concentrations of quinimine was then compared with the percentage inhibition of xanthine oxidase. As the concentration of quinimine was decreased, the liver inhibition decreased more rapidly than the xanthine oxidase inhibition until at a concentration of 0.00001 M the liver was only 16 per cent inhibited but the xanthine oxidase was still 45 per cent inhibited. These results are shown in Table I. In each case the percentage inhibition remained constant during the experiment.

The effect of pH on the inhibition of liver by quinimine was compared with its effect on the inhibition of the xanthine oxidase preparation. As the liver suspension was made more alkaline, the inhibition of the oxygen uptake decreased from 66 per cent
at pH 6.7 (with 0.0001 M quinimine) to 22 per cent at pH 7.8. Correspondingly the xanthine oxidase inhibition decreased from 100 to 69 per cent. This is shown in Table II. There are two possible reasons for the decreasing inhibition with increasing pH. Either there is a dissociation of the quinimine-enzyme complex or the drug is inactivated or destroyed in alkaline solutions. If the latter alternative were correct, then there should be a progressive decrease of the inhibition with time when the drug is used at pH 7.8. The inhibition, however, of both the liver and the xanthine oxidase remains constant for a period of at least 3 hours, which proves that no progressive destruction of the quinimine was taking place. The pH effect must be caused by the dissociation of the drug enzyme complex in alkaline solutions and this is also indicated by the fact that it is possible to obtain complete

### Table II

<table>
<thead>
<tr>
<th>pH</th>
<th>6.7</th>
<th>7.0</th>
<th>7.3</th>
<th>7.5</th>
<th>7.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of xanthine oxidase</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>93</td>
<td>84</td>
<td>77</td>
<td>68</td>
</tr>
<tr>
<td>“ “ liver</td>
<td>66</td>
<td>58</td>
<td>48</td>
<td>43</td>
<td>22</td>
</tr>
</tbody>
</table>

inhibition of the xanthine oxidase at pH 7.8 with concentrations of quinimine varying between 0.0003 M and 0.0002 M.

The rate of oxidation of hypoxanthine at pH 7.3 to 7.8 was about 90 to 100 c.mm. per hour and at pH 6.7 to 7.3 it was 80 to 90 c.mm. per hour. There was therefore no correlation between rate of oxidation and percentage inhibition. Moreover, the concentration of xanthine or hypoxanthine present had no effect on the inhibition; at pH 6.7 complete inhibition of the xanthine oxidation was obtained when amounts varying from 0.25 to 3.0 mg. of xanthine were being oxidized.

*p*-Aminophenol autoxidizes in solution and the rate increases with the pH. It is in equilibrium with quinimine, and its potential is close to that of the quinhydrone system. Theoretically, then, it should not matter whether the reduced or the quinimine form is added to the liver or the xanthine oxidase. Reduction or
oxidation of the quinimine-\(p\)-aminophenol system should occur until it is in equilibrium with any other systems that happen to be present in the preparation. In the presence of liver, however, whether it is freshly prepared or dialyzed, this does not occur. Addition of \(p\)-aminophenol does not inhibit the oxygen uptake of the liver at all at \(pH\) 7.8, whereas the addition of the quinimine\(^{1}\) inhibits it 20 per cent. It is at this \(pH\) that autoxidation should occur most readily. At \(pH\) 6.7 the reduced form inhibits the liver only 30 per cent while the quinimine inhibits 60 per cent. The xanthine oxidase is not inhibited at all at either \(pH\) by the reduced form. The probable explanation for the non-interchangeability of the two forms in the presence of liver is that the receptors in the liver immediately combine with the drug so that it can no longer act as a reversible oxidation-reduction system.

