A New Assay for Diaphorase Activity in Reagent Formulations, Based on the Reduction of Thiazolyl Blue

Robert S. Boehling and Terry L. Weaver

This new assay procedure for diaphorase eliminates problems of high blank rates and nonlinear kinetics associated with other methods. The dye thiazolyl blue tetrazolium bromide is reduced in the presence of NADH and diaphorase to yield a colored formazan, which has maximum absorbance at 560 nm.

Additional Keyphrase: enzymatic method

“Diaphorase” is the generic name for a group of enzymes widely used in clinical diagnostic reagents to couple pyridine nucleotide-dependent anlyte oxidation to reduction of chromogenic electron acceptors. This allows many different assays to be done with measurements within the visible range of the spectrum, rather than in the ultraviolet. Diaphorase activity is commonly measured spectrophotometrically, with use of various electron acceptors such as 2,6-dichlorophenolindophenol (DCPIP). Unfortunately, there are several problems with these techniques, including nonlinearity, high blank rates, and measurement of decreasing rather than increasing absorbance.

Tetrazolium salts have been increasingly applied in recent years, particularly in oxidation-reduction histochemistry (1). Enzymic reduction of tetrazolium salts by microbial diaphorase has been reported (2). In this communication, we describe a new diaphorase assay procedure based on the reduction of thiazolyl blue tetrazolium bromide (MTT). The method is simple, sensitive, and avoids difficulties associated with the use of many other electron acceptors. This work has been presented in preliminary form.

Materials and Methods

Reagents

Tris(hydroxymethyl)aminomethane (Tris), MTT, DCPIP, and FMN were obtained from Calbiochem, La Jolla, CA 92112. Disodium β-NADH was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250, and bovine serum albumin was obtained as Pentex Bovine Albumin Fraction V Powder from Miles Labs., Elkhart, IN 46514. Purified diaphorase from Clostridium kluyveri (EC no. not established) was obtained from Biocentrics, Gibbstown, NJ 08027. Distilled, de-ionized water was used throughout.

The purity of the MTT was assessed by thin-layer chromatography on precoated silica-gel plates (Silica Gel 60, layer thickness 0.2 mm, from E. Merck, Darmstadt, F.R.G.), with n-butanol/acetic acid/water (78/5/27 by vol) as the solvent system (3). Only one chromatographic component was observed either before or after reduction of MTT by alkaline sodium ascorbate.

DCPIP Diaphorase Assay

Diaphorase activity on DCPIP was determined by measuring the decline in absorbance at 600 nm, resulting from the reduction of DCPIP by NADH. Each assay mixture contained the following: 0.3 mL of 0.2 mol/L Tris buffer, pH 7.5; 0.1 mL of 1.2 mmol/L DCPIP; 0.1 mL of 6 mmol/L NADH; 0.1 mL of diaphorase in 0.2 mol/L Tris buffer; and water to a total volume of 3.0 mL. The reactions, carried out at 25 °C, were started by adding the diaphorase. We measured the change in absorbance (ΔA) during the first 60 s after the enzyme was added, from the slope of the recorder tracing made with a Model 240 spectrophotometer (Gilford Instrument Labs., Oberlin, OH 44074), and expressed it as ΔA/min. Blank rates, obtained by measuring ΔA/min in the absence of diaphorase, were subtracted from values of ΔA/min obtained with enzyme. One unit (U) of activity is defined as the enzyme activity producing a ΔA/min of 1.0 under the specified conditions.

MTT Diaphorase Assay

Diaphorase activity on MTT was determined by measuring the increase in absorbance at the absorption maximum of MTT formazan, 560 nm, resulting from the reduction of MTT by NADH. Each assay mixture contained the following: 0.3 mL of 1.0 mol/L potassium phosphate buffer, pH 7.6; 0.5 mL of 2.4 mmol/L MTT; 0.1 mL of 13.4 mmol/L NADH; 0.1 mL of diaphorase in an aqueous solution containing 40 g of bovine serum albumin per liter; and water to a total volume of 3.0 mL. The assay temperature was 30 °C, and reactions were initiated by adding the diaphorase. We measured ΔA/min from the linear portion of the recorder tracing with a Model EU-700 spectrophotometer (GCA/McPherson Instrument, Acton, MA 01720). The rate of MTT reduction in the absence of enzyme was measured for at least 1 min before enzyme was added, but this rate was always zero. One unit of activity (U) is the activity necessary to reduce 1 μmol of MTT to MTT formazan per minute under the specified conditions. Calculate specific activity as follows:

$$U/mg = \frac{\Delta A/\min}{c/1000 \times mg/mL \text{ in assay}}$$

where $c$ is the molar absorbitivity of MTT formazan at 560 nm, 14,000.

The pH values at the assay temperature (30 °C) were determined with a Model 101 pH meter (Corning Scientific Instruments, Medfield, MA 02052). Enzyme weights in grams or milligrams are the actual weights of samples of lyophilized
diaphorase; they are not based on milligrams of total protein in the sample.

