Inhibitor-contaminated NADH: Its Influence on Dehydrogenases and Dehydrogenase-coupled Reactions

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Preparations of reduced NAD are known to be prone to contain a substance which inhibits the catalytic activity of LDH. (L-Lactate: NAD oxidoreductase, EC 1.1.1.27).

This publication presents data concerning the relative quality of commercially available reduced NAD preparations for LDH assays. Data on the influence of the inhibitor on other dehydrogenases are presented, as well as data concerning the inhibitor in coupled enzyme tests with dehydrogenases as indicator enzymes.

Freshly prepared solutions of various commercially available reduced NAD preparations were found to be essentially free of inhibitors acting on dehydrogenases. It is shown that the dehydrogenases GLDH (L-glutamate:NAD oxidoreductase, deaminating, EC 1.4.1.2), "α-HBDH" ("α-hydroxybutyrate dehydrogenase"), LDH (L-lactate: NAD oxidoreductase, EC 1.1.1.27), MDH (L-malate:NAD oxidoreductase, EC 1.1.1.37), and SDH (L-iditol:NAD oxidoreductase, EC 1.1.1.14) are quite different in their sensitivity against the inhibitor.

Experiments with coupled enzyme systems using dehydrogenases as indicator enzymes have shown that the inhibitor does not affect kinetic assays of ALD (Ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7), CPK (ATP:creatine phosphotransferase, EC 2.7.3.2), GOT (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1), GPT (L-alanine:2-oxoglutarate amino transferase, EC 2.6.1.2), and PK (ATP:pyruvate phosphotransferase, EC 2.7.1.40), if the kinetic requirements for coupled enzyme systems are met.

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Materials and Methods

Preparations of reduced NAD were obtained from BMC (Boehringer Mannheim Corporation), Dade, Fermco, and Sigma as part of their reagent sets for the determination of the activity of LDH in serum and compared with reduced NAD-chromatopure from PL-Laboratories.

All other reagents were obtained from BMC. Samples were human sera having normal and slightly elevated enzyme activity. In the case of GLDH, PK, and SDH, purified enzymes were added to normal human serum.

Controls were performed with commercially available control materials (Versatol E and Enzatrol) where applicable, or with known amounts of purified enzymes (from BMC).

"Inhibitor-enriched" reduced NAD was prepared by repeated freezing and thawing of a slightly alkaline solution.

All enzyme activities were measured at 340 nm in a Zeiss PMQ-II Spectrophotometer with the temperature regulated cell compartment. Activities are expressed in International Units (U) according to the recommendation of the International Union of Biochemistry; however, a temperature of 25° was maintained throughout the experiments.

Reagents

Assay conditions for the determination of the activity of:

LDH (Lactate Dehydrogenase, EC 1.1.1.27) 3.1 × 10⁻⁴ mol/liter pyruvate, 1.5 × 10⁻⁴ mol/liter red. NAD, 0.05 mol/liter phosphate buffer, pH 7.5.

α-HBDH (α-Hydroxybutyrate Dehydrogenase) 3.3 × 10⁻³ mol/liter α-ketobutyrate, 1.3 × 10⁻⁴ mol/liter red. NAD, 0.05 mol/liter phosphate buffer, pH 7.4.

MDH (Malate Dehydrogenase, EC 1.1.1.37) 4.2 × 10⁻² mol/liter L-aspartate, 1.1 × 10⁻³ mol/liter α-ketoglutarate, 1 U (25°) GOT, 2 × 10⁻⁴ mol/liter red. NAD, 0.1 mol/liter phosphate buffer, pH 7.4.

SDH (Sorbitol Dehydrogenase, EC 1.1.1.14) 0.12 mol/liter D-fructose, 2.5 × 10⁻⁴ mol/liter red. NAD, 0.2 mol/liter triethanolamine buffer, pH 7.4.

GLDH (Glutamate Dehydrogenase, EC 1.4.1.3) 6.8 × 10⁻³ mol/liter α-ketoglutarate, 0.1 mol/liter ammonium acetate, 2.5 × 10⁻³ mol/liter EDTA, 2 × 10⁻⁴ mol/liter red. NAD, 0.05 mol/liter triethanolamine buffer, pH 8.0.

CPK (Creatine Phosphokinase, EC 2.7.3.2) 3.2 × 10⁻² mol/liter creatine, 1.2 × 10⁻⁸ mol/liter ATP (Adenosine-5'-Triphosphate) 4 × 10⁻⁴ mol/liter, PEP (Phosphoenol Pyruvate) 7 × 10⁻³ mol/liter
MgCl₂, 2 × 10⁻⁴ mol/liter red. NAD, 40 U (25°) LDH, 15 U (25°) PK, 0.45 mol/liter glycine buffer, pH 9.0.