That some such combination of the quinimine with liver occurs is indicated by the following experiment. In order to prove the reversibility of the inhibition of the xanthine oxidase a series of Warburg vessels was put up in the usual way with hypoxanthine, freshly prepared liver at \(pH\) 6.7, and 0.0001 \(M\) quinimine. As shown by Curve 6 in Fig. 1, the inhibition by the drug was almost complete. At intervals a control vessel and one containing hypoxanthine were taken off and the contents transferred to Thunberg tubes. These were then evacuated and incubated at 37°. Even though the drug when added to liver and shaken in air is not oxidized or reduced, the results as shown in Fig. 1 indicate that the quinimine is reduced \textit{in vacuo} by the liver. Once the reduction had taken place, the solutions were placed again in the Warburg vessels and the oxidation of hypoxanthine which before the reduction was almost completely inhibited now proceeded at the same rate as the control with no drug added. But as Curve 5 shows, it requires a reduction period of at least an hour before complete recovery takes place. The vessels which Curve 5 represents were taken off after the inhibition had been allowed to run only half an hour. They remained \textit{in vacuo} for half an hour and the subsequent recovery was not complete, indicating that there was still quinimine present. For Curve 3 the inhibition was allowed

\(^{1}\) This is prepared by warming a solution of \(p\)-aminophenol and then allowing it to stand at room temperature for half an hour. The \(p\)-aminophenol was recrystallised from alcohol and water.
to run for 1 hour before reduction but the reduction was carried on for an hour. This time, despite the longer period of inhibition,

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

**Fig. 1.** The oxidation of 2 mg. of hypoxanthine by rat liver at pH 6.7. Curve 1 control, Curve 2 with 0.0001 M p-aminophenol, Curve 3 with 0.0001 M quinimine after 1 hour's incubation anaerobically, Curve 4 same as Curve 3 after 1½ hours' incubation anaerobically, Curve 5 same as Curve 3 after ½ hour's incubation anaerobically, Curve 6 same as Curve 3 except that there was no anaerobic incubation. The dotted lines are extrapolations representing the time of anaerobic incubation. Each curve represents the oxygen uptake of liver plus 2 mg. of hypoxanthine minus the uptake of the liver alone.

the recovery was complete, as shown by the slope of the curve. For Curve 4 the inhibition ran for 2 hours, and complete recovery occurred after 1½ hours reduction. This experiment shows that
liver which will reduce 200 micrograms of methylene blue in 3 to 5 minutes under these conditions takes over half an hour to reduce 20 micrograms of quinimine, although the latter belongs to an oxidation-reduction system with a higher potential than the methylene blue system. The indications therefore are that the drug is not in solution but closely bound to the receptors in the liver. Fig. 1 also shows that the reduced form is without effect (Curve 2) and that complete reversibility is possible no matter how long the inhibition has run. Further evidence for the combination of the drug with liver receptors is the fact that a 4 hour dialysis at pH 6.7 of liver to which quinimine had been added does not restore the oxidation of xanthine. This experiment was carried out by placing liver and quinimine in a dialyzing bag and dialyzing against a buffer in a flask which was shaken in air at 37°. A control without quinimine was similarly shaken and showed the usual xanthine oxidase activity.

Because quinimine had no effect on any of the other dehydrogenases tried (1), it is possible that its action on the xanthine oxidase is due to some peculiarity of the enzyme structure. It was therefore of interest to try its effect on guanase and uricase, enzymes which attack substrates the chemical structure of which is closely related to xanthine. The rat liver contains an active guanase. Its activity could be measured by the ammonia liberated and also by the rate of oxygen uptake due to the xanthine formed from the deamination of the guanine. This latter method also served to check the activity of the added quinimine, for in its presence the oxidation of xanthine was completely suppressed. Warburg vessels were set up in the usual way with either freshly prepared or dialyzed rat liver suspension and guanine. When the theoretical amount of xanthine had been formed from the guanine as shown by the oxygen uptake of the control, the amount of free ammonia was estimated by a vacuum distillation method. A large number of experiments were carried out and the results showed that guanase was inhibited from 30 to 50 per cent at pH 6.7 by the quinimine. Increasing the concentration of the drug did not increase the percentage inhibition nor did varying the relative concentrations of drug, guanine, and guanase. The pH effect was similar to that on the xanthine oxidase. In a typical
experiment the inhibition was 48 per cent at pH 6.7 and 12 per cent at pH 7.8. These experiments also showed that quinimine had no effect on the ammonia production of the liver suspension alone. It has already been shown (1) that the drug has no effect on CO₂ production. Adenase was not tried because no active source of the enzyme could be obtained.

