Further Studies with Xanthine Oxidase Inhibitors*

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The oxidase and dehydrogenase activities in the enzymes oxidizing xanthine can be differentiated by the use of certain inhibitors (1). The present study was designed to see if such inhibitors are specific for the reaction with oxygen, or whether these agents also block the transfer of electrons to cytochrome c and nitrate. Both reduced diphenolphosphorine nucleotide and hypoxanthine were studied as substrates because they represent separable activities in the xanthine enzymes.

EXPERIMENTAL

Milk and rat liver xanthine oxidase as well as chicken liver xanthine dehydrogenase were studied by previously described procedures (1, 2). The manometric oxygen consumption was measured (1) in the presence and absence of methylene blue, phosphate buffer, pH 7.4, and 3 mg. of albumin stabilizer for the milk enzyme; the substrate was 0.15 ml. of 0.05 M hypoxanthine or DPNH, and the total volume was 2 ml.

The reduction of cytochrome c was measured (2) by the change in optical density at 550 mp in a system containing tris(hydroxymethyl)aminomethane buffer, pH 8, catalase, 1.6 mg. of cytochrome c, and 2.5 µmoles of hypoxanthine or DPNH; when present, 0.1 ml. of a saturated solution of 8-hydroxyquinoline and 0.37 µmole of FeCl₃ were part of the total 3 ml. volume. The reaction with the milk and chicken liver enzyme was exposed to air, while the reduction of cytochrome c by the rat liver oxidase proceeded better when protected from air.

The reduction of nitrate (3) was carried out in Thunberg tubes under N₂ at the optimal pH of 4.6 for the milk oxidase and at the optimal pH of 5.3 for chicken liver xanthine dehydrogenase; the rat liver oxidase was also tested at pH 5.3. The tube contained 1 ml. of 0.6 M KNO₃, 0.5 ml. of 1 M acetate buffer, pH 4.6 or 5.3, and 3.2 ml. of enzyme in water; the side arm contained 0.3 ml. of 0.05 M hypoxanthine in 0.05 M NaOH. After equilibration for 10 minutes at 37°C, the contents were mixed and incubated for 1 hour. An aliquot was then deproteinized with trichloroacetic acid and analyzed for nitrite (4). Nitrite was not reduced further by any of the xanthine enzymes.

The inhibitors were prepared and tested as previously described (1, 2). 6-Pteridylaldehyde and 3,3′,4,4′-tetracyanohydroxychalcone (chalcone) were added to the test system in a final concentration of 0.015 and 0.01 mg. per ml., respectively, except that 0.01 mg. per ml. of 6-pteridylaldehyde was used in all studies of nitrate reduction and in the reduction of cytochrome c by milk enzyme.

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Semicarbazide was added to the test system in a final concentration of 10⁻³ M for all studies with the milk enzyme, and in a concentration of 0.1 M for all studies with rat and chicken liver enzymes. Each enzyme was incubated at 37°C for 45 minutes in 0.1 M hydroxylamine or 2.5 × 10⁻³ M cyanide before it was added to the test system in all studies of these inhibitors.

The rat liver enzyme preparation oxidized DPNH very rapidly, and this was presumably due to the presence of a contaminant in the DPNH or the enzyme; hence the oxidation of DPNH by rat liver xanthine oxidase was not studied. Similarly, the combination of methylene blue and DPNH was omitted because the dye was reduced by DPNH in the absence of enzyme.

RESULTS

Table I shows the comparative activities of the different xanthine enzymes with hypoxanthine or DPNH used as substrates, and four different electron acceptors (O₂, methylene blue, cytochrome c, and nitrate). The outstanding features of this comparison are the following: (a) milk xanthine oxidase reacted very slowly with cytochrome c or nitrate; (b) rat liver xanthine oxidase reacted well with cytochrome c; (c) neither milk nor the chicken liver enzymes utilized DPNH substrate readily with any electron acceptor, and could not utilize DPNH at all for nitrate reduction; (d) rat liver xanthine oxidase was similar to the milk enzyme in those activities which were studied; (d) in all cases where the initial reaction with cytochrome c was sluggish, the rate was speeded appreciably by the addition of 8-hydroxyquinoline and Fe⁺⁺⁺; however, the maximal rate achieved with stimulation was still small when compared with the reduction of cytochrome c by the chicken liver dehydrogenase, and the latter reaction was stimulated only slightly by 8-hydroxyquinoline plus iron. Since 1 mg. of purified milk or rat liver enzyme (5) and 1 mg. of chicken liver enzyme will reduce approximately 1 and 3 µmoles of oxygen per minute respectively in the presence of methylene blue, the values in Table I represent the activities of about 1 mg. of milk xanthine oxidase, 1 mg. of rat liver xanthine oxidase, and 3 mg. of chicken liver xanthine dehydrogenase, respectively.

