The Reduction of Nicotinamide N-Oxide by Xanthine Oxidase*

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SUMMARY

The following evidence has led to the conclusion that xanthine oxidase, with its usual electron donors, is capable of reducing nicotinamide N-oxide to nicotinamide.

1. The purest enzyme fraction from hog liver which catalyzes the reduction has the spectrum of a metalloflavoprotein and has xanthine oxidase activity.

2. The enzyme responsible for the reduction cofractionates with hog liver xanthine oxidase.

3. Milk xanthine oxidase also catalyzes the reduction of nicotinamide N-oxide.

4. The electron donors for the reduction are known substrates of xanthine oxidase.

5. Both milk and liver xanthine oxidases exhibit separate pH optima for the reductions dependent on reduced diphosphopyridine nucleotide and xanthine.

6. Xanthine oxidase activity and nicotinamide N-oxide reduction activity are destroyed by heat at the same rate.

7. Each activity decreases to the same extent in the livers of rats maintained on protein-deficient diets.


It is proposed that xanthine oxidase might be responsible for other N-oxide reductions.

Some of the properties of the purified hog liver system which reduces nicotinamide N-oxide to nicotinamide were described in a recent communication (1). It was concluded that both reduced diphosphopyridine nucleotide and boiled supernant could serve as reductants in the reaction. Although these factors appeared to be involved in the expression of two distinct activities, there were several indications that the two activities might be associated with a single enzymatic unit. Recently, when it was found that xanthine could function as a reducing agent in the enzymatic reduction of nicotinamide N-oxide, the possibility arose that xanthine oxidase (EC 1.2.3.2) was responsible for both modes of nicotinamide N-oxide reduction. Evidence which supports this contention is presented in this paper.

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EXPERIMENTAL PROCEDURE

Substrates—Nicotinamide N-oxide was synthesized according to the method of Taylor and Crovetti (2). N-Methylnicotinamide iodide was synthesized according to the procedure of Holman and Wiegand (3). DPNH, xanthine, and hypoxanthine were purchased from Sigma. The preparation of boiled supernant has been described previously (1). Xanthine-8-14C was obtained from Calbiochem and nicotinamide-7-14C from New England Nuclear.

Enzymes—The hog liver enzyme catalyzes the reduction of nicotinamide N-oxide was purified through the carboxymethyl cellulose chromatographic step according to the procedure of Murray and Chaykin (1). In the experiments which follow it will be referred to as the CM-cellulose fraction. Milk xanthine oxidase was obtained from Worthington. Hog liver xanthine oxidase was prepared by the method of Murashige, McDaniel, and Chaykin (4). It was purified further by subjecting it to diethylaminoethyl cellulose chromatography according to the same procedure used in the purification of the enzyme which catalyzes the reduction of nicotinamide N-oxide (1). A crude xanthine oxidase preparation was obtained from rat liver as follows. Each gram of rat liver was homogenized with 3 ml of 0.01 M Tris buffer, pH 8, containing 0.14 M 2-mercaptoethanol. The homogenate was centrifuged for 30 min at 27,000 X g. The supernatant was decanted and passed through a Sephadex G-25 column which had been equilibrated with the Tris-mercaptoethanol buffer used in the homogenization procedure. The Sephadex eluate was used in those experiments which specify rat liver supernatant.

Assays—The assay procedure for the reduction of nicotinamide N-oxide has been described previously (1). The assay mixtures were effectively anaerobic since they contained buffer at pH 8.0 and 2-mercaptoethanol. When applicable the boiled supernant was replaced by 0.65 mmole of xanthine or by 0.75 mmole of N-methylnicotinamide iodide. Xanthine oxidase was prepared by the method of Murashige, McDaniel, and Chaykin (4). It was purified further by subjecting it to diethylaminoethyl cellulose chromatography according to the same procedure used in the purification of the enzyme which catalyzes the reduction of nicotinamide N-oxide (1). A crude xanthine oxidase preparation was obtained from rat liver as follows. Each gram of rat liver was homogenized with 3 ml of 0.01 M Tris buffer, pH 8, containing 0.14 M 2-mercaptoethanol. The homogenate was centrifuged for 30 min at 27,000 X g. The supernatant was decanted and passed through a Sephadex G-25 column which had been equilibrated with the Tris-mercaptoethanol buffer used in the homogenization procedure. The Sephadex eluate was used in those experiments which specify rat liver supernatant.

