The Enzymatic Reduction of Nicotinamide N-Oxide*

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SUMMARY

An enzymatic system which reduces nicotinamide N-oxide to nicotinamide was isolated from hog liver and purified about 280-fold. The reduction is dependent on the presence of reduced diphenophosphorylamine nucleotide or a low molecular weight constituent found in boiled liver supernatant. The two cofactors appear to be involved in the expression of two distinct enzymatic activities. Evidence has been presented which indicates both activities may be associated with a single protein or enzymatic unit. The DPNH-dependent reaction is the following.

\[
H^+ + DPNH + \text{nicotinamide N-oxide} \rightarrow DPN^+ + \text{nicotinamide} + H_2O
\]

The nature of the supernatant-dependent reaction is not completely understood, since the identity of the compound which is oxidized in the course of reduction of the N-oxide is not known.

Recently, nicotinamide N-oxide has been recognized as an excretory product of nicotinamide in the mouse (1, 2) and the hog. The oxidation of nicotinamide to nicotinamide N-oxide has been demonstrated in vitro with use of the microsomal fraction of livers from rats, mice, and rabbits (3). The biological reduction of various N-oxide compounds has also been reported, although very little is known about the enzyme, or enzymes, involved. May (4) demonstrated the reduction of pyridine N-oxide to pyridine by fermenting yeast. Dunn, Maguire, and Brown (5) administered adenine 1-N-oxide-8-14C to rats and found radioactivity in a number of metabolites, including adenine, guanine, and pyridine nucleotides. These compounds, as well as several radioactive compounds found in urine, lacked the N-oxide group. After intravenous administration of nicotinic acid N-oxide to rats, Tatsumi and Kanamitsu (6) detected nicotinic acid in the urine. They were able to demonstrate the reduction of nicotinic acid N-oxide in perfused rabbit liver, but were unable to do so with rat liver (7). In other studies (8), they found that nicotinic acid-requiring strains of Lactobacillus arabinosus and Escherichia coli could use nicotinic acid N-oxide instead of nicotinic acid, but with somewhat reduced efficiency.

While studying the role of nicotinamide in the squalene oxidocyclase system, Chaykin and Bloch (9) noted that nicotinamide N-oxide occasionally produced a marked stimulation of the oxidocyclase activity. They demonstrated not only that rats formed nicotinamide N-oxide in vivo, but also that hog liver homogenates catalyzed its reduction to nicotinamide. On the basis of these observations Chaykin and Bloch proposed the involvement of nicotinamide N-oxide in biological oxidations. As part of a study designed to evaluate this hypothesis, an active fraction from the hog liver system which produced nicotinamide N-oxide has been partially purified, and some of its properties have been examined.

EXPERIMENTAL PROCEDURE

Materials and Methods—Hog liver was obtained at the local abattoir and stored frozen until used. DEAE-cellulose and carboxymethyl cellulose were purchased from Gallard-Schlesinger and Bio-Rad, respectively. Crystalline alcohol dehydrogenase was the product of Sigma. Sephadex G-25 was purchased from Pharmacia. Nicotinamide-7-14C (42.1 mCi per mmole) was purchased from the New England Nuclear Corporation. The N-oxides of nicotinamide and nicotinic acid were synthesized according to the procedure of Taylor and Crovetti (10).

Spectrophotometric measurements were made with a Zeiss PMQ II spectrophotometer. Nicotinamide and nicotinic acid were determined by the cyanogen bromide method of Mueller and Fox (11) in a 5.0-ml reaction volume. Neither the N-oxide of nicotinamide nor that of nicotinic acid interfered with the assay of the parent compounds by the cyanogen bromide method. Radioactivity was measured with a Vanguard paper chromatogram counter, a Nuclear-Chicago low background counter, or a Packard Tri-Carb liquid scintillation counter. For the last instrument, the scintillation solvent that was used contained 6 g of 2,5-diphenyloxazole (PPO), and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethylPOPOP) per liter of toluene.

Protein was determined by the biuret method (12) or the A280:A410 method (13). Because 2-mercaptoethanol interfered with the biuret method, protein was first precipitated from mercaptoethanol-containing solutions with trichloroacetic acid. The precipitated protein was recovered by centrifugation and redis-
solved in 3% NaOH (w/v) before being subjected to the biuret
determination.

