The Cytochemical Localization of Oxidative Enzymes

I. Diphosphopyridine Nucleotide Diaphorase and Triphosphopyridine Nucleotide Diaphorase

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(Received for publication, July 7, 1958)

ABSTRACT

Cytochemical methods involving metal chelation of the formazan of an N-thiazol-2-yl tetrazolium salt are described for the localization of diphosphopyridine nucleotide diaphorase (DPND) and triphosphopyridine nucleotide diaphorase (TPND) in mitochondria. These methods utilize the reduced coenzymes DPNH or TPNH as substrate. The reaction involves a direct transfer of electrons from reduced coenzyme to the respective diaphorase which in turn transfers the electrons to tetrazolium salt, reducing it to the insoluble formazan. Competition for electrons by preferential acceptors in the respiratory chain was prevented by various inhibitors. In the presence of respiratory inhibitors the rate of tetrazolium reduction was markedly increased. The greatest reduction was observed when amytyl was used.

Sites of diaphorase activity appeared as deposits of blue-black metal formazan chelate measuring 0.2 to 0.3 μ in diameter. Small mitochondria contained 2 deposits, while larger ones contained up to 6.

Considerable differences were observed in the rate of tetrazolium reduction and cellular localization of diaphorase activity when DPNH was used as substrate as compared to TPNH. In each instance DPNH was oxidized more rapidly by tissues than TPNH. These findings support the concept that the oxidation of coenzymes I and II is mediated through separate diaphorases.

An enzyme capable of catalyzing the oxidation of reduced diphosphopyridine nucleotide (DPNH) by methylene blue or cytochrome was independently discovered by Green el al. (13) and von Euler and Hellström (10, 11). Several years later Straub (27) and Corran et al. (8) succeeded in purifying the enzyme and characterized it as a flavoprotein, which has since been referred to as DPN diaphorase (DPND). Since various tissues catalyze the oxidation of DPNH and reduced triphosphopyridine nucleotide (TPNH) at considerably different rates, Adler et al. (1) concluded that the oxidation of TPNH is mediated through a separate diaphorase (TPND). Further investigations (24) have firmly established that DPND and TPND function in the electron transport system of the cell as a link between DPNH and TPNH and the cytochrome system.

The histochemical localization of DPND and TPND was first accomplished by Farber et al. (12), utilizing an exogenous dehydrogenase and DPN to transfer electrons from substrate to DPND. Triphosphopyridine nucleotide diaphorase, on the other hand, was demonstrable without the necessity of exogenous dehydrogenase. A histochemical method for DPND developed recently by Nachlas and his coworkers (19) is based on the sample principle. Although the latter method allows improved localization of diaphorase, it does not demonstrate all sites of DPND activity unless exogenous enzyme is added.

The present communication concerns the fine...
hypothesis localization and cytometry of DPND and TPND by a method utilizing an N-thiazol-2-yl monobenzotetrazolium salt (21), which does not require exogenous dehydrogenase for the optimal demonstration of diaphorase activity.

Materials and Methods

Male and female stock hooded rats weighing between 100 to 250 grams were used. Tissues were removed as rapidly as possible after the death of the animal, mounted on metal chucks, and frozen within 20 seconds by dipping the base of the chuck in an acetone-solid CO2 mixture. Sections of salivary gland, stomach, and kidney were cut at 4 and 8 μm in a cryostat and mounted on coverslips. Incubation medium containing 3,5-diphenyl-2-(4,5-dimethylthiazol-2-yl) tetrazolium bromide (MTT) was freshly prepared from the following stock solution:

DPND, TPND Stock Solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Cobaltous chloride 0.5 M</td>
<td>0.9 ml</td>
</tr>
<tr>
<td>0.06 M phosphate buffer pH 8.2</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4.1 ml</td>
</tr>
<tr>
<td>Polyvinylpyrrolidine</td>
<td>0.75 gm</td>
</tr>
</tbody>
</table>

Phosphate buffer and cobaltous chloride should be mixed and the precipitate formed should be removed by filtration prior to addition of the remaining constituents. Since the addition of cobalt results in marked lowering of pH, this solution must be adjusted to 7.2 with 0.2 M tris (hydroxymethyl) aminomethane pH 10.4 buffer, using a glass electrode. This solution can be stored for 3 to 4 weeks at 0°-4°C.

