Formation of Reduced Nicotinamide Adenine Dinucleotide Peroxide*  

(Received for publication, February 1, 1982)  

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Incubation of NADH at neutral and slightly alkaline pH leads to the gradual absorption of 1 mol of H+. This uptake of acid requires oxygen and mainly yields anomarized NAD+ (NAD+*), with only minimal formation of acid-modified NADH. The overall stoichiometry of the reaction is: NADH + H+ + ½O2 → H2O + NAD+. With NADH peroxide (HO2-NADH*) serving as the intermediate that anomerizes and breaks down to give NAD+* and H2O2. The final reaction mixture contains less than 0.1% of the generated H2O2, which is nonenzymically reduced by NADH. The latter reaction is inhibited by catalase, leading to a decrease in the overall rate of acid absorption, and stimulated by peroxidase, leading to an increase in the overall rate of acid absorption.

Although oxygen can attack NADH at either N-1 or C-5 of the dihydropyridine ring, the attack appears to occur primarily at N-1. This assignment is based on the inability of the C-5 peroxide to anomerize, whereas the N-1 peroxide, being a quaternary pyridinium compound, can anomerize via reversible dissociation of H2O2. The peroxidase-catalyzed oxidation of NADH by H2O2 does not lead to anomerization, indicating that anomerization occurs prior to the release of H2O2. Chromatography of reaction mixtures on Dowex 1 formate shows the presence of two major and several minor neutral and cationic degradation products. One of the major products is nicotinamide, which possibly arises from breakdown of nicotinamide-1-peroxide. The other products have not been identified, but may be derived from other isomeric nicotinamide peroxides.

While studying the stability of NADH under various conditions, we attempted to neutralize a solution by the cautious addition of acid and found that, between pH 6 and 8, the acid was continuously absorbed in a stoichiometric manner with concomitant oxidation of the NADH. The present communication describes these observations and demonstrates the requirement for O2 and the intermediate formation of H2O2. The results indicate that, at physiological pH, NADH is unstable in the presence of oxygen and gives rise to an NADH-peroxide that can serve as a source of H2O2. A preliminary account of this work has appeared (1).

**MATERIALS AND METHODS**

NADH—All experiments were conducted with the same lot of NADH, which was obtained preweighed (106 mg; 128 pmol) in evacuated ampules from Sigma, and stored at room temperature in a desiccator. Before use, the contents of each vial was dissolved in 1.28 ml of 0.15 M NaCl.

**pH Measurement and Titration**—pH was measured with a Metrohm, Model E-512 pH meter and EA-147 electrode (Brinkmann Instruments, Cantigue, NY), and the output (100 mV/pH units) recorded on a Linear, Model 555 recorder (Linear Instruments Corp., Irvine, CA), adjusted to spans of 2 to 7 pH units.

Titrations were conducted in a closed, water-jacketed, 1-ml glass vessel kept at 30 °C by the circulation of dilute sodium dichromate, the latter to shield the NADH from ambient fluorescent lighting (2). Titrant (0.1 M HCl in 0.15 M NaCl) was delivered with a Model E-415 Multi-Dosimat (Brinkmann), equipped with a 1-ml burette and strip chart recorder. In the "timer-operated" mode, the burette delivered a discrete 10-µl aliquot in response to brief electrical contact closure. In this mode, intervals between pulses were controlled by connecting the event-marker output of a Buchler, Alpha 200 fraction collector (Buchler Instruments, Inc., Fort Lee, NJ), operated without the mechanical mechanism, to a relay that provided 0.5-s contact closure.

In the pH-stat mode, the burette was controlled by a Model E-473 Impulsomat (Brinkmann), which delivered variable amounts of acid on demand and was usually adjusted to maintain a fixed pH of 7.04 to 7.38 by minimal additions of acid. In certain instances, as noted, the instrument was adjusted to deliver maximal additions of acid (approximately 3 µl), to deliberately provide large deviations from the setpoint. Kinetics of acid absorption were obtained from a continuous record of the quantity of acid discharged from the burette.

**Analysis of NADH and NAD+ in Mixtures**—Aliquots were diluted into 20 mM Na Bicine, pH 7.6, and the A260 and A280 determined against a buffer blank. Analysis was based on millimolar extinction coefficients for NADH and NAD+ of 6.22 and 0.04 at 340 nm, respectively, and 14.3 and 17.8 at 260 nm, respectively. These extinction coefficients are derived from various literature sources (3-9), except for NAD+ at 340 nm, which was determined experimentally. Millimolar concentrations were calculated from the equations given below, which were verified with known mixtures of pure NADH and NADPH. Although the absorption maximum of a-α-NADH occurs at 346 nm, its 340-nm extinction was assumed to be the same as that of β-NADH.

\[
[NADH] = \frac{17.8 A_{260} - 6.04 A_{280}}{(6.22 \times 17.8) - (14.3 \times 0.04)}
\]

\[
[NAD+] = \frac{6.22 A_{340} - 14.3 A_{280}}{(6.22 \times 17.8) - (14.3 \times 0.04)}
\]

