COMPARATIVE STUDIES OF VARIOUS INHIBITORS ON XANTHINE OXIDASE AND RELATED ENZYMES*

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Many substances have been examined (1, 2) as possible inhibitors of xanthine oxidase. Included among the more effective inhibitors are 2-amino-4-hydroxy-6-pteridinecarboxaldehyde (hereafter referred to as 6-ppteridylaldehyde) (1, 3-7) and other pteridine derivatives (5, 6); chalcones such as 3,3',4,4'-tetrahydroxylchalcone (8, 7); cyanide (9-14); the quinimine or oxidized form of p-aminophenol (2, 14-16); tetraethylthiuram disulfide or Antabuse (17, 18); arsenite (16, 19); ascorbic acid (20); pyrogallol (2); and bromoaecetophenone (21). Most of these studies have been made on the well known milk enzyme, while a few have been based on rat liver homogenates (4, 6-8, 15-17) and occasionally on other mammalian tissues (7, 14, 18, 19). The inhibitions have been measured by an aerobic manometric procedure (2, 6, 8-10, 12, 15, 16, 20) in the presence of methylene blue (7, 17, IS), by methylene blue reduction (1, 9-11, 13, 19-21), and by fluorometric (3, 4) or spectrophotometric changes (3-5, 20) due to an alteration in the spectrum of the substrate. The manometric and substrate spectral procedures usually measure the over-all process of reduction and autoxidation of the enzyme, while the methylene blue methods are concerned with a study of the reduction step only. The reduction and autoxidation of the enzyme represent two separable activities, since the latter can be inhibited by Antabuse without affecting the former (17).

The present report is a systematic study of five selected inhibitors of xanthine oxidase. By using a manometric procedure in the presence and absence of methylene blue, a distinction could be made between those inhibitors which affected the oxidase activity of the enzyme only and those inhibitors which blocked the dehydrogenating ability of the enzyme. Both a purine and an aldehyde substrate were used. The inhibitors were studied with respect to milk and rat liver xanthine oxidases, chicken liver xanthine dehydrogenase, and a flavoprotein enzyme having a typical riboflavin absorption curve, d-amino acid oxidase. In addition, a known (22, 23) inhibitor of the latter, Atabrine, was tested for its effect on the xanthine enzymes. The reduction of cytochrome c by the milk and chicken liver xanthine oxidases was also studied with selected inhibitors.

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Milk xanthine oxidase and chicken liver xanthine dehydrogenase were prepared by the methods of Ball (12) and Remy et al. (24) respectively. D-Amino acid oxidase was prepared from a pig kidney cortex acetone powder by extraction with water (25). Rat liver xanthine oxidase was prepared from a high speed supernatant solution of a 0.04 M, pH 7.4, phosphate buffer homogenate by precipitating between 30 and 60 per cent saturation with ammonium sulfate.

All enzyme activities were measured manometrically in air at 37°. The Warburg flask contained an appropriate amount of enzyme in 0.04 M phosphate buffer, pH 7.4. Twelve flasks were used for the milk and rat liver xanthine oxidase studies; six contained 0.15 ml. of 0.0113 M methylene blue, and six were run without methylene blue. Three flasks in each series were run with and without 0.15 ml. of the inhibitor solution; one flask contained no substrate (blank), while the other two had 0.15 ml. of 0.05 M hypoxanthine and 0.15 ml. of 0.05 M p-hydroxybenzaldehyde, respectively, in the side arm. The total volume of the reaction mixture was made to 2 ml. with the phosphate buffer; the center well contained 0.2 ml. of 10 per cent KOH and a filter paper. The substrate was tipped in after a 10 minute equilibration period, and readings were taken every 10 minutes. For the studies with chicken liver xanthine dehydrogenase the series without methylene blue was omitted, since this enzyme had no significant oxygen uptake in its absence; 0.2 ml. (5 mg.) of crystalline bovine serum albumin was also added to the Warburg flask to stabilize the enzyme. For the d-amino acid oxidase studies only eight flasks were required, four with and without methylene blue, two in each series with and two without inhibitor, and one of each two flasks containing 0.15 ml. of 0.1 M DL-alanine in the side arm.