Boiled supernatant was prepared by homogenizing 33 g of
frozen hog liver with 100 ml of deionized water and then heating
the homogenate for 15 min in a boiling water bath. Precipitated
protein was removed by centrifugation for 10 min at 27,000 × g.
The supernatant was heated in a boiling water bath for an addi-
tional 10 min, centrifuged, and lyophilized. The residue was
stored in the freezer. It was dissolved in deionized water (0.25 g
per ml) when needed.

Enzyme Assay—In the assay procedure, the nicotinamide
N-oxide-dependent formation of nicotinamide was measured.
One unit of activity was defined as that amount of enzyme which
catalyzed the formation of 1.0 µg of nicotinamide in 10 min at
50°. Unless otherwise noted, the standard incubation mixture
(0.5 ml) contained 5.43 µmoles of nicotinamide N-oxide, 0.65
µmole of DPNH, 100 µmoles of potassium phosphate (pH 6.5),
and 35 µmoles of 2-mercaptoethanol; or the DPNH was replaced
by 0.05 ml of boiled supernatant, and the potassium phosphate
was replaced by 100 µmoles of Tris, pH 8.0. The size of the
reaction mixture was doubled when a larger sample was needed
for the nicotinamide assay. Either the enzyme or substrate was
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The enzyme could be further purified by chromatography on carboxymethyl cellulose. In preparation for this step, 0.01 M citrate, pH 5.5, was substituted for the 0.01 M Tris buffer when the ammonium sulfate-precipitated DEAE-cellulose fractions were redissolved and desalted. Ordinarily, two preparations were carried through DEAE cellulose chromatography and then combined and applied to a carboxymethyl cellulose column. A column (1.3 x 14 cm) of carboxymethyl cellulose-KCl which had been equilibrated with the citrate buffer was used. The activity was eluted with a linear salt gradient, 0 to 0.25 M KCl in the pH 5.5 citrate buffer in a total volume of 200 ml (Fig. 2); 3 ml-fractions were collected at the rate of 1 ml per min. The pH of the buffer with which the column was equilibrated was critical because the activity was not adsorbed at pH 5.8 or higher. The activity was eluted at a KCl concentration of 0.1 M. The active fractions were pooled and concentrated in the same manner as the DEAE-cellulose fractions. The precipitated protein was redissolved in 0.01 M Tris, pH 8, and freed of salt as described previously.

Cofactors—DPNH was found to stimulate the activity in enzyme fractions up to the ammonium sulfate fraction, but it was not an absolute requirement. On the other hand, after Sephadex treatment, no activity could be demonstrated unless DPNH was added. Even with excess DPNH, boiled supernatant was needed to obtain complete recovery of activity. It was possible to show that the boiled supernatant was not acting as an additional source of DPNH. Boiled supernatant which had been titrated to pH 2 in order to destroy DPNH was, after neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material. Mild treatment with base (pH 10.5, 3 min in boiling water, and neutralization, as active as the untreated material.

Boiled supernatant was also capable of supporting nicotinamide N-oxide reduction without the addition of DPNH. Further, it appeared that the boiled supernatant cofactor, or cofactors, was consumed during the reaction rather than acting catalytically; that is, after the rate of nicotinamide formation had decreased significantly, the addition of more boiled supernatant increased the rate of nicotinamide formation. Nevertheless, at saturating levels of boiled supernatant, DPNH stimulated the activity further. The sum of the DPNH-dependent activity and the boiled supernatant activity was slightly higher than the activity obtained in the presence of both, presumably owing to some degree of competition for an enzyme site or substrate. Since the competition was of minor proportions, it was concluded that the two cofactors functioned independently. Therefore, in order to obtain the purification data in Table II, all cofactors were removed from the various protein fractions by chromatography on Sephadex prior to assay, and all of the fractions were assayed in the presence of either DPNH or boiled supernatant.

