The Histochemical Localization of Triphosphopyridine Nucleotide Diaphorase*‡

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ABSTRACT

A histochemical method is described for the localization of triphosphopyridine nucleotide diaphorase using a recently synthesized tetrazolium salt (Nitro-BT). By virtue of the favorable histochemical properties of this reagent, it has been possible to demonstrate that whereas DPN diaphorase is usually restricted to the mitochondria, the TPN diaphorase activity of corresponding cells was distributed throughout the cytoplasm in granules too fine to be considered mitochondria. Furthermore, although the diaphorase alone is responsible for the passage of electrons from TPNH to the tetrazole, it has been found that sites of activity of different TPN-linked dehydrogenases can be visualized in tissue sections, and characteristic loci for each enzyme may be observed. For example, whereas TPN diaphorase and isocitric dehydrogenase have an extensive distribution in the kidney cortex, 6-phosphogluconic dehydrogenase is limited to the cells of the macula densa.

INTRODUCTION

During the past six years, tetrazolium salts have received widespread attention and use as electron acceptors in both biochemical and histochemical investigations (2, 13–15, 19–21, 25). Their value derives primarily from their color and solubility change when the tetrazole is reduced to the formazan. Evidence from several laboratories (1, 3, 4, 24) has indicated that these colored indicators are not reduced directly by the dehydrogenases, but rather by flavoprotein enzyme intermediates. The existence of two flavoenzyme systems has been postulated, one which transfers electrons to a redox dye alone (diaphorase) and one which effects the reduction of oxidized cytochrome *c* (cytochrome *c* reductase). Knowledge concerning the nature and properties of the diaphorases is indeed meager. Only DPN diaphorase has been satisfactorily prepared and purified from animal tissues (heart muscle) (24). That this enzyme differs from DPN cytochrome *c* reductase is substantiated by the observation that the enzyme can react with a wide variety of dyes, but is inert with cytochrome *c* (16). On the other hand, occurrence of the analogous enzyme, TPN diaphorase, as distinguished from TPN cytochrome *c* reductase, is not supported by such incontrovertible experimental evidence. The findings of von Euler *et al.* (5), and Abraham and Adler. (1) which lend support to the existence of TPN diaphorase have been criticized by Mahler (16). His reservations are based upon two facts; (1) since TPN cytochrome *c* reductase also has dye-reducing activities, the observations of these investigators could be explained by the presence of the cytochrome *c* reductase in their diaphorase preparation, and (2) the reported findings could have

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resulted from the operation of transhydrogenase plus DPN diaphorase. More recently an enzyme preparation has been obtained from chloroplasts, which has been shown to transfer electrons from TPNH to a redox dye, but not to oxidized cytochrome *c*.[1] As yet, no such enzyme has been obtained from animal tissues. Farber *et al.* (6) reported demonstrating the histochemical sites of TPN diaphorase, but they did not offer any experimental evidence to eliminate the possibility that TPN cytochrome *c* reductase was also being visualized by the precipitated diformazan. For purposes of simplicity, the enzyme system described in the following experiments, which passes electrons from reduced TPN to the tetrazolium salt, will be designated as TPN diaphorase. It is conceded that the identification of this enzyme system as distinct from TPN cytochrome *c* reductase has not been established (8–12).

The introduction of the new ditetrazole, 2,2′-di-(*p* - nitrophenyl) - 5,5′ - diphenyl - 3,3′ - (3,3′ -dimethoxy-4,4′-biphenylene) ditetrazolium chloride (Nitro-BT) (26) with its particularly favorable properties for histochemistry, has permitted a significant improvement in the methods (17) for visualizing in tissue sections the sites of the succinic dehydrogenase system (22) and DPN diaphorase (18). An evaluation of this new tetrazole for the cytochemical visualization of several TPN-linked dehydrogenases, and the intermediate reductase, forms the basis of the investigations reported herein.

Materials and Methods

Histological Material.—The kidney, pancreas, and stomach of young adult white rats were used in the majority of the experiments. Sections were prepared in the cryostat after quick-freezing the tissues as described in the preceding paper (18). Unless otherwise stated, all the reactions were run aerobically at 37°C. Sections inactivated by exposure to boiling water for 5 minutes were used routinely for control purposes. Of the pair of serial sections incubated simultaneously, one was mounted in glycerol jelly and the other in permount, following formalin fixation, dehydration in graded alcohol solutions, and clearing in xylene.

Incubation Media.—Several of the TPN-linked dehydrogenase systems were studied. In general, the pH of the buffer, the concentration of TPN, and the amount of the tetrazolium salt used were kept constant in the studies with the different substrates. The substrates employed, and the sources of each are as follows: DL-isocitric acid lactone (California Foundation for Biochemical Research); DL-isocitric acid-sodium salt (H & M Chemical Co.); L-glutamic and L-malic acids (Eastman Kodak Co.); barium salt of glucose-6-phosphate (Mann Research Laboratories); barium salt of 6-phosphogluconate and sodium salt of glucose-6-phosphate (Sigma Chemical Company); and calcium salt of phosphogluconic acid (Delta Chemical Works, Inc.). Triphosphopyridine nucleotide and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company. Blue tetrazolium (Dajac Laboratories), neotetrazolium (Nutritional Biochemicals Corporation), and Nitro-BT were compared as histochemical indicators of dehydrogenase activity.