**Enzymic Analysis of Anomers**—Assays of the β- and α- anomers of NADH and NAD+ were based on the oxidation of β-NADH but not α-NADH with lactate dehydrogenase and the reduction of β-NAD+ but not α-NAD+ with alcohol dehydrogenase. The lower and upper \( A_{260} \) limits expected from these procedures were determined from the known concentration of β-NADH in the starting mixture or from analysis of \( A_{260} \) and \( A_{280} \) as described above. The latter analysis is valid when NADH and NAD+ are the only uv-absorbing species; however, the accumulation of 260-nm-absorbing degradation products

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* This work was supported by grants from the Elizabeth W. Hill Foundation, Ladies Leukemia League, National Science Foundation (PCM77-22793), and National Institutes of Health (HL-24192). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S. C. Section 1734 solely to indicate this fact.

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leads to overestimation of the total NAD$^+$ and thus of the α-NAD$^+$ component, which is determined by difference.

To determine β-NADH in reaction mixtures initially containing 50 to 100 mM β-NADH, 1.5- to 30-μl aliquots were added to 1 ml of 100 mM Na Bicine, pH 7.8, containing 5 mM Na pyruvate, and the A$_{340}$ recorded versus the appropriate blank. For samples of 5 μl or less, a positive displacement micropipette was used (Scientific Manufacturing Industries, Emeryville, CA). Lactate dehydrogenase (5 μl, 22 μg) was added to both cuvettes and the decrease in A$_{340}$ used to calculate the β-NADH. The difference between the observed A$_{340}$ and the A$_{340}$ expected if all the NADH were oxidized was used to calculate the α-NADH component.

To determine β-NAD$^+$ in reaction mixtures initially containing 50 to 100 mM β-NADH, 1.5- to 2.0-μl aliquots were added to 1 ml of 50 mM Na$_2$CO$_3$, 50 mM NaHCO$_3$, pH 9.6, containing 600 mM ethanol, and the A$_{340}$ recorded versus the appropriate blank. Alcohol dehydrogenase (5 μl, 80 μg) was added to both cuvettes and the increase in A$_{340}$ used to calculate the β-NAD$^+$. The difference between the observed A$_{340}$ and the A$_{340}$ expected if all the NAD$^+$ were reduced was used to calculate the α-NAD$,^+$ component.

Chromatographic Analysis of Anomers—Chromatographic separation of the β- and α-anomers of NAD$^+$ on Dowex 1 formate is the most reliable method of analysis as it requires no assumptions about the presence of other components in the same mixture (6). No direct method presently exists for separating the β- and α-anomers of NADH.

Dowex 1 formate causes anomerization and oxidation of NADH (7); therefore, NADH was first separated from NAD$^+$ by chromatography on a column (0.9 × 100 cm) of DEAE-cellulose-bicarbonate, using a gradient of ammonium bicarbonate and the same apparatus and conditions previously described (8). The NAD$^+$ peak was lyophilized and rechromatographed on a column (0.9 × 65 cm) of Dowex 1 formate using a formic acid gradient (chambers 1 to 5 of Gradient I) as previously described (9), but collecting fractions of 10 ml.

Hydrogen Peroxide Assay—H$_2$O$_2$ was determined with an Aminco-Beckman Acta C-I11 spectrophotometer using quartz cuvettes of 1 cm. Water was visibly deionized. All other chemicals were of reagent grade quality, and from bovine liver was a suspension in water (82 mg/ml; 2.8 units/ml). Crystalline alcohol dehydrogenase from yeast was reconstituted with 0.1 M ethanol, and 1.5- to 30-μl aliquots were added to 1 ml of 100 mM Na Bicine, pH 7.8, containing 5 mM Na pyruvate, and the A$_{340}$ was excited at 375 nm. Standard curves were prepared with H$_2$O$_2$, which was assayed using a molar extinction coefficient of 10.0 at 266 nm (11). Before use, the peroxidase was diluted to 2 mg/ml with 50 mM Na Bicine, pH 7.8, and stored at -20 °C in small aliquots. Crystalline catalase (5 pl, 80 pg) was added to both cuvettes and the decrease in A$_{340}$ used to calculate the β-NADH. The difference between the observed A$_{340}$ and the A$_{340}$ expected if all the NADH were oxidized was used to calculate the α-NAD$^+$ component.

The assay was initiated by adding 5 μl (10 μg) of horseradish peroxidase to a mixture containing H$_2$O$_2$ and 0.5 nmol of scopoletin in 1 ml of 10 mM BES, 10 mM HEPES, pH 7.2, and followed as a decrease in the 465-nm fluorescence emission of scopoletin, which was excited at 375 nm. Standard curves were prepared with H$_2$O$_2$, which was assayed using a molar extinction coefficient of 10.0 at 266 nm (11). Before use, the peroxidase dilute was 2 mg/ml with 50 mM KH$_2$PO$_4$, K$_2$HPO$_4$, pH 7.0, and kept in the cold (0–4 °C).