The identity of the boiled supernatant factor, or factors, is not known. Its properties are as follows. It was adsorbed to activated charcoal but could not be efficiently eluted by either 0.03% or 0.1% NH₄OH in 50% ethanol. After 2 hours at room temperature in 1.0 M HCl or 1.0 M NaOH, 58% and 82%, respectively, of the activity remained. The stimulatory activity seemed to fractionate when the boiled supernatant was chromatographed on paper, Dowex 1, and Dowex 50; and when the lyophilized material was extracted with ethanol. It could not be extracted by ether and was not precipitated by barium ion. Ashed, boiled supernatant did not support the reduction, indicating that the activator, or activators, is an organic compound.

FIG. 2. Distribution of the DPNH- and boiled supernatant-dependent activities on carboxymethyl cellulose. The preparation of the column and the conditions of elution are described in "Results." The fractions were assayed as described in "Experimental Procedure." • • •, DPNH activity; O—O, boiled supernatant activity; — — , optical density at 280 μμ..

TABLE I

<table>
<thead>
<tr>
<th>Assay mixture</th>
<th>Nicotinamide (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>1.4</td>
</tr>
<tr>
<td>+ DPNH</td>
<td>0.8</td>
</tr>
<tr>
<td>+ base-treated boiled supernatant</td>
<td>18.0</td>
</tr>
<tr>
<td>+ boiled supernatant</td>
<td>47.2</td>
</tr>
<tr>
<td>+ acid-treated boiled supernatant</td>
<td>48.2</td>
</tr>
<tr>
<td>+ boiled supernatant</td>
<td>36.1</td>
</tr>
<tr>
<td>+ base-treated boiled supernatant</td>
<td>32.0</td>
</tr>
</tbody>
</table>
TABLE II

Purification of enzyme from hog liver homogenate

Details of the successive steps are described in "Results." The DPNH activity was measured at pH 6.5; the boiled supernatant activity, at pH 8.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>DPNH activity</th>
<th>DPNH total activity</th>
<th>DPNH specific activity</th>
<th>Ratio of DPNH activity to boiled supernatant activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>ml</td>
<td>units/ml</td>
<td>units X 10^2</td>
<td>units/mg</td>
<td></td>
</tr>
<tr>
<td>27,000 X g supernatant</td>
<td>470</td>
<td>107</td>
<td>5.02</td>
<td>1.8</td>
<td>0.85</td>
</tr>
<tr>
<td>pH 5 supernatant</td>
<td>444</td>
<td>48</td>
<td>2.11</td>
<td>2.3</td>
<td>0.64</td>
</tr>
<tr>
<td>60° heat-treated</td>
<td>397</td>
<td>48</td>
<td>1.84</td>
<td>4.4</td>
<td>0.70</td>
</tr>
<tr>
<td>0.40 to 0.55 (NH₄)SO₄</td>
<td>45</td>
<td>372</td>
<td>1.07</td>
<td>12.0</td>
<td>1.38</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>17.5</td>
<td>475</td>
<td>0.83</td>
<td>52.2</td>
<td>1.67</td>
</tr>
<tr>
<td>(after (NH₄)SO₄ concentration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM-cellulose eluate</td>
<td>4.8</td>
<td>977</td>
<td>0.47</td>
<td>276.7</td>
<td>1.69</td>
</tr>
</tbody>
</table>

* The homogenate could not be chromatographed on Sephadex to remove endogenous cofactors.

In addition, it was probably of low molecular weight since it was retained on Sephadex G-25 and was active after boiling the solution. Studies directed toward its identification are still in progress. Several known cofactors have been tested for activity: ATP (7.3 X 10⁻⁴ M), TPN (5 X 10⁻⁴ M), DPN (10⁻³ M), FAD (2 X 10⁻⁴ M), GSH (10⁻³ M), dihydrodipyridine (10⁻² M), and ascorbate (2 X 10⁻² M) were inactive. TPNH was only 40% as effective as DPNH.

The effect of 2-mercaptoethanol is not fully understood. Alone, it did not support the reduction. However, it had a definite stimulatory effect which favored the boiled supernatant activity more than the DPNH-dependent activity. When the concentrated DEAE-cellulose fraction was passed through Sephadex G-25 to remove the 2-mercaptoethanol and then assayed in the absence of 2-mercaptoethanol, 80% of the DPNH activity was retained, while only 32% of the boiled supernatant activity remained. Virtually all of the activity was regained when 2-mercaptoethanol (8.4 X 10⁻⁵ M to 1.4 X 10⁻⁴ M, final concentration) was added to the reaction mixture.