The reduction of cytochrome c by milk xanthine oxidase and chicken liver xanthine dehydrogenase was measured in a system composed of 2.5 × 10⁻⁶ mole of hypoxanthine, 1 × 10⁻⁷ mole of cytochrome c, and the enzyme in enough 0.04 M phosphate buffer, pH 7.4, to give a total volume of 3 ml. Catalase was also added when testing the milk enzyme in order to destroy any peroxide formed, but was omitted from the dehydrogenase assays, since the latter had no significant direct reaction with oxygen. The reaction was carried out at room temperature in a Beckman spectrophotometer, and the optical density at 550 mp was measured at 1 minute intervals. The average change in optical density for the initial four readings was proportional to the amount of enzyme added.

The inhibitor solutions were prepared as follows: 6-Pteridylaldehyde and 3,3',4,4'-tetrahydroxylchalcone were dissolved with the aid of a few drops
of 0.01 N NaOH and diluted to 0.03 and 0.02 mg. per 0.15 ml. respectively. A solution of p-aminophenol containing 0.044 mg. per 0.15 ml. was prepared in a hot water bath, shaken vigorously, and allowed to stand at room temperature for 30 minutes to produce the quinimine form (15). The Atabrine was dissolved in water, neutralized to pH 7.4 with NaOH, and diluted to a concentration of 3.75 mg. per 0.15 ml. Each of the above inhibitors was added to the body of the Warburg flask along with the enzyme just prior to the measurement of enzyme activity.

The KCN and ascorbic acid were incubated with the enzyme prior to adding the enzyme solution to the Warburg flask. 0.04 M KCN freshly neutralized with HCl to pH 7.4 was mixed with the buffered enzyme solution in a ratio of 1:16 to give a concentration of cyanide of 0.0025 M. The mixture was incubated at 37° for 45 minutes; an aliquot of this solution was tested for enzyme activity and compared with another solution of the enzyme treated identically except that no cyanide was added. Ascorbic acid was dissolved in phosphate buffer, pH 7.4, enough NaOH was added to restore the pH to 7.4, and the solution was diluted to a concentration of 2 mg. per ml. The buffered enzyme and ascorbic acid solutions were mixed in a 10:1 ratio to give an ascorbic acid concentration of 0.2 mg. per ml., and the mixture was incubated at 37° for 45 minutes before testing the enzyme activity.

Results

The effect of the various inhibitors on these enzymes is shown in Table I. Pteridylaldehyde was a potent inhibitor of both the milk and rat liver xanthine oxidases as well as of the chicken liver xanthine dehydrogenase. The rat liver enzyme tended to "escape" from this inhibition with time in the presence of methylene blue, possibly because the inhibitor itself was oxidized. Since pteridylaldehyde was an effective inhibitor of the dehydrogenase and since the inhibition of the xanthine oxidases was not reversed by methylene blue, this inhibitor undoubtedly blocked the dehydrogenase portion of all these enzymes. The oxidation of both p-hydroxybenzaldehyde and hypoxanthine was inhibited completely.

Incubation of the milk xanthine oxidase with cyanide also inactivated the dehydrogenase portion of the enzyme, since the treated enzyme was still inactive in the presence of methylene blue. The chicken dehydrogenase enzyme was similarly inactivated by cyanide, while the less pure rat liver oxidase was affected in the same direction but to a lesser degree. In the latter case the ratio of dehydrogenase to oxidase activity (with and without methylene blue) was the same, 1.6, that was found for the untreated enzyme.