Enzymes and Other Chemicals—All enzymes and their cited activities were supplied by Sigma. Lactate dehydrogenase, Type XI, from rabbit muscle, was reconstituted with water (26 mg/ml; 20,000 units/ml) and stored at -20 °C in small aliquots. Before use, the enzyme was diluted 6-fold with 100 mM Na Bicine, pH 7.8, 10 mg/ml of bovine serum albumin, and 1 mM EDTA, and kept in the cold. Crystalline alcohol dehydrogenase from yeast was reconstituted with water (15 mg/ml; 7,500 units/ml) and kept in the cold. Horseradish peroxidase, Type VI, was reconstituted with water (39 mg/ml; 10,000 units/ml) and stored at -20 °C in small aliquots. Crystalline catalase from bovine liver was a suspension in water (82 mg/ml; 2.8 × 10$^8$ units/ml). All other chemicals were of reagent grade quality, and solutions were prepared with Pyrex-distilled water that was previously deionized.

Spectrophotometry—Absorption measurements were made with a Beckman, Acta C-III spectrophotometer using quartz cuvettes of either 1- or 10-mm lightpath and recorded at ambient temperature (23 °C), unless otherwise noted.

RESULTS

Uptake of Acid by NADH

Fig. 1 illustrates the uptake of acid by NADH under conditions where acid is added at regular intervals by means of a timer. The pH falls with each aliquot of acid and rises during the intervals, producing an oscillatory pattern of pH excursions. Overall, there is a gradual acidification because the rate of acid absorption is less than the rate of acid addition. In the example shown (Fig. 1), the last aliquot given causes the pH to fall to 6.34, after which hydrogen ion continues to be absorbed until the pH reaches a stable equilibrium value of 7.98. Resumption of acid addition (not shown) results in a continuation of the oscillatory pH excursions. By measuring the rates of acid uptake from various pH values, the greatest rate of acid absorption was found to occur at pH 7.01 ± 0.04 (mean ± S.E.; n = 4).

The absorption of acid by NADH at pH 7.04 under pH-stat conditions is shown in Fig. 2. The starting pH of the NADH solution was 9.39 ± 0.01 (mean ± S.E.; n = 21), and approximately 3.4 μmol of HCl was required to lower it to the setpoint initially. The titrator, controlled by Impulsomat instead of a timer, was adjusted to deliver a maximal pulse of acid (about 3 μl; 0.3 μmol) whenever the setpoint was reached. After 25.7 μmol of acid had been delivered, the titrator was turned off, permitting the pH to gradually rise to a final equilibrium value of 7.90 (off-scale in Fig. 2). Spectrophotometric examination after equilibrium showed the presence of only NADH and NADH, with no detectable 290-nm shoulder that would be indicative of acid modification.

To survey the effect of pH on the absorption of acid, reaction mixtures containing 100 μmol of NADH in 1 ml of 0.15 M NaCl were pulsed via timer with 100 consecutive 1-μmol aliquots of HCl at various rates, all of which exceeded the rate of acid uptake and thus caused eventual acidification. As shown in Fig. 3 (24 min between pulses), the rate of acid uptake diminishes as the pH falls and ceases altogether at about pH 5.0. Fig. 3 also shows that, if the titration is rapid enough (0.1 min between pulses), little absorption of acid occurs, resulting in the greatest overall decrease in pH. Spectrophotometric examination of the acidified NADH showed
that acid modification had occurred (disappearance of the 340 nm peak and appearance of a prominent 290 nm shoulder). Further titration studies (not shown) indicated that acid-modified NADH is incapable of acid absorption.

**Dependence on Oxygen**

The dependence of acid uptake by NADH on O₂ is illustrated in Fig. 4, which shows the rate of acid uptake by 50 pmol of NADH in the presence and absence of O₂. In an atmosphere of N₂ (Fig. 4, lower curve), the rate of acid uptake gradually decreases to zero, and when O₂ is readmitted, the uptake of acid resumes. The pH responses to this titration sequence are shown in Fig. 4 (inset).

In the anaerobic titration, it is noteworthy that pregassing with N₂ does not eliminate the initial ability of the NADH solution to absorb acid. This initial absorption of acid indicates that NADH has an extremely high affinity for O₂ and suggests either the pre-existence of an oxygen-NADH complex, or its immediate formation when NADH is dissolved in saline that is incompletely deoxygenated. The affinity of NADH for O₂ is further indicated by experiments (not shown) demonstrating that the uptake of acid by NADH in the presence of air is only slightly enhanced by changing to an atmosphere of pure O₂.

**Dependence on NADH Concentration**

The dependence of acid uptake on NADH concentration is shown in Fig. 5. The kinetics of the reaction is complex, and semilog plots of acid uptake versus time show pseudo-first order kinetics for less than 1 h. Nevertheless, the acid uptake is proportional to NADH concentration, with the uptake of acid per mole of NADH increasing somewhat with higher concentrations of NADH. This may occur because a product of the initial reaction (i.e. H₂O₂) reacts with NADH in a second reaction that also absorbs acid.

