A Histochemical Method for the Demonstration of Diphosphopyridine Nucleotide Diaphorase*†

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PLATES 11 TO 13

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ABSTRACT

The present investigation concerning the histochemical demonstration of DPN diaphorase follows the development of a new reagent, Nitro-BT, which has already been used successfully for the cytochemical localization of the succinic dehydrogenase system. The most consistently favorable results were obtained with the lactate-lactic dehydrogenase system buffered at pH 7.4. Using sections of rat kidney and stomach, it was found that the intensity of stain was optimal after 15 minutes incubation at 37°C., conducted aerobically.

By appropriate variations in the substrate mixture it was possible to selectively demonstrate the histochemical distribution of certain DPN-linked dehydrogenases in addition to DPN diaphorase. This was made possible by the special distribution of some of these dehydrogenases which distinguished them from one another. Of the dehydrogenases studied the distribution pattern of β-hydroxybutyric dehydrogenase was the most singular. In the gastric mucosa β-hydroxybutyric dehydrogenase was restricted to the cells of the mucous lining epithelium and the gland necks; and in the kidney the enzyme was limited to the cells of the proximal convoluted tubule and thick limbs of Henle’s loop. In contrast, lactic dehydrogenase like DPN diaphorase was demonstrable in almost all cytologic elements of both the stomach and the kidney.

During the latter part of the 1930’s, biochemical investigations in several laboratories (1, 4-6) established the fact that the redox dyes which were used as indicators of dehydrogenase activity were not reduced by the specific enzyme or either coenzyme, but rather by a flavoprotein intermediate. Straub (18) were the first to isolate the flavoprotein which transferred enzymatically liberated hydrogen from reduced diphosphopyridine nucleotide (DPNH) to a redox dye. This enzyme has been designated as DPN diaphorase. Kun (12)

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and Jensen, et al. (10) suggested the use of tetrazolium salts for the determination of several DPN and TPN-linked dehydrogenases. At about the same time, Kuhn (11) and Brodie and Gots (2) demonstrated that the flavoprotein intermediate, DPN diaphorase, was also necessary for the enzymatic reduction of the tetrazolium salts.

Following the introduction of a new ditetrazolium salt 2,2′,5,5′-tetraphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (BT) by Seligman and Rutenburg (15) for the histochemical demonstration of the succinic dehydrogenase system in 1951, Farber, Sternberg, and Dunlap (7, 8) applied this reagent in 1956 to the development of histochemical methods for the localization of the diaphorases. In a series of carefully planned and beautifully executed experiments the latter authors presented data which
correlated biochemical findings with their histochemical observations. Although they used a variety of substrates in an effort to demonstrate the specific dehydrogenases, only two distinctive staining patterns were obtained; one common to all the DPN-linked enzymes and one for those which were TPN-linked. Thus, they showed that the visualizing pigment, the diformazan, demonstrated consistently the sites of DPN or TPN diaphorase, regardless of the substrate utilized, as long as the latter was dehydrogenated by an enzyme linked to either DPN or TPN. The sensitivity of their methods was greatly increased by the ingenious procedure of introducing into the incubation media catalytic amounts of appropriate redox dyes which served as intermediates in the transfer of electrons from the diaphorases to the tetrazolium salt at the enzymic sites (9).

Several shortcomings exist, however, in the histochemical methods for the diaphorases as recommended by Farber and associates; namely (1) anaerobic conditions are required during the incubation period, (2) the incubation medium consists of a complicated mixture of reagents, (3) the visualizing pigment appears as two colors (red and blue), the explanation for which is not clearly understood, and (4) the diformazan produced from blue tetrazolium by enzymatic activity is not satisfactory for cytochemical localization.

Recently, the introduction of two nitro groups into blue tetrazolium, 2,2'-di-(p-nitrophenyl)-5,5'-diphenyl-3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride (Nitro-BT) has been reduced from blue tetrazolium by enzymatic activity clearly understood, and (4) the diformazan produced from blue tetrazolium by enzymatic activity is not satisfactory for cytochemical localization.