The Preparation of Boiled Supernatant

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measurements were made with a Zeiss PMQ II spectrophotometer and absorption spectra were recorded with a Cary model 14 spectrophotometer.

RESULTS

Early attempts to isolate the factor or factors in boiled supernatant which could serve as reducing agent or agents in the reduction of nicotinamide N-oxide invariably led to a separation of the activity into a number of different fractions. A variety of methods were used including paper chromatography, ion exchange chromatography, ethanol extraction, and barium precipitation; all gave the same result. The lack of promise shown by the isolation experiments prompted the examination of a number of oxidizable compounds for their ability to substitute for the boiled supernatant factor or factors. It was found that in the presence of 4 mM xanthine about 1.5 times as much nicotinamide was formed from nicotinamide N-oxide as was produced by saturating levels of boiled supernatant. Hypoxanthine could also replace boiled supernatant. These observations suggested that the 280-fold purified CM-cellulose fraction might contain xanthine oxidase, and that xanthine oxidase could be the enzyme involved in the reduction of nicotinamide N-oxide.

Xanthine Oxidase Activity—The presence of xanthine oxidase in that fraction of hog liver which is capable of nicotinamide N-oxide reduction was established by a variety of means. Spectral evidence for the conversion of xanthine to uric acid by the CM-cellulose fraction is presented in Fig. 1. The 270 mμ absorption band of xanthine disappeared upon incubation with the CM-cellulose fraction and the two bands characteristic of uric acid appeared at 230 and 290 mμ. Because xanthine oxidase has a characteristic metalloflavoprotein absorption spectrum in the 400- to 500-mμ region, the spectrum of the CM-cellulose fraction was examined. The absorption spectrum of the CM-cellulose fraction (Fig. 2) is similar to that of xanthine oxidase from hog (10), calf (11), or chicken (12) livers. It is not completely identical with the spectrum of hog liver xanthine oxidase, perhaps because the CM-cellulose fraction is not as highly purified. The state of purity of xanthine oxidase is indicated by its A 280 : A 450 ratio. This ratio was 11.6 for the CM-cellulose fraction. For comparison, the lowest A 280 : A 450 ratios reported for xanthine oxidase are about 5 (10, 13).

Murashige et al. (4) have recently shown that hog liver xanthine oxidase will oxidize N-methylnicotinamide to its 6-pyridone at pH 10. The CM-cellulose fraction will also oxidize N-methylnicotinamide at pH 10, but little, if at all, at pH 8. These data are not only indicative of the presence of xanthine oxidase but also of the absence of liver aldehyde oxidase which is able to oxidize N-methylnicotinamide at pH 7.8 (14).

The similarities in these properties of the xanthine oxidase activity and of nicotinamide N-oxide-reducing activity are compatible with the hypothesis that xanthine oxidase can catalyze nicotinamide N-oxide reduction.

Cofractionation—The presence of xanthine oxidase in the CM-cellulose fraction does not necessarily mean that it had cofractionated with the enzyme which reduces nicotinamide N-
oxide. However, it was reasoned that if one enzyme were responsible for both activities, then both activities should also cofractionate throughout a different series of purification steps designed to isolate xanthine oxidase. In order to evaluate this concept the various fractions obtained in the purification of hog liver xanthine oxidase were assayed for their capacity to reduce nicotinamide N-oxide. It is clear from a consideration of the data presented in Table I that both the DPNH- and boiled supernatant-dependent reducing activities fractionated with xanthine oxidase. The ratio of the xanthine oxidase activity to the boiled supernatant-dependent activity in the purer fractions was essentially constant. The ratios of xanthine oxidation to nicotinamide reduction found in both the CM-cellulose fraction and the xanthine oxidase isolated by the procedure of Murashige et al. were the same.