**Stoichiometry**

Table I compares the amount of acid absorbed by NADH at pH 7.08 with the amount of NAD⁺ appearing in the reaction mixture. The data clearly indicate a 1:1 relationship between the HCl consumed and NAD⁺ produced, although with increasing times, a trend toward higher NAD⁺/HCl ratios is apparent. This trend is caused by the gradual accumulation of 260-nm absorbing degradation products, which exaggerates the calculated amount of NAD⁺ present.

**Anomerization**

Enzymic Analysis—The spectrophotometric analysis on which the data in Table I are based provides no information on the anomeric composition of the NADH reaction mixture. Therefore, both enzymic and chromatographic methods were used to determine the degree of anomerization.

The data in Table II indicate the occurrence of anomerization. One interesting result is that the quantity of α-NADH in the reaction mixture remains relatively constant at 3.2 ± 0.5\% of the initial NADH sample (mean ± S.E.; n = 8). This result suggests that anomerization does not occur primarily at the level of NADH, but rather at the level of an NADH derivative, i.e. 1-HO₂-NAD⁺. It may also mean that α-NADH is less reactive than β-NADH with O₂, or that α-1-HO₂-NAD⁺ is
more readily oxidized than \( \beta \)-1-HO\(_2\)-NADH\(^+\). These possibilities will require further experimental clarification.

**Chromatographic Analysis**—The analysis of \( \alpha \)-NAD\(^+\) assumes that the total pyridine nucleotide content of the reaction mixture remains constant, an assumption that is valid as long as degradation is minor. However, with increasing times of incubation, degradation products accumulate that cause the enzymically determined \( \alpha \)-NAD\(^+\) component to be increasingly overestimated. For this reason, the \( \beta \) and \( \alpha \)-NAD\(^+\) composition of two NADH reaction mixtures was determined by direct chromatographic analysis.

Table III shows that, after 51.1 h of incubation, there is a 33.9\% degradation of the original NADH, primarily to ADP-ribose and nicotinamide and its derivatives, but also to some acid-modified NADH. Both NADH and 1-HO\(_2\)-NADH\(^+\) probably contribute to the formation of ADP-ribose. Both experiments in Table III show that \( \alpha \)-NAD\(^+\) represents about 20\% of the total NAD\(^+\) in the sample. This figure may reflect the anomeric composition of the 1-HO\(_2\)-NADH\(^+\) intermediate.

**Determination of Peroxide**

The fluorometric assay of H\(_2\)O\(_2\) based on peroxidase-catalyzed oxidation of scopoletin, was employed as described under "Materials and Methods." Reaction mixtures containing NADH could not be assayed by this method because of the net quenching of fluorescence caused by NADH. However, fully oxidized NADH-titration mixtures could be assayed, and these were found to contain only about 0.1\% of the expected peroxide, based on a 1:1 formation of H\(_2\)O\(_2\) from the starting

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**Fig. 4. Dependence of acid absorption on O\(_2\).** NADH (50 \( \mu \)mol), in 1.5 ml of 0.15 M NaCl and 5 mM EDTA, was titrated with 0.1 M HCl in 0.15 M NaCl at 30 °C in the presence (upper curve) or absence (first part of lower curve) of O\(_2\). Rates of acid uptake were obtained from burette recordings. The O\(_2\) atmosphere was maintained by gassing with hydrated O\(_2\) at 1 liter/min, and then at 0.1 liter/min. At the point indicated (arrow), N\(_2\) was replaced by O\(_2\). In both experiments, the titrator delivered maximal pulses of acid at a setpoint of pH 7.04. Inset, pH responses to the anaerobic titration shown in lower curve. At the point indicated (arrow), N\(_2\) was replaced by O\(_2\).

**Fig. 5. Dependence of acid absorption on NADH concentration.** NADH, as indicated in figure, in 1.5 ml of 0.15 M NaCl and 5 mM EDTA, was titrated with 0.1 M HCl in 0.15 M NaCl at 30 °C in the presence of hydrated O\(_2\) (50 ml/min). The titrator delivered maximal pulses of acid at a setpoint of pH 7.08. Rates of acid uptake were obtained from burette recordings. Note that all rates are decreased by approximately 50\% in the absence of EDTA (not shown).

