Colorimetry of Diaphorase in Commercial Preparations and Clinical Chemical Reagents by Use of Tetrazolium Salts

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We describe a procedure for assay of diaphorase activity in commercial purified preparations and in clinical chemical reagents by use of iodonitrotetrazolium chloride or other tetrazolium salts. The method is based on measurement of the formazan produced by enzymic reduction of tetrazolium salts in the presence of NADH. The assay procedure has been optimized for linear kinetics, simplicity of operation, nondetectable blank rates, and extended activity/enzyme concentration proportionality. The proposed method has several advantages over the older assay by use of dichlorophenolindophenol.

Enzymes called "diaphorases," which catalyze the NADH- or NADPH-dependent reduction of dyes, have been shown to be flavoproteins containing either FAD or FMN (1, 2). The enzyme reacts with numerous electron acceptors: oxidized lipoyl derivatives, ferricyanide, 2,6-dichlorophenolindophenol, tetrazolium salts, and many other dyes. Diaphorase can be used in clinical chemical assays in any reaction where NADH (or NADPH) is the final product. The inclusion of diaphorase and a chromogenic electron acceptor in the system converts what was ultraviolet spectrophotometry to colorimetry, and increases by threefold the sensitivity of the test.

Diaphorase activity is commonly measured by NADH-dependent reduction of 2,6-dichlorophenolindophenol (DCPIP). On reduction, DCPIP is decolorized, giving a reaction measurable by visible spectrophotometry (3). However, this assay presents several shortcomings: high blank rates, color intensity and time are linearly related for only a few seconds, and decreasing rather than increasing absorbance is being measured. Recently a new assay procedure, based on the reduction of thiazolyl blue tetrazolium bromide (MTT) in the presence of NADH, has been reported (4). This procedure improved the linearity of the assay and decreased the blank rate.

Here we present results of our study on a diaphorase assay in which tetrazolium salts are used. The method is suitable for use in standardization of the activity of this enzyme in clinical diagnostic reagents. We optimized the components of the assay system to obtain linear kinetics, maximum specific activity, proportional activity/enzyme concentration, minimum blank rate, and simplicity of operation.

Materials and Methods

Reagents

Tris(hydroxymethyl)aminomethane and DCPIP were obtained from Merck, Darmstadt, G.F.R.; bovine serum albumin (Cohn Fraction V powder) was from Nutritional Biochemicals Co., Cleveland, OH 44128; disodium β-NADH from PL Bio-

chemicals GmbH, St. Goar, G.F.R.; INT from Loba Chemie, Vienna, Austria; MTT from Sigma Chemical Co., St. Louis, MO 63178; Cremophor EL (polyoxyethylene castor oil ether) from BASF Wyandotte Co., Wyandotte, MI 48192; and NBT from ICN, Cleveland, OH 44128. Purified diaphorase from microbial source was obtained from Whatman Biochemicals, Maidstone, U.K., and Fermco Biochemicals Inc., Elk Grove Village, IL 60007.

Diaphorase Assays

The spectrophotometer used in the assays was a Beckman Model 25. The cuvettes had a 1-cm light path.

Diaphorase activity on MTT and on DCPIP was determined as described by Bothling and Weaver (4). The assay temperature was 25 °C.

Diaphorase activity on INT and on NBT was determined by measuring the increase in absorbance at 500 nm (INT) or 580 nm (NBT), resulting from the reduction of these dyes by NADH in the presence of the enzyme. The reaction was started by mixing 0.2 mL of diaphorase (0.1–0.2 kU/L in a 50 g/L solution of bovine serum albumin) with 2.0 mL of an assay mixture containing, per liter, 0.55 mmol of INT or NBT, 0.55 mmol of NADH (from a freshly prepared 10-fold concentrated solution in 20 mmol/L Tris buffer, pH 8.5), 2.75 g of Cremophor EL, and 55 mmol of the Tris buffer.