As will be seen in the results, the medium considered optimum for demonstrating the TPN diaphorase activity of most organs is composed of the following reagents:

Sodium DL-isocitrate (0.1 M)	0.6 ml.
Sodium L-malate (2.5 M)	0.5 ml.
Manganese chloride (0.005 M)	0.3 ml.
TPN (5 mg./ml.)	0.2 ml.
Nitro-BT (5 mg./ml.)	0.3 ml.
Veronal buffer (0.05 M) pH 7.4	1.1 ml.

At the time of their preparation, the pH of all stock solutions of substrates was adjusted to 7.4. Except when actually in use, these stock solutions were stored at about 4°C. Enzyme preparations were stored at −20°C. Incubation of two sections at a time was carried out on a half cover slip placed in a test tube. A reagent mixture prepared in the morning can be used throughout the day for repeated incubations with as many as 10 sections.

EXPERIMENTAL

Enzyme Localization with Different Substrates.—Farber *et al.* (6) emphasized the fact that the staining patterns in the rat kidney using three different substrates (isocitrate, glucose-6-phosphate, and malate) were identical. This finding supported their argument that only the flavoprotein intermediate was reducing the tetrazole, and hence the histochemical sites visualized were those of the intermediate enzyme. In previous work, it has been shown that several of the specific dehydrogenases that are DPN-linked can be demonstrated separately in tissue sections because of their unique localization, despite the fact that the DPN diaphorase alone is responsible for the reduction of the tetrazole (18). This was possible because the greater ease of reduction of Nitro-BT as compared to blue tetrazolium (BT) or neotetrazolium (NT)

[1] This observation which was recently brought to our attention, was made by Avron and Jagendorf, *Arch. Biochem. and Biophysics*, 1956, **65**, 475.

enabled demonstration of dehydrogenases in certain sites not reactive before. Similarly, in the present investigations differences in the histologic pattern of diformazan deposition were noted when several TPN-linked dehydrogenases were studied. In decreasing order, the relative activities were isocitric dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and TPN-linked malic dehydrogenase (Ochoa's "malic enzyme"). Of the qualitative differences in the staining pattern, the most striking was that disclosed in the rat kidney sections after incubation for 10 minutes in the 6-phosphogluconate medium, as compared with the result obtained using the isocitrate medium. In the latter instance, the diformazan particles were deposited in all components of the nephron. However, when the 6-phosphogluconate medium was used, only the cells comprising the macula densa responded with intense activity. Elsewhere in the cortex, no activity was seen unless the period of incubation was very much prolonged. The staining in the rat kidney for "malic enzyme" was much weaker than that seen with the other enzymes studied, but its pattern was similar to that of isocitric dehydrogenase. As will be reported elsewhere, other qualitative staining differences were noted between the three dehydrogenases when several tissues were examined.

Optimal Demonstration of TPN Diaphorase.—While the activity of isocitric dehydrogenase in general was fairly intense, the possibility exists that TPN diaphorase might be present in certain cells unassociated with that particular dehydrogenase, but associated with some other dehydrogenase. Thus, for the optimal demonstration of all TPN diaphorase sites, two alternatives were considered; namely, (1) the use of two substrates in the incubation medium, or (2) the addition of an exogenous enzyme source, such as isocitric dehydrogenase, to a medium containing only isocitrate. In the latter situation, the soluble enzyme would transfer hydrogens or electrons to the diaphorase at all sites irrespective of the sections' content of intrinsic isocitric dehydrogenase. Because isocitric dehydrogenase was not available to us, and the glucose-6-phosphate dehydrogenase commercially obtainable was unsatisfactory (see discussion below), we elected to use the first alternative described. Experiments were performed with the four substrates in various combinations. The mixtures which appeared to result in a summation of enzymatic activity were isocitrate plus malate, isocitrate plus glucose-6-phosphate, and 6-phosphogluconate plus malate. An unexplained finding was the failure to note summation with the combination of glucose-6-phosphate plus malate, and glucose-6-phosphate plus phosphogluconate. From these studies, the isocitrate-malate mixture appeared to give the most satisfactory and consistent staining pattern. Indeed, the effect of these two substrates in combination seemed to be even greater than could be accounted for in terms of a simple summation of the activities observed when the substrates were used individually. The recommended incubation medium for the demonstration of TPN diaphorase with these two substrates is given above.

Studies of Isocitric Dehydrogenase.—The substitution of citrate for isocitrate has been suggested by others (6, 7) in the histochemical demonstration of TPN diaphorase, since citrate is readily converted to isocitrate by aconitase. This maneuver has the two theoretical objections that (1) it assumes the existence of aconitase in all loci which contain isocitric dehydrogenase and (2) the substrate (isocitrate) concentration would depend on the activity of the aconitase. This appears to be too precarious an arrangement, and for these reasons we have recommended the use of DL-isocitrate which is readily available commerically.