Ratios of DPNH and Boiled Supernatant Activities — The ratio of the DPNH activity to the boiled supernatant activity varied about 1 unit within or between different enzyme preparations, although it was not necessarily consistent as might be inferred from the data in Table II. Of particular interest in this regard are the DEAE-cellulose and carboxymethyl cellulose chromatograms although not evident in Fig. 1. However, these same fractions, after concentration, had a different ratio at the pH of optimal activity (Fig. 3).

pH Optima — Both the DPNH- and boiled supernatant-dependent activities were studied as a function of pH (Fig. 3). The pH optimum of the DPNH-dependent activity fell in the range 6.4 to 6.8, whereas the pH optimum of the boiled supernatant-dependent activity fell in the range 7.8 to 8.2. The individual activities were assayed throughout this work at their pH optima, 6.5 and 8, respectively.

Effect of Temperature — The dependence of initial rate upon the temperature of incubation for both activities is shown in Fig. 4. The residual activity observed above 70° probably reflected limited action of the enzyme during the time course of its inactivation. This suppression is born out by the data in Fig. 5, which suggest that the two activities were lost at the same rate when the enzyme was incubated at 70° and pH 8. As a result of the increased activity observed at temperatures above 37° (Fig. 4), it was decided that the sensitivity of the assay procedure would benefit by the adoption of 50° as the incubation temperature for routine assays.

Identification of Product of N-Oxide Reduction — In order to confirm the observation that the nicotinamide produced during the reduction was formed from its N-oxide, nicotinamide N-oxide-7,3C was used in the standard assay procedure. Controls were run with boiled enzyme. The incubation was terminated by heating the reaction vessels in a boiling water bath, rather than with trichloroacetic acid; the denatured protein was removed by centrifugation, and the supernatant was applied to 1.5 inch Whatman No. 1 paper strips. These were chromatographed in chromagrams although not evident in Fig. 1. However, these same fractions, after concentration, had a different ratio at the pH of optimal activity (Fig. 3).
I I I I I t I I 1 I

TEMPERATURE (°C)

FIG. 4. The effect of temperature on the rate of the DPNH- and the boiled supernatant-dependent reactions. The reaction mixtures were prepared as described in “Experimental Procedure” and were incubated at the temperatures indicated. O---O, boiled supernatant activity; O---O, DPNH activity.

I I I I I I I

TIME at 70° (minutes)

FIG. 5. The parallel heat inactivation of DPNH- and boiled supernatant-dependent activities. The DEAE-cellulose eluate, after (NH₄)₂SO₄ concentration, was incubated at 70° in 0.01 M Tris buffer which was 0.14 M in 2-mercaptoethanol, pH 8.0. At the times indicated, samples were removed, added to reaction vessels, and kept on ice to be assayed as described in “Experimental Procedure.” O---O, boiled supernatant activity; O---O, DPNH activity.

1-butanol saturated with water (1, 2). The radioactive component with a Rₚₙ identical with nicotinamide was eluted from the strips. It was the only radioactive material which was formed in the course of the reaction. The nicotinamide content of an aliquot of the eluate was determined calorimetrically with the cyanogen bromide procedure, and a second aliquot was counted in the scintillation counter with Bray's scintillation solvent (14).

The specific activity of the product, nicotinamide, was the same as that of the substrate, nicotinamide N-oxide. Verification of the identity of the product as nicotinamide was obtained when the radioactive material was recrystallized with authentic nicotinamide through three recrystallizations. Similar results were obtained for both the boiled supernatant and the DPNH-dependent activities.

**Stoichiometry**—With the evidence that the nicotinamide produced in the reaction had arisen entirely from the nicotinamide N-oxide and that nicotinamide is the only product of the reaction, it was possible to establish a stoichiometric relationship for the reactants involved in the DPNH-dependent reduction. Fig. 6 shows that DPNH oxidation and the nicotinamide formation are identical in both amount and rate.

