Formation and Properties of Lactate Dehydrogenase Inhibitors in NADH

C. A. Loshon,1 Robert B. McComb, Lawrence W. Bond,2 George N. Bowers, Jr., W. H. Coleman,3 and R. H. Gwynn3

We describe some characteristics of the mode of formation of inhibitors of lactate dehydrogenase from commercial NADH. Inhibitor formation is time- and concentration-dependent and also varies with the commercial source of the NADH. At least two inhibitory components can form in concentrated NADH solutions. One of these can be separated from NADH by chromatography on either diethylaminoethyl-cellulose or diethylaminoethyl-Sephadex; the second cannot. The NADH-associated inhibitor appeared to be present in each of the three commercial NADH preparations studied. The 260 nm/340 nm absorbance ratio was of no help in locating this inhibitor during chromatography.

The presence of lactate dehydrogenase (LDH; EC 1.1.1.27) inhibitors in commercial NADH is well documented (1–8). An earlier study (4) from this laboratory demonstrated considerable variation in the quality of NADH and attributed the low LDH activities supported by some preparations to the presence of an inhibitor. The amount of inhibitor in even relatively pure preparations of NADH was shown to markedly increase on exposure of these preparations to water vapor, after which an inhibitory fraction could be isolated. In the present studies we have continued our investigations of the properties of NADH and its associated LDH inhibitor.

Materials and Methods

NADH was obtained from the same commercial sources as used previously (4): Boehringer Mannheim Corp., New York, N. Y. 10017; Sigma Chemical Co., St. Louis, Mo. 63178; and P-L Biochemicals, Inc., Milwaukee, Wis. 53205. To avoid implied endorsement of any one manufacturer, these preparations have been randomly coded A, B, and C. NAD+, AMP, adenosine 5′-diphosphoribose, nicotinamide mononucleotide, tris(hydroxymethyl)methylamine, and sodium pyruvate were obtained from Sigma Chemical Co. Diethylaminoethyl-cellulose, Type 20, was obtained from Schleicher and Schuell, Keene, N. H. 03431 and diethylaminoethyl-Sephadex A-25 from Pharmacia, Piscataway, N. J. 08854.

Pooled human serum was used as the source of the LDH activity. "Inhibitor" was generated by adding either 10 or 20 μl of water (or aqueous reagent) directly to 10 mg of NADH. The freshly weighed dry salt dissolved quickly to give a colorless or pale-yellow solution in which the NADH concentration was about 0.6 mol/liter. This concentrated NADH solution was kept in a capped, polystyrene vial at room temperature for the indicated time. When larger amounts of inhibitor were called for, the procedure was scaled upward 10- to 50-fold.

Production of inhibitor by this technique was more convenient than that used previously (4) and allowed better control of reaction conditions. The LDH inhibition that developed was quite reproducible for any one lot of NADH, but varied considerably among lots.

Diethylaminoethyl-cellulose was processed and packed (9) into a glass column to final dimensions of 28 × 625 mm. A solution of 100 mg of fresh NADH (or NADH reaction product) in 1.0 ml of "starting" buffer, tris(hydroxymethyl)methylamine chloride buffer (20 mmol/liter, pH at 30 °C = 7.35), was added directly to the column, which had previously been equilibrated at 4 °C with starting buffer. The column was eluted step-wise, first with a 350 ml of starting buffer, followed by 650 ml of tris(hydroxymethyl)methylamine (0.1 mol/liter, pH 7.35). Ten-milliliter fractions were collected and the absorbances at 340 nm and 260 nm were determined for each with a Cary 16 spectrophotometer.

Column chromatography on diethylaminoethyl-Sephadex was essentially as suggested by Strandjord.4 The exchanger, suspended in "starting" buffer, tris(hydroxymethyl)methylamine chloride buffer (50

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4 Strandjord, P.E., personal communication.
Table 1. Comparison of LDH Activity in the Presence of Three Commercial NADH Preparations

<table>
<thead>
<tr>
<th>NADH source</th>
<th>n</th>
<th>Relative acy</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50</td>
<td>97.6</td>
<td>2.8</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>100</td>
<td>1.2</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>95.3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Assay by method C.