Cysteine or a suitable substitute (thioglycollate) has been found essential for isocitric dehydrogenase activity in the histochemical studies of Farber *et al.* (6). In fact, this reagent was thought to be a requirement for all three of the TPN-linked enzymes which these workers used. An explanation for this fact was not suggested. Early in our studies, it became apparent that cysteine effected a non-enzymatic reduction of the tetrazole in concentrations well below those recommended by Farber *et al.* (6) and thus was not satisfactory for enzymatic reactions visualized by Nitro-BT reduction. The tetrazoles used by Farber's group (BT and NT) are more resistant to chemical as well as to enzymatic reduction, so that non-enzymatic reduction did not offer a serious problem with the concentration of cysteine they used. Cysteine (0.2 M) did not reduce BT after 1 hour's exposure; NT was reduced slowly by cysteine (0.1 M); while Nitro-BT was reduced rapidly by cysteine at 0.01 M concentration. Somewhat higher concentrations of thioglycollate were necessary to reduce both BT and NT, but Nitro-BT was converted to

the diformazan by as little as 10^{-4} M of this reducing agent. It is not known whether these reducing agents exert an activating effect upon the enzyme systems or in some way alter the rate of reduction of the tetrazoles. The minimal increase in the intensity of the stain which was obtained with cysteine and Nitro-BT, in contrast to the markedly intensifying effect of cysteine when used with BT, suggested that the activation in the latter situation is more likely to be related to an effect on the tetrazole (BT) rather than on the enzyme itself. If the tetrazole oxidized the cysteine to cystine, the latter compound might accept hydrogens more readily from the diaphorase, and in this manner more formazan would be produced. This supposition was not borne out by experimentation in that the oxidized form of the reducing agent showed no activating effect. It can only be concluded that cysteine does activate isocitric dehydrogenase, but for some unexplained reason the activation is more pronounced when BT serves as the hydrogen acceptor from the diaphorase than when Nitro-BT is used. Fortunately, the presence of such reducing agents was not necessary when the activity of the several TPN-linked dehydrogenases was demonstrated by means of the reduction of Nitro-BT.

Studies of Glucose-6-Phosphate Dehydrogenase.— The kidney sections were adequately stained after a 20 minute incubation period when glucose-6-phosphate served as the substrate. The poor solubility of the barium salt, which was used in our earlier studies, limited the range of substrate concentrations that might be tested. Therefore, the sodium salt of glucose-6-phosphate was obtained, but with this reagent the staining was quite poor. The possibility that this particular substrate was at fault was eliminated by the observation that adequate staining was once again noted when $BaCl_2$ was added. The activating effect of Ba^{++} on glucose-6-phosphate dehydrogenase was somewhat surprising since this has not been reported previously, and inasmuch as others have obtained satisfactory activity using the potassium salt (6). The possibility that the Ba^{++} might simply serve to maintain a very low concentration of glucose-6-phosphate in solution was examined. Sections incubated in a medium containing 3×10^{-3} M of substrate (sodium salt) and no $BaCl_2$ were much less intensely stained than those sections incubated in a similar medium but containing 0.067 M $BaCl_2$. Lesser concentrations of Ba^{++} were ineffective. Thus, the data support the viewpoint that barium ions activate glucose-6-phosphate dehydrogenase. Mg^{++} at the same concentration (0.067 M) also exerted an activating effect, but this was only about one-half as effective as Ba^{++}. Mn^{++} activated but slightly. Other ions tested at the same molar concentrations (K^+, Ca^{++}, Al^{+++}) had no favorable influence on this dehydrogenase.

The previously referred to theoretical advantage of adding an exogenous enzyme source to the reaction mixture was tested with glucose-6-phosphate dehydrogenase (Sigma Chemical Company). In concentrations as low as 0.1 mg. per ml. of solution, the exogenous enzyme almost doubled the intensity of the stain. However, that the tetrazole was reduced directly by the exogenous enzyme source was shown by the fact that bluish coloration appeared before any sections were introduced into the medium. Reduction of the Nitro-BT would not occur if either TPN or glucose-6-phosphate were omitted from the solution. The most likely explanation for these observations is that the exogenous enzyme preparation contained a significant amount of TPN diaphorase. If a purer sample could be obtained, its addition to the incubation medium would be useful in accelerating the histochemical reaction.

Studies of 6-Phosphogluconic Dehydrogenase.— Using the barium salt of 6-phosphogluconic acid, the distribution of formazan in the kidney was a more limited one than that obtained with glucose-6-phosphate or isocitrate. In the cortex only the cells of the macula densa were readily stained; while in the medulla the thin limb of Henle's loop, the stromal cells, and the papillary portion of the collecting ducts showed activity. When the calcium salt was used no staining was noted. The possibility that Ca^{++} inhibited this dehydrogenase was demonstrated by the finding that the staining of the kidney section was markedly decreased when Ca^{++} (0.1 M) was added to the reaction mixture containing the barium salt of 6-phosphogluconic acid. Several other ions Mn^{++}, Mg^{++}, and Al^{+++}) were tested at 0.33 M concentration in the incubation mixture, but in no instance was any activating effect observed. Formazan deposition was greatest when the incubation solution was saturated with the substrate. The optimal substrate concentrations for the histochemical demonstration of this enzyme and each of the other TPN-linked dehydrogenases studied is shown in Table I.