DPND and TPND Incubating Medium:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution</td>
<td>1 ml</td>
</tr>
<tr>
<td>DPNH 95 per cent or TPNH1</td>
<td>0.006 gm</td>
</tr>
</tbody>
</table>

Final concentration of reduced pyridine nucleotide is 1 x 10^-2 M. These solutions are stable for 1½ to 2 hours.

The effect of hydrogen ion concentration on the diaphorase reaction was investigated by incubating tissue sections in media at pH 5.3.

The effect of various inhibitors of the respiratory chain on the DPND and TPND reactions was studied by addition of amobarbital (amytal), sodium azide, and quinacrine hydrochloride so that their final concentrations were 1 x 10^-5 M or 1 x 10^-4 M. Duplicate sections were incubated in identical solutions to which cytochrome c had been added to make a final concentration of 1 x 10^-3 M or 1 x 10^-4 M.

To ascertain whether oxidative phosphorylation was present in our preparations the following uncoupling agents were added to the incubating medium prepared with tris buffer at pH 7.2 (15): (1) 1 x 10^-3 m, 2,4-dinitrophenol (DNP), and (2) 1 x 10^-4 m dicoumarol.

The role of vitamin E as a cofactor (20) in DPNH oxidation was studied by addition of 1 x 10^-3 m of d-α-tocopherol acetate (suspended in a 10 per cent ethanol - 0.2 per cent bovine albumin solution) in 0.15 M phosphate buffer at pH 7.2.

Incubating medium was placed on tissue sections in volumes of 0.1 to 0.2 ml. Incubation was performed at 37°C under the following conditions: (1) pure nitrogen, (2) pure oxygen, and (3) air. Following incubation, sections were washed successively in (a) 1 per cent HCl, (b) distilled water, and (c) fixed in 10 per cent formaldehyde for 5 minutes. They were then thoroughly rinsed in distilled water and mounted in glycercin jelly containing 0.5 M-cobaltous chloride.

RESULTS

Diphosphopyridine Nucleotide Diaphorase:

Intracellular sites of DPND activity were demonstrable in most instances after 12 to 15 minutes incubation in air. These appeared as intramitochondrial formazan deposits 0.2 to 0.3 μm in diameter separated from each other by a distance of 0.2 to 0.4 μm (Fig. 1). These appearances are compatible with the idea that small mitochondria contain 2 deposits while larger ones may contain as many as 6. In very active cells longer incubation resulted in mitochondrial swelling and growth of the formazan deposits with a loss of fine localization of DPND.

In rat salivary gland the sites of highest activity were localized in the excretory and intercalated ducts (Fig. 2). Serous cells showed a weak reaction. DPND activity in the squamous portion of the stomach was localized mainly in the basal M epithelial layer (Fig. 3). In the parietal portion of the stomach, on the other hand, sites of enzyme activity were distributed throughout the mucosa (Fig. 4). An intense activity was localized in parietal cells especially those in the upper one-third of the gastric glands, while the surface epithelium and zymogen cells showed a weak reaction. Smooth muscle and vessels throughout the stomach exhibited an intense activity.

In the kidney, DPND activity was highest in the inner cortex (Fig. 5). The descending limb of Henle's loop showed an intense reaction, whereas the proximal tubules, ascending limb and distal tubules exhibited a moderate degree of activity. A weak reaction was present in the mesangium of the glomerular tuft, collecting ducts and renal...
papilla. The terms descending and ascending limbs refer here, and elsewhere, solely to the thick parts of Henle’s loop.

A slight increase in the rate of reduction of tetrazolium was observed when incubation was performed in an atmosphere of nitrogen as compared with air or oxygen. The addition of various inhibitors resulted in marked changes in the rate of reduction. An optimal effect was obtained only when high concentrations of inhibitor were used. Amytal produced the greatest increase in reduction, so that the DPND reaction was usually complete in 5 minutes. Azide showed an intermediate effect and shortened the incubation time to 10 minutes. Quinacrine hydrochloride, on the other hand, inhibited the tetrazolium reduction and increased the incubation time to 25 minutes.

At pH 5.3 the rate and intensity of the DPND reaction was approximately one-half that observed at pH 7.2.

A $1 \times 10^{-5}$ M cytochrome $c$ markedly inhibited tetrazolium reduction in the presence of amytal. No inhibitory effect was observed when $1 \times 10^{-6}$ M cytochrome $c$ was added.