Even though the xanthine oxidase and the reductive activities remained associated, there was still the possibility that the co-fractionation resulted from unusually strong protein-protein interactions between two liver proteins, each possessing one of the two activities. Since it is unlikely that two such proteins would remain associated during the purification of xanthine oxidase from another source, milk xanthine oxidase was examined for its ability to reduce nicotinamide N-oxide. As would be expected if one enzyme catalyzed both xanthine oxidation and nicotinamide N-oxide reduction, purified milk xanthine oxidase was capable of reducing nicotinamide N-oxide with xanthine, DPNH, or boiled supernatant as electron donors. However, it should be mentioned that the milk xanthine oxidase and the liver xanthine oxidase were not identical. They differed in both pH optima (see below) and in their relative abilities to catalyze nicotinamide N-oxide reduction by boiled supernatant. When the capacity for DPNH-dependent reduction by the two enzymes was equal, the boiled supernatant-dependent reduction by milk xanthine oxidase was only 25% of the reduction by the liver enzyme.

### Table I

**Cofractionation of enzyme responsible for reduction of nicotinamide N-oxide and of hog liver xanthine oxidase**

The purification of xanthine oxidase was as described by Murashige et al. (4). The additional DEAE-cellulose chromatography step and the enzymic assays are described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Xanthine oxidation (A)</th>
<th>Nicotinamide N-oxide reduction</th>
<th>Ratio (X 10)^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.7</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Crude supernatant</td>
<td>2.4</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Heat-treated supernatant</td>
<td>0.008</td>
<td>2.1</td>
<td>0.02</td>
</tr>
<tr>
<td>50% (NH₄)₂SO₄ precipitate</td>
<td>0.017</td>
<td>14.6</td>
<td>2.7</td>
</tr>
<tr>
<td>35 to 45% acetone precipitate</td>
<td>0.038</td>
<td>21.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Alumina Cy eluate in 0.5 m phosphate</td>
<td>0.210</td>
<td>64.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Cs₂(PO₄)₂ gel eluate in 0.5 m phosphate</td>
<td>0.505</td>
<td>138.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Cs₂(PO₄)₂ gel eluate, heated at 70°C</td>
<td>0.79</td>
<td>210.8</td>
<td>2.7</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>1.45</td>
<td>473.0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Increase in optical density at 295 mμ.

**Minor criterion used earlier to establish the identity of uric acid was chromatographed in a 1-butanol-acetic acid-water (4:1:1, v/v) solvent. The R_f values of uric acid, xanthine, nicotinamide N-oxide, and xanthine were 0.17, 0.29, 0.45, and 0.70, respectively. The uric acid band appeared only when both xanthine and nicotinamide N-oxide were present. Such an experiment was carried out with xanthine-8-'⁴C and nicotinamide N-oxide-7-'⁴C of known specific activities. Determination of the radioactivity associated with the products indicated that uric acid and nicotinamide were produced in equimolar amounts, 2.16 and 2.37 μmoles, respectively. Furthermore, as might be predicted from the hypothesis that xanthine oxidase catalyzes the reduction of nicotinamide N-oxide, hypoxanthine reduced 2 eq of nicotinamide N-oxide just as it reduces 2 eq of oxygen during its two-step conversion to uric acid.

Since the boiled supernatant activity could not be attributed to either DPN or DPNH (1), it was possible that hypoxanthine and xanthine might be responsible. Milk xanthine oxidase was used to assay the boiled supernatant for these substrates. Their presence was inferred from a significant increase in radioactivity at 295 mμ which presumably accompanied their oxidation to uric acid.