TABLE I

Concentrations of Substrates and Enzymes Tested in Media Prepared for Demonstrating TPN Diaphorase Activity

Enzyme or substrate	Final concentration in medium		Activator	Buffer preferred	Relative activity	Color of supernatant
	Range tested	Optimal				
Isocitrate, sodium	0.005–0.05 M	0.017 M	10^{-2} M $MgCl_2$ 5×10^{-4} M $MnCl_2$	Veronal	+++	None
Malate, sodium	0.025–0.75 M	0.5 M	10^{-2} M $MgCl_2$	Veronal	+	None
Glucose-6-phosphate, sodium	0.0003–0.04 M	0.0033 M	0.067 M $BaCl_2$	Veronal	+++	None
Glucose-6-PO_4 dehydrogenase	0.05–1.0 per cent	0.1 per cent	None	Veronal	+++++	Blue
6-phospho-gluconate, barium	1.0 to 20 mg./ml.	5.0 mg./ml. (super saturated)	None	Veronal	++	None
TPN	0.01–0.1 per cent	0.03 per cent	None	Veronal	−	None

As in the previously mentioned use of glucose-6-phosphate dehydrogenase, when 6-phosphogluconate dehydrogenase (Sigma Chemical Company) was added to the 6-phosphogluconate containing medium, the tetrazole was rapidly reduced in the absence of histologic sections.

Studies of TPN Diaphorase.—The influence of agitation, anaerobiosis, temperature, and pH on the staining of kidney sections was tested. No increase in formazan deposition was noted with agitation of the solution or under anaerobic conditions. Sections incubated at room temperature were less intensely stained than those incubated at 37°C. However, with an active tissue such as kidney, it was possible to obtain satisfactory staining at room temperature by prolonging the incubation period from 20 minutes (routine time) to 30 minutes. The influence of the hydrogen-ion concentration was tested over a range of pH 7 to 12. At pH 12, spontaneous reduction of Nitro-BT was observed. Sections incubated in solutions with the pH between 7.0 and 7.8 were alike with respect to the pattern and intensity of the stain, while at higher pH levels there was a progressive decrease in activity. At pH 8.8., the amount of formazan deposition was about one-half that obtained at the recommended pH (7.4); while at pH 11.0 the sections were about one-fourth as intense as those stained optimally.

The effect of certain metallic ions upon the activity of the individual dehydrogenases has been referred to above. The only activating reagents found for the demonstration of TPN diaphorase as recommended were magnesium and manganese. These ions at a concentration of 10^{-3} M approximately doubled the intensity of the stain. Equimolar concentrations of Ca^{++}, Ba^{++}, and Al^{+++} did not accelerate TPN diaphorase activity.

Methylene blue was reported by Farber and Louviere (7) to exert a marked effect on the staining of tissue sections for TPN diaphorase when BT was used as the tetrazolium salt. The soluble redox dye was thought to act as an intermediate hydrogen acceptor which catalyzed the transfer between the diaphorase and the tetrazole. If this were the mechanism, one might not expect such a favorable influence of the redox dye when a more easily reduced tetrazole was used. To test this point, sections were incubated both aerobically and anaerobically in the recommended medium containing Nitro-BT with and without added methylene blue. There was no significant increase in formazan deposition in any of the sections. This finding is consistent with the explanation given by Farber and Louviere (7) for the beneficial effect of methylene blue with BT.

Histochemical Demonstration of TPN Diaphorase in the Rat Stomach, Pancreas, and Kidney.—In the rat *stomach* the surface cells (Fig. 1), the intermediate third of the stratified squamous

epithelium, the ganglion cells of Auerbach's and Meisner's plexi, and large ovoid elements of the stroma (perhaps macrophages) were intensely stained and therefore comparatively rich in TPN diaphorase. When the incubation period was prolonged to an hour or more, a weak reaction was usually demonstrable in the parietal and zymogenic cells. The epithelial cells of the islets of Langerhans (Fig. 3), ganglion cells (Fig. 4), and elements resembling macrophages (Fig. 5), both seen in the stroma of the *pancreas*, were very intensely stained in the medium recommended for the demonstration of TPN diaphorase. The acinar cells, and ductile epithelium (Fig. 4), on the other hand, were only faintly stained. The pattern of TPN diaphorase distribution in the pancreas is distinctly different from that of DPN diaphorase, the latter being shown in Fig. 6. A moderately intense staining reaction due to presence of DPN diaphorase was elicited from the acinar as well as islets' cells. Rat *kidney* sections that had been incubated for 20 minutes showed the most intense TPN diaphorase activity in the cells of the proximal convoluted tubules and macula densa (Fig. 7), thick limbs of Henle's loop (Fig. 9), and the stromal cells of the papilla (Fig. 8). Somewhat less intensely stained were the thin limbs of Henle (Fig. 9) and the papillary portions of the collecting ducts (Fig. 8), and still less, the distal convoluted tubules, save the macula densa. The glomeruli and the collecting ducts of the cortex and outer medulla were but weakly stained, if at all (Fig. 7).