In order to test the action of quinimine on uricase an acetone preparation from pig liver was made according to the method of Keilin and Hartree (3). Although the conditions were varied in all possible ways, the oxidation of uric acid by the enzyme was not affected at all by the drug even in concentrations of 0.001 M. The uricase preparation still had a small xanthine oxidase activity. This was completely inhibited by small concentrations of quinimine, showing that there was nothing in the preparation that interfered with the action of the drug.

**DISCUSSION**

The evidence indicates that both the reduced and oxidized forms of p-aminophenol combine with some substance in the liver and once in such a combination are no longer readily oxidized or reduced. The nature of the substance in the liver is not known. p-Aminophenol is not a very reactive compound. Except for its oxidizing and reducing properties, it reacts readily only with aldehydes. Consequently addition of small amounts of aldehyde to liver completely protects the liver and the xanthine oxidase from the action of quinimine (1). The inhibitory effect of cyanide on xanthine oxidase as shown by Dixon and Keilin (4) is of interest in this connection. Its action differs in that the presence of the substrate protects the enzyme against cyanide but not against quinimine. The difference in the action of the oxidized and reduced p-aminophenol may be due to a different type of complex formation. Once the reduced complex is formed, shaking in oxygen at 37° for at least 4 hours does not oxidize a significant amount of it. The oxidized complex, however, can be reduced in vacuo after incubation at 37° for about 1 hour.

The fact that quinimine is effective in such small concentrations makes it a more powerful inhibitor of oxygen uptake in vitro than cyanide, arsenite, pyrophosphate, or urethane. It also shows
greater specificity of action, for xanthine oxidase is the only one of the common dehydrogenases that is inhibited, whereas the other drugs act on groups of dehydrogenases. It is possible that there are other systems that are inhibited; in fact, unless xanthine and hypoxanthine play some cyclic rôle in the cell, the inhibition of xanthine oxidase alone cannot account for a 60 per cent inhibition in the oxygen uptake of liver. But until this question is decided, quinimine is the most specific of the substances that act on the known oxidation systems. As already shown (1) it has no effect on the autoxidation of sulfhydryl compounds or on carbon dioxide or ammonia production.

Addition of quinimine to liver slices suspended in Ringer's solution caused only small inhibitions of the oxygen uptake. This indicates that the drug does not readily penetrate the cell membrane. It has been shown (5) that quinimine is formed when such antipyretics as acetanilide and acetophenetidin are taken. Possibly the effectiveness of these substances may be due to their ability to penetrate through the cell membrane and thereby allow the quinimine to be formed inside the cell.

SUMMARY

1. The quinimine and probably also the reduced form of \( p \)-aminophenol make non-dialyzable complexes with receptors in the rat liver. Only the quinimine is active in inhibiting the oxygen uptake of the liver and the oxidation of hypoxanthine and xanthine by the xanthine oxidase.

2. The inhibition of the xanthine oxidase by the quinimine is reversible if the mixture is incubated anaerobically for an hour at \( 37^\circ \). The reduced \( p \)-aminophenol shaken in air at \( 37^\circ \) with liver is not oxidized to the quinimine.

3. Varying the concentration of the quinimine affects differently the inhibition of the liver uptake and the xanthine oxidase. The \( \text{pH} \) effect on the inhibition is different in both cases also.

4. The xanthine oxidase of milk is usually not affected by the quinimine. This result has been shown to be due to an interfering substance in the milk oxidase preparation.

5. The guanase of rat liver is inhibited but not completely by the quinimine. Uricase from pig liver is not affected by the drug.
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