Tetrazolium Stains for Diphosphopyridine Nucleotide (DPN) Diaphorase and Triphosphopyridine Nucleotide (TPN) Diaphorase in Animal Tissue.* (21156)

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Seligman and coworkers(1,2) have recently described a method of staining for the succinic dehydrogenase system using tetrazolium salts as indicators. Their method has proved satisfactory in the hands of several other investigators(3,4). For the past 2 years, we have been attempting to develop technics for the histochemical demonstration of other oxidative enzymes. Methods employing tetrazolium salts have now been perfected for the demonstration of 2 enzymes, diphosphopyridine nucleotide (DPN) diaphorase and triphosphopyridine nucleotide (TPN) diaphorase.

In our original experiments, frozen sections of animal tissues (mainly rat kidney) were incubated in solutions containing tetrazolium salts together with factors which should permit the activity of individual tissue dehydrogenases. Excellent staining was observed in many instances. For example, when sections were incubated in a medium containing malate as substrate with cofactors and the coenzyme (DPN) essential for malic dehydrogenase activity, distinctive staining patterns were observed in the sections. Omission of any one of these components prevented staining. The localization of formazan in the tissue was highly reproducible and was different from that observed with the succinic dehydrogenase system. However, when the composition of the incubating medium was changed so as to permit the action of lactic dehydrogenase, which is also a DPN-linked enzyme, the staining pattern was identical to that observed with the malic system. Again, when sections were incubated in media containing alcohol as substrate and soluble yeast alcohol dehydrogenase (crystalline) as the only active DPN-linked dehydrogenase in the medium or tissue, the staining patterns were identical to those obtained with the malate and lactate systems.

It soon became clear that the action of the dehydrogenases was not directly responsible for the staining and probably served only to produce a common factor essential to the activity of some other enzyme in the tissue. This theory gained support when identical staining patterns were obtained on incubating sections in media that contained no dehydrogenase substrate but only reduced DPN.

Similarly, with TPN-linked system, it was found that each of 3 different dehydrogenases (isocitric, glucose-6-phosphate, and Ochoa's "malic enzyme") gave identical staining patterns which were highly reproducible but differed from those obtained with the DPN systems or the succinic dehydrogenase system. Here too, it appeared that the role of the dehydrogenases might be merely to produce a substrate for some other enzyme.

The enzymes responsible for these 2 distinct staining patterns have been tentatively identified as DPN diaphorase and TPN diaphorase. This conclusion is in agreement with that of Brodie and Gots(5,6) who demonstrated that a flavoprotein enzyme is essential for reduction of tetrazolium salts by several dehydrogenase systems.

Frozen sections of tissue prepared in the routine manner contain insufficient amounts of the dehydrogenase substrates and the coenzymes DPN or TPN to allow significant dehydrogenase activity but do retain the diaphorases and effective amounts of many dehydrogenase apoenzymes. Therefore, staining specific for DPN diaphorase may be obtained

* Supported in part by a research grant from the National Cancer Institute, United States Public Health Service. (No. C-1462)

† Scholar in Cancer Research of the American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

by incubating frozen sections in a medium containing DPN and tetrazolium together with substrates and cofactors for one or more DPN-linked dehydrogenases. In like fashion TPN diaphorase is selectively stained by supplying TPN and the factors necessary for the action of a TPN-linked dehydrogenase. Increased efficiency of tetrazolium reduction may be obtained by permitting 2 dehydrogenases to react simultaneously with the same coenzyme. In this way, the DPN or TPN is reduced at a more rapid rate to furnish a richer substrate for the action of the diaphorase.

The following incubation medium has yielded reproducible and satisfactory staining for DPN diaphorase in frozen sections of rat kidney and other tissues: Solution of crystalline alcohol dehydrogenase (Mann) (ca. 1.5 mg per ml)—0.1 ml, ethyl alcohol (1.09M)—0.2 ml, DPN (5 mg per ml)—0.2 ml, Na L-malate (0.5M)—0.3 ml, Na L-glutamate (0.5M)—0.5 ml, semicarbazide (0.1M)—0.2 ml, blue tetrazolium (1 mg per ml)—0.7 ml, and phosphate buffer (0.1M, pH 7.4)—0.8 ml. All solutions are adjusted to pH 7.4 before use. The alcohol is supplied as substrate for the added alcohol dehydrogenase. The malate serves as substrate for the endogenous malic dehydrogenase. Oxalacetate, the product of malate oxidation, will "poison" the enzyme system if allowed to accumulate. Glutamate added to the medium removes oxalacetate by converting it to aspartate through the action of transaminase. The semicarbazide removes the acetaldehyde formed by oxidation of the ethanol as well as reacting with oxalacetate.

This system is planned so as to permit both endogenous (malic) and exogenous (alcohol) dehydrogenases to contribute to the supply of reduced DPN. Although neotetrazolium can be used in place of blue tetrazolium, we have found blue tetrazolium more satisfactory. The medium usually remains colorless if the alcohol dehydrogenase solution is made up not more than a few weeks before it is used.