**Results**

We studied the effect of pH on the reduction of MTT by diaphorase, and the data in Figure 1 summarize the effects of pH on enzyme activity, duration of linearity, and the duration of the initial period of nonlinearity. Although MTT was reduced most quickly at a pH of 8.5, in agreement with the data of Kaplan et al. (4), the reaction was linear for a longer time at lower pH values. The kinetics almost immediately became linear in the pH range of 7.5-7.8, but the initial nonlinearity was progressively longer at higher or lower pH values. There was no reduction of MTT in the absence of enzyme at any pH value within the interval of the assay. Neither the buffer itself nor added KCl substantially affected the rate or kinetics of the reaction over a range in total concentration of 33-200 mmol/L.

MTT diaphorase activity was also tested for the effects of ethylenediaminetetraacetic acid, Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, and FMN. The first three had no effect on the assay when separately included in the incubation mixture at 3.33 mmol/L. Co$^{2+}$ has been used in histochemical applications to stabilize MTT formazan (1); addition of 0.33 mmol of it per liter not only depressed the rate of MTT reduction by more than half but also altered the color of the formazan produced. Addition of 0.1 mmol of the diaphorase cofactor FMN per liter increased the blank rate, but did not change the duration of linearity of the assay.

As shown in Figure 2, enzyme activity was directly proportional to micrograms of diaphorase per assay up to $\Delta A$/min values of at least 0.2. MTT reduction was linearly related to time for at least 3 min at the highest values of $\Delta A$/min and for longer intervals at lower values. The molar absorbptivity $\varepsilon$ of MTT formazan at 560 nm was determined under conditions essentially the same as those described in Materials and Methods. Its value, measured over a range of initial MTT concentrations, was 14 000 L/mol$^{-1}$cm$^{-1}$ at the concentration used in the final assay procedure.

Figure 3 shows the effect of temperature on enzyme activity, presented as an Arrhenius plot of the log of enzyme activity vs the inverse of the absolute assay temperature. The relationship was linear between 25 and 37°C.

We assayed 25 different lots of diaphorase by the DCPIP-based and MTT procedures and analyzed the results by least-squares linear regression (Figure 4). The data indicate an acceptable correlation between the two methods over a range of enzyme activities of approximately 20-120 DCPIP kilounits/g, or 3-16 MTT kU/g. The coefficient of correlation was 0.93. We made nine replicate determinations of enzyme activity in a single diaphorase dilution and also determined the activity in 11 weighed samples, as measures of within-day and day-to-day reproducibility of the MTT method. The results showed coefficients of variation of 1.44 and 4.32%, respectively.

**Discussion**

Our data show that the MTT-based diaphorase assay fulfills the following criteria: low blank rate, linear kinetics, direct proportionality of activity to enzyme concentration, reproducibility, and ease of performance. The high blank rates and nonlinearity of DCPIP- and ferricyanide-based methods are well known (5). Typical assay procedures with the electron acceptors nitro blue tetrazolium chloride, iodonitrotetrazolium chloride, and 2-(2'-benzothiazolyl)-5-styryl-3(4'-phthalaldehydrazyl)tetrizolium chloride also failed with respect to one or more of these criteria (unpublished data). Nonlinear rates of formazan formation were obtained with all three of these tetrazolium salts as substrates under conditions similar to those described for the MTT-based assay, a result in agreement with an earlier study (6) in which the reduction of MTT, nitro blue tetrazolium chloride, and iodonitrotetrazolium chloride by succinate dehydrogenase was examined with
the use of liver homogenates. In that study, the reduction of nitro blue tetrazolium chloride and iodonitrotetrazolium chloride was nonlinear, but apparent zero-order rates of reduction were obtained with MTT. A report on the MTT-hydroquinone lipoprotein reaction in tissue sections also showed that the production of MTT formazan was linear with respect to time of incubation (7). In the present study, MTT reduction by diaphorase was linearly related to time for at least several minutes over a wide range of enzyme activity. These differences in kinetics may be due to the ability of some tetrazolium salts, including iodonitrotetrazolium chloride and nitro blue tetrazolium chloride, but not MTT, to bind non-specifically to protein. This property has been called "substantivity" (1).

Altman (1) reported that MTT, nitro blue tetrazolium chloride, and iodonitrotetrazolium chloride can react nonenzymically with NADH. Under the assay conditions described in our study, we detected no reduction of MTT with a boiled diaphorase preparation, with or without bovine serum albumin. Moreover, assay solutions that were complete except for the final addition of enzyme solution could be stored at 4 °C for several days with no effect on reaction rate or kinetics and little or no increase in blank absorbance at 580 nm. This negligible blank rate provides substantial benefits in both precision and assay facility over DCPIP-based procedures.

The apparent absence of significant impurities in the MTT preparation we used in this study and in several others that we examined by thin-layer chromatography supports published data (8). The relative absence of impurities in commercial preparations of MTT, in marked contrast to many other commonly used tetrazolium salts, may be considered an important advantage of this compound. In addition, many tetrazolium salts are sensitive to light, but MTT is exceptionally light-stable (9).

The linearity of the Arrhenius plot relating enzyme activity and assay temperature suggests that the Arrhenius equation is an adequate description of the effects of temperature on the rate of the diaphorase-catalyzed reduction of MTT. Thus, enzyme denaturation, though not precluded by the data, is not thermodynamically favored over the range of 25–37 °C. An activation energy of 60 kcal/mol (14.2 kcal/mol) and a Q₁₀ value of approximately 1.9 for the reaction were calculated from the temperature data. The proportional nature of the temperature response of this assay between 25 and 37 °C suggests that this procedure could be satisfactorily adapted for use at any temperature in that range.

References