**pH Optima**—Further support for the idea that xanthine is one of the active principles of boiled supernatant was obtained from the pH dependence of the nicotinamide N-oxide reductions. The DPNH-dependent reduction of nicotinamide N-oxide by the CM-cellulose fraction has an optimum at pH 6.5 and the boiled supernatant-dependent reduction has an optimum at about pH 8 (1). If xanthine is partially responsible for the boiled supernatant-dependent reaction, it would be expected that its pH optimum would be similar when either cofactor was used. When xanthine was substituted for boiled supernatant, the pH optimum was 8.7 (Fig. 3). The fact that the liver nicotinamide N-oxide reduction has two distinct pH optima served as another means for equating xanthine oxidase with the nicotinamide N-oxide reduction activity. Thus, if nicotinamide N-oxide reduction were a general property of xanthine oxidases, milk xanthine oxidase should also display two pH optima for the xanthine- and DPNH-dependent reduction reactions. Milk xanthine oxidase was found to possess the expected dual pH optima when xanthine and DPNH were used as electron donors for nicotinamide N-oxide reduction (Fig. 4). The pH optima of the milk enzyme were 0.5 to 1.0 pH unit higher than those of the liver enzyme; an observation which is explicable, perhaps, by the different origins of the two enzymes.

**Heat Denaturation**—The similar rates of the heat inactivation of the xanthine oxidase activity and of nicotinamide N-oxide reduction give further support to the idea that they are associated with a single protein (Fig. 5). The DPNH-dependent activity, which was lost at the same rate as the boiled supernatant activity when the enzyme was incubated at 70°C, pH 8 (1), was also lost.

**Substrates in Common**—DPNH, xanthine, hypoxanthine, and N-methylnicotinamide, which are all known to be oxidized by xanthine oxidase with oxygen as the terminal electron acceptor, were also oxidized by the CM-cellulose fraction or xanthine oxidase with nicotinamide N-oxide as the terminal electron acceptor. The identity of the product of xanthine oxidation by the CM-cellulose fraction with the N-oxide as electron acceptor was shown to be uric acid by paper chromatography. The spectral criterion used earlier to establish the identity of uric acid was not applied in this case because of the interfering absorption of nicotinamide N-oxide. The deproteinized reaction mixture was chromatographed in a 1-butanol-acetic acid-water (4:1:1, v/v) solvent. The R_f values of uric acid, xanthine, nicotinamide N-oxide, and nicotinamide were 0.17, 0.29, 0.45, and 0.70, respectively. The uric acid band appeared only when both xanthine and nicotinamide N-oxide were present. Such an experiment was carried out with xanthine-8-'⁴C and nicotinamide N-oxide-7-'⁴C of known specific activities. Determination of the radioactivity associated with the products indicated that uric acid and nicotinamide were produced in equimolar amounts, 2.16 and 2.37 μmoles, respectively. Furthermore, as might be predicted from the hypothesis that xanthine oxidase catalyzes the reduction of nicotinamide N-oxide, hypoxanthine reduced 2 eq of nicotinamide N-oxide just as it reduces 2 eq of oxygen during its two-step conversion to uric acid.

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at the same rate as were the xanthine-dependent activity and the xanthine oxidase activity under the same conditions.

**Cyanide Inhibition**—The inhibition by cyanide showed that there was a distinct relationship between the two types of activity as well. After a 5-min preincubation of the CM-cellulose fraction with various concentrations of cyanide, both the xan-

![Fig. 3](image-url)

**Fig. 3.** Xanthine-dependent reduction as a function of pH. The Tris-phosphate buffer which was used had a final concentration of 0.25 M for each salt. It was substituted for the buffers specified for the assay procedures described in the "Experimental Procedure."