By means of the alcohol dehydrogenase and the cyanogen bromide assay procedures, it was also possible to show that equivalent amounts of DPN and nicotinamide are formed in the reactions. Samples taken after 10 min of reaction showed that 115 mμmoles of DPN and 121 mμmoles of nicotinamide had been formed. Therefore, the DPNH-dependent reaction can be written as shown in Scheme 1.

**Substrate Specificity and Affinity**—For both activities, nicotinic acid N-oxide was as effective a substrate as nicotinamide N-oxide. The Kₘ for nicotinamide N-oxide in the DPNH-dependent reaction was 2 × 10⁻³ M and about 5 × 10⁻³ M for the boiled supernatant-dependent reaction.

**Product Inhibition**—By the use of nicotinamide N-oxide-7-¹⁴C in the standard assay, it was possible to demonstrate a product inhibition by nicotinamide for both activities. The radioactive substrate was incubated with various amounts of nicotinamide. At the termination of the reaction, a 100-μl aliquot of the reaction supernatant was chromatographed in the water-saturated 1-butanol solvent. The portions of the chromatograms containing nicotinamide were cut out and counted in the scintillation counter. The results presented in Table III show that decreasing amounts of nicotinamide N-oxide were reduced in the presence

![.Scheme 1](https://example.com/scheme1.png)

**Scheme 1**
tinamide had been added. The product was isolated as described in "Results" and counted.

DPNH oxidation was measured at 340 mp; as the amount of nicotinamide was increased, the rate of DPNH oxidation decreased.

The physiological significance of the reduction of nicotinamide N-oxide in rat liver homogenates (7) is not at present understood. In the case of the DPNH dependent activity, at least, the nature of the reaction has been elucidated. That is, the coordinately linked oxygen can oxidize DPNH, with the oxygen presumably becoming reduced to water.

of increasing amounts of nicotinamide. Similar results were obtained for the DPNH-dependent activity when the rate of DPNH oxidation was measured at 340 mp; as the amount of nicotinamide was increased, the rate of DPNH oxidation decreased.

**DISCUSSION**

The DPNH-dependent activity and the boiled supernatant activity seem to operate through independent mechanisms. In particular, the two activities can be additive, and each displays its own pH optimum. The varying ratio of the two activities—the potassium stimulation, which affects only the DPNH activity, and the mercaptoethanol stimulation, which favors the boiled supernatant activity—also indicates two distinct processes. However, the cochromatography on both DEAE- and CM-celluloses and the similar effects of incubation temperature, as well as the parallel heat inactivation of the two activities, suggest a single enzymatic unit. Systems are known (e.g. Reference 15) in which a single enzyme exhibits separate pH optima for separate activities. Further, the variation in the ratio of the two activities, which might be interpreted as being indicative of two separate proteins having the two activities, did not exceed 1.1. Although no completely satisfactory explanation for this phenomenon is available, the variation in ratio could be attributed, in part, to the changing concentration of 2-mercaptoethanol, since the 2-mercaptoethanol was constantly undergoing oxidation, particularly at basic pH values. The ratio might also tend to vary because 2-mercaptoethanol affected the two activities differently. At present, then, it seems that the evidence more strongly favors a single enzymatic unit, rather than two distinct enzymes, as being responsible for the two activities.

Tatsumi and Kanamitsu have studied the reduction of nicotinic acid N-oxide and nicotinamide N-oxide in rat liver homogenates (7). In contrast to the hog liver system, they found that the partially active supernatant fraction had to be combined with the mitochondrial fraction in order to obtain the full activity originally exhibited by the unfractionated homogenate. The rat liver system was similar to the hog liver system in that the enzymatic activity of the combined fractions was dependent on the presence of a dialyzable factor, or factors. However, the compounds which could substitute for the dialyzable factor, or factors, in the two systems differed somewhat. Whereas ATP, ADP, DPNH, and DPN were active and TPNH and TPN were inactive in the rat system, ATP, DPN, and TPN were inactive in the hog system and both DPNH and TPNH were active. It is not clear whether the differences in the properties of the two systems are real or are the result of the particular experimental designs used in the two investigations.

The authors are indebted to David Edwards for technical assistance during the early phases of this work, and to Dr. Shirley Su for helpful discussions of the Japanese literature.

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Keith N. Murray and Sterling Chaykin


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