Intermediate Electron-Acceptors in Quantitative Cytochemistry

Comparison of Phenazine Methosulphate and Meldola Blue

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Summary. The efficacy of Meldola Blue (MB), a new intermediate electron-acceptor, has been compared with that of phenazine methosulphate (PMS) in the assay of oxidoreductase activity in cryostat sections; various tetrazolium salts have been used as the final electron-acceptors. Three enzymes: succinate dehydrogenase, glucose 6-phosphate dehydrogenase and lactate dehydrogenase were investigated, the activity in sections being quantitated by scanning and integrating microdensitometry. Phenazine methosulphate was superior to Meldola Blue in transferring reducing equivalents from reduced coenzyme to all the tetrazolium salts examined.

Introduction

Meldola Blue (8-dimethylamino-2,3-benzophenoxazine), which has recently become commercially available as an electron-transfer reagent, has been claimed to be equal to, or even superior than, phenazine methosulphate in transferring electrons from reduced coenzymes to tetrazolium salts (Möllering et al. 1978; Kugler and Wrobel 1978; Turner and Hopkinson 1979). No quantitative cytochemical evidence supporting this claim has been published. This communication is concerned with the quantitative assessment of the relative values of PMS and Meldola Blue as intermediate electron-acceptors for the assay of NAD⁺, NADP⁺- and flavoprotein-dependent oxidoreductase enzymes in unfixed tissue sections.

Materials and Methods

Adult albino Wistar rats of either sex (weight 200–300 g) were killed by asphyxiation in nitrogen and the livers and hearts were removed. Small pieces (0.5 cm³) were chilled by precipitate immersion in n-hexane (BDH 'low in aromatic hydrocarbons' grade, boiling range 67–70°C) at −70°C. After not more than 1 min they were removed and stored in dry glass tubes at −70°C. The tissues were sectioned at 10 μm in a Bright's cryostat in which the ambient temperature was −25°C.

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to $-30^\circ$ C and with the knife cooled to $-70^\circ$ C by having its haft packed in solid carbon dioxide. The sections were picked off the knife on to warm slides (Chayen et al. 1973).

**Enzyme Assays.** Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by the method of Altman (1972); lactate dehydrogenase (EC 1.1.1.27) by the method described by Chayen et al. (1973) and succinate dehydrogenase (EC 1.3.99.1) by the method of Butcher (1970). All assays were done in 50 mM glycylglycine buffer (BDH) at pH 8.0, containing 30% (w/v) polyvinyl alcohol (PVA; grade G04/140; obtained from Wacker-Chemie GmbH, München West Germany), plus the appropriate tetrazolium salt.

**Preparation of Electron Acceptors.** Phenazine methosulphate (Sigma) and Meldola Blue (Boehringer) were dissolved in distilled water and dimethylformamide respectively, prior to being added to the substrate media. The final concentration of dimethylformamide, in the substrate media, was 5%; this had no effect on the three enzymes assayed.

**Preparation of Tetrazolium Salts.** Nitroblue tetrazolium (NBT); MTT; blue tetrazolium (BT) (Sigma); neotetrazolium chloride (NT; Serva) and INT (BDH) were dissolved in 30% PVA-glycylglycine buffer (pH 8.0) at a concentration of 4.5 mM.

**Measurements of Enzyme Activity.** Enzyme activity was measured by scanning and integrating microdensitometry with a Vickers M85 microdensitometer (Vickers Instruments Ltd., Haxby Road, York, England). The machine settings used were: $\times 40$ objective; optical mask B3 (area scanned for each measurement was 1,500 $\mu$m$^2$) and a flying spot of 0.5 $\mu$m in diameter. The absorption maxima of each of the formazans were determined and subsequent measurements were made at these wavelengths (Table 1). For each enzyme assay seven measurements were made in each of four serial sections and the results (mean $\pm$ SEM) were expressed as the mean integrated absorbance $\times 100$ (MIA $\times 100$) by reference to a standard calibration graph (Bitensky 1980).

**Results**

**Binding of Meldola Blue to Tissue Sections**

When used at concentrations greater than $10^{-4}$ M, Meldola Blue bound to tissue sections staining them blue. The absorption spectrum for Meldola Blue bound to liver sections is shown in Fig. 1. It should be noted that the absorption maximum for MB is identical to that of most of the formazans. This binding to tissue was both time and concentration dependent and was decreased if polyvinyl alcohol was included in the assay media (Fig. 1). Because the binding of Meldola Blue was so significant, when used at $10^{-3}$ and $10^{-2}$ M, in all subsequent experiments this was controlled for by measuring the amount of MB staining in serial sections (inactivated by being placed in boiling water), and subtracting this value from the “activity” in normal sections reacted with MB in the assay medium.

**Comparison of the Electron-Transferring Activities of PMS and Meldola Blue**

Substrate media for glucose 6-phosphate dehydrogenase, lactate dehydrogenase and succinate dehydrogenase assays were prepared in polyvinyl alcohol-buffer

\[ NBT = \text{(2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride)); } \]
\[ 
\text{MTT = (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)); } \]
\[ 
\text{BT = (3,3'-(3,3'-dimethoxy-4,4'-biphenylene)-bis-(2,5-diphenyl-2H-tetrazolium chloride)); } \]
\[ 
\text{NT = (3,3'-(4,4'-biphenylene)-bis-(2,5-diphenyl-2H-tetrazolium chloride)); } \]
\[ 
\text{INT = (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl-2H-tetrazolium chloride). } \]