**Table I**

**Stoichiometry of acid absorption and NADH oxidation.**

NADH (50 \( \mu \)mol), in 1.5 ml of 0.15 M NaCl and 5 mM EDTA, was titrated with 0.1 M HCl in 0.15 M NaCl at 30 °C in an atmosphere of hydrated O\(_2\) (50 ml/min). The titrator delivered maximal pulses of acid at a set-point of pH 7.08. At the times indicated, 2-\( \mu \)l aliquots were diluted with 1 ml of 20 mM Na Bicine, pH 7.6, and examined spectrophotometrically. Because the spectra indicated essentially pure forms of NAD\(^+\) and NADH, the percentages of these substances were calculated directly, using the extinction coefficients given under "Materials and Methods." No corrections were made to the raw data; NAD\(^+\)/HCl ratios were obtained prior to rounding.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>HCl (( \mu )mol)</th>
<th>NAD(^+) present</th>
<th>NAD(^+)/HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>3.1</td>
<td>2.7</td>
<td>0.88</td>
</tr>
<tr>
<td>1.0</td>
<td>5.1</td>
<td>4.3</td>
<td>0.85</td>
</tr>
<tr>
<td>2.0</td>
<td>8.2</td>
<td>7.5</td>
<td>0.91</td>
</tr>
<tr>
<td>3.0</td>
<td>11.0</td>
<td>10.4</td>
<td>0.95</td>
</tr>
<tr>
<td>4.0</td>
<td>13.5</td>
<td>13.3</td>
<td>0.99</td>
</tr>
<tr>
<td>5.0</td>
<td>15.6</td>
<td>16.1</td>
<td>1.04</td>
</tr>
<tr>
<td>6.0</td>
<td>17.9</td>
<td>19.2</td>
<td>1.07</td>
</tr>
<tr>
<td>8.8</td>
<td>21.2</td>
<td>23.6</td>
<td>1.12</td>
</tr>
<tr>
<td>11.8</td>
<td>24.5</td>
<td>27.7</td>
<td>1.13</td>
</tr>
<tr>
<td>23.9</td>
<td>30.9</td>
<td>36.1</td>
<td>1.17</td>
</tr>
</tbody>
</table>

NADH. Because of the small quantity of H\(_2\)O\(_2\) found, indirect evidence for its formation was sought.

**Effects of Catalase and Peroxidase**

Fig. 6 shows the kinetics of acid absorption of 100 \( \mu \)mol of NADH under three different conditions. The uptake of acid is seen to be partially inhibited by catalase and considerably stimulated by peroxidase (Fig. 6). The partial inhibition by

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\(^{2}\) Studies with NADPH under the same conditions showed similar, although not identical results. The starting pH of the NADPH was 8.46 ± 0.06 (mean ± S.E.; \( n = 8 \)), and approximately 15.6 \( \mu \)mol of HCl/100 \( \mu \)mol of NADPH was required to initially lower it to the setpoint (pH 7.08). Part of the initially absorbed HCl reflects partial titration of the 2'-phosphate.
catalase indicates that free \( \text{H}_2\text{O}_2 \) is not required by one of the component reactions responsible for the acid uptake by NADH. This component appears to be the \( \text{O}_2 \)-dependent uptake of acid that results in the formation of NADH peroxide as indicated by: NADH + \( \text{O}_2 \) + \( \text{H}^+ \) → HO₂-NADH. The reaction that is inhibited by catalase is assumed to be dependent on free \( \text{H}_2\text{O}_2 \) and may be represented as: NADH + \( \text{H}_2\text{O}_2 \) + \( \text{H}^+ \) → NAD⁺ + 2\( \text{H}_2\text{O} \). These reactions are discussed more fully below.

The occurrence of the latter reaction is confirmed by the data in Fig. 7, which shows that NADH is nonenzymically oxidized by \( \text{H}_2\text{O}_2 \). The stimulation by peroxidase of acid absorption by NADH is thus explained by the catalysis of this reaction by peroxidase. This was verified experimentally by treating NADH (0.13 \( \mu \text{mol} \)) and horseradish peroxidase (400 \( \mu\text{g} \)) in 1 ml of 20 mM BES, 20 mM HEPES, pH 7.2, with a series of 1.5-\( \mu\text{l} \) additions of 20 mM \( \text{H}_2\text{O}_2 \) and monitoring the reaction at 340 nm. Under these conditions, oxidation of NADH by \( \text{H}_2\text{O}_2 \) was stoichiometric.

**Table II**

Enzymically determined changes in NADH during acid absorption

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>HCl ( \mu\text{mol} )</th>
<th>Per cent total NADH + NAD⁺ present as</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td></td>
<td>NADH</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>99.1</td>
</tr>
<tr>
<td>2.7</td>
<td>10.5</td>
<td>92.6</td>
</tr>
<tr>
<td>5.7</td>
<td>15.1</td>
<td>88.8</td>
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<tr>
<td>28.3</td>
<td>40.5</td>
<td>52.1</td>
</tr>
<tr>
<td>52.6</td>
<td>61.6</td>
<td>20.0</td>
</tr>
<tr>
<td>74.2</td>
<td>65.9</td>
<td>8.8</td>
</tr>
</tbody>
</table>

* The presence of ADP-ribose and other products causes overestimation of α-NAD⁺, which is probably more accurately represented as 20% of NAD⁺ (see Table III).

**Table III**

Chromatographically determined anomerization of NADH during acid absorption

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time (h)</th>
<th>HCl ( \mu\text{mol} )</th>
<th>Per cent initial NADH as</th>
<th>Per cent NAD⁺ as</th>
<th>Other products as</th>
<th>β-NAD⁺</th>
<th>α-NAD⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>25.0</td>
<td>41.4</td>
<td>46.7</td>
<td>31.2</td>
<td>20.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>51.1</td>
<td>59.4</td>
<td>15.8</td>
<td>50.3</td>
<td>33.9</td>
<td>79.7</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>95.3</td>
<td>66.2</td>
<td></td>
<td></td>
<td>80.4</td>
<td>19.6</td>
<td></td>
</tr>
</tbody>
</table>

* Primarily ADP-ribose, nicotinamide, and several other unknown neutral and cationic compounds. Absorption spectra of the ADP-ribose fractions (Experiment A, 51.1 h) revealed a barely discernible shoulder at 290 nm that disappeared upon treatment with 0.5 M HCl, thus indicating the presence of a small quantity of acid-modified NADH (8).