In no instance was an increase in the rate of DPND reaction observed when either DNP or dicoumarol were added to the incubating medium. $1 \times 10^{-5}$ M vitamin E likewise did not change the reaction rate.

**Triphosphopyridine Nucleotide Diaphorase:**

Although the TPND reaction was sufficiently rapid in the presence of amytal to show mitochondrial localization of diaphorase activity after 15 to 20 minutes of incubation, it was noticeably more sluggish than the DPND reaction. The distribution of TPND activity in the salivary gland and squamous portion of the stomach was identical with that of DPND. In addition, ganglion cells and nerve fibers showed a strong reaction especially in Auerbach’s and Meissner’s plexus. The parietal portion of the stomach showed a moderate reaction in the parietal cells localized in the middle third of the gastric gland (Fig. 6). A weak reaction was observed in the surface epithelium and zymogen cells. Smooth muscle and vessels showed a high activity.

In contrast to the DPND reaction, TPND activity was distributed throughout the entire renal cortex. Highest activity was localized in the descending limb of Henle’s loop (Fig. 7). The proximal tubules, ascending limb, and distal tubules showed a moderate reaction. A moderately intense activity was observed in the macula densa while the glomerulus showed little activity (Fig. 8). A weak reaction was exhibited by the collecting ducts and papilla (Fig. 9). At pH 5.3, the TPND reaction was markedly inhibited and such active tissues as kidney showed an activity approximately one-third the activity observed at pH 7.2. The effects of incubation in nitrogen, air, and oxygen were similar to those observed in the DPND reaction. Amytal and azide produced increases in tetrazolium reduction which were identical to their effects on the DPND system, while quinacrine hydrochloride completely inhibits the TPND reaction.

Although $1 \times 10^{-4}$ M cytochrome $c$ inhibited the amytal effect, this was less marked than in the instance of the DPND reaction. No change in the rate of the TPND reaction was observed in the presence of DNP or dicoumarol.

**DISCUSSION**

The present experiments have shown that a rapid and fine histochemical localization of DPND and TPND can be achieved with DPNH and TPNH as substrates. The intramitochondrial localization of DPND and TPND is similar to that observed for succinic dehydrogenase (21, 22) and suggests a basic pattern of enzyme localization in the mitochondrion. The reaction involves a direct transfer of electrons from reduced pyridine nucleotide to the respective diaphorase which in turn presumably transfers the electrons to the tetrazolium salt, reducing it to the insoluble formazan. Since the reaction does not require the addition of exogenous dehydrogenase, the method offers several distinct advantages over diaphorase methods in current use. First, the incubating medium can be prepared very easily. Secondly, spontaneous reduction of the incubating medium due to the presence of diaphorase in the chosen exogenous enzyme preparation is avoided. This is especially true in the case of alcohol dehydrogenase which may contain DPND as a contaminant, according to Racker (24).

The ability of the DPNH and TPNH to enter mitochondria signifies that some alteration has occurred to the mitochondrial membrane, as shown by Lehninger (18). In addition, the failure of 4,5-dinitrophenol and dicoumarol to increase the rate of tetrazolium reduction in the presence of reduced coenzyme is further evidence that our preparative
techniques of freezing, cold microtomy, and thawing, have uncoupled oxidative phosphorylation. The marked inhibitory effect of a hydrogen ion concentration on the oxidation of TPNH by its diaphorase may be due to isomeric conversion of the adenylic acid ribose moiety of TPNH, since Shuster and Kaplan (26) have shown that the monophosphate group of adenylic acid ribose migrates from the 2' to 3' position at an acid pH, and that the 3' TPN formed is incapable of functioning with enzymes that are strictly specific for the naturally occurring 2' TPN. The respiratory chain involved in DPNH and TPNH oxidation may be represented (incompletely) as follows:

\[
\text{TPNH} \rightarrow \text{TPND (flavoprotein)} \\
\text{Tetrazolium} \rightarrow \text{cvt} \rightarrow \text{cvt} \rightarrow \text{cvt} \rightarrow \text{O}_2 \\
\text{DPNH} \rightarrow \text{DPND (flavoprotein)}
\]

The use of respiratory inhibitors to block the electron transport chain at different points shows that those substances which block near diaphorase increase the rate of tetrazolium reduction to a greater extent than those which exert their effect on terminal components of the chain. Since interruption of electron transport results in a reduction of components on the substrate side of the block and oxidation of components on the oxygen side, our results show that alternate pathways of electron transport may exist. These in effect “drain” electrons from the main transport pathway and thus decrease the rate of reduction of the blocked portion of the chain, leading to longer incubation periods, which, in turn, cause modifications of mitochondrial structure and function (16, 25).