At the intracellular level of study a basic difference in the localization of TPN diaphorase as compared with DPN diaphorase was observed. DPN diaphorase apparently was principally located in the mitochondria since these organelles were clearly defined and very numerous in sections (Figs. 2 and 12) incubated in the medium containing lactate, lactic dehydrogenase, and DPN. On the other hand, in similar sections that were incubated for TPN diaphorase the activity appeared as fine granules, never rods or filaments, often closely packed in the cytoplasm (Figs. 11 and 13.)

DISCUSSION

Farber and coworkers (6) were the first to localize diaphorase activities in the various components of the mammalian nephron (rat). This was accomplished with a rather wide variety of reagents in their incubation media, and the reactions necessitated some degree of anaerobiosis. The replacement of the tetrazolium salt (BT), which they used, with the more easily reducible Nitro-BT permitted a simplification of the reaction mixture, the elimination of anaerobiosis, the use of thinner sections, and a shortening of the incubation period. In addition, the substantive properties of Nitro-BT prevented crystallization of the pigment and allowed the mounting of sections in an organic medium, thus improving the optical characteristics of the section, which permitted localization of the enzymic sites at a higher degree of resolution. By means of this improved technic it was demonstrated that whereas DPN diaphorase was often restricted to the mitochondria (Figs. 2 and 12), the TPN diaphorase activity of the corresponding cells of adjacent sections was typically distributed throughout the cytoplasm in granules too fine to be considered mitochrondria (Fig. 13). Despite the diffuse character of its intracellular distribution, TPN diaphorase was not seen to escape extracellularly (Figs. 9 and 10), and therefore the activity of individual cells such as the stromal cells of the renal papilla (Fig. 8) was decidedly more evident than with the method employing BT.

The histologic pattern of TPN diaphorase in the rat kidney as revealed by the medium recommended in this paper is essentially the same as that reported by Sternberg, Farber, and Dunlap (23) who employed BT. However, in contrast to the findings of the latter investigators, certain characteristic variations of the staining pattern were obtained, depending upon the particular substrate employed. For example, when barium-6-phosphogluconate was used as the only dehydrogenase substrate, in rat kidney sections the macula densa became intensely stained while the glomeruli and all other tubular elements of the cortex remained unstained (Figs. 10 and 11). The latter variation in staining pattern must be due to the fact that whereas TPN diaphorase and isocitric dehydrogenase have an extensive distribution in the kidney cortex, 6-phosphogluconic dehydrogenase is limited to the cells of the macula densa. Sternberg *et al.* (23) also identified TPN diaphorase-containing cells in the stroma of the renal papilla. The cytochemical distribution of TPN diaphorase is shown for these cells in Fig. 8, in

detail that was not possible with BT. The granular distribution of TPN diaphorase is also shown in proximal convoluted tubular epithelium at high magnification in Fig. 13. The non-mitochondrial distribution of TPN diaphorase may be compared with the mitochrondrial distribution of DPN diaphorase in Fig. 12. It is suggested that TPN diaphorase is associated with the microsomes.

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Explanation of Plates

Plate 223

Fig. 1. Rat stomach. Isocitrate and malate as substrates. The surface epithelial cells gave a moderately intense reaction for TPN diaphorase. In contrast the parietal and zymogenic cells were negative. Compare with Fig. 2, reference 18, showing similar magnification of stomach stained for DPN diaphorase. × 150.

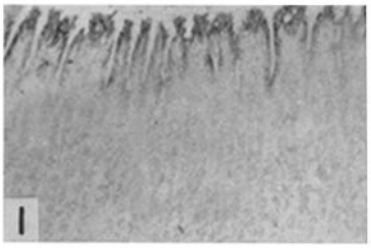
Fig. 2. Rat stomach. Lactate, lactic dehydrogenase, and *DPN* as substrates. Rod-shaped mitochondria are distinctly seen in the cells lining the gastric pit shown in the cross-section by virtue of their *DPN diaphorase* activity. In the more intensely stained parietal cells the mitochondria are densely packed in the cytoplasm except where the intracellular canaliculi course. Localization of DPN diaphorase in the mitochondria is to be contrasted with the more diffuse cytoplasmic distribution of TPN diaphorase (Figs. 3 to 5, and 13). × 1500.