**Materials and Methods**

**Histological Preparations.**—Tissues of freshly killed rats were cut into small blocks and quickly frozen by immersion in isopentane kept at −70°C with a mixture of acetone and dry ice. Blocks were stored at −20°C. They were then mounted on half coverslips (11 x 22 mm.) so that the incubations could be carried out in a small volume of reagents. To insure adherence of the section to the coverslip, a jet of air was directed over the mounted section for about 5 seconds just before it was to be inserted into the incubation solution. The sections were incubated aerobically at room temperature for 5 to 30 minutes. They were then rinsed briefly in saline, fixed in formalin for 10 minutes, carried through the graded alcoholic solutions, xylol, and mounted in Canada balsam.

**Incubation Solutions.**—Several substrates were studied both with and without added exogenous enzyme preparations. These included sodium lactate (Fisher Scientific Company), lactic dehydrogenase, malic dehydrogenase, and diphosphopyridine nucleotide (Sigma Chemical Company), l-glutamic and l-malic acids (Eastman Kodak Company), and o-nitro-4-hydroxybutyric acid and alcohol dehydrogenase (Mann Research Laboratories). Nitro-BT was synthesized in our laboratory as described elsewhere (19). The incubating solutions were prepared identically except for the substrate variations and consisted of the following reagents:

1. DPN (5 mg/ml stock) 0.3 ml.
2. Nitro-BT (5 mg/ml stock) 0.3 ml.
3. Phosphate buffer pH 7.4 (0.2 M) 1.0 ml.
4. Substrate and water (with or without exogenous dehydrogenase) to make 3 ml.

Five different substrates were studied. These were added individually to the reagents listed above in the following amounts:

1. Sodium lactate (0.5 m) 0.6 ml.
2. Sodium malate (2.5 m) 0.3 ml.

1 The capacity to bind tenaciously without requiring a mordant.
binding agents, redox dyes, and metallic ions were stored at -20°C. when not in use. In addition to the reagents used in the incubation media, a number of ketone reagents were adjusted to pH 7.4 at the time of preparation and refrigerated at 6°C. for as long as 2 to 3 months. The solutions of DPN and the dehydrogenases were made up in small quantities (see Table II) without pH adjustment and stored for as long as a month at -20°C. when not in use. In addition to the reagents used in the incubation media, a number of ketone binding agents, redox dyes, and metallic ions were tested in the course of this study.

Control sections were prepared by immersion in boiling water for 5 minutes. This procedure uniformly destroyed all enzymatic activity. Exposure to absolute ethanol or formaldehyde required longer periods for effective enzymatic inactivation, and hence was not used. As an additional safeguard to rule out non-enzymatic tetrazole reduction, fresh sections were incubated in solutions containing all the reagents of the incubation mixture except the substrate.

Conditions for the Demonstration of DPN Diaphorase.—The experiments which led to the recommended incubation medium are recorded in the section on results. The reaction mixture includes the following reagents:

- Sodium lactate (0.5 M) 0.6 ml.
- Lactic dehydrogenase (1.5 per cent solution) 0.2 ml.
- DPN (5 mg./ml.) 0.3 ml.
- Nitro-BT (5 mg./ml.) 0.3 ml.
- Phosphate buffer pH 7.4 (0.2 M) 1.0 ml.
- Water to make 3.0 ml.

EXPERIMENTAL

Enzymatic Localization with Different Substrates.—The viewpoint was expressed by Farber and associates (7) that the precipitated diformazan identifies the site of DPN diaphorase, rather than that of a specific dehydrogenase. To substantiate this argument they cited the observation that irrespective of the substrate employed, the enzymatic localization was identical. Since the resolution of their method was limited to a histochemical level, the possibility arose that at a cytochemical level differences might be noted in the intracellular localization of the various dehydrogenases which would be expected to be found in the same tissue.

Fresh frozen sections of rat kidney and stomach were incubated in a series of similar solutions (as described above) into which the various substrates were added in the specified amounts. The intensity of the staining varied with the different substrates, indicating differences in enzymatic activity. In general, the activity in decreasing order was: lactic, malic, glutamic, β-hydroxybutyric, and alcohol dehydrogenase. By contrast, Farber et al. (7), using blue tetrazolium in media lacking exogenous dehydrogenase, were unable to obtain enzymatic staining with ethanol, sodium glutamate, and sodium β-hydroxybutyrate. But more important than this variation in intensity of stain, was the finding of qualitative differences in the staining patterns. Table I summarizes some of these differences in the rat kidney and stomach. The pattern of formazan distribution seen in sections that had been incubated in either the lactate or the malate medium was closely similar to that obtained with either of the media that contained exogenous lactic or malic dehydrogenase. On the other hand, incubation in the glutamate, ethanol, and particularly the β-hydroxybutyrate media, yielded highly singular patterns of stain. Presumably, the distribution of lactic and malic dehydrogenases in the rat kidney and stomach is coextensive with that of DPN diaphorase, while the other dehydrogenases have a more restricted distribution than DPN diaphorase.