As reported for the milk enzyme (10), the addition of hypoxanthine sub-
strate to the chicken liver enzyme prior to treatment with cyanide prevented the inhibition and allowed normal reduction of cytochrome c. The cyanide-inactivated dehydrogenase, dialyzed 48 hours, could not be reactivated by the addition of Fe** or molybdenum trioxide as tested by cytochrome reduction. Dialysis of the chicken liver enzyme against 0.015 M ammonia and then tris(hydroxymethyl)aminomethane buffer caused no loss of cytochrome reductase activity. The oxidation of reduced diphosphopyridine nucleotide (DPN) by either the milk, rat liver, or chicken liver enzyme was relatively unaffected by cyanide, pteridylaldehyde, chalcone, or Antabuse as measured by the change in optical density at 340 μ or by dye reduction with reduced DPN as substrate.

The chalcone had little effect on the oxidation of hypoxanthine, but did inhibit the oxidation of p-hydroxybenzaldehyde by the milk oxidase; the latter inhibition was overcome by methylene blue. The rat liver oxidase was very effectively inhibited by the chalcone, and this inhibition was

### Table I

**Effect of Various Inhibitors on Milk and Rat Liver Xanthine Oxidases and Chicken Liver Xanthine Dehydrogenase**

Enzyme activities have been recorded as c.mm. of oxygen consumed per 10 minutes per Warburg flask.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration (mg per ml)</th>
<th>Milk xanthine oxidase</th>
<th>Rat liver xanthine oxidase</th>
<th>Xanthine dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypoxanthine (p-Hydroxybenzaldehyde)</td>
<td>Hypoxanthine (p-Hydroxybenzaldehyde)</td>
<td>Hypoxanthine (p-Hydroxybenzaldehyde)</td>
<td></td>
</tr>
<tr>
<td>No inhibitor</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6-Pteridylaldehyde</td>
<td>0.015</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>KCN</td>
<td>0.16†</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3,3',4,4'-Tetrahydroxychalcone</td>
<td>0.010</td>
<td>21</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Atabrine</td>
<td>1.88</td>
<td>15</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>p-Aminophenol (oxidized)</td>
<td>0.022</td>
<td>24</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.20†</td>
<td>19</td>
<td>29</td>
<td>26</td>
</tr>
</tbody>
</table>

* -, without methylene blue; +, with methylene blue.
† Increasing activity with time, a tendency to escape from the inhibition.
‡ Concentration of inhibitor in the enzyme solution which was incubated prior to the testing of its activity in the Warburg apparatus.
again reversed almost completely by methylene blue. The dehydrogenase, tested in the presence of methylene blue, was unaffected by this inhibitor. The chalcone inhibition was essentially identical with that previously reported (17) for Antabuse, inasmuch as both inhibitors were effective with the rat liver but not the milk enzyme, and both inhibitors blocked the oxidase portion of the enzyme without interfering appreciably with the dehydrogenase activity. Treatment of the crude rat liver enzyme with Pangestin destroyed its susceptibility to both Antabuse and the chalcone. The crude enzyme with an activity of 7 and 14, respectively, in the absence and presence of methylene blue had corresponding activities of 0 and 12 when treated with either Antabuse or the chalcone. For the Pangestin-purified enzyme the corresponding activities were 7 and 13, and these values were unaffected by Antabuse or the chalcone. It cannot be assumed that Antabuse or the chalcone would be effective in vivo, since the inhibition of the oxidase portion only of the enzyme might not interfere with the passage of electrons from the reduced enzyme to cytochrome c and eventually to oxygen.

Atabrine was the only one of the six inhibitors tested which inhibited \( \text{d-} \) amino acid oxidase; this inhibition amounted to about 50 per cent and was not reversed by methylene blue. Atabrine had relatively little effect (10 to 25 per cent) on the dehydrogenase or the xanthine oxidases when each was tested in the presence of methylene blue. The inhibition of the oxidases by Atabrine was greater (35 to 40 per cent) in the absence of methylene blue.

