Residual Fluorescence as an Index of Purity of Reduced Nicotinamide Adenine Dinucleotide

Barbara F. Howell, Sam Margolis, and Robert Schaffer

Determination of fluorescence remaining after reduced nicotinamide adenine dinucleotide (NADH) has reacted with excess acetaldehyde in the presence of alcohol dehydrogenase (EC 1.1.1.1) is useful as a criterion of NADH purity when used in conjunction with other methods for determining purity such as the rate of reaction, the ratio of ultraviolet absorbances at 260 nm and 340 nm, the color, and the chromatographic homogeneity of the preparation. Measurement of residual fluorescence monitors the enzymatically inactive material which absorbs at 340 nm. The specific optical rotations of NADH at several wavelengths are also reported.

Additional Keyphrase: specific optical rotation of NADH

Demand for more accurate measurement of blood enzyme activities in the clinical laboratory has created a need for an NADH preparation that is more nearly pure than preparations currently available. Determinations of enzymatic activity in blood depend on measuring the rate at which blood serum reacts with NADH and substrate, and enzymatic reaction rates in which NADH functions as the coenzyme may be greatly affected by small amounts of inhibitory or accelerative materials present in some NADH preparations. Descriptions of commercial preparations appearing in supply catalogs indicate that these materials contain as much as 10 to 20% impurity; the effect of these impurities on the activity of the coenzyme is largely unknown and not easily determined. For this reason, development of the capability for detecting and identifying impurities in existing NADH preparations is essential for the production of NADH preparations with accurately known, reproducible activities.

Traditional methods for determining NADH purity include:

(a) Determination of the ratio of absorbances at 260 and 340 nm. The ratio of 2.32 has been widely cited as the criterion (1).

(b) Determination of the molar absorptivities at 338 and 259 nm. The value usually cited for 338 is 6.22 × 10³ liter- mol⁻¹- cm⁻¹, and for 259 it is 14.4 × 10³ liter-mol⁻¹- cm⁻¹ (2).

(c) Measurement of the kinetic coefficients for the enzyme-catalyzed reaction of NADH with the appropriate substrate (3).

(d) Elemental analysis for sodium and phosphorus (1).

(e) Chromatographic homogeneity (1).

(f) Determination of the percentage of the initial spectrophotometric absorbance at 340 nm remaining after the NADH–enzyme–substrate mixture has reached equilibrium (1).

We describe the use of residual fluorescence to monitor NADH purity as an additional criterion. Preliminary attempts to use residual fluorescence for this purpose have been reported by James et al. (4).

Materials and Methods

NADH preparations were obtained from Boehringer Mannheim Corp., New York, N.Y. 10017; P-L Biochemicals, Inc., Milwaukee, Wis. 53205; Searle Diagnostic Inc., Des Plaines, Ill. 60018; and Sigma Chemical Co., St. Louis, Mo. 63178. NAD⁺, lot No. 368, was obtained from Worthington Biochemical Corp., Freehold, N. J. 07728. Horse liver alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1), lot 1JB, was obtained in crystalline form from

In this report, in order to describe procedures adequately, it has occasionally been necessary to identify commercial products and equipment. In no case does such identification imply NBS recommendation or endorsement, nor does it imply that the item identified is necessarily the best available for the purpose.

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Worthington Biochemical Corp. All other chemicals were of the highest quality available commercially and were used without further purification, except for acetaldehyde, which was distilled within a few hours of its use, and cacodylic acid, which was recrystallized twice from ethanol, so that it melted in the range 198–200 °C.

A Farrand Optical Co. (Valhalla, N. Y. 10695) spectrofluorometer with a thermostatted cell compartment was used for fluorescence measurements. The fluorescence intensity of quinine sulfate, at a concentration of 0.25 mg/liter, in sulfuric acid (50 mmol/liter) was used to normalize all measured values of fluorescence intensity. Because the data were obtained on a spectrofluorometer that was uncorrected for instrumental perturbations—such as the dependencies of PM response, monochromator transmission, and source intensity as functions of wavelength—the excitation and emission wavelengths for quinine sulfate and NADH differ from those normally reported. For example, λex and λem for quinine sulfate were 385 nm and 446 nm rather than the commonly accepted values of 348 and 452–461 nm, respectively (5). The effect of these factors on observed emission and excitation wavelengths has been discussed by Parker (5).