The assay mixture can be prepared in large volume and used for several tests. There is a slow but constant color development of approximately 0.03 absorbance unit/h at 25 °C, but it does not interfere. After enzyme was mixed with assay mixture, color development at 25 °C was immediately recorded and thereafter for 3 min. The rate of dye reduction (ΔA/min) was always constant within this period of time. Color development in the absence of enzyme (blank rate) was too low to be measured at the concentrations of these dyes used in the standard assay. In experiments with different assay conditions where blank rate was higher than 0.001 A/min, it was subtracted from the reaction rate.

Calculations

One unit of diaphorase activity is defined as the amount of enzyme necessary to reduce 1 μmol of the substrate (INT, NBT, MTT, or DCPIP) per minute at 25 °C under the conditions of the assay. Maximum absorption wavelengths for INT and NBT formazans under the assay conditions are 497 and 580 nm, respectively.

INT and NBT molar absorptivities were evaluated under the actual reaction conditions by completely reducing a known amount of NADH in the presence of excess diaphorase and excess of dye, and found to be 19 000 and 20 000, respectively. Molar absorptivities for MTT and DCPIP under our assay conditions were 14 000 (4) and 19 900 (5), respectively.

Electrophoresis on Acrylamide Gel

We used the technique proposed by Davis (6). Gels were 10 cm long and 0.5 cm in diameter. Acrylamide concentration was

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1 Nonstandard abbreviations used: DCPIP, 2,6-dichlorophenolindophenol; INT, iodonitrotetrazolium chloride; MTT, thiazolyl blue tetrazolium bromide; NBT, nitroblue tetrazolium chloride; Tris, tris(hydroxymethyl)aminomethane.

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2 The function of the Cremophor EL is to solubilize and stabilize the formazan formed by the reduction of the tetrazolium salts.
75 g/L. Forty microliters of the sample solution, containing 20 μg of diaphorase, was mixed with 10 μL of 500 g/L sucrose and the solution was then layered on top of the gel. Electrophoresis was at 1.5 mA per gel.

Diaphorase activity in the gels was identified as follows: gels were incubated for 1 h in a diaphorase assay mixture containing, per liter, 0.55 mmol of INT, 0.55 mmol of NADH, and 55 mmol of Tris buffer (pH 8.5). Red bands of precipitated INT-formazan appeared. At the end of the incubation period, the gels were stored immersed in water.

Results

Analytical Variables

Effect of pH and buffer ion concentration. The effect of varying the pH of the INT diaphorase assay was determined in three different buffers: Tris, triethanolamine, and phosphate, each at a final concentration of 50 mmol/L. The results are shown in Figure 1. The enzyme activity in Tris and triethanolamine buffers increased with pH up to pH 9.0. The activity was lower in phosphate buffer than in the other buffers and reached a maximum at pH 8.2.

Reduction of INT in the absence of enzyme was negligible at low pH values but became significant (higher than 0.001 A/min), and was subtracted, at pH values above 8.5. The molar absorptivity of the INT-formazan was constant over the pH range tested.

To assay the effect of the buffer ion concentration, we determined diaphorase activity in different buffer concentrations at pH 8.5. The results (Figure 2) show that maximum activities were obtained at low buffer concentrations. The maximum activity in Tris buffer was higher than in the other two systems. Therefore we selected Tris buffer (50 mmol/L, pH 8.5) for the standardized assay.

Effect of the tetrazolium salt concentration. The INT, NBT, or MTT concentration of the diaphorase assay was varied between 0.1 and 1 mmol/L. Measurements were carried out under the conditions shown in Methods for the INT-assay. Blank rates increased with substrate concentration, becoming significant when concentrations of the tetrazolium salts exceeded 0.70 mmol/L. Measured blank rates were subtracted from enzyme reaction rates. The results are shown in Figure 3. Michaelis constants, calculated from the reciprocal plots, were 0.45 mmol/L for INT, 1.00 mmol/L for NBT, and 0.71 mmol/L for MTT.

The dye concentration chosen for the standard assay, 0.50 mmol/L, is far from the saturating concentration, but it was preferred to higher molarities as a trade-off against substantial blank rates.

Effect of NADH concentration. The NADH concentration in the assay system was varied between 10 and 600 μmol/L.

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**Fig. 1.** Effect of pH on diaphorase activity
Tris (△), triethanolamine (●), and phosphate (○) buffers, each at a concentration of 50 mmol/L.