The quinimine form of \( \text{p-} \) aminophenol was not a particularly effective inhibitor of any of these purified enzymes. This inhibitor was previously found (15, 16) to be effective in crude liver homogenates, but produced variable results with the milk enzyme (2, 15). Ascorbic acid was also not a very effective inhibitor of any of these enzymes when the incubation was carried out in a buffered medium.

*8-Hydroxyquinoline and Other Chelating Agents*- For these studies the cytochrome c reduction measurements were made in tris(hydroxymethyl)-aminomethane buffer, pH 8, to keep the iron in solution. The various additions were substituted for an equivalent volume of buffer. These additions, when appropriate, were as follows: 0.1 ml. of chelating agent, 0.1 ml. (0.37 \( \mu \)mole) of ferric chloride, 0.1 ml. (0.45 \( \mu \)mole) of ferrous ammonium sulfate, 0.2 ml. (0.8 \( \mu \)mole) of molybdenum as molybdenum tri-

Ethylenediaminetetraacetic acid (disodium Versenate) and Versene ferric-speci-

1 Ethylenediaminetetraacetic acid (disodium Versenate) and Versene ferric-specific, supplied by the Bersworth Chemical Company, were dissolved in water to make 0.1 M solutions. Saturated solutions of 1,10-phenanthroline, 8-hydroxyquinoline, 2-thenoyltrifluoroacetone, and benzoylacetaone were used; the last two were obtained from the Midcontinent Chemicals Corporation.
oxide or molybdic acid in water, and 0.1 ml. (0.5 μmole) of the other metallic ions tested. In testing the ferrous iron plus chelator, the enzyme was added to the cuvette last and only after there was essentially no reduction of cytochrome c by the reagents themselves. Chelated ferrous iron by itself had very little reducing effect.

The rate of cytochrome c reduction by milk xanthine oxidase was increased 2- to 3-fold by the addition of 8-hydroxyquinoline. The simultaneous addition of 20 μg of ferric or ferrous iron with the 8-hydroxyquinoline gave approximately 5- and 7-fold increases. Ferric ions in the absence of 8-hydroxyquinoline had little effect; ferrous ions alone reduced cytochrome c and could not be tested. Typical results are presented in Table II. When a concentrated solution of milk xanthine oxidase was allowed to react with hypoxanthine (3 to 5 μmoles) under anaerobic conditions in the presence

### Table II

<table>
<thead>
<tr>
<th>Enzyme alone</th>
<th>8-Hydroxyquinoline added</th>
<th>Incubated*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Fe**</td>
<td>+ Fe**</td>
</tr>
<tr>
<td>0.012</td>
<td>0.034</td>
<td>0.054</td>
</tr>
</tbody>
</table>

*The enzyme was incubated anaerobically with hypoxanthine substrate and 8-hydroxyquinoline for 1 hour prior to measuring cytochrome c reduction.

of 8-hydroxyquinoline for 1 hour, then further diluted and tested for its ability to reduce cytochrome c by the usual procedure, the activity was increased as much as 10-fold. Dialysis of such a preparation for 17 hours against ion-free water produced no loss of iron from the enzyme. Incubation of the enzyme with substrate in the absence of 8-hydroxyquinoline did not activate the cytochrome reduction. The results suggest that 8-hydroxyquinoline reacts with ferrous iron in the enzyme without removing it, and that this complex is more effective in reducing cytochrome c than is the original enzyme. Addition of or incubation with 8-hydroxyquinoline had no comparable effect on milk xanthine oxidase when the activity of the latter was measured manometrically in the presence or absence of methylene blue.