The manometric oxidation of DPNH substrate by the milk enzyme was stimulated at least 2.6-fold by the addition of 25 µg. or more of 2-methyl-1,4-naphthoquinone (menadione) per ml; no reaction took place in the absence of the enzyme. 8-Hydroxyquinoline, 0.01 M, with or without FeCl₃ also stimulated the oxidation of DPNH by at least 70 per cent.

Table II shows that those inhibitors which blocked the dehydrogenase activity of the xanthine enzymes with respect to hypoxanthine substrate and methylene blue also blocked this
Oxidation of hypoxanthine (Hx) and DPNH substrates by three xanthine enzymes with different electron acceptors

All values have been recorded as amoles of oxygen, cytochrome c, or nitrate reduced per minute per ml. of each enzyme, and are directly comparable for any one enzyme. These activities represent 1 mg. of milk or rat liver xanthine oxidase and 1 mg. chicken liver xanthine dehydrogenase.

Oxygen consumption was measured manometrically in the presence and absence of methylene blue (m.b.). Cytochrome reduction was measured by the change in optical density at 550 mp in the absence and presence of 8-hydroxyquinoline (HQ) or 8-hydroxyquinoline plus ferric chloride. Nitrate reduction was measured as nitrite formed anaerobically.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Oxygen</th>
<th>Cytochrome c</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>- + mb</td>
<td>- + HQ</td>
</tr>
<tr>
<td>Milk xanthine oxidase</td>
<td>Hx</td>
<td>0.82</td>
<td>1.00</td>
<td>0.020</td>
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<tr>
<td></td>
<td>DPNH</td>
<td>0.044</td>
<td></td>
<td>0.010</td>
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<td>Hx</td>
<td>1.00</td>
<td>1.00</td>
<td>0.553</td>
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<tr>
<td></td>
<td>DPNH</td>
<td>1.00</td>
<td></td>
<td>0.026</td>
</tr>
<tr>
<td>Rat liver xanthine oxidase</td>
<td>Hx</td>
<td>0.74</td>
<td>1.00</td>
<td>0.019</td>
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</tbody>
</table>

**Table II**

Percentage inhibition of xanthine enzymes by various inhibitors with hypoxanthine (Hx) and DPNH substrates and four different electron acceptors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>O2 uptake</th>
<th>Cytochrome c reduction</th>
<th>Nitrate reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>- + mb</td>
<td>+ FO + FeCl3</td>
<td>+ FO + FeCl3</td>
</tr>
<tr>
<td>Milk xanthine oxidase</td>
<td>Hx</td>
<td>CN, PA, HA*</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chalcone†</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC‡</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>DPNH</td>
<td>CN, PA, HA*</td>
<td>50</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chalcone</td>
<td>0</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Chicken liver xanthine dehydrogenase</td>
<td>Hx</td>
<td>CN, PA, HA*</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chalcone</td>
<td>0</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
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<tr>
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<td>CN, PA, HA*</td>
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<td></td>
<td></td>
<td>Chalcone</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rat liver xanthine oxidase</td>
<td>Hx</td>
<td>CN, PA, IIA</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chalcone</td>
<td>100</td>
<td>5</td>
<td>100</td>
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<td></td>
<td></td>
<td>SC</td>
<td>80</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

* Similar results were obtained by adding 6-pteridylaldehyde (PA) or by incubating the enzyme with cyanide (CN) or hydroxylamine (HA). All inhibitions between 90 and 100 per cent were blocked. CN gave 90 per cent inhibition instead of 100. 6-PA gave 25 per cent and HA gave no inhibition, while CN gave 50 per cent.
† 3,3',4,4'-Tetrahydroxychalcone.
‡ Semicarbazide.

**Pyridoxal**—Pyridoxal was found to be a good substrate for liver aldehyde oxidase (6, 7), but neither pyridoxal nor pyridoxal phosphate was oxidized by milk xanthine oxidase. Both pyridoxal and pyridoxal phosphate inhibited the oxidation of hypoxanthine by the milk enzyme. Pyridoxal concentrations of 0.05, 0.25, and 1.0 mg. per ml. gave inhibitions of about 30, 65, and 75 per cent, respectively; identical results were obtained when the amount of substrate added to the flask was varied from 0.05 to 0.3 ml. of 0.05 M hypoxanthine. A 50 per cent inhibition of activity was produced by a pyridoxal concentration of 0.1 to 0.15 mg. per ml., and this was restored to 70 per cent of the initial rate by 0.5 to 1.5 mg. per ml. of glutathione. Methylene blue, 8-hydroxyquinoline, and Fe+++-specific Versene did not overcome the inhibition by pyridoxal or pyridoxal phosphate. Pyridoxine was not oxidized and did not inhibit the aerobic oxidation of hypoxanthine by milk xanthine oxidase. Pyridoxal phosphate (0.5 to 1.0 mg. per ml.) had little or no inhibitory effect on urease, uricase, tyrosinase, alcohol dehydrogenase, or carbonic anhydrase.