To determine values of relative fluorescence intensity, we made fluorescence measurements on solutions containing accurately weighed amounts of the various commercial NADH preparations at concentrations ranging from 2 to 90 μmol.l−1. The percentages of fluorescent intensity appearing in Table 1 were calculated from comparisons between a particular NADH preparation and the commercial material E-1, which rated best according to our evaluation.

To determine the percent residual fluorescence, we measured fluorescent intensity for the mixture of the reactants at the beginning of the enzymatic reaction and corrected for concentration quenching. Fluorescence was remeasured at kinetic equilibrium, and the ratio of the second intensity divided by the first is what we call the “residual fluorescence.”

Optical activity was measured with a Model 141 polarimeter (Perkin-Elmer Corp., Norwalk, Conn. 06856), and ultraviolet absorbances were measured with a Model 14 recording spectrophotometer (Cary, Monrovia, Calif. 91016). These instruments were each equipped with thermostatted cell compartments.

The velocity of the enzymatic oxidation of NADH was measured fluorometrically (excitation wavelength, 353 nm, emission wavelength, 460 nm) at several concentrations of NADH in the presence of, per liter, 444 μmol of acetaldehyde and 5.6 nmol of alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1). Reactions were run in one of two buffer systems: 0.1 mol/liter cacodylic acid buffer that had been adjusted to pH 7.5 by addition of 1.0 mol/liter tris(hydroxymethyl)aminomethane, or 0.11 mol/liter ammonium bicarbonate. Buffer solutions, which constituted 97% of the final solution volume, were equilibrated at 25 °C before the chilled reactants were added. Duplicate determinations were performed at each NADH concentration except with column-purified NADH.

Columns containing diethylaminoethyl cellulose, as described by Strandjord and Clayson (6), were used to prepare chromatographically purified NADH. Results of tests run on these column-purified materials are designated as “col” in Table 1.

**Results**

A summary of the properties of the NADH preparations as they relate to purity is given in Table 1. A pure white color appears to indicate high-purity NADH (1). Determined molar absorptivity values at 340 nm vary for the materials tested, but the more

<table>
<thead>
<tr>
<th>Sample</th>
<th>Color</th>
<th>ε × 10⁻³ at 340 nm</th>
<th>Absorbance ratio, 260/340 nm</th>
<th>Fluorescence intensity</th>
<th>NADH, μmol/liter</th>
<th>Residual fluorescence, % ± SD</th>
<th>Spectrophotometric residual, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>pale yellow</td>
<td>5.67</td>
<td>2.47</td>
<td>99.7</td>
<td>6.8–10.0</td>
<td>2.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>cream</td>
<td>5.52</td>
<td>2.51</td>
<td>96.7</td>
<td>3.84–79.1</td>
<td>3.0 ± 0.2</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>D</td>
<td>cream</td>
<td>5.5</td>
<td>2.45</td>
<td>94.4</td>
<td>6.6–79.7</td>
<td>0.75 ± 0.21</td>
<td>1.0 ± 0.21</td>
</tr>
<tr>
<td>D col</td>
<td>white</td>
<td>5.9</td>
<td>2.29</td>
<td>100</td>
<td>0.70–62</td>
<td>0.31 ± 0.21</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>E-1</td>
<td>white</td>
<td>5.99</td>
<td>2.33</td>
<td>99.2</td>
<td>2.0–87.0</td>
<td>0.61 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>E-2</td>
<td>white</td>
<td>2.26</td>
<td>2.3–58.5</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-2 col</td>
<td>white</td>
<td>2.26</td>
<td>2.3–58.5</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Based on a molecular weight of 709.0; samples were used as obtained from commercial supplier. Molar absorptivity (ε) measurements were made in NH₄HCO₃ buffer (0.11 mol/liter, pH 7.9), except as noted.