GOT (Glutamate-Oxalacetate Transaminase, EC 2.6.1.1) 4.2 × 10⁻² mol/liter L-aspartate, 8 × 10⁻⁸ mol/liter α-ketoglutarate, 2 × 10⁻⁴ mol/liter red. NAD, 10 U (25°) MDH, 0.1 mol/liter phosphate buffer, pH 7.4.

GPT (Glutamate-Pyruvate Transaminase, EC 2.6.1.2) 8 × 10⁻² mol/liter DL-alanine, 8 × 10⁻³ mol/liter α-ketoglutarate, 2 × 10⁻⁴ mol/liter red. NAD, 5 U (25°) LDH, 0.1 mol/liter phosphate buffer, pH 7.4.

ALD (Aldolase, EC 4.1.2.7) 3 × 10⁻³ mol/liter fructose-1,6-diphosphate, 3 × 10⁻⁴ mol/liter monooiodoacetate, 3.6 × 10⁻⁴ mol/liter red. NAD, 0.8 U (25°) GDH (Glycerophosphate Dehydrogenase, EC 1.1.1.8) 4.8 U (25°) TIM, 0.05 mol/liter collidine buffer, pH 7.4.

PK (Pyruvate Kinase, EC 2.7.1.40) 3.7 × 10⁻³ mol/liter ADP (Adenosine-5'-Diphosphate) 1.2 × 10⁻² mol/liter PEP, 9.2 × 10⁻² mol/liter KCl, 1.6 × 10⁻² mol/liter MgSO₄, 1 × 10⁻² mol/liter EDTA, 2.2 × 10⁻⁴ mol/liter red. NAD, 10 U (25°) LDH, 0.12 mol/liter triethanolamine buffer, pH 7.5.

Total volume for all assays was 3.0 ml. All solutions of reduced NAD used were standardized to the same molarity.

Results

All five brands of commercially available red. NAD gave similar results in the LDH test using the so-called reverse reaction:

LDH
Pyruvate + NADH + H⁺ ——> Lactate + NAD⁺

In order to avoid any commercial leanings all brands of reduced NAD were given arbitrary numbers (Table 1).

Inhibitor-enriched red. NAD has different effects on various dehydrogenases in direct kinetic tests (Table 2).

None of the five enzymes, which were not dehydrogenases, showed

Table 1. Comparison of Fresh Commercially Reduced NAD Preparations

<table>
<thead>
<tr>
<th>Sample</th>
<th>NADH Preparations (mU LDH activity/ml at 25°)</th>
<th>Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Serum 1</td>
<td>89</td>
<td>84.5</td>
</tr>
<tr>
<td>Serum 2</td>
<td>227</td>
<td>207</td>
</tr>
<tr>
<td>Versatol E</td>
<td>560</td>
<td>524</td>
</tr>
<tr>
<td>Enzatrol</td>
<td>344</td>
<td>333</td>
</tr>
<tr>
<td>Cryst LDH from rabbit muscle</td>
<td>410</td>
<td>374</td>
</tr>
</tbody>
</table>
any effect of the inhibitor on the kinetics of the systems. All five systems used as indicator enzyme a dehydrogenase which, in its direct assay, is inhibited by the "enriched" reduced NAD. In the case of aldolase the indicator enzyme GDH—even though it is not mentioned in Table 2—is also subject to inhibition (Table 3).

**Discussion**

The instability of reduced NAD preparations in storage was first reported by Lowry et al (1). In 1961 Dalziel (2) observed an inhibitor of liver alcohol dehydrogenase in preparations of reduced NAD, and Strandjord et al (3) reported in the same year the inhibition of lactate dehydrogenase with frozen preparations of reduced NAD. Also, in 1961 Fawcett et al (4) reported that this inhibitor was formed by freezing and thawing of reduced NAD solutions. It was Fine et al (5) who reported in 1962 that the inhibitor is also formed in solid reduced NAD as a result of its exposure to moisture.

Applegarth and Eden (6) investigated the stability of reduced NAD under various conditions and concluded that it is more hazardous to keep reduced NAD solutions frozen or at room temperature than to store them at 4°. Research workers have since pursued the problem and tried to identify the structure and kinetics of the inhibitor (7, 8).