For staining for TPN diaphorase, the following medium has proved satisfactory: A mixture of Na L-malate and Na citrate (each 0.5M)—0.3 ml, TPN (2 mg per ml)—0.1 ml, $MnCl_2$ (0.005M)—0.3 ml, cysteine hydrochloride (0.1M)—0.2 ml, veronal buffer

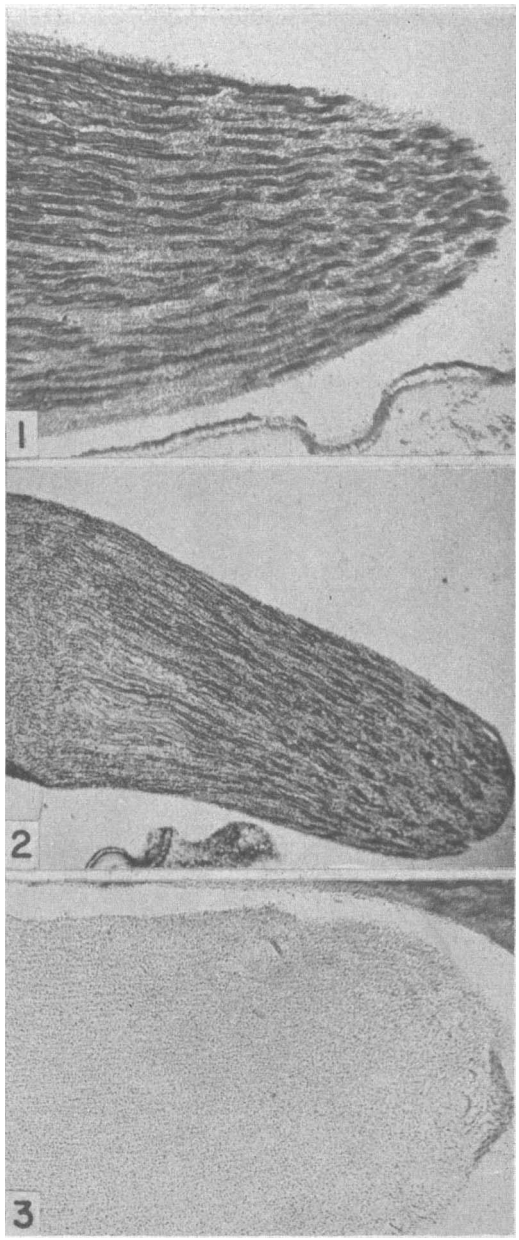

FIG. 1-3. Papilla of the rat kidney stained for DPN diaphorase, TPN diaphorase, and succinic dehydrogenase system.

FIG. 1. DPN diaphorase. Collecting tubules stain intensely throughout their length. Moderate staining of epithelium of renal pelvis.

FIG. 2. TPN diaphorase. Note increasing intensity of staining of collecting tubules as tip of papilla is approached.

FIG. 3. Succinic dehydrogenase system. Virtually no staining of the rat kidney papilla.

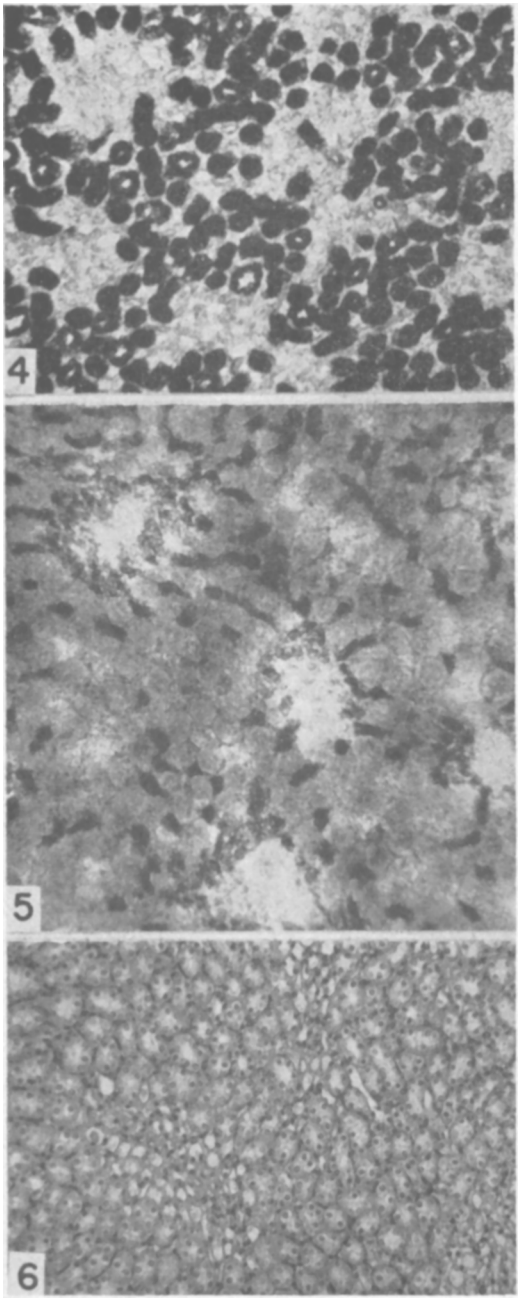

FIG. 4-6. Cross-sections through outer medullary zone of rat kidney, stained for DPN and TPN diaphorase, with a hematoxylin and eosin stained paraffin section for comparison.