Sections that had been incubated in the ethanol medium were unstained except for the glomeruli, proximal convoluted tube, and stroma of the papilla in the kidney, and the surface epithelium in the stomach. The pattern of formazan deposition in the kidney sections that had been exposed to the glutamate medium resembled that obtained with lactate; while in the glutamate-treated stomach sections, the surface epithelial cells became darkly stained, but the parietal and zymogenic cells were almost negative. After incubation in the β-hydroxybutyrate mixture, the components of the nephron that showed intense activity were the loops of proximal convoluted tubules of the juxtedudillary portion of cortex and the thick limb of Henle’s loop. Sections of stomach mucosa showed β-hydroxybutyric dehydrogenase activity in the surface epithelium and superficial half of the glands. The highly selective staining pattern obtained with the β-hydroxybutyrate medium may be contrasted with the more widely distributed stain obtained with lactate in Figs. 1 and 2.

Since the different patterns of dehydrogenase activities in the kidney were somewhat more striking in the mouse than in the rat, the former are illustrated in Figs. 3 and 4. Here it may be seen that in the specimen incubated in the lactate medium all of the cellular elements show some...
Demonstration of DPN Diaphorase

Table I

Enzymatic Distribution in Rat Kidney and Stomach of DPN Diaphorase and Several DPN-Linked Dehydrogenases

<table>
<thead>
<tr>
<th>Organ</th>
<th>Histologic element</th>
<th>DPN diaphorase</th>
<th>Specific DPN-linked dehydrogenases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lactic</td>
<td>Malic</td>
</tr>
<tr>
<td>Kidney</td>
<td>Glomerulus</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Proximal convoluted tubule</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Distal convoluted tubule</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Thick limb (Henle's loop)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Thin limb (Henle's loop)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Collecting tubules (cortex)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Collecting tubules (medulla)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Stromal cells (papilla)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle (arterial)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Stomach</td>
<td>Surface epithelium</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Parietal cells (superficial)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Parietal cells (deep)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Zymogenic cells</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Serial sections 6 μ thick were incubated aerobically at room temperature for 20 minutes (except for 4 hour incubation with ethanolic dehydrogenase) in the various substrate media as described in the method section. The intensity of staining was arbitrarily graded from 0 to 4. The relative differences between various cytologic elements with the individual substrates resulted in several distinctive histological staining patterns (see figures).

The degree of activity (Fig. 3). Note that the epithelial cells of the entire cortex are uniformly and intensely stained and that the tubules traversing the papilla are stained, though not intensely. In contrast to the latter distribution pattern representing lactic dehydrogenase system, the consecutive section incubated in the β-hydroxybutyrate medium remained unstained throughout the papilla and most of the outer cortex (Fig. 4). In fact, the diformazan particles were restricted to the proximal convoluted tubules and thick limbs of Henle's loop. These findings suggest that the diformazan is deposited at the site of the diaphorase which is intimately associated with the specific dehydrogenase. Thus, it is possible to demonstrate the site of the various dehydrogenase systems, despite the fact that the diaphorase rather than the dehydrogenase is responsible for the reduction of the tetrazole (shown below).

Combinations of Substrate and Exogenous Dehydrogenase.—Since the diaphorase can accept the electrons from the dehydrogenase as rapidly as they are provided, Farber et al. (7) recommended the addition of exogenous enzyme to the incubation media. By this maneuver, staining was produced more rapidly. These findings were confirmed in our laboratory by adding lactic, malic, and ethanolic dehydrogenase to the three incubation media containing those substrates, respectively. Since the stain appeared rapidly in sections incubated with sodium lactate alone, the added dehydrogenase made little difference in the incubation time. With sodium malate, however, the exogenous enzyme resulted in a slight, but definitely increased rate of staining, while with ethanol, the added alcoholic dehydrogenase produced a very marked increased in rate of diformazan formation. In contrast to the qualitative differences found above when the substrates were used in the absence of exogenous dehydrogenase, it was noted that such differences were effaced when incubations were conducted in the presence of exogenous dehydrogenase. Thus, the dehydrogenase when present in abundance together with its specific substrate is able to saturate the diaphorase throughout the section with the enzymatically liberated hydrogens, and the tetrazolium salt is reduced at all these sites. Because the optimal concentrations of the various reagents we employed in this study differed from those of Farber et al. (7) this information has been summarized in Table II.