* Measurement made in NH₄HCO₃ buffer (0.11 mol/liter, pH 7.9). These figures are percentage of the intensity of sample E-1, the most nearly pure specimen.

* Corrected for absorption at 340 nm caused by tailing from the principal NAD⁺ absorbance maximum at 260 nm. Spectrophotometric measurements were possible only at the highest concentrations shown, whereas fluorometric measurements involved 10–20 measurements made over the entire concentration range.

* Measured in tris(hydroxymethyl)aminomethane HCl buffer (0.10 mol/liter, pH 7.5).

* The abbreviation “col” indicates that the NADH preparation was purified by diethylaminoethyl cellulose chromatography.
nearly pure commercial products have molar absorptivities closer to the best literature value of $6.22 \times 10^3 \text{ liter}^{-1} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Low values for the $A_{360}/A_{340}$ ratio are also characteristic of the more nearly pure materials from commercial sources.

We did kinetic assays of the various commercial NADH materials with alcohol dehydrogenase and acetaldehyde, by monitoring NADH concentrations fluorometrically, and the data were used to produce Lineweaver-Burke plots. For the materials tested, the intercepts of these plots ranged from 0.056 to 0.074 s. These intercept values indicate that the commercial preparations used were virtually free of “inhibitor.” For the most concentrated NADH solutions, about 2 h was required for equilibrium to be attained; for the most dilute solutions, equilibrium was reached within 5 min after the reactants were mixed.

After each kinetic assay, residual fluorescence was measured. Typically, fluorescent residual measurements were obtained for a series of 10 to 20 mixtures in which there were increments of about $5 \mu\text{mol/liter}$ in the initial concentration of NADH.

Values for the residual fluorescence must be corrected for the contribution to fluorescence owing to the products expected (such as NAD$^+$.adducts) from the enzymatic reaction. Their contribution over the range of concentrations of NADH and acetaldehyde used in the enzymatic reaction was determined by measuring the fluorescence of synthetic equilibrium mixtures consisting of NAD$^+$, alcohol, and acetaldehyde in buffer. Contributions to the residual fluorescence from the reaction products agreed very closely with the values for the residual fluorescence as determined directly on the column-purified samples of E-1 NADH at enzymatic equilibrium. Hence, values from the synthetic mixtures were used to correct the measured residual fluorescence intensities. The corrected value was used in the calculation of percent of residual fluorescence.

Nonreproducible, high values for residual fluorescence were obtained if the cuvets used for the measurements had been dried with acetone or alcohol shortly before use, the fluorescence from this source often amounting to 5 to 10 times the value of residual fluorescence from the reaction mixture. Figure 1 shows the effects of acetone and alcohol on the residual fluorescence spectra as compared with a valid spectrum obtained by using a cuvet that was washed only with water and vacuum-dried. The prominent peak observed at 400 nm (uncorrected) in Figure 1 is the Raman band of water.

The spectra obtained for the residual fluorescence of equilibrium mixtures from reactions performed in ammonium bicarbonate buffer showed little evidence of spurious fluorescence, even after storage for 5 h in the dark at room temperature. On the other hand, reactions performed in the tris(hydroxymethyl)aminomethane–cacodylate buffer sometimes showed large increases in fluorescence with time. Fluorescence measurements were therefore made as soon as possible after equilibrium was attained in mixtures for kinetic assay.

After column chromatography of the two best commercial NADH materials, the value for the residual fluorescence was negligible. Since NADH eluted from the column was dissolved in buffer solution, it was not possible to determine color or to measure molar absorptivity or relative fluorescence intensity for these samples.

**Optical Activity**

As another basis for evaluating purity, optical activity was measured for two commercial preparations having different purities. The measurements at several wavelengths appear in Table 2.