Table 2. Relation between NADH Concentration and Inhibitor Production

<table>
<thead>
<tr>
<th>NADH concn, mol/liter</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00019</td>
<td>6</td>
</tr>
<tr>
<td>0.0064</td>
<td>9</td>
</tr>
<tr>
<td>0.064</td>
<td>30</td>
</tr>
<tr>
<td>0.2</td>
<td>62</td>
</tr>
<tr>
<td>0.8</td>
<td>87</td>
</tr>
</tbody>
</table>

* Incubated at this concentration for 50 h at 25 °C in the presence of buffer (pH 7.5 at 25 °C). Just before assay, all solutions were adjusted to 0.19 mol/liter in terms of the initial NADH concentration and assayed for LDH activity by method B.

mmol/liter, pH at 25 °C = 8.0), was packed into a glass column to final dimensions of 20 × 625 mm. About 500 mg of NADH (or its reaction-product equivalent), dissolved in 10 ml of starting buffer, was added to the top of the column. The NADH and accompanying impurities were eluted at 4 °C with a gradient that ranged from 50 mmol/liter (starting buffer) up to 0.5 mol/liter pH at 25 °C = 8.0.

For high-pressure liquid chromatography we used a Model 1240 (Perkin-Elmer Corp., Norwalk, Conn. 06852) chromatograph equipped with a 2.6 × 50 mm column packed with the strong anion exchanger (Ion X-SA; Perkin-Elmer Corp.). The 5-μl sample containing 100 μg of NADH (or its reaction-product equivalent) was injected directly onto the column and eluted with 0.1 mol/liter tris(hydroxymethyl)methylamine phosphate buffer (pH at 25 °C = 7.2). The column effluent was monitored at 254 nm with a fixed-wavelength detector.

LDH activity was determined at 30 °C by one of three procedures. Method A was identical to a procedure described previously (10). Methods B and C were modifications of Method A. In Method B, 50 μl of LDH solutions (pooled serum) was added to 1.00 ml of the test fraction that was to be evaluated for inhibition. To this diluted enzyme was added 0.3 ml of 0.8 mmol/liter NADH in tris(hydroxymethyl)methylamine chloride buffer (0.1 mol/liter, pH at 30 °C = 7.35). The reaction was started with 0.1 ml of 18 mmol/liter sodium pyruvate in the buffer.

Method C is Method A slightly modified and adapted to the Model KA-150 kinetic analyzer (Perkin-Elmer Corp.). In this method, 10 μl of enzyme sample is diluted with 90 μl of water; the mixture is further diluted with 50 μl of 0.72 mmol/liter NADH in tris(hydroxymethyl)methylamine chloride buffer (0.2 mol/liter, pH at 30 °C = 7.35); and the reaction is started with 50 μl of 4.8 mmol/liter sodium pyruvate in the same buffer. NADH or NADH reaction products were evaluated by substituting them in place of either the sample diluent (90 μl) or the NADH reagent (50 μl). In the latter case the NADH and buffer were adjusted to the required conditions before assay.

Results

LDH Activity with Different NADH Preparations

Table 1 shows the relative LDH activity obtained in the presence of NADH preparations from three suppliers. All of these were assayed within one month of receipt. Each was stored at 4 °C in its original container, unopened, until the day of analysis. No two preparations supported the same LDH activity, but the total spread of activities was less than 5%.

Effect of Reaction Conditions on the Generation of LDH Inhibitor

As shown in Table 2, the development of inhibition in NADH solutions was concentration dependent. In this experiment, the different NADH solutions were made up with tris(hydroxymethyl)methylamine buffer instead of water, so as to maintain a uniform pH in the incubation mixtures. The initial pH of unbuffered, 0.6 mol/liter solutions of the disodium salt of NADH was 9.19 at 25 °C. Substitution of 0.4 mol/liter buffer (pH 7.5) for water decreased the pH to 7.85. The degree of inhibition produced in the presence of water or buffer was the same. Replacement of water or buffer with 0.1
or 1.0 mol/liter NaOH led to significantly greater inhibition of LDH activity by the NADH reaction product.

Figure 1 shows the time course of inhibitor formation in unbuffered aqueous solutions of NADH in about 0.6 and 1.2 mol/liter concentrations. The concentration dependence of inhibitor production is clearly illustrated, as is an apparent destruction of the inhibitor toward the end of the incubation period.