Recommended Conditions for the Demonstration of DPN Diaphorase.—Since the blue tetrazolium...
is not as readily reduced as Nitro-BT, Farber and associates (7, 8) found it necessary to use an incubation medium composed of several substrates (malate and ethanol), one exogenous enzyme source, and other reagents, even when utilizing the catalytic effect of a redox dye. Furthermore, they found anaerobiosis necessary to obtain a satisfactory stain. While it seems likely that sodium lactate alone would serve to visualize all the diaphorase sites when Nitro-BT is used, the possibility does exist that certain cells might contain DPN diaphorase in association with an enzyme other than lactic dehydrogenase. Therefore, in order to demonstrate all of the diaphorase loci, the addition of exogenous enzyme is advised. Significant improvement in staining was not obtained by the addition of substrates or enzymes other than those in the recommended incubation solution, which has been given in the Method section above.

Enzymatic Reduction of Nitro-BT.—Several workers have demonstrated that the tetrazolium salts are reduced by a flavoprotein (the diaphorase) rather than by the specific dehydrogenase (2, 11). Since this new tetrazolium is much more readily reduced than those studied by others, it seemed advisable to reaffirm this fact. That only the diaphorase transfers the hydrogens to Nitro-BT was shown by the following observations:

1. Histologic sections became faintly stained, if at all, when incubated in several substrate solutions lacking extrinsic DPN. This was true even if the specific dehydrogenase was added to the solution. The weak stain that developed was attributed to the presence of intrinsic DPN. This was proven by the experiment in which sections were allowed to soak for 1 hour in saline prior to incubation, thus washing out the intrinsic DPN. Such pre-soaked sections remained entirely unstained during the subsequent incubation in a medium which lacked DPN. On the other hand, presoaked sections did become stained in the presence of extrinsic DPN. Thus, the specific dehydrogenases could not reduce the tetrazolium salt.

2. Incubating solutions containing a dehydrogenase (lactic or malic), its substrate, and DPN yielded no coloration in the absence of tissue. Furthermore, when reduced DPN is allowed to incubate with the tetrazolium salt, no color developed. Thus, reduced DPN, whether produced chemically or enzymatically, did not reduce the tetrazole.

3. Sections incubated in the various substrates

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration in medium</th>
<th>Range tested</th>
<th>Optimal concentration</th>
<th>Reduction color of supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium lactate</td>
<td>0.05-0.25 mg./ml.</td>
<td>0.1 mg./ml.</td>
<td>4+</td>
<td>0</td>
</tr>
<tr>
<td>Lactic acid dehydrogenase</td>
<td>0.025-0.25 mg./ml.</td>
<td>0.1 mg./ml.</td>
<td>4+</td>
<td>0</td>
</tr>
<tr>
<td>Sodium malate</td>
<td>0.025-0.8 mg./ml.</td>
<td>0.75 mg.</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>Malic acid dehydrogenase</td>
<td>300-3000 units/ml.</td>
<td>1000 units/ml.</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.2-4.0 per cent/ml.</td>
<td>1.0 per cent/ml.</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>0.025-0.5 mg./ml.</td>
<td>0.1 mg./ml.</td>
<td>4+</td>
<td>Pink</td>
</tr>
<tr>
<td>Sodium β-hydroxybutyrate</td>
<td>0.01-1.0 mg./ml.</td>
<td>0.4 mg.</td>
<td>±</td>
<td>0</td>
</tr>
<tr>
<td>Sodium glutamate</td>
<td>0.04-0.1 mg./ml.</td>
<td>0.1 mg.</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>DPN</td>
<td>0.2-0.8 mg./ml.</td>
<td>0.5 mg./ml.</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

