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Rapid Electrophoretic Examination of Diaphorases in *Mycobacterium tuberculosis* (34027)

THOMAS P. O'BARR

Microbiology Division, U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado 80240

Although diaphorases occur widely in nature, their role in respiration remains obscure. Renewed interest in this area resulted from the finding that extracts of *Mycobacterium tuberculosis* which were resistant to isoniazid contained greater NADH-diaphorase activity than did extracts of drug-susceptible *M. tuberculosis* (1). In the course of these studies a technique was developed which allowed rapid visualization of diaphorases in cell extracts after electrophoretic separation on cellulose acetate membranes. The present report is concerned with describing this technique in detail and illustrating its application to the study of specific tetrazolium reductases.

Methods. *Mycobacterium tuberculosis* H37Rv was maintained by weekly transfer in Middlebrook's 7H10 OA liquid medium. Large numbers of cells were obtained by growth in fermentors containing 14 liters of the same medium. All incubations were carried out at 37°. Cells were harvested while in the logarithmic phase of growth by aerosol-free centrifugation.

In the preparation of cell extracts, recovered cells were washed and made up in thick suspension with Tris buffer, 0.05 *M*, pH 7.0. The suspension was sonicated at icewater temperature for three 10-min intervals with a 5-min rest between each treatment. The sonicate was centrifuged first at 20,000*g* for 30 min and then at 105,000*g* for 90 min. The supernatant fraction from the high-speed centrifugation was removed and filtered through a 0.22-μ filter. This filtrate constituted the cell extract used for all assays. Protein was determined by the Lowry method.

Diaphorases were measured by coupling the oxidation of NADH or NADPH with the reduction of *p*-nitrobluetetrazolium and measuring the increase in absorbancy at 540 mμ. Electrophoretic examination of the extracts was carried out with Sepraphore III as the support medium. Strips were subjected to 450 V for 50–60 min at 5° employing Gelman's high-resolution buffer. Diaphorases were visualized by overlaying the strip with a similar Sepraphore III strip saturated with NADH, buffer, and *p*-nitrobluetetrazolium.

TABLE I. Some Properties of Diaphorases Present in Extracts of *Mycobacterium tuberculosis*.[a]

Addition	Percentage of control rate
Control	100
No menadione	59
No menadione or Triton X-100	21
Menadione replaced by menadione sodium bisulfite	72
NADH replaced by NADPH	19

[a] Reaction tube contained 1.02 mg of protein, 1 ml of 0.1 *M* phosphate buffer, pH 7.4, 0.4 μmole of *p*-nitrobluetetrazolium, 0.5 μmole of NADH, 0.5 μmole of menadione sodium bisulfite or Triton X-100 solubilized menadione in a total volume of 3 ml. Incubation at 37°.

When present, menadione was solubilized with the Triton X-100. The two strips were then placed in a stoppered tube containing a moistened piece of filter paper to maintain an atmosphere of water vapor. After a short incubation at 37°, colored bands of reduced dye served to locate sites of diaphorase activity.

Results and Discussion. Some properties of *p*-nitrobluetetrazolium reductases present in extracts of *M. tuberculosis* are shown in Table I. Both menadione and Triton X-100 greatly enhanced the reduction of *p*-nitrobluetetrazolium. The reactivity of these compounds in other NADH-diaphorase systems has been described (2, 3). Reductase activity was reduced significantly by the replacement of menadione with menadione sodium bisulfite. In addition, the extent of dye reduction with NADPH was 80% lower than that observed with NADH.

By subjecting the extracts to electrophoresis on Sepraphore III, and using an overlay mixture of buffer, NADH, and *p*-nitrobluetetrazolium, the total reductase activity was shown to be the resultant effect of a group of related enzymes. The pattern obtained is shown in Fig. 1, strip C. It was possible to distinguish clearly some five different tetrazolium reductases. The relative ease of this operation may be compared to other studies in which extensive chemical fractionation was employed for the characterization of individual diaphorases (4). In an attempt to correlate the properties of specific reductases with data presented in Table I, additional electrophoretic strips were examined with modified overlay mixtures. The addition of Triton X-100 resulted in an overall intensification of the colored bands of reduced dye (Fig. 1, strip E), and showed its effect to be nonspecific. Although some of the observed effect was lost in photography, a further stimulation was brought about by menadione (Fig. 1, strip D). This stimulation was most apparent for the reductase farthest from the origin. By replacing NADH in the overlay mixture with NADPH it was found that the ability to mediate the transfer of electrons from NADPH to *p*-nitrobluetetrazolium did not extent to all reductases (Fig. 1, strip A). In a like manner the lowered reductase activity observed in the presence of menadione sodium bisulfite (Table I) was found to reflect its suppressive effect on specific reductases (Fig. 1, strip B). It may be speculated that this finding is related to the reported inhibitory effect of menadione sodium bisulfite on the growth of *M. tuberculosis* (5).

These studies have demonstrated the considerable potential afforded by the technique of modifying the overlay mixture in the char-

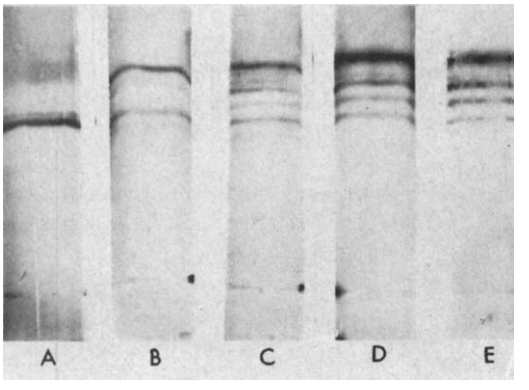

FIG. 1. Electrophoretic pattern of diaphorases present in cell extracts of *Mycobacterium tuberculosis*. (A) NADPH, menadione, Triton X-100; (B) NADH, menadione sodium bisulfite; (C) NADH; (D) NADH, menadione, Triton X-100; (E) NADH, Triton X-100. Overlay mixture contained in 6 ml:1 mmole phosphate buffer, pH 7.4, 2.44 μmole *p*-nitrobuletetrazolium. Menadione (1.5 μmole and 2.5 μmole of NADH or NADPH added as indicated. 0.1 mg of protein placed on Sepraphore III.

acterization of specific reductases. Results obtained in test-tube assay may be related to specific enzymes which could be eluted from the electrophoretic strip for additional study. It is hoped in future work to determine the diaphorase patterns of medically important Mycobacteria, not only for classification purposes, but to explore further the relationship of the soluble tetrazolium reductases to the growth and survival of these organisms.

Summary. *p*-Nitrobluetetrazolium reductases in *Mycobacterium tuberculosis* were examined by a rapid electrophoretic technique. This procedure not only allowed the demonstration that total diaphorase activity was the resultant effect of multiple enzymes but was found to be adaptable to the study of individual tetrazolium reductases. In this manner, Triton X-100 was shown to have an overall stimulatory effect for all reductases, while the effect of menadione was most pronounced for a single reductase. Menadione sodium bisulfite not only could not replace menadione for full activity but was shown to be inhibitory for specific reductases. Most of the reductases were unable to utilize NADPH for the reduction of *p*-nitrobluetetrazolium.

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