Most efficient of the blocking agents tested on the DPND and TPND reactions was amytal, which according to Chance (6, 7) blocks electron transfer between DPN and DPND (flavoprotein) in intact liver mitochondria. Thus, one would expect amytal completely to inhibit the DPN-diaphorase reaction in tissues, since it has been shown by numerous investigators (3, 4, 12, 17, 19) that reduction of tetrazolium salts and other dyes is mediated by flavoprotein. However, contrary to Chance's biochemical findings, the rapid rate of tetrazolium reduction by tissues in the presence of reduced pyridine nucleotide and amytal, suggests that the site of the amytal effect is beyond flavoprotein. Recently, Estabrook (9) has shown a similar site of inhibition with amytal in the DPNH cytochrome c system and suggests that this may be due to a structural alteration of the electron transfer chain. Our results may possibly be explained on a similar basis, since freezing and thawing exert a deleterious effect on tissues.

Although oxidative phosphorylation is uncoupled by narcotics, as shown by Brody and Bain (5), the amytal effect on our preparations cannot be explained on this basis, since the phosphorylation mechanism has been uncoupled during preparation of the tissue sections prior to the addition of amytal.

The DPND and TPND reactions with DPNH and TPNH as substrates demonstrate all sites of diaphorase activity and the results are in agreement with Farber's observations (12). However, subsequent studies (15) utilizing substrates for various DPN and TPN-linked dehydrogenases showed variations in the cellular distribution of diaphorase activity. These results suggest that although the DPN and TPN diaphorase systems are widely distributed, they are associated with different DPN and TPN-linked dehydrogenases which may vary from cell to cell. This will be reported on more fully in the following communication.

The failure of some cells to oxidize DPNH and TPNH after long incubation periods may be interpreted to mean either that some mitochondria do not contain the respective diaphorase or that the N-thiazol mononetrazolium salt is not sufficiently sensitive to demonstrate sites of low enzyme activity. The latter explanation, however,
seems unlikely in view of the redox potential of -0.11 volts for the reduction of MTT to its formazan (23).

The diaphorase reactions as described here provide simple and inexpensive methods for demonstrating DPND and TPND containing mitochondria. In addition, the rapidity and clarity of mitochondrial localization afforded by these methods represent a distinct advantage over current cytological methods (modified Altmann methods such as those of Wigglesworth) for demonstrating mitochondrial morphology.

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EXPLANATION OF PLATES

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Fig. 1. Rat kidney. Tubular cell from descending limb of Henle's loop. Sites of DPN-diaphorase activity were localized as discreet 0.2 to 0.3 \( \mu \) cobalt-formazan deposits in rod-shaped mitochondria. \( \times \) 4,450.

Fig. 2. Rat salivary gland (serous part). DPN-diaphorase activity localized in the secretory and intercalated portions of the salivary ducts. \( \times \) 395.

Fig. 3. Rat stomach (squamous). Moderately intense DPN-diaphorase activity localized in the basal cells. Enzyme activity on the surface of the stratum corneum was due to bacteria. \( \times \) 395.

Fig. 4. Rat stomach (parietal). The most intense DPN-diaphorase activity was localized in the upper one-third of the gastric glands. \( \times \) 100.

Fig. 5. Rat kidney (inner cortex). Intense DPN-diaphorase activity localized in the descending (thick) limbs of Henle's loop. \( \times \) 100.
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Fig. 6. Rat stomach (parietal). TPN-diaphorase activity was localized in the midportion of the gastric glands. × 100.

Fig. 7. Rat kidney. TPN-diaphorase activity in the cortex. Sites of highest activity were localized in the descending (thick) limbs of Henle's loop. × 100.

Fig. 8. Rat kidney. TPN-diaphorase activity in proximal convoluted tubules and in a distal tubule, including the macula densa. × 400.

Fig. 9. Rat kidney. Distribution of TPN-diaphorase activity at the cortico-medullary junction. Note the strong reaction in the descending (thick) limbs of Henle's loop as contrasted to the weak activity in the collecting ducts. × 100.
(Scarpelli et al.: Localization of oxidative enzymes. I)