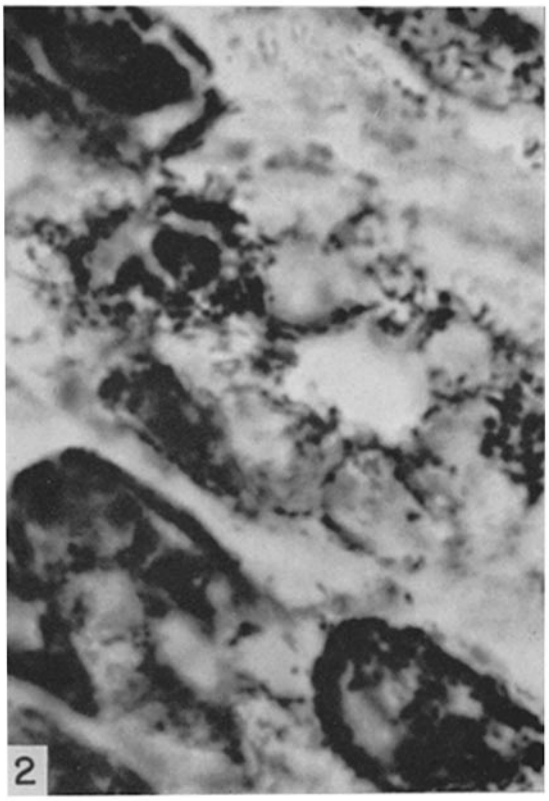
Fig. 3. Rat pancreas. Isocitrate and malate as substrates. The very intensely stained cells are those of an islet of Langerhans. Surrounding the islet are the faintly stained acinar cells. × 500.

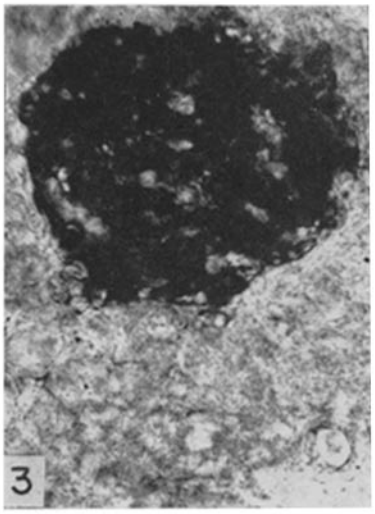
Fig. 4. Same preparation as that shown in Fig. 3. The large, deeply stained cells represent a small cluster of neurons of a minute parasympathetic ganglion. Included among the faintly stained elements flanking the ganglion are acinar cells and an interlobular duct. Nerve cells of the peripheral nervous system consistently showed very intense TPN diaphorase activity. × 500.

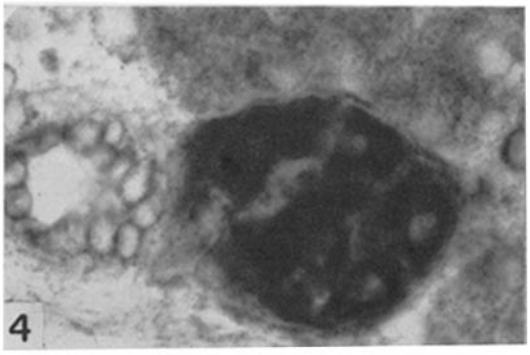
Fig. 5. Same preparation as that shown in Fig. 3. Throughout the stroma of the pancreas large, ovoid, frequently vacuolated cells (presumably macrophages) are particularly conspicuous by virtue of their very intense reaction for TPN diaphorase activity. Less intensely stained were elements with attenuated processes (presumably fibroblasts). × 500.

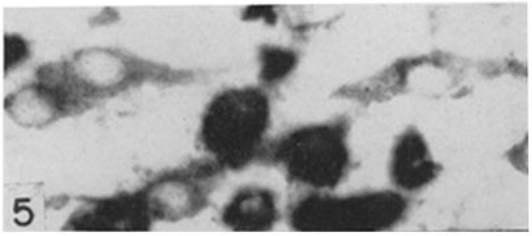
Fig. 6. Rat pancreas. Lactate, lactic dehydrogenase, and DPN as substrates. A single islet is shown enclosed on three sides by acinar cells. In contrast to the pattern of stain disclosed in the pancreas after incubation in the TPN media (Fig. 3), all elements of the parenchyma show intense *DPN diaphorase* activity. × 500.