![Fig. 4](image-url)

**Fig. 4.** The pH dependence of nicotinamide N-oxide reduction by milk xanthine oxidase. The Tris-phosphate (○) and Tris-phosphate-carbonate (×) buffers which were used had a final concentration of 0.25 M for each salt. They replaced the individual buffers in the assay procedures described in the "Experimental Procedure." ——, DPNH activity; ——, xanthine activity.

![Fig. 5](image-url)

**Fig. 5.** The heat inactivation of xanthine oxidase and of the DPNH- and xanthine-dependent reduction activities. The DEAE-cellulose eluate, 3.8 mg per ml, was incubated at 70° in 0.01 M Tris, pH 8, which was 0.14 M in 2-mercaptoethanol. At the times indicated, samples were removed and assayed as described in the "Experimental Procedure." ---●---●, DPNH activity; ×---X, xanthine activity; ○—○, xanthine oxidase activity.

![Fig. 6](image-url)

**Fig. 6.** The inhibition of the xanthine oxidase activity and the xanthine-dependent reduction of nicotinamide N-oxide by cyanide. The CM-cellulose fraction was preincubated for 5 min with the amount of cyanide indicated in the figure and then assayed for activity according to the assay procedures described in the "Experimental Procedure." ×---X, xanthine activity; ○---○, xanthine oxidase activity; the difference between the two activities, ●—●.

At the same time as were the xanthine-dependent activity and the xanthine oxidase activity under the same conditions.

**Cyanide Inhibition**—The inhibition by cyanide showed that there was a distinct relationship between the two types of activity as well. After a 5-min preincubation of the CM-cellulose fraction with various concentrations of cyanide, both the xanthine-dependent activity and the xanthine oxidase activity were inhibited in a parallel fashion (Fig. 6).

**Effect of 2-Mercaptoethanol**—It was reported previously that both the boiled supernatant- and the DPNH-dependent reduction of nicotinamide N-oxide required 2-mercaptoethanol for maximal activity (1). Although xanthine oxidase contains sensitive sulfhydryl groups (10, 15, 16), the presence of a sulfhydryl reagent is not normally required for activity. The relatively high levels of mercaptoethanol which were necessary for the maximal expression of the nicotinamide N-oxide-reducing activities appeared to be required in order to effect anaerobiosis. As
shown in Table II, anaerobic conditions stimulated the boiled supernatant activity to the same extent as did 2-mercaptoethanol. Anaerobiosis accounted for at least 60% of the stimulation of the DPNH activity by mercaptoethanol. Two other observations also indicated that the 2-mercaptoethanol does produce anaerobiosis. When the Tris buffer containing 0.14 M 2-mercaptoethanol was tested with the oxygen electrode no oxygen could be detected. The xanthine oxidase reaction was used to check the operation of the oxygen electrode both before and after these measurements. Another index of the anaerobic state of the Tris-mercaptoethanol buffer was its ability to maintain FAD in the reduced state for at least an hour. FADH₂ was rapidly oxidized in buffer solutions without 2-mercaptoethanol. The conclusion that anaerobiosis was the main reason for mercaptoethanol stimulation was plausible because oxygen and nicotinamide N-oxide might be expected to compete as electron acceptors. Although oxygen appeared to antagonize the N-oxide reduction, concentrations of up to 6.2 mm nicotinamide N-oxide did not inhibit the utilization of oxygen by xanthine oxidase.

Dietary Effects—Several groups of workers have shown that xanthine oxidase activity in rat liver is a good indicator of the amount and the nutritional value of dietary protein (17–19). This property was used as another criterion for demonstrating the relationship between the enzyme responsible for xanthine oxidation and the enzyme responsible for nicotinamide N-oxide reduction. Xanthine oxidase and the nicotinamide N-oxide reduction activities were compared in rat livers from animals which had been maintained for 4 days on the protein-free or 90% protein diets described by Freedland (20). The protein-free diets contained glucose in place of the 90% casein component of the high protein diets. In each case, rat liver supernatant was prepared as described in “Experimental Procedure.” It was assayed for xanthine oxidase activity and for DPNH, boiled supernatant, and xanthine-dependent nicotinamide N-oxide reduction. The results, which are summarized in Table III, show that the maintenance of rats on a protein-free diet caused all the activities to decrease to the same extent.