The only exception was encountered in assay systems containing alcohol and alcohol dehydrogenase, in which a faint pink to blue coloration appeared in the solution after repeated incubations with several sections. In the absence of sections, the coloration of the solution occurred more slowly. The presence of inactivated sections had no influence on the slow development of the color in solution, and the sections were invariably unstained. The conclusion which appears justified is that the commercial preparation of alcohol dehydrogenase also contains a trace of diaphorase. For this reason, it is not recommended as the substrate-enzyme combination best suited to demonstrate DPN diaphorase.
inhibitory effect of oxalacetate, Farber et al. (7) found it necessary to introduce semicarbazide to bind the inhibitor, or sodium glutamate to remove it by enzymatic transamination to aspartate. Without one of these manipulations, these workers obtained no stain in the malate incubation solution, and although the reaction proceeded in the presence of either semicarbazide or glutamate, they suggested adding both. Reliance on a second enzyme to remove the inhibitory product of the one being studied appears to us to be a precarious arrangement since it postulates that transaminase is present in all of those tissues which contain malic dehydrogenase and DPN diaphorase. Our experiments indicated that oxalacetate at $5 \times 10^{-4}$ M concentration did not inhibit the staining of rat kidney and stomach significantly. Higher concentrations did produce some inhibition ($10^{-3}$ M). However, since the staining of the sections was quite adequate in 15 to 30 minutes without adding any reagent to remove the oxalacetate which appeared enzymatically, we must conclude that the concentration of oxalacetate attained is not sufficient to inhibit malic dehydrogenase. Furthermore, the addition of a ketone binding agent such as semicarbazide to our incubation solution inhibited the staining reaction significantly. As reported by Farber et al. (7) the addition of sodium glutamate did enhance staining. However, since glutamate serves as a substrate for the DPN-linked glutamic dehydrogenase, and produced a stain in the tissue section when it alone served as the substrate, we can only conclude that the enhancement of staining seen when glutamate is added to the malate incubation solution represents the additive effect of two dehydrogenases simultaneously transferring electrons to the carriers, rather than an effect attributable to the removal of the inhibitor, oxalacetate, by enzymatic transamination.

**Significance of the Red Color seen on Reduction of Ditetrazoles.**—Two colors (red and blue) have been observed frequently (3, 8, 15) in investigations using blue tetrazolium as an indicator of dehydrogenase activity. The red component could result from either monoreduction of the ditetrazole, or simply the reduction of a monotetrazole which is present as a contaminant. In an earlier paper (14), one of us (A. M. S.) suggested that the blue granules represent sites of high, and the red areas low enzymatic activity. Farber and coworkers (7, 8) agree with this suggestion. The red color was considered to be the monoformazan resulting from partial reduction of the ditetrazole. This issue was discussed and investigated further by Burtner et al. (3), who studied the ditetrazole, neotetrazolium (NT). Their experiments indicated that the red pigment is not the result of monoreduction of the ditetrazole, but rather that of the reduction of a monotetrazole contaminant. During the synthesis of Nitro-BT, Tsou et al. (19) found that a monoformazan was obtained as a contaminant in high yield. This red monoformazan was shown to be a product of monocupling together with replacement of the other diazonium group with hydrogen. It was relatively simple to separate the bulk of this monoformazan from the diformazan by virtue of their great difference in solubility in organic solvents. However, the two tetrazoles obtained on oxidation could not be separated. It is probable that the commercial preparations of BT and NT contain some monotetrazole produced in the same way, which on reduction is responsible for the red color obtained both colorimetrically and histochemically.

Using a less pure sample of Nitro-BT it was found that red pigment appeared in the sections. This observation again raised the question as to whether the sample was contaminated with some of the monotetrazole or whether actual monoreduction of the ditetrazole was being produced at weaker sites of enzymatic activity. The former explanation was shown to be correct by the following experiments:

1. Sections of kidney and stomach which contain cytologic elements of both low and high activity were examined at intervals of 5 minutes or less. The elements of low activity that required a prolonged period of incubation were nevertheless blue from the onset of appearance of the stain. The red areas which were evident early in the incubation period remained this color, and did not shift to blue on longer incubation.

2. Several sections containing the red color were placed in 95 per cent alcohol which extracted the color. This alcoholic solution did not change color upon the addition of a reducing agent. If the red color had represented monoreduction of the ditetrazole, a shift to the blue would have been expected.

3. After preparing a sample of Nitro-BT free of all but a slight trace of the monotetrazole, and repeating the experiment above, the red color was largely eliminated.