FIG. 4. DPN diaphorase. Two types of tubules are readily identified and stain deeply. Predominant tubules are the ascending thick limbs of Henle with small or indistinguishable lumens. Collecting tubules are larger and their lumens wider. Delicate staining of endothelium of the vascular bundles *(vasa recta)* is also seen.

FIG. 5. TPN diaphorase. Larger, pale-staining tubules (pink in the original preparation) are ascending thick limbs of Henle. Intensely stained small-caliber tubules are thin limbs of Henle (deep blue in the original preparation). Relatively clear areas are vascular bundles *(vasa recta)*.

FIG. 6. Hematoxylin and eosin stain, same magnification as Fig. 5. Thick limbs of Henle predominate. Thin limbs of Henle are difficult to identify or to differentiate from blood vessels.

0.005M, pH 7.4)—0.9 ml, methyl cellulose (15 cps. 3%)—0.5 ml, and blue tetrazolium (1 mg per ml)—0.7 ml.

The malate is supplied as substrate for the TPN-linked "malic enzyme" of Ochoa (malic decarboxylase); the citrate is converted to isocitrate by the active tissue aconitase and the isocitrate then serves as substrate for the tissue isocitric dehydrogenase. Mn^{++} is an essential activator for the decarboxylation of malate and also oxalsuccinate, the product of isocitrate oxidation. Cysteine or a suitable substitute is essential in the system and no reduction of tetrazolium occurs in its absence. In the absence of substrate, cysteine produces no staining or at most a very faint pink. Fe^{++} cannot replace cysteine. Thioglycollate but not glutathione can effectively substitute for cysteine but a higher concentration is required. The cysteine must be freshly prepared and neutralized. Veronal buffer was used in place of phosphate buffer to prevent any possible inhibition of aconitase by phosphate. The methyl cellulose is not essential but its presence gives better stained preparations. With all TPN systems, the medium tends to become colored due probably to leaching out of the TPN diaphorase from the tissue into the incubating solution. By adding the viscous methylated celluloses, coloration of the medium is much reduced, and staining of the tissue is considerably enhanced. Different methyl celluloses (Methocel, Dow Chemical Co.) or carboxymethyl celluloses (Hercules Powder Co.) give somewhat different degrees of improvement of the staining but all enhance it to at least some degree.

In the staining for both diaphorases, frozen sections are floated in cold phosphate buffer (0.1M, pH 7.4) and then transferred to 50 ml glass stoppered Erlenmeyer flasks containing the cold incubating media. The flasks

FIG. 7. TPN diaphorase stain of distal third of rat papilla. Higher magnification of same section as Fig. 2. Branching collecting ducts stain well. Numerous elongate cells with processes are arrayed in parallel rows in the interstitial tissue. Cells are oriented at right angles to the direction of the collecting ducts.

are briefly (0.5 to one minute) flushed with N_2 and then incubated at 37.5°C for from 0.5 to 2 hours. They are washed in 4% formaldehyde, 1% acetic acid and then H_2O and mounted in glycerine jelly.

In hundreds of slides the staining patterns of rat kidney proved to be highly reproducible and were distinctly different for each of the 2 diaphorases and for the succinic dehydrogenase system. For example, the renal papilla, unstained with the succinic dehydrogenase system, shows intense staining of the entire length of the collecting tubules with DPN diaphorase (Fig. 1,3). On the other hand only the terminal portions of the collecting tubules in the papilla stain deeply with TPN diaphorase (Fig. 2). The thick ascending portions of Henle's loop seen in the outer medullary zone, stain intensely both with the succinic dehydrogenase system and DPN diaphorase (Fig. 4), but less intensely with TPN diaphorase (Fig. 5). In contrast, the thin limbs of Henle stain brilliantly only with TPN diaphorase (Fig. 5). With this method thin limbs, so difficult to visualize with conventional histologic methods, are beautifully delineated. Capillary endothelium as well as glomerular tufts, virtually unstained with succinic dehydrogenase system, are delicately stained with TPN diaphorase, and to a lesser extent with DPN diaphorase. Similarly epithelium of the renal pelvis shows staining with both TPN and DPN diaphorase.

In sections stained for TPN diaphorase, rows of darkly stained fusiform and stellate cells are seen in the distal half of the papilla. These cells are oriented transversely to the direction of the collecting ducts, thin limbs and capillaries (Fig. 7). Work is in progress to determine whether these cells have contractile properties and whether they play any part in the regulation of urinary flow.

Summary. Methods for the specific staining for DPN diaphorase and TPN diaphorase in frozen sections of fresh tissue are presented. The staining patterns of rat kidney are highly reproducible and are distinctly different for each of the 2 diaphorases and for the succinic dehydrogenase system.

We wish to thank the Hercules Powder Co. and Dow Chemical Co. for supplying samples of the cellulose derivatives.

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Received June 14, 1954. P.S.E.B.M., 1954, v86.