Chicken liver xanthine dehydrogenase reduced 0.5 to 1.0 μmole of cytochrome c per mg. per minute and was therefore 40 times as active as the untreated milk enzyme in this test. The reduction of cytochrome c by the liver dehydrogenase was stimulated only slightly by the addition of 8-hy-
droxyquinoline, and iron caused no added effect. The low cytochrome c reductase activity of our milk enzyme may have been fortuitous for the demonstration of any iron effect in cytochrome reduction and probably accounts for the different results obtained with the liver dehydrogenase. The low activity of the milk enzyme was not increased by the use of phosphate buffer.

Other metal ions which were tested, but found to be incapable of replacing iron, were uranyl, zinc, magnesium, chromium, and cobalt. Molybdate and molybdenum trioxide by themselves usually gave a 50 to 75 per cent increase in activity.

None of the other chelating agents tested were as effective as 8-hydroxyquinoline. 1,10-Phenanthroline, alone or with iron, produced a 1- to 2-fold stimulation. Disodium Versenate and ferric-specific Versene, together with ferrous iron, were about as effective as 8-hydroxyquinoline alone; without ferrous iron they were less effective. Benzoylacetone, with or without ferric or ferrous iron, caused no more than a 50 per cent increase in activity. 2-Thenoyltrifluoroacetone was ineffective.

**SH Inhibitors**—The addition of some commonly used sulfhydryl inhibitors, i.e. Hg++, Cu++, arsenite, and p-chloromercuribenzoate, all caused inhibition of the chicken liver dehydrogenase by either the manometric or cytochrome assay. At a Cu++ concentration of $2.5 \times 10^{-6}$ M the methylene blue reduction was inhibited approximately 100 per cent, while cytochrome reduction was inhibited approximately 60 per cent. At a concentration of $7 \times 10^{-7}$ M p-chloromercuribenzoate, the methylene blue reduction was inhibited approximately 40 per cent, while the cytochrome reduction was inhibited 90 per cent; the enzyme tended to escape from the p-chloromercuribenzoate inhibition with time in the methylene blue assay. The addition of substrate did not protect against the SH inhibitors.

**Other Substances Tested**—In agreement with Dixon and Thurlow (26) and contrary to a previous report (27), we have not observed any inhibition of the manometric activity of milk xanthine oxidase or chicken liver xanthine dehydrogenase when they were tested in 0.1 M borate buffers of pH 8.0 and 8.4 and compared with their activities in corresponding phosphate buffer.

The manometric oxidation of hypoxanthine and p-hydroxybenzaldehyde by either the milk or rat liver xanthine oxidase in the presence or absence of methylene blue was not inhibited by butazolidine in a final concentration of 5 mg. per ml. Butazolidine was not oxidized by milk xanthine oxidase, but the rat liver preparation contained an enzyme which did oxidize butazolidine, and this oxidation was inhibited by methylene blue.

Fluoroacetate (final concentration = 0.01 M) had no effect on the xanthine oxidase activity or the endogenous oxygen consumption of a rat liver homogenate in the presence or absence of methylene blue. This was true
of normal chow-fed rats in which the liver xanthine oxidase activity was 40 (c.mm. of O₂ per 20 minutes per 283 mg. of fresh liver) or rats fed a protein-free diet for 1 week with a liver xanthine oxidase activity of 4.

**DISCUSSION**

Both 6-pteridylaldehyde and cyanide block the purine- and aldehyde-dehydrogenating prosthetic group of all three xanthine enzymes; inhibition by the former is reversible (3). Both the cyanide-inactivated (13) and pteridylaldehyde-inhibited (3) enzymes are still capable of oxidizing reduced DPN. The same dehydrogenase prosthetic group appears to be involved in removing hydrogen from purine and aldehyde substrates (and probably xanthopterin), while the oxidation of reduced DPN appears to involve a different prosthetic group.