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**Table 2. Influence of NADH Inhibitor on Various Dehydrogenases in Direct (one step) Optical Assays**

<table>
<thead>
<tr>
<th>Dehydrogenases</th>
<th>Reduced NAD (mU enzyme activity/ml at 25°)</th>
<th>Fresh</th>
<th>Inhibitor</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>13.34</td>
<td>9.75</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>α-Hydroxy butyrate</td>
<td>201</td>
<td>15.9</td>
<td>92.1</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>197</td>
<td>27.8</td>
<td>85.9</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>205</td>
<td>63</td>
<td>69.3</td>
<td></td>
</tr>
<tr>
<td>Sorbitol</td>
<td>15.0</td>
<td>15.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Influence of NADH Inhibitor on Various Dehydrogenases in Coupled Kinetic Tests**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reduced NAD (mU enzyme activity/ml at 25°)</th>
<th>Fresh</th>
<th>Inhibitor</th>
<th>Inhibition (%)</th>
<th>(U/test at 25°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldolase</td>
<td>5.5</td>
<td>5.5</td>
<td>0</td>
<td>0.8 (GDH)</td>
<td></td>
</tr>
<tr>
<td>Creatine phosphokinase</td>
<td>9.3</td>
<td>9.5</td>
<td>0</td>
<td>40 (LDH)</td>
<td></td>
</tr>
<tr>
<td>GOT</td>
<td>32.7</td>
<td>32.4</td>
<td>0</td>
<td>10 (MDH)</td>
<td></td>
</tr>
<tr>
<td>GPT</td>
<td>38.4</td>
<td>38.9</td>
<td>0</td>
<td>5 (LDH)</td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>25.3</td>
<td>25.5</td>
<td>0</td>
<td>10 (LDH)</td>
<td></td>
</tr>
</tbody>
</table>
McComb and Gay pointed out that the inhibitor was always present in reduced NAD preparations that were yellow in color. It may therefore be useful to simply use only white or slightly yellow preparations for assays which can be influenced by the inhibitor.

Since the discovery of the inhibitor, progress in applied enzymology has often been hampered by suspicion of enzyme reactions based on the absorbency of reduced NAD. It was assumed that such NAD-linked reactions may generally result in false low values. Advocators of colorimetric procedures have continuously mentioned the hazards of UV methods because of the inhibitor.

Freshly prepared solutions of reduced NAD, formulated from freshly received preparations, have proven to contain only small amounts of the so-called "inhibitor." Since differences of the LDH tests were less than 10% for the various preparations (Table 1), it can be concluded that commercially available reduced NAD from major suppliers is of similar quality with respect to the inhibitor.

It is well known that the most crucial factor in the stability of reduced NAD preparations is the reconstituted solution. The stability may be influenced by either chemical destruction in acid solutions (1) or to inhibitor formation in slightly alkaline solutions (6). However, it is also known that even the solid product is not of unlimited stability (9). The stability is influenced by the amounts of moisture within the preparation or packaging. It is therefore hard to understand why all suppliers but one fail to make a defined statement about the stability of reduced NAD preparations, thus neglecting an A.A.C.C. requirement (10).

It is shown in Table 2 that 4 out of 5 dehydrogenases assayed in direct 1-step systems are subject to inhibition. The effect of the inhibitor on "α-HBDH" is more than 90%. It is therefore of utmost importance that assays for dehydrogenases are carried out with fresh, colorless solutions of reduced NAD which have been prepared from reasonably fresh material if false low results are to be avoided.

The coupled enzymatic reactions for ALD CPK, GOT, GPT, and PK are all not affected by the inhibitor (Table 3), although all indicator enzymes GDH, MDH, and LDH had shown great sensitivity towards the inhibitor.

In kinetic assay procedures it is necessary that auxiliary and indicator enzymes be present in excess. Following Bergmeyer's theory (11) on enzyme concentrations for coupled reactions we have used large amounts of such enzymes with the result that no influence of the inhibitor can be detected in coupled systems.
To what extent commercially available kits do follow the requirements according to Bergmeyer will be published in a future paper.

Conclusions

The practical conclusions which can be drawn from this study are the following:
1. LDH activity can be measured with reduced NAD and pyruvate if the coenzyme is reasonably fresh and kept free from moisture. Solutions of reduced NAD are of limited stability for LDH assays and should not be kept for longer periods of time.
2. Arguments against the UV procedures for ALD, CPK, GOT, GPT, and PK are not valid. It has been proven that even solutions of reduced NAD which contain enough inhibitor to inhibit approximately 90% of LDH have no influence on any of the above mentioned coupled reactions, under the assay conditions used in this paper.

References