**Discussion**

Enzymatically inactive substances that exhibit fluorescence when excited at 340 nm, and which might occur in the commercial preparations of NADH, include $\alpha$-NADH, reduced nicotinamide, reduced nicotinamide riboside, or other reduced nicotinamide derivatives. Of these, reduced nicotinamide, which has an absorbance maximum at 360 nm (7), was apparently not present in quantities detectable by fluorometric analysis of the products of the reaction. NADH itself contributes negligibly to residual fluorescence because the equilibrium concentration of this substance lies below the detection limit of the fluorometer.

Some of the nicotinamide moieties mentioned may be presumed to be present as a consequence of cleavage of NADH molecules. If this process had occurred
and adenine-containing portions were present in an equimolar proportion, the 260/340 ratio would not be a sensitive reflection of such cleavage. However, cleavage is accompanied by an increase in molar absorptivity at both 260 and 340 nm (9), so that measured increases in absorptivity at these wavelengths reflect cleavage. The presence of significant amounts of residual fluorescence further indicates that cleavage has occurred.

The relative fluorescence intensities of the different NADH preparations appearing in Table 1 compared with that of preparation E-1, do not correlate with their measured residual fluorescences because low values of relative fluorescence intensity in commercial NADH preparations are due to nonfluorescent impurities that contribute to weight but not to fluorescence. Substances contributing to residual fluorescence, on the other hand, are initially fluorescent, but are incapable of enzymatic reduction to nonfluorescent substances. They therefore produce fluorescence in kinetic equilibrium mixtures in excess of the background level (residual fluorescence), and no correlation between the two types of impurities would be expected.

Comparison of the results for residuals as measured by spectrophotometry and by fluorescence shows some discrepancy. A source of difference is the fact that the residual absorption at 340 nm measured by spectrophotometry is superimposed on the tail of the 260-nm absorption band of the NAD+. Overlap occurs because of the large concentrations of NAD+ produced by nearly complete conversion of NADH to NAD+. This overlap, shown in Figure 2, coupled with the very low 340-nm absorbance values, makes it difficult to obtain accurate residual measurements by spectrophotometry.

By contrast, measurement of residual fluorescences for testing NADH purity eliminates this interference caused by tailing of the NAD+ absorption band, and provides the advantages of a 10-fold increase in sensitivity, in addition to the inherent selectivity of fluorometry. Because several of the compounds that may arise from cleavage of NADH are optically active, as are α- and β-NADH, the optical rotation of sample B, considered moderately impure, and of sample E-2, which was considerably more nearly pure, were measured. The measured values for the two samples show only small differences in optical rotation. Sample E-2 is slightly more levorotatory at the shorter wavelengths than is sample B, as may be seen from Table 2. The small differences in measured optical rotation between samples B and E-2, contrasted with their considerable differences in residual fluorescence indicate that the residual fluorescent material is not α-NADH. The possible presence of cleavage fragments of NADH, however, is not eliminated by optical rotation measurements as possible contributors to the fluorescent residual.

In view of the small differences observed between relatively impure and more nearly pure preparations and the relatively large amounts (10 mg) of material required to make the measurement, optical rotation may be of limited usefulness as a monitor of NADH purity. We are unaware of other literature values for NADH, although optical rotary dispersion measurements at shorter wavelengths have been made (8, 9).

We conclude that the determination of residual fluorescence for assessing the purity of NADH is best made soon after equilibrium is attained and must be corrected for the fluorescence from the reaction products in the equilibrium mixture and for quenching. The fluorometric method for making residual measurements is both more selective and more sensitive than the spectrophotometric method, and complements other methods used for monitoring NADH purity. We have reported here our measurements of the optical rotation of NADH at several wavelengths; however, this method may be of limited usefulness as an index to NADH purity.
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References


ª May be obtained by writing Customer Services, Clearinghouse, U. S. Dept. of Commerce, Springfield, Va. 22151.