Figure 2 shows the time course of selected components in the 0.6 mol/liter NADH reaction mixture. The chromatograms are from high-pressure liquid chromatographic analyses of lower concentrations of NADH in Figure 1. NAD+, adenosine monophosphate, and adenosine-5'-diphosphoribose, originally present in small amounts in the unexposed NADH, progressively increased in concentration during the 96-h exposure. At 48 h, when the rate of inhibitor production had declined (see Figure 1), the NAD+ concentration was near maximum and the NADH concentration had decreased to half the starting value. By 96 h, nearly all the original NADH had disappeared.

Inhibitor formed at different rates with different batches of NADH. As shown in Table 3, the degree of inhibition of LDH activity by four different preparations of water-exposed NADH ranged from 21 to 51%. Inhibitor production appeared to parallel loss of absorbance at 340 nm. The greatest loss of NADH and the greatest inhibition was found in preparation B, 1969. During this 96-h incubation we saw surprisingly little loss of absorbance at 340 nm in the newer NADH preparation A as compared with that found in other lots of NADH (cf. Figure 2).

The striking increase of NAD+ in some of the NADH preparations (see Figure 2) and the presence of this nucleotide as a contaminant of all commercial NADH preparations had indicated that NAD+ might be a component in inhibitor production. We therefore added NAD+ to a concentrated aqueous NADH solution to determine its effect on inhibitor production: the results (Table 4) indicate a significant increase in inhibition. No inhibition was produced by NAD+ incubated alone under the same conditions.

Column Chromatography of NADH

Previous studies by ourselves (4) and others (2, 6, 7) had indicated that an LDH inhibitor could be isolated from impure NADH by anion-exchange chromatography. An inhibitory fraction always eluted after the main NADH peak. However, in subsequent efforts to purify NADH preparations by chromatography on diethylaminoethyl-cellulose, techniques that clearly resolved NADH from this late-running inhibitor still left a nonhomogeneous NADH fraction, regardless of the source of starting material.

Results of several of these attempts to further purify commercial NADH are summarized in Table 5, and the results of one attempt are illustrated in Figure 3. When effluent fractions containing NADH were used as the source of cofactor in the LDH assay, the highest activity was always associated with the peak fractions (corre-

Table 3. Inhibitor Production in Different NADH Preparations

<table>
<thead>
<tr>
<th>NADH source</th>
<th>Year purchased</th>
<th>LDH inhibition % after water exposure</th>
<th>Final A at 340 nm</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>1977</td>
<td>29.0</td>
<td>0.314</td>
</tr>
<tr>
<td>A</td>
<td>1967</td>
<td>32.2</td>
<td>0.291</td>
</tr>
<tr>
<td>B</td>
<td>1977</td>
<td>21.0</td>
<td>0.377</td>
</tr>
<tr>
<td>B</td>
<td>1969</td>
<td>51.2</td>
<td>0.090</td>
</tr>
<tr>
<td>B</td>
<td>1977</td>
<td>control</td>
<td>0.394</td>
</tr>
</tbody>
</table>

(No exposure)

NADH was neutralized with 2 mol/liter tri(hydroxymethyl)methylamine to pH 7.5 before being added to the NADH. Each mixture was incubated for 20 h at 25 °C, pH 7.5

≥96 h and at 25 °C LDH analysis by Method C.

Absorbance contributed by water-exposed NADH to absorbance of the assay mixture.

Electrolyte analysis of this preparation indicated that it was the di-lithium rather than the disodium salt.

Table 4. Effect of NAD+ on Inhibitor Production

<table>
<thead>
<tr>
<th>Nucleotide concn. (mol/liter)</th>
<th>NADH</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.011</td>
<td>0.6</td>
<td>31</td>
</tr>
<tr>
<td>0.16</td>
<td>0.6</td>
<td>49</td>
</tr>
<tr>
<td>0.15</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Endogenous (estimated).

NAD+ was neutralized with 2 mol/liter tris(hydroxymethyl)methylamine to pH 7.5 before being added to the NADH. Each mixture was incubated for 20 h at 25 °C, pH 7.5.