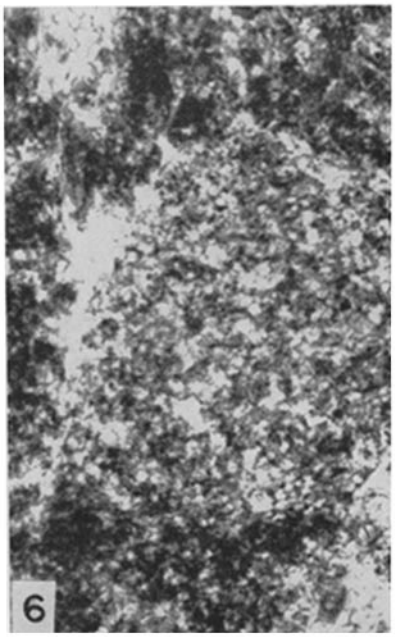


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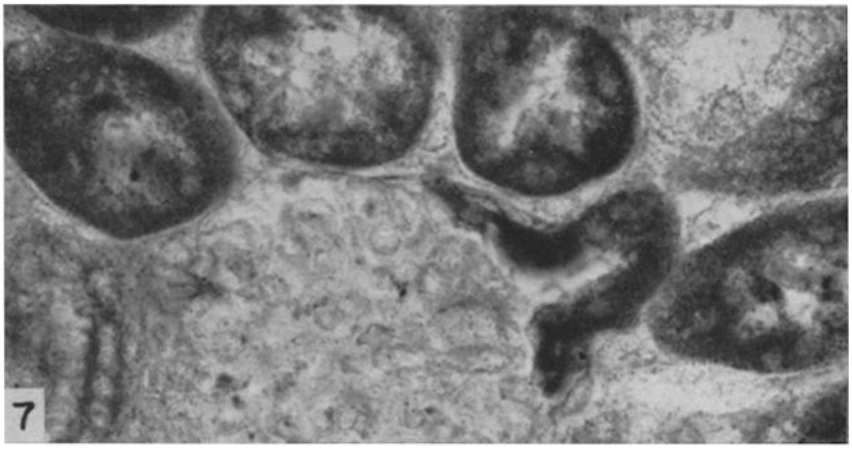
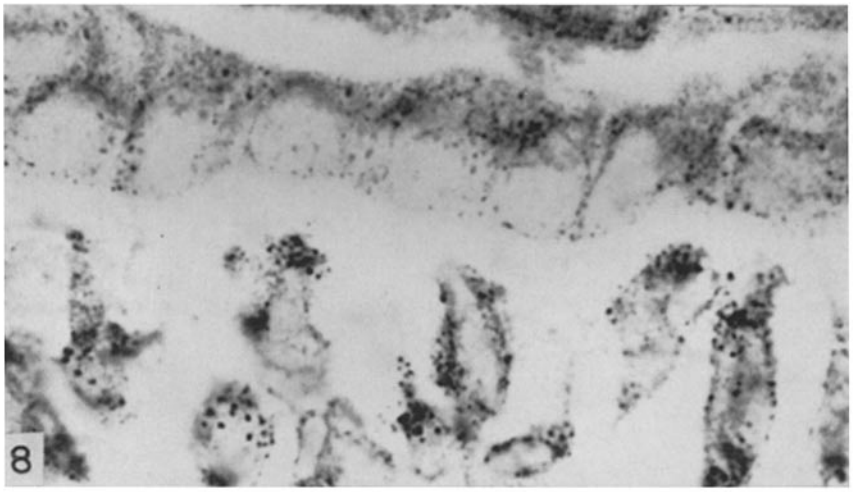
PLATE 224

FIG. 7. Rat kidney. Isocitrate and malate as substrates. At one pole of this renal corpuscle the funnel-shaped origin of the proximal convoluted tubule may be seen. At the opposite pole (left) the renal corpuscle is attached to the distal convoluted tubule at the macula densa. Intense TPN diaphorase activity was seen throughout the entire length of the proximal convoluted tubule, but in the case of the distal convoluted tubule only those cells composing the macula densa showed marked activity. The glomerulus showed only a faint degree of TPN diaphorase activity. × 500.

FIG. 8. Papilla of rat kidney. Isocitrate and malate as substrates. The supranuclear portion of the epithelial cells of the terminal portion of the collecting duct, seen in longitudinal section (top), are filled with fine particles of formazan. In the stromal cells, coarse as well as fine granules of formazan are seen distributed throughout the cytoplasm. The coarse granules may be due to overstaining. × 1500.

FIG. 9. Outer medulla of rat kidney. Isocitrate and malate as substrates. The thick (*A*) and thin limb (*B*) of Henle's loop and a neighboring collecting duct (*C*) are shown in cross-section. Intense activity was revealed in the thick limbs of Henle's loop, and TPN diaphorase activity of moderate intensity was elicited from the cells composing the thin limbs and the collecting ducts of the outer medulla. × 1500.





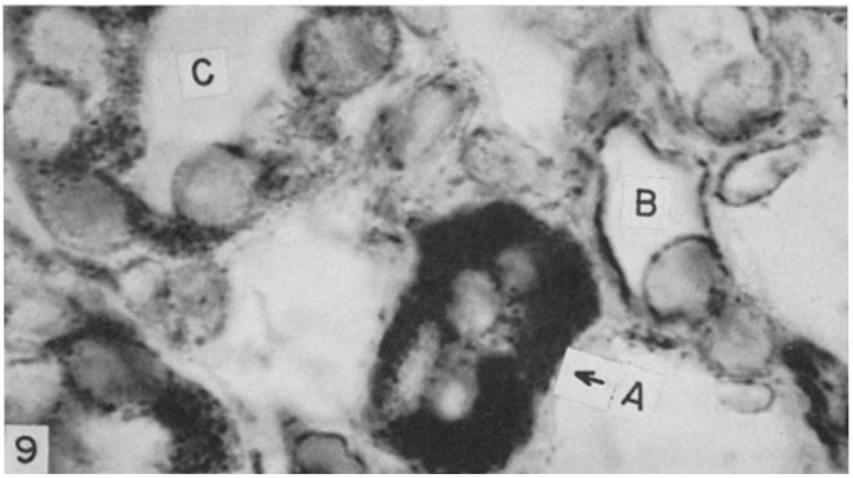


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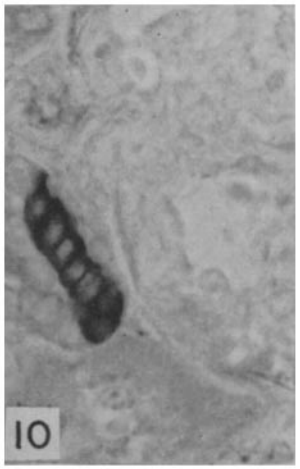
PLATE 225