**Fig. 6. Effect of catalase and peroxidase on acid absorption.** NADH (100 \( \mu\text{mol} \)), in 1 ml of 0.15 M NaCl, was titrated with 0.1 M HCl in 0.15 M NaCl at 30 °C in the presence of air. The titrator delivered minimal pulses of acid at a setpoint of pH 7.08. At the times indicated, aliquots were assayed enzymically for β- and α-NAD⁺ and β- and α-NAD⁺ as described under "Materials and Methods." The titrator delivered minimal pulses of acid at a setpoint of pH 7.08. Rates of acid uptake were obtained from burette recordings. Values in the control curve are means ± S.D. (n = 5). Other values are means of duplicate experiments conducted with either 0.8 mg of horseradish peroxidase or 1.6 mg of bovine liver catalase. Min⁺⁺ (0.2 mm) was without effect in the presence or absence of peroxidase.

**Fig. 7. Nonenzymic oxidation of NADH by \( \text{H}_2\text{O}_2 \).** Two cuvettes of 1-mm lightpath, each containing 3 mm NADH in 50 mM BES, 50 mM HEPES, pH 7.2, with one containing 3 mm \( \text{H}_2\text{O}_2 \), were incubated at 30 °C, and difference spectra taken at the times indicated in figure. The cuvette with \( \text{H}_2\text{O}_2 \) was placed in the reference beam so that oxidation of NADH would be recorded as a positive pen deflection. The net disappearance of NADH in this cuvette is identified by the 340 nm absorption maximum of the difference spectrum.

**Effect of Peroxidase on Anomerization**

Because the \( \text{O}_2 \)-dependent oxidation of NADH yields anomerized NAD⁺ and proceeds with the intermediate formation of \( \text{H}_2\text{O}_2 \), it was of interest to determine whether the peroxidase-catalyzed oxidation of NADH by \( \text{H}_2\text{O}_2 \) would also lead to anomerization. The chromatographic results described below indicate that peroxidase does not cause anomerization. A reaction mixture containing 6 \( \mu\text{mol} \) of NADH, 20 \( \mu\text{mol} \) of \( \text{H}_2\text{O}_2 \), and 3.2 mg of horseradish peroxidase in 4 ml of 20 mM triethanolamine acetate, pH 7.4, was incubated at 30 °C and monitored at 340 nm in a 1-mm lightpath cuvette. After

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The origin of the nicotinamide is uncertain, but one possibility was confirmed by assaying the ultrafiltrate for P-NAD' as reported, but under different conditions (12). 98% recovery of p-NADH. Similar results were previously described under "Materials and Methods" and observing degradation products that appear to arise by hydrolysis of 1.5 pmol of the original NADH was chromatographed on a column of Dowex 1 formate as described under "Materials and Methods," the a-NAD' was observed to be 2.0% of the total NADH+ intermediates. These products are illustrated by the chromatogram shown in Fig. 8. With the exception of nicotinamide, these substances are unidentifed but are different from those arising from the acid degradation of NADH (13). The origin of the nicotinamide is uncertain, but one possibility is that it arises from breakdown of nicotinamide-1-peroxide, a hydrolysis product of 1-H02-NADH+. In a similar fashion, the other degradation compounds may be derived from other isomeric HO2-NADH+ intermediates. The major unknown degradation compound (Fig. 8), has an absorption maximum at 261 nm in acid and water, shifting to 267 nm in alkali. The structure of this substance is under investigation.

Degradation Products

During the O2-dependent absorption of acid by NADH, there is a gradual accumulation of neutral and cationic degradation products that appear to arise by hydrolysis of HO2-NADH+ intermediates. These products are illustrated by the chromatogram shown in Fig. 8. With the exception of nicotinamide, these substances are unidentifed but are different from those arising from the acid degradation of NADH (13). The origin of the nicotinamide is uncertain, but one possibility is that it arises from breakdown of nicotinamide-1-peroxide, a hydrolysis product of 1-HO2-NADH+. In a similar fashion, the other degradation compounds may be derived from other isomeric HO2-NADH+ intermediates. The major unknown degradation compound (Fig. 8), has an absorption maximum at 261 nm in acid and water, shifting to 267 nm in alkali. The structure of this substance is under investigation.

\[
\text{NADH Peroxide}
\]

\[
\text{NADH + O}_2 \rightarrow \text{O}_2\text{-NADH}^+ \quad (1)
\]

\[
\text{O}_2\text{-NADH}^+ + \text{H}^+ \rightarrow \text{HO2-NADH}^+ \quad (2)
\]

\[
\text{HO2-NADH}^+ \rightarrow \text{HO2-NAD}^+ + \text{H}_2\text{O} \quad (3)
\]

\[
\text{HO2-NAD}^+ \rightarrow \text{HO2-NAD}^+ + \text{H}_2\text{O} \quad (4)
\]

\[
\text{NADH + H}_2\text{O} + \text{H}^+ \rightarrow \text{NAD}^+ + 2\text{H}_2\text{O} \quad (5)
\]