### TABLE II

**Effects of 2-mercaptoethanol and anaerobiosis**

The standard assay was performed with the CM-cellulose fraction which had been passed through Sephadex G-25 to remove mercaptoethanol. The Sephadex G-25 column was equilibrated and developed with 0.01 M Tris pH 8.0. The anaerobic experiments were carried out in Thunberg tubes with a helium atmosphere.

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Nicotinamide formed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPNH activity</td>
<td>Boiled supernatant activity</td>
</tr>
<tr>
<td>Complete</td>
<td>136.1</td>
<td>58.2</td>
</tr>
<tr>
<td>2-Mercaptoethanol omitted</td>
<td>84.4</td>
<td>19.7</td>
</tr>
<tr>
<td>2-Mercaptoethanol omitted; anaerobic conditions</td>
<td>115.6</td>
<td>62.7</td>
</tr>
</tbody>
</table>

### TABLE III

**Effect of diet on rat liver xanthine oxidase**

Rats were maintained for 4 days on a protein-free diet or a 90% protein diet. Liver supernatant was prepared from the rat livers and assayed by the methods described in “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Activity</th>
<th>High protein diet</th>
<th>Protein-free diet</th>
<th>Decrease in activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidation</td>
<td>0.036</td>
<td>0.013</td>
<td>64</td>
</tr>
<tr>
<td>Nicotinamide N-oxide reduction</td>
<td>55.7</td>
<td>19.3</td>
<td>66</td>
</tr>
<tr>
<td>DPNH-dependent</td>
<td>58.8</td>
<td>20.5</td>
<td>65</td>
</tr>
<tr>
<td>Boiled supernatant-dependent</td>
<td>25.8</td>
<td>7.0</td>
<td>73</td>
</tr>
</tbody>
</table>

* The rates of xanthine oxidation at 38° are expressed as the change in optical density at 250 μm per min.

* The rates of nicotinamide N-oxide reduction are expressed as micromoles of nicotinamide formed per 10 min.

**DISCUSSION**

The identity of xanthine oxidase and the enzyme responsible for the reduction of nicotinamide N-oxide is compatible with previous published work on the reductive activity (1) and with the known properties of xanthine oxidase. Xanthine oxidase exhibits a relatively wide temperature range (21), product inhibition (19), and variable pH optima depending on the substrates used (14). These same properties are also characteristic of the nicotinamide N-oxide reduction system. The slight variation in the ratio of the boiled supernatant- and DPNH-dependent activities has a parallel in the properties of xanthine oxidase as well. During the controversy over the identity of Schardinger's enzyme and xanthine oxidase, one of the main arguments for the nonidentity of the two was variation in the ratio of rate of aldehyde oxidation by the former to that of xanthine oxidation by the latter. Booth (22, 23) presented strong evidence that this argument, as well as others favoring nonidentity, were unsound and that, in fact, xanthine oxidase could oxidize both aldehydes and purines. He went on to imply that xanthine oxidase had multiple sites which could be affected differentially. The multiple sites and lack of specificity in xanthine oxidase have since been generally accepted. With the knowledge that different sites of xanthine oxidase can be affected separately, the varying ratio of the boiled supernatant- and DPNH-dependent activities is not incongruous with a single enzyme being responsible for both. The differential behavior of the two activities at low cytochrome concentration (Fig. 6) could also be interpreted on these grounds; that is, two different sites might not be inhibited equally.