The NADH preparation used in the chromatographic studies illustrated in Figure 3 was estimated to contain 1.2 mol of NAD+ per 100 moles of the preparation.
Table 5. Properties of Several Commercial NADH Preparations before and after Chromatography on Diethylaminoethyl-cellulose

<table>
<thead>
<tr>
<th>NADH source</th>
<th>Before chromatography</th>
<th>Leading edge</th>
<th>Peak max.</th>
<th>Relative activity</th>
<th>Trailing edge</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>90</td>
<td>95</td>
<td>100</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>91</td>
<td>94</td>
<td>100</td>
<td>94</td>
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<td>A</td>
<td>90</td>
<td>97</td>
<td>100</td>
<td>96</td>
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<tr>
<td>A</td>
<td>2.52</td>
<td>3.17</td>
<td>2.32</td>
<td>2.35</td>
<td></td>
</tr>
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</table>

260 nm/340 nm ratio

Corresponding to an elution volume of 625 ml in Figure 3.

Similar results were obtained with use of an alternative chromatographic system, diethylaminoethyl-Sephadex, although resolution of noninhibitory components absorbing at 260 nm near the NADH peak was improved (see Figure 4). NADH from the peak fraction supported a slightly higher LDH activity than did the starting commercial material (Table 6). Late-eluting material (trailing edge, Figure 4) supported significantly less LDH activity than did material from peak fractions despite its still favorable 260 nm/340 nm absorbance ratio.

These results suggested that one or more inhibitory component(s) co-chromatograph with NADH under these conditions. The apparent inhibition of LDH activity was considerably enhanced when water-exposed NADH was chromatographed. Figure 5 illustrates the locations of the two sets of inhibitory fractions. We saw little change in the 260-nm absorbance curve relative to the elution pattern of unexposed NADH (see Figure 4) except for small increases in several of the 260-nm absorbing substances eluting before the NADH peak and the appearance of a small shoulder on the trailing edge of the NADH peak. An association of this shoulder with “inhibitor” has not been demonstrated. The expected post-inhibitory component was clearly separated from both NADH and the early eluting inhibitor.

Table 7 summarizes the relative activities and 240 nm/340 nm ratios of key fractions from this chromatographic run. The NADH fraction corresponding to the peak maximum exhibits a favorable 260 nm/340 nm absorption ratio despite supporting only half as much LDH activity as did the unexposed material.

Discussion

The LDH activity comparisons (Table 1) for different NADH preparations, as well as results of chromatography on diethylaminoethyl-cellulose (Table 5, Figure 3) suggest an improvement over the years in the quality of commercially-supplied NADH, at least from three

Fig. 3. Chromatography of NADH (from source C) on diethylaminoethyl-cellulose

The solid line represents 260-nm absorbance; the dashed line, 340-nm absorbance. Elution was isocratic with 20 mmol/liter buffer used up to the vertical arrow, 0.1 mol/liter buffer thereafter. Peaks I and II are predominantly nicotinamide mononucleotide and NADH but may contain other compounds absorbing at 260 nm. Peak VI is largely NADH. The dotted line indicates the 260 nm/340 nm absorbance ratio in the various fractions making up peak VI. In this particular preparation, no significant inhibition was detected in fractions eluting after peak VI.

Fig. 4. Chromatography of NADH (from source B) on diethylaminoethyl-Sephadex

The peaks immediately preceding the main NADH peak probably include AMP and adenosine-5'-diphosphorbose. Peaks I, II, and VI are as in Figure 3. The solid and dashed lines represent absorbance at 260 nm and 340 nm, respectively. The solid line with filled dots represents the elution gradient (right-hand ordinate).
major suppliers. Nevertheless, all the NADH preparations we have tested to date contain inhibitory components.

We suspected the presence of the second LDH inhibitor in commercial NADH preparations after we found that selected fractions of the chromatographed NADH supported slightly different LDH activities. If the starting NADH were pure, all chromatographic fractions when adjusted to identical concentration should support equal activity.

Differences in the rate of inhibitor production found with different lots of NADH suggest that there may be more than one impurity involved in the various preparations. At least one impurity, NAD⁺, influenced the rate of inhibitor production. Others have noted that inhibitor production can be decreased by purifying the starting NADH (2, 6).

The extent to which a particular reaction condition applies to one or the other inhibitor has not been determined, although these preliminary studies indicate that both are formed in concentrated aqueous solutions of NADH. We hope that high-pressure chromatographic techniques, which have been used successfully to characterize NADH (13), will lead to a more thorough characterization of these important and interesting LDH inhibitor (14).

We thank Mrs. Marcia Montwell for technical assistance with some of these experiments.

References