The cyanide effect is not characteristic of the inhibition of an iron porphyrin enzyme and is not observed with many other dehydrogenases (11); there is no reason to believe that cyanide affects the flavin adenine dinucleotide (FAD) moiety. Cyanide inactivation of the purine dehydrogenase group suggests that a metal such as molybdenum may be associated with it. The atypical absorption spectra of the xanthine enzymes may also be related to the purine dehydrogenase group, since the aldehyde-quinine oxidase has a similar spectrum and is likewise inhibited by pteridylaldehyde (4).

Atabrine inhibited D-amino acid oxidase, which has a typical flavin spectrum, but it had little effect on the dehydrogenating activity of the xanthine enzymes. None of the xanthine oxidase inhibitors affected D-amino acid oxidase. Such evidence suggests that the FAD moiety of xanthine oxidase might be concerned with the oxidation of reduced DPN, while an unidentified prosthetic group is actually the center of dehydrogenase activity for aldehydes and purines. FAD has always been assumed to be the hydrogen acceptor of the enzyme, because its spectrum is reduced by the addition of substrate, but such reduction shows only that the hydrogen is eventually passed to the FAD component and provides no information about possible earlier reactions in which other parts of the enzyme might be involved.

Reoxidation of xanthine oxidase by air involves a component of the enzyme other than the dehydrogenase group, since certain inhibitors, *i.e.* Antabuse and the chalcone, can block the autoxidation of the liver enzyme without affecting its dehydrogenase activity. This type of inhibition has been observed so far only with the rat liver xanthine oxidase, but the separation of oxidase and dehydrogenase activities in this enzyme by two different inhibitors is unambiguous. A comparison of chicken liver xanthine dehydrogenase (24) with milk xanthine oxidase did not identify the component of the latter, which is responsible for its reaction with oxygen.
The portion of the enzyme responsible for the reduction of cytochrome c is not clearly established. Mackler et al. (28) have assigned this activity in milk xanthine oxidase to molybdenum largely on the basis of a stimulation of this reaction by added MoO₃. We also have observed some stimulation by molybdenum, but this effect is minor in comparison with the very marked response obtained with iron in the presence of 8-hydroxyquinoline. The rôle of the quinoline in this reaction is ambiguous; hence some doubt must be attached to the significance of the iron complex in this reaction. However, the possibility that iron mediates the transfer of electrons from the enzyme to cytochrome c is obvious.

SUMMARY

Pteridylaldehyde and cyanide inhibited the dehydrogenase activity of milk and rat liver xanthine oxidases and chicken liver xanthine dehydrogenase toward purines and aldehydes but not toward reduced DPN. 3,3',4,4'-Tetrahydroxychalcone, like Antabuse, blocked the oxidase portion of the rat liver enzyme without interfering appreciably with its dehydrogenase activity; it had little or no effect on milk xanthine oxidase, and the susceptibility of the liver enzyme was destroyed by incubation with Pangestin. The oxidation of purine and aldehyde substrates was affected similarly by the inhibitors. Atabrine was the only effective inhibitor of D-amino acid oxidase; it had little effect on the dehydrogenating activity of the xanthine enzymes, but was somewhat more effective against their reoxidation by air. Ascorbic acid and the oxidized form of p-aminophenol were not very effective inhibitors of any of these purified enzymes.

8-Hydroxyquinoline activated the cytochrome reductase activity of milk xanthine oxidase 2- to 3-fold, and this was increased to 10-fold by the simultaneous addition of ferrous ions or by preincubation of the enzyme with 8-hydroxyquinoline and substrate. Chicken liver xanthine dehydrogenase was more active than the milk enzyme in reducing cytochrome c and was stimulated only slightly by 8-hydroxyquinoline with or without iron. Cu⁺⁺ was somewhat more effective in inhibiting xanthine dehydrogenase in the methylene blue test than in cytochrome reduction, while the reverse was true for p-chloromercuribenzoate.

It is suggested that an unidentified prosthetic group is the center of dehydrogenase activity for aldehydes and purines, while FAD is responsible for the oxidation of reduced DPN. Iron may mediate the transfer of electrons to cytochrome c.

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