Since aldehydes and purines are activated at the same site on the enzyme, it seems probable that the inhibition produced by pyridoxal can be attributed to a loose blocking of this site by an inert aldehyde. 6-Pteridylaldehyde was a more effective inhibitor of this site, with inhibition starting at about 4 \( \times 10^{-4} \) M, and being reasonably complete at 4 \( \times 10^{-4} \) M in the presence or absence of methylene blue. Irrespective of the order of addition, the inhibition produced by 2.5 \( \times 10^{-4} \) M 6-pteridylaldehyde was not overcome at all in the aerobic manometric assay by glutathione (0.5 mg. per ml.) or by 0.001 M concentrations of...
ferrous ammonium sulfate, sodium molybdate (with or without silicic acid), or by 0.01 M 8-hydroxyquinoline.

Others—10⁻⁴ M 2,3-mercaptopyrrolopol (BAL) inhibits liver aldehyde oxidase by 50 per cent (7), but 10⁻¹ to 10⁻⁴ M 2,3-mercaptopyrrolopol did not inhibit either the milk or rat liver xanthine oxidase when tested manometrically in the presence or absence of methylene blue. Incubation of the enzyme with 2,3-mercaptopyrrolopol for 1 hour before testing also did not produce any inhibition.

Concentrations of less than 0.5 mg. of dicumarol per flask had no inhibitory effect on the aerobic activity of milk xanthine oxidase with hypoxanthine substrate; 1 and 10 mg. per flask decreased this activity to 75 and 65 per cent of the original, respectively. When the flask contained 10 mg. of dicumarol, the addition of 0.5 to 10 mg. of menadione restored the activity to the original 100 per cent, but did not give the additional 50 per cent stimulation observed in the absence of dicumarol. 2-Methyl-3-phytyl-1,4-naphthoquinone did not overcome the dicumarol inhibition. Similar concentrations of dicumarol inhibited the succinic oxidase system to about the same extent, but the inhibition produced by 10 mg. of dicumarol per flask was not overcome by 0.1 to 10 mg. per flask of menadione.

A concentration of 0.1 or 0.5 mg. of antimycin per flask had no inhibitory effect on the aerobic activity of milk xanthine oxidase with hypoxanthine substrate.

p-Chloromercuribenzoate, 4.4 x 10⁻⁴ M, inhibited the oxidation of hypoxanthine by milk xanthine oxidase almost completely, while it inhibited the initial oxidation of DPNH by only 50 per cent; the inhibition of DPNH oxidation by p-chloromercuribenzoate tended to decrease with time. In both cases, 0.5 mg. per ml. of glutathione completely prevented the inhibition. These findings are in essential agreement with the report by Weber and Kaplan (8) that an appropriate concentration of this substance will inhibit the oxidation of hypoxanthine without affecting the oxidation of DPNH.

Nitrate Reduction

The reduction of nitrate by chicken liver xanthine dehydrogenase with hypoxanthine as substrate was completely inhibited by 2 x 10⁻⁴ M CuSO₄ or HgCl₂. Complete inhibition was also effected by 0.02 m arsenite, tetraborate, ferricyanide, ferrocyanide, pyrophosphate, and bisulfite; citrate had no effect.

When 0.02 M molybdate replaced nitrate in this test procedure with the chicken liver enzyme, a colloidal molybdenum blue color could be detected within 5 minutes, and became intense within 1 hour. The molybdate did not inhibit the reduction of nitrate when both were present together, and the solution remained clear with only a slight tinge of blue. Thus, in the absence of other electron acceptors, the added molybdate was reduced. These observations agree in general with the concept that the enzymatic Mo is reduced in the process of nitrate reduction (9). However, commercial molybdenum blue could not be substituted for hypoxanthine in the enzymatic reduction of nitrate to nitrite, and the added molybdate acted as an external electron acceptor in the nitrate test system.

The rate of reduction of nitrate by milk xanthine oxidase was doubled by the addition of 0.2 ml. of a saturated solution of 8-hydroxyquinoline to the test system. A 3-fold increase was obtained with the quinoline plus 50 μg. of ferrous or ferric ions. Ferrous iron alone doubled the rate of reduction of nitrate by the chicken liver dehydrogenase, and stimulated the reduction of nitrate 5-fold by the milk enzyme. Ferrous ions did not reduce nitrate in the absence of enzyme, would not substitute for hypoxanthine as a source of electrons for nitrate reduction by either enzyme, and did not overcome or protect against the semicarbazide inhibition.