In the above sequence, the initial formation of a peroxide is indicated by the requirement of O2 for the absorption of acid (Fig. 4). Thus, formation of the NADH-peroxide anion (Equation 1) would cause immediate uptake of acid (Equation 2) because the pK of most peroxides is above 10 (14). The second reaction involving absorption of acid is the oxidation of NADH by H2O2 (Equation 5), which is inhibited by catalase (Fig. 6) and stimulated by peroxidase (Fig. 6). The overall absorption of acid occurs in a 1:1 relationship with NADH oxidation (Table I), and results in formation of anomerized NAD+ (Tables II and III). These findings are consistent with the net result shown (Equation 6) and indicate the general validity of the reaction sequence. The formation of NADH peroxide appears to explain the manometric finding of da Silva Araujo and Cilento (15) that oxygen is absorbed steadily by NADH that is buffered at pH 6.8.

Formation of NADH Peroxide—Based on the known chemical reactivity of NADH, there are three possible anionic sites on the dihydropyridine nucleus that are vulnerable to attack by oxygen: N-1, C-3, and C-5. Formation of the C-5 peroxide would entail the sequence of reactions shown in Scheme 1, while formation of the N-1 peroxide would entail the sequence of reactions shown in Scheme 2. We believe that the C-3 peroxide is less likely to be formed because the carboxamide group would act as an electron sink to destabilize the C-3 carbanion (Scheme 3).

\[
\text{Scheme 1. Reaction mechanism for the formation and breakdown of 5-HO2-NADH+}. \quad R = 5\text{-adenosine diphosphate ribose.}
\]

\[
\text{Scheme 2. Reaction mechanism for the formation and breakdown of 1-HO2-NADH+}. \quad R = 5\text{-adenosine diphosphate ribose.}
\]

\[
\text{Scheme 3. Reaction mechanism for the formation and breakdown of 5-HO2-NADH+}. \quad R = 5\text{-adenosine diphosphate ribose.}
\]
Significance of Anomerization—The formation of NADH peroxide appears to proceed mainly, although not exclusively, by the mechanism shown in Scheme 2 because of the fact that the NADH product is anomerized. Thus, there is no clear way that the C-5 peroxide of NADH can anomerize, whereas the N-1 peroxide would be expected to anomerize, as shown in Scheme 4.

The anomerization observed during anaerobiosis (Table IV) deserves special comment. The \( \alpha \)-NADH that is measured enzymically represents that fraction of the NADH in the reaction mixture that is not oxidized by lactate dehydrogenase. However, that fraction could contain other \( \beta \)-40 nm absorbing species that are also not oxidized by lactate dehydrogenase. One such compound would be \( 1-HO_2-NADH^+ \) which, because it has a 1,4-dihydropyridine structure, would be expected to have spectral properties similar to NADH.

We have found that, when NADH is dissolved under aerobic or anaerobic conditions, the solution can immediately absorb about 3.4 \( \mu \)mol of acid/100 \( \mu \)mol of NADH to bring the pH to neutrality, and even under anaerobic conditions, there is a continued uptake of acid to the extent of 8 to 9 \( \mu \)mol of acid/100 \( \mu \)mol of NADH (Table IV). These results may reflect the difficulty of removing residual oxygen by means of \( N_2 \) purging. Alternatively, they suggest that part of the initial NADH sample may exist as the 340 nm-absorbing peroxide anion which, upon dissolution, can combine with acid to form the undissociated peroxide.

NADH peroxide, when present in even small amounts, appears to catalyze the anomerization of the entire NADH sample. For example, in Table IV (Experiment A), very little acid was absorbed between 4.2 and 24.2 h, indicating that there had been a reasonable, although not absolute, exclusion of oxygen. Nevertheless, there was a very large increase in the \( \alpha \)-NADH component. We believe that the \( \alpha \)-NADH component present at zero time consisted, in part, of \( 1-HO_2-NADH^+ \), which tended to anomerize the NADH sample. This anomerization occurs because \( \beta \)- and \( \alpha \)-1-HO_2-NADH^+ exist in equilibrium with \( \beta \)- and \( \alpha \)-NADH^+, respectively, a condition that is a consequence of the reversibility of Equations 1 to 3 (see Scheme 4).

During anaerobiosis, when there is little net conversion of NADH to NADH^+, \( \alpha \)-NADH apparently accumulates in solution (Table IV), reflecting, in part, the buildup of 1-HO_2-NADH^+. However, in the presence of oxygen, the amount of \( \alpha \)-NADH present remains small and constant (Table II), possibly because steady state levels of 1-HO_2-NADH^+ are reduced. In support of this idea, we have found that, when NADH absorbs acid in the presence of peroxidase, the extent of anomerization is also reduced, presumably because \( H_2O_2 \) is rapidly removed. Thus, chromatography on Dowex 1 formate of the (fully oxidized) peroxidase-treated reaction mixture described in Fig. 6 revealed the NADH^+ to contain 10% \( \alpha \)-NAD^+, in comparison to the 20% \( \alpha \)-NAD^+ content of controls without peroxidase.