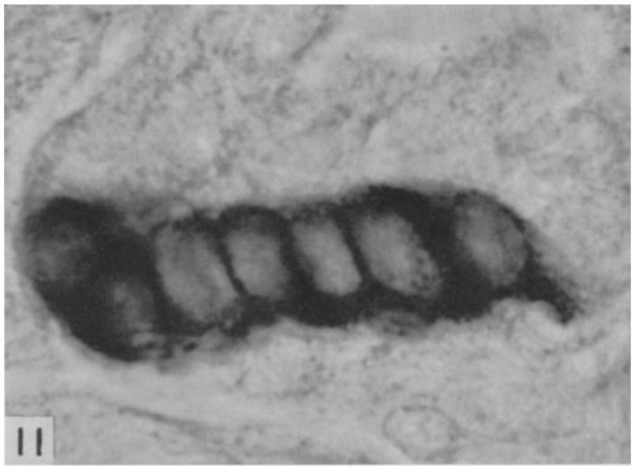
FIG. 10. Rat kidney. Barium-6-phosphogluconate as substrate. The plane of section of this renal corpuscle approximates that depicted in Fig. 7. However, TPN diaphorase activity was elicited only from the cells of the macula densa and not from other cortical epithelial elements. Weakly stained endothelium of the afferent arteriole is seen directly above the macula densa, and unstained are the glomerulus to the right and proximal convoluted tubule below. Apparently, isocitric dehydrogenase and TPN diaphorase are present in abundance in the cells of the proximal convoluted tubules as well as those of the macula densa, but only the latter possess 6-phosphogluconic dehydrogenase activity. × 500.

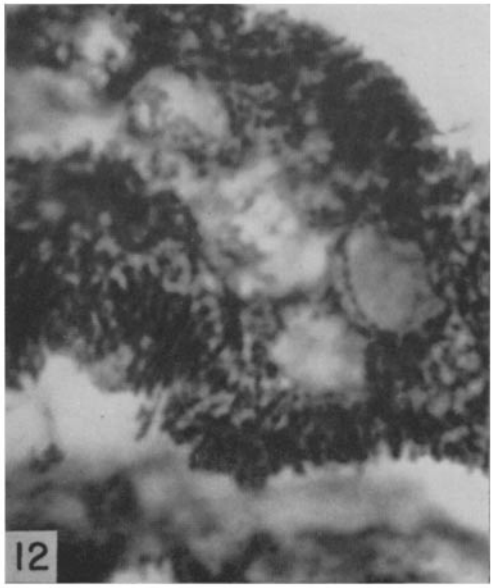
FIG. 11. Rat kidney. Barium-6-phosphogluconate as substrate. The macula densa cells of Fig. 10 are shown at a higher magnification in order to resolve the fine blue granules of formazan which are found throughout the cytoplasm of these cells. The stained particles cannot be regarded as mitochondria or the Golgi apparatus, but may be associated with the microsomal fraction of the cells. × 2000.

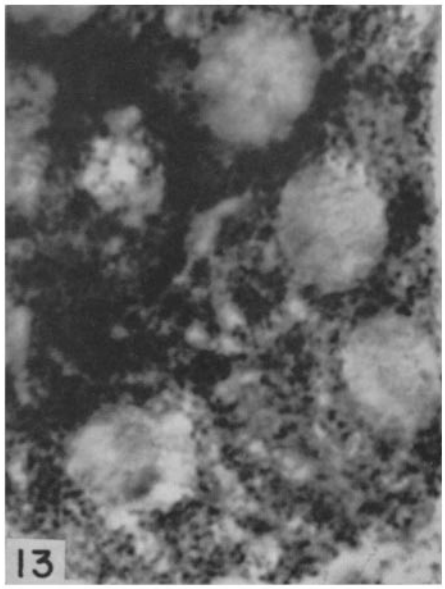
FIG. 12. Rat kidney. Lactate, lactic dehydrogenase, and DPN as substrates. One of the most striking displays of mitochondria as revealed by virtue of their *DPN diaphorase* activity is demonstrated in the basal portion of the cells lining the proximal convoluted tubules. The localization of *DPN diaphorase* in the mitochondria is in contrast to the more diffuse granular distribution of *TPN* diaphorase in Fig. 13. × 2000.

FIG. 13. Rat kidney. Isocitrate and malate as substrates. The proximal convoluted tubule has been somewhat tangentially cut. Throughout the cytoplasm of the cells, the sites of *TPN diaphorase* activity appear as granules, roughly 0.3 μ in diameter. The proximal convoluted tubules are also very rich in *DPN* diaphorase (Fig. 12), but in the latter instance all of the activity is located in the mitochondria which is in striking contrast to the granular intracellular distribution of TPN diaphorase seen here. This difference was noted irrespective of the plane of section of the cells. × 2000.









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