DISCUSSION

The factors affecting the oxidation of DPNH by both the milk and chicken liver xanthine enzymes are so different from those affecting the oxidation of hypoxanthine, that two separate systems seem to be involved. The evidence can be summarized as follows: (a) both enzymes have little activity toward DPNH as compared with hypoxanthine; (b) DPNH can act for hypoxanthine as a substrate for nitrate reduction; (c) cyanide, 6-pteridyldaldehyde, and hydroxylamine all block the dehydrogenase activity of the enzyme with hypoxanthine as substrate, but have no effect on the oxidation of DPNH; (d) the aerobic oxidation of hypoxanthine by the milk enzyme is stimulated 50 per cent by menadione, while the corresponding oxidation of DPNH is stimulated much more; (f) p-chloromercuribenzoate in appropriate concentration inhibits the oxidation of hypoxanthine without affecting the oxidation of DPNH (8); (g) aldehydes compete with purines for an active site on rat liver xanthine oxidase, but DPNH does not (5). It is also interesting to note that the cyanide-treated enzyme is still capable of reacting with cytochrome c when DPNH is the substrate.

Whether these two separable systems are different enzymes or different portions of the same enzyme is not clearly established. Numerous flavoproteins can oxidize DPNH, and this would not be an unusual reaction for the xanthine enzymes. All three of the highly purified xanthine-oxidizing enzymes from milk, rat liver, and chicken liver retain some ability to oxidize DPNH, and it seems unlikely that a DPNH-oxidase contaminant would be carried along with these three enzymes from three different sources through three different purification procedures. When measured aerobically, the crystalline milk enzyme had about 1 per cent of the activity toward DPNH substrate that it exhibited toward hypoxanthine substrate, and it was about 3 per cent as active toward DPNH as hypoxanthine when both reactions were measured by dye reduction (10). However, the crystalline enzyme may contain some "inactive" flavoprotein. The evidence favors the concept of a single enzyme with two different active sites for hypoxanthine and DPNH, but it is hardly conclusive.

There are not only two different sites involved in the dehydrogenation of hypoxanthine and DPNH, but two different groups are also responsible for the transfer of electrons to oxygen. It is tempting to believe that one of the flavin groups in milk xanthine oxidase is associated with purine activation while the other flavin is responsible for the oxidation of DPNH; but it should be noted that the chicken liver enzyme carries out both oxidations in approximately the same way with only one flavin group.

The purine dehydrogenase activity of all three xanthine enzymes was blocked by cyanide, 6-pteridyldaldehyde, and hydroxylamine, and there could be no reduction of any of the electron acceptors. The other inhibitors were less consistent in their action. With the rat liver enzyme, both the chalcone and semicarbazide blocked all activities excepting the dehydrogenation of the substrate. With the milk enzyme, semicarbazide ap-
proached the category of a specific inhibitor of the oxidase group, since it had no effect on dye or cytochrome $c$ reduction and gave only partial inhibition of nitrate reduction.

The reduction of nitrate is thought to be a function of the molybdenum in the xanthine enzymes as well as in nitrate reductase (11). However, the stimulation of this reaction by the addition of 8-hydroxyquinoline or ferrous ions to milk xanthine oxidase and the inhibition of this reaction by semicarbazide may also implicate the enzymatic iron in this reaction. Wainwright (12) has described a nitrate reductase system from a coliform organism which requires menadione and ferrous ions as cofactors. Westerfeld et al. (13) found that the reduction of organic nitro groups by Aspergillus niger was independent of molybdenum, inhibited by iron chelators, and stimulated by ferrous ions.

SUMMARY

1. The oxidation of hypoxanthine by xanthine oxidase was independent of the oxidation of reduced diphosphopyridine nucleotide. Hydroxylamine, 6-pteridylaldehyde, and cyanide blocked the purine dehydrogenase group of milk xanthine oxidase and chicken liver xanthine dehydrogenase, but had little or no effect on the oxidation of reduced diphosphopyridine nucleotide substrate with any electron acceptor. Semicarbazide blocked the reaction with oxygen, when hypoxanthine was the substrate for the milk enzyme, but it had no effect on this reaction with reduced diphosphopyridine nucleotide as substrate.

2. 3,3',4,4'-Tetrahydroxychalcone and semicarbazide inhibited the reduction of oxygen, cytochrome $c$, and nitrate by rat liver xanthine oxidase with hypoxanthine, but had no effect on the dehydrogenation of the substrate and the reduction of methylene blue. With the milk enzyme, semicarbazide was a relatively specific inhibitor of the reaction with oxygen.

3. While the reduction of sodium molybdate by chicken liver xanthine dehydrogenase indirectly supports the catalytic function of Mo in nitrate reduction, a possible role of iron in this reaction was also indicated by the stimulation produced by 8-hydroxyquinoline, ferrous ions, or both together.

REFERENCES

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