Immediate Uptake of \( O_2 \)—When horseradish peroxidase is added to a solution of NADH, there is an immediate uptake of \( O_2 \) that can be followed by means of an oxygen electrode (16). Under controlled conditions, the decrease in dissolved oxygen is proportional to the NADH present, with the rate of \( O_2 \) depletion reaching a peak in about 10 s. Because removal of NADH by the action of dehydrogenases causes a decrease in the \( O_2 \)-uptake signal, Nikolelis et al. (17) have proposed using this reaction to measure serum lactate dehydrogenase in an automated flow system.

The time frame of the \( O_2 \) uptake is so short that the reaction appears to represent the peroxidase-catalyzed discharge of preformed \( H_2O_2-NADH^+ \) followed by the immediate reaction of \( O_2 \) with NADH to form additional \( H_2O_2-NADH^+ \). Thus, the abrupt removal of \( H_2O_2-NADH^+ \) (Equations 4 and 5) upon the addition of peroxidase would perturb the equilibria represented by Equations 1 to 3.

Cheng and Christian (16) found that the peroxidase-catalyzed burst of \( O_2 \) uptake by NADH is inhibited by \( H_2O_2 \) and catalase. The former effect is understandable because the peroxidase-catalyzed oxidation of NADH by \( H_2O_2 \) would remove the NADH required for reaction with \( O_2 \). The inhibition by catalase (16) appears to reflect the return of \( O_2 \) to the solution from breakdown of the \( H_2O_2 \) formed in Equation 4. In these short term reactions, it appears that the \( O_2 \) uptake represents the initial capacity of NADH to absorb \( O_2 \), as expressed in Equation 1. Only a small part of the NADH present, perhaps 10% or less, would be involved in peroxide anion formation because of equilibrium considerations.

Oxygen Stoichiometry and Other Cofactors—The aerobic oxidation of NADH, catalyzed by horseradish peroxidase, was first studied in detail by Akazawa and Conn (12). Under the
source of intracellular H$_2$O$_2$ is thought to be the dismutation of O$_2^-$, catalyzed by superoxide dismutase (25, 26). Such O$_2^-$ would be formed by interaction of O$_2$ with certain electron-donating sites in mitochondria, microsomes, peroxisomes, and cytosolic enzymes (21). In mitochondria, the greatest O$_2^-$ and H$_2$O$_2$ production occurs under conditions where terminal respiration is inhibited and electron carriers are in a reduced state (27-30). These same conditions would also favor HO$_2$-NADH formation because inhibition of the electron transport chain would cause an elevation of reduced pyridine nucleotides as well as an increase in O$_2$ availability. If 1-HO$_2$-NADH$^+$ is formed, the release of H$_2$O$_2$ and its immediate removal would be imperative to prevent anomerization of NADH, for such anomerization would result in formation of α-NADH, a dehydrogenase inhibitor that may have serious metabolic effects.

The potential of the peroxides described in this paper to participate in various oxidative and peroxidative processes that require oxygen and reduced pyridine nucleotides is a subject that merits exploration. One example is the possible involvement of HO$_2$-NADH$^+$ and HO$_2$-NADPH$^+$ in the production of H$_2$O$_2$ by phagocytic leukocytes. The activation of these cells during phagocytosis is accompanied by changes in oxidative metabolism characterized by marked increases in the hexose monophosphate shunt, NADH and NADPH oxidation, consumption of O$_2$, and production of O$_2^-$ and H$_2$O$_2$ (31-33). The ability of NADPH to combine with O$_2$ (Equation 7), suggests that this reaction could be involved in H$_2$O$_2$ production. Key steps would be the increased availability of NADPH, catalyzed by activated glucose-6-phosphate dehydrogenase (Equation 9), and the discharge and transfer to another environment of H$_2$O$_2$ from HO$_2$-NADPH$^+$, catalyzed by activated NADPH oxidase (Equation 8). This speculative mechanism is summarized by the following equations.

\[
\text{NADPH} + \text{O}_2 + \text{H}^+ \rightleftharpoons \text{HO}_2\text{-NADPH}^+ \quad (7)
\]
\[
\text{HO}_2\text{-NADPH}^+ \xrightarrow{\text{oxidase}} \text{NADP}^+ + \text{H}_2\text{O}_2 \quad (8)
\]
\[
\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-P-glucuronolactone} + \text{NADPH} + \text{H}^+ \quad (9)
\]
\[
\text{Sum:} \quad \text{G-6-P} + \text{O}_2 \rightarrow \text{6-P-glucuronolactone} + \text{H}_2\text{O}_2 \quad (10)
\]

Acknowledgment—We are grateful to Dr. R. H. Steele for use of the Amino-Bowman spectrophotofluorometer.

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Formation of reduced nicotinamide adenine dinucleotide peroxide.
C Bernofsky and S Y Wanda


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