The relative lack of substrate specificity of xanthine oxidase applies to both its electron acceptors and its electron donors (19, 24). Therefore, it is reasonable to believe that nicotinamide N-oxide deoxidizes xanthine oxidase just as oxygen, cytochrome c, and various dyes do. Furthermore, it is quite significant that the same compounds, DPNH, xanthine, hypoxanthine, and N-methyl nicotinamide, act as electron donors for xanthine oxidase and for nicotinamide N-oxide reduction. This plurality of substrates also explains the earlier difficulties in identifying the boiled supernatant cofactor or cofactors. It is presumed that several compounds in the boiled supernatant can act as electron donors.

Although the heat inactivation experiment showed a parallel loss of all activities at 70° (Fig. 5), there was evidence of individuality in the active sites during the first few minutes of the heat treatment. Nicotinamide N-oxide reduction was constant during the first 2 to 3 min of heating. The xanthine oxidase activity, on the other hand, was activated. This effect might be compared to the properties of the action of Antabuse on rat liver xanthine oxidase (25). Antabuse can inhibit the autoxidation...
of the enzyme while leaving the dehydrogenase activity unaffected. This sensitivity to Antabuse can be lifted by heating the enzyme to 56° for 5 min. In a similar manner, the nicotinamide N-oxide reduction, which is independent of oxygen (Table II), and the xanthine oxidase activity, which involves an autoxidation step, were affected differentially by heat. The reduction of nicotinamide N-oxide behaved as a dehydrogenase activity; that is, it was not activated by heat under the same conditions and at the same time as the xanthine oxidase activity was activated. A similar heat effect was observed with an endonuclease which catalyzes the hydrolysis of DNA and RNA (26). The rate of hydrolysis of DNA was constant and the rate of hydrolysis of RNA was activated before the parallel heat inactivation of both began.

The foregoing results imply that oxygen and nicotinamide N-oxide act at different sites. This conclusion is not incompatible with the observation that oxygen partially inhibits nicotinamide N-oxide reduction. A higher affinity of oxygen would still give it an advantage over nicotinamide N-oxide because they still compete for the reducing agent even if not for the same site.

In spite of the differences in the enzymatic sites, several observations strongly suggest that xanthine oxidase is responsible for the reduction of nicotinamide N-oxide. Both activities, nicotinamide N-oxide reduction and xanthine oxidation, remain associated during the purification of the enzyme by three different procedures and from two different sources. Milk xanthine oxidase exhibits the same binary pH optima for the two types of nicotinamide N-oxide reduction as the hog liver enzyme does. All the activities have the same stability to heat, and there is an amazing similarity in the substrates for both reaction types. Lastly, when rats are maintained on protein-deficient diets both xanthine oxidase activity and the capacity to reduce nicotinamide N-oxide decrease to the same extent.

In concluding that xanthine oxidase catalyzes the reduction of nicotinamide N-oxide, it is conceivable that this widely distributed enzyme is also responsible for other N-oxide reductions such as those observed in rats (27, 28), bacteria (29), and yeast (30). It is possible that N-oxide compounds represent a unique class of oxidants for reactions catalyzed by xanthine oxidase, a class which is distinguished by virtue of the unusual nitrogen-oxygen bond cleavage involved in the oxidative process.

One of our reasons for investigating the enzymatic reduction of nicotinamide N-oxide was the proposed involvement of this compound in biological oxidations which result in the incorporation of oxygen into the product (31). Xanthine oxidase has been shown to utilize a variety of oxidizing agents as electron acceptors with water serving as oxygen donor, when a donor is required. It would appear likely that nicotinamide N-oxide interacts with xanthine oxidase as an electron acceptor as do the other oxidizing agents. This is certainly the case when DPNH is the electron donor. Despite the likelihood that water is the origin of the oxygen atom introduced into xanthine upon its oxidation with nicotinamide N-oxide, the origin of that oxygen atom is still unknown. The question which led to this investigation of nicotinamide N-oxide reduction, i.e. whether or not oxygen can be directly transferred from nicotinamide N-oxide to another organic molecule, also remains unanswered.

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