These findings indicate that any red pigment
seen during the histochemical demonstration of DPN diaphorase with Nitro-BT represents the presence of the mononitrotrazole contaminant in the sample. As a precautionary measure, each new sample should be tested for this constituent by examining the sections in an aqueous, rather than an organic mounting medium in order to preserve the red pigment. A significant amount of red is undesirable; trace amounts, however, are removed when the sections are carried through the graded alcohol solutions and mounted in Canada balsam.

Histochemical Demonstration of DPN Diaphorase in Rat Kidney and Stomach.—DPN diaphorase activity was elicited from all epithelial and most mesenchymal components of the rat kidney and stomach. In the kidney, the cells most intensely stained were those of the proximal convoluted tubules (Fig. 5) and thick limb of Henle’s loops (Fig. 6). Fine, densely packed dye particles filled out the cytoplasm of the latter cells. The glomeruli (Fig. 5) were weakly stained. In the thin cells lining the thin limbs of Henle’s loop (Fig. 6) the stain was seen concentrated in the area of cytoplasm adjacent to the nucleus. A distal convoluted tubule may be seen in Fig. 5 at the pole of the renal corpuscle, opposite to that from which the proximal convoluted tubule takes origin. The cells lining the distal convoluted tubules and the collecting tubules (Figs. 6 and 7) were stained with moderate intensity. The stromal cells of the renal papilla contained rather coarse dye particles which were sparsely distributed throughout their elongated cytoplasmic processes (Fig. 7). The foregoing observations are consistent with those reported by Sternberg et al. (17).

DPN diaphorase is also intensely distributed throughout the gastric mucosa. As seen in Figs. 8 and 9, the surface epithelium and the parietal cells throughout the entire extent of the glands are intensely stained, while the zymogenic cells (Fig. 9) at the base of the glands are weakly stained. The formazan particles are distributed rather uniformly throughout the parietal and zymogenic cells, but are confined to the supranuclear portion of the cytoplasm in the case of surface epithelial cells. The unstained nuclei of the cells are readily seen in Figs. 8 and 9.

DISCUSSION

The work reported here confirms the generally accepted viewpoint that tetrazolium salts are not reduced by either the specific dehydrogenase or reduced DPN, but rather by a flavoprotein intermediate. This enzyme which has been designated DPN diaphorase is bound to the structural constituents of the cell (7), thus permitting its intracellular localization by the use of the new tetrazole indicator, Nitro-BT. The favorable properties of this tetrazolium salt make possible the identification of the diaphorase sites in a short incubation period using a simple incubation medium.

Farber and associates (7, 8) used blue tetrazolium (BT) as their indicator of dehydrogenase activity, and concluded that since the diaphorase alone was responsible for the reduction of the tetrazole, the localization of formazan in tissue sections represented the sites of diaphorase activity and not that of the specific dehydrogenase. This conclusion was supported by their inability to obtain a distinctive staining pattern for each dehydrogenase which they studied. The greater ease of reduction of Nitro-BT with its more favorable properties for intracellular localization has resulted in variations of the diformazan deposit which are related to the substrate employed. Thus, the specific dehydrogenases with their accompanying diaphorase can be demonstrated selectively. This does not imply that each dehydrogenase is capable of directly reducing the tetrazole, but rather that some of the dehydrogenases have a more limited distribution than that of DPN diaphorase. Our experiments are in agreement with the reported work of others that the diaphorase alone is responsible for production of the diformazan. It appears that the diaphorases are distributed abundantly in many organs, in association with certain dehydrogenases but not all dehydrogenases are present at each site. A given cell might contain the diaphorase together with lactic and malic dehydrogenases, whereas another cell might only have lactic dehydrogenase in association with the diaphorase. Hence, the substrate solution containing sodium malate would only visualize the one site, while the incubation medium having sodium lactate would result in diformazan deposition in both sites. Although the diaphorase would be responsible for the pigment production, the histochemical localization would be related to the distribution of the specific dehydrogenase. Furthermore, as the illustrations indicate, DPN reduced by the dehydrogenase at one site does not diffuse to more distant sites to act as a substrate for the diaphorase present there. Studies
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demonstrating the cytochemical localization of the individual dehydrogenases are in progress in our laboratory and will be presented elsewhere.

SUMMARY

A histochemical method for the demonstration of DPN diaphorase has been presented using the lactate-lactic dehydrogenase system and Nitro-BT.

Other DPN-linked dehydrogenases have been shown to identify the sites of the diaphorase used in the appropriate incubation solution, but were considered to be inferior to the one recommended for routine use.