**Fig. 2.** Effect of buffer ion concentration on diaphorase activity
Tris (△), triethanolamine (●), and phosphate (○) buffers, each at pH 8.5.

**Fig. 3.** Effect of tetrazolium salt concentration on diaphorase activity
Double-reciprocal plots of initial velocities as a function of substrate concentration. INT (●), MTT (△), and NBT (○).
Reaction conditions were those shown in Methods for the INT assay. As shown in Figure 4, there was little increase in activity at NADH concentrations above 0.40 mmol/L. The $K_m$ was 14 $\mu$mol/L. We chose to use a 0.50 mmol/L concentration in the proposed assay system.

*Linearity range of the assay.* Diaphorase activity was directly proportional to enzyme concentration up to 0.23 kU/L (0.4 $A$/min) under the standard assay conditions described in Methods (Figure 5).

*Stability of diaphorases.* Solutions of diaphorase in water or buffer are remarkably unstable. This lack of stability imposed a study of the conservation of the enzyme dissolved in different solutions and stored at 4 °C (Figure 6). Diaphorase activity, assayed a few minutes after reconstitution, was greatly diminished when diluted in water or in buffer solutions. High concentrations of bovine serum albumin protected the enzyme against loss of activity so that 80% of enzyme activity remained after six days at 4 °C in the presence of 50 g of albumin per liter.

**Comparative Studies**

Different lots of diaphorase obtained from two manufacturers were assayed by the DCPIP- and the MTT-based procedures (4) as well as by the INT standardized procedure as described in Methods. Diaphorase activity was measured by duplicate in at least six different dilutions of the enzyme in 50 g/L bovine serum albumin prepared on different days. Table 1 summarize the results. It can be seen that the measured enzyme activity depends on the method used to assay it, without a defined correlation between methods, even when different lots from a same manufacturer were used. This was specially noticeable with the enzyme purchased from Fermco, which showed much less activity with the DCPIP method than with the tetrazolium-salt methods. When NBT was substituted for INT, the measured enzyme activities were very similar (under the same standardized conditions). The striking differences in the activities obtained by these distinct methods prompted us to investigate the nature of such diaphorases. Polyacrylamide disc-ger electrophoresis of the enzymes showed that the diaphorase from Whatman differs from the enzyme obtained from Fermco, because the diaphorase activity migrated differently in the gels (Figure 7).

![Table 1. Comparison of Methods and Enzyme Sources](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Enzyme supplier</th>
<th>Lot no.</th>
<th>DCPIP</th>
<th>MTT</th>
<th>INT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whatman</td>
<td>127</td>
<td>5.35</td>
<td>6.95</td>
<td>6.30</td>
</tr>
<tr>
<td>Whatman</td>
<td>R135</td>
<td>7.05</td>
<td>6.25</td>
<td>5.95</td>
</tr>
<tr>
<td>Fermco</td>
<td>4479</td>
<td>1.05</td>
<td>3.50</td>
<td>6.10</td>
</tr>
</tbody>
</table>

*Activities are expressed as IUB units (U) per mg of powder.
Diaphorases from different manufacturers were found to behave quite differently when assayed with distinct substrates. The enzyme purchased from Fermlco showed an activity as much as sixfold lower with the DCPIP method than with the assay described here. This fact probably reflects the different molecular structure of the enzyme as shown by electrophoresis of the tested diaphorases on polyacrylamide gel. Massey and Veeger (11, 12) reported that diaphorase is probably a denatured lipoamide dehydrogenase activity, and that decrease in lipoamide dehydrogenase activity by denaturation is associated with an increase in diaphorase activity. These observations suggest that several diaphorase forms with different ratios of lipoamide dehydrogenase:diaphorase activities may exist, with distinct affinities for electron acceptors such DCPIP or tetrazolium salts. Diaphorase is included in many current clinical chemical assays coupled with INT or NBT, to convert ultraviolet to colorimetric tests (e.g., 13–17) and to improve the sensitivity of the test. Because diaphorase activity in these systems could be an important factor in the behavior of the test, it seems necessary to measure and to standardize diaphorase activity, utilizing the same substrate that will be part of the reagent.

**References**