An explanation has been offered for the presence of both red and blue pigment when the ditetrazolium salts are employed. It has been possible largely to eliminate the red component, by using ditetrazole free of all but traces of monotetrazole.

The findings of other workers that only DPN diaphorase is responsible for the reduction of the ditetrazolium salt to the diformazan have been confirmed. However, reduced DPN produced in one site transfers its hydrogens to Nitro-BT so rapidly in the presence of diaphorase, that distant sites of diaphorase are not demonstrated histochemically. Therefore, it is possible in certain instances to demonstrate the histochemical localization of several DPN-linked dehydrogenase systems.

BIBLIOGRAPHY

11. Kuhn, R., Quoted by Brodie and Gots (2).
EXPLANATION OF PLATES
DEMONSTRATION OF DPN DIAPHORASE

PLATE 11

Fig. 1. Rat stomach with $\beta$-hydroxybutyrate as substrate. In the gastric mucosa the stain is limited to the surface epithelial cells and the parietal cells of the superficial half of the glands. This same sharp demarcation between stained and unstained components existed throughout the length of the specimen. $\times$ 150.

Fig. 2. Rat stomach with lactate as substrate. In the corresponding area of a section serially cut from the same block as that illustrated in Fig. 1, it is seen that not only are the surface epithelial cells stained, but the zymogenic cells at the base of the glands and all of the parietal cells are stained as well. $\times$ 150.

Fig. 3. Mouse kidney with lactate as substrate. All epithelial components of this specimen are stained, including the collecting ducts and the thin limb of Henle's loops that traverse the inner medulla. $\times$ 40.

Fig. 4. Mouse kidney with $\beta$-hydroxybutyrate as substrate. In the corresponding area of a section cut in series with that shown in Fig. 3 a strikingly different staining pattern is evident. The inner medulla is entirely unstained, and in the outer cortex only the first turn of the proximal convoluted tubule is stained. The very intensely stained saw-toothed area represents the agglomerular or inner cortex composed chiefly of the terminal loops of the proximal convoluted tubules. In the outer medulla only the thick limb of Henle's loops are stained. $\times$ 40.
(Nachlas et al.: Demonstration of DPN diaphorase)
Fig. 5. DPN diaphorase of rat kidney, using the lactate-lactic dehydrogenase incubation medium. At one pole of this renal corpuscle the funnel-shaped origin of a proximal convoluted tubule is seen at lower right, and at the opposite pole a section of the distal convoluted tubule is discernible. Most of the other tubules seen in this field are of the proximal convoluted variety. Although DPN diaphorase activity may be elicited from all portions of the nephron, the cells of the proximal convoluted tubule and thick limb of Henle’s loop show the most intense degree of activity. × 270.

Fig. 6. DPN diaphorase of rat kidney, using the lactate-lactic dehydrogenase incubation medium. In longitudinal section the junction of a thick limb (right) with a thin limb of Henle is shown. Just below the latter a collecting duct may be seen. Cells of all three tubular types show DPN diaphorase activity. × 270.

Fig. 7. DPN diaphorase of rat kidney, using the lactate-lactic dehydrogenase incubation medium. Several collecting ducts transversely cut and intervening stellate-shaped stromal cells are seen in this section, which transects the tip of a renal papilla. Rather coarse formazan particles representing DPN diaphorase activity are sparsely distributed throughout the protoplasmic processes of the stromal cells. The cells of the transitional epithelium of the renal pelvis as well as those of the collecting duct contained a moderately heavy deposit of fine formazan particles. × 270.
PLATE 13

Fig. 8. DPN diaphorase of *rat stomach*, using the lactate-lactic dehydrogenase incubation medium. Two gastric pits and the necks of the adjacent glands are shown. The dye is distributed throughout the cytoplasm of the parietal cells, but appears more concentrated in the supranuclear portion of the cytoplasm in the case of the surface epithelial cells. All nuclei are unstained. × 270.

Fig. 9. DPN diaphorase of *rat stomach*, using the lactate-lactic dehydrogenase incubation medium. The deep aspect of two gastric glands are shown. The parietal cells here are very intensely stained as were those in the gland necks. The zymogenic cells are comparatively weakly stained by the fine dust-like formazan particles distributed throughout the protoplasm. × 270.
(Nachlas et al.; Demonstration of DPN diaphorase)