The Cytochemical Localization of Oxidative Enzymes

II. Pyridine Nucleotide–Linked Dehydrogenases

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ABSTRACT

Methods are presented for the intramitochondrial localization of various diphosphopyridine nucleotide and triphosphopyridine nucleotide-linked dehydrogenases in tissue sections. The cytochemical reactions studied involve the oxidation of the substrates by a specific pyridino-protein. The electron transfer of tetrazolium salt is mediated by the diaphorase system associated with the dehydrogenase. The final electron acceptor was either *p*-nitrophenyl substituted ditetrazole (nitro-BT) or *N*-thiazol-2-yl monotetrazole (MTT), the latter giving rise to metal formazan in the presence of cobaltous ions.

Mitochondrial localization of the formazan precipitate could be achieved by using hypertonic incubating media containing high concentrations of substrate and co-enzyme. A fast reduction of tetrazolium salt was obtained by chemically blocking the respiratory chain enzymes beyond the flavoproteins. Although diaphorase systems are implicated in the reduction of tetrazolium salts, specific dehydrogenases are solely responsible for the distinct distribution pattern obtained in tissues with various substrates.

The present findings in tissue sections are discussed in conjunction with existing biochemical evidence from differential centrifugation experiments.

The study of mitochondrial function in homogenates prepared by differential centrifugation (5, 16) has yielded a large amount of information on the pattern of oxidative enzymes within these organelles. Interpretation of the quantitative results is beset with difficulty, however, because many limiting factors, such as diffusion of soluble enzymes and co-enzymes, are involved in the preparation of the homogenates. Although isolated mitochondria are generally believed to contain all the enzymes of the tricarboxylic acid cycle, only succinic dehydrogenase has been shown to be almost exclusively bound to the mitochondrial fraction (15). The histochemical demonstration of pyridine nucleotide–linked dehydrogenases in thin tissue sections represents a qualitative alternate approach to homogenate studies. The localization of the enzymes within the complex environment of the cytoplasm can reasonably be expected to yield additional information.

Earlier studies on the histochemical localization of dehydrogenase-diaphorase systems in fresh tissue sections by tetrazolium methods (10, 37) resulted only in the demonstration of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) diaphorases independent of the substrates used for the reduction of the pyridine nucleotide. This technique has recently been improved by Nachlas, Walker, and Seligman (23, 24), using 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (Nitro-BT) (22) as their final electron acceptor, and extended to the demonstration of a number of dehydrogenases. The present paper deals with a further development and modification of the Nitro-BT method and demon-

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strates the application of the metal formazan technique (27) to the study of certain dehydrogenases. The methods developed enabled us accurately to demonstrate that a considerable number of pyridine nucleotide–linked dehydrogenases are localized in mitochondria.

In confirmation of the view already expressed by Nachlas *et al.*, it has been found that when oxidized pyridine nucleotide is added to the system, the site of reduction of the tetrazolium salt reflects not only the activity of the diaphorase, but is dependent on the specific pyridine-protein enzyme involved in the dehydrogenation of the substrate. In acting as an indicator of the substrate specific dehydrogenase, therefore, the electron transfer from substrate to tetrazolium involves at least two enzymatic steps. This conclusion is based mainly on the different distribution pattern in the tissues, which is exhibited by each dehydrogenase-diaphorase system. Our method has been the subject of a preliminary report (12).

Materials and Methods

The present studies have been carried out with rat tissues, stomach, kidney, and submaxillary gland being used for most of the experiments. The tissues were prepared as described in the previous communication (35). The time required for freezing of the tissues did not exceed 1 minute. Considerable decrease of enzymatic activity together with loss of precise localization was observed after slow freezing of tissue blocks to −15°. Repeated freezing and thawing resulted in complete loss of enzymatic activity. After mounting on warm (room temperature) coverslips, the thawed sections were covered with the incubating medium and incubation was carried out for 15 minutes at 37°. The demonstration of weak enzymatic activity required an incubation up to 30 minutes More usually the time required was 10 to 15 minutes.

Incubating Media:

The various incubating media were based on the original metal formazan technique and on suggestions by Nachlas *et al.* with modifications given below. The media were prepared freshly before use and had the following standard composition:[1]

Substrate (1.0 M)	0.1 ml.
DPN or TPN (1.0 M to 0.1 M)	0.1 ml.
Respiratory inhibitor (amytal, sodium azide, or sodium cyanide) (0.1 M)	0.1 ml.
Magnesium chloride (0.05 M)	0.1 ml.
Buffer (phosphate 0.06 M, or tris (hydroxymethyl) aminomethane 0.2 M, pH 6.8–7.0)	0.25 ml.
Tetrazolium salt (1 mg./ml.)	0.25 ml.
Distilled water to make	1.0 ml.
Polyvinylpyrrolidone (M.W. 11,000)	75 mg.

For demonstrating the different dehydrogenase systems the components of this standard mixture were varied as follows:

A. Using MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide):

1. β-Hydroxybutyric Dehydrogenase.—substrate DL β-hydroxybutyric acid sodium salt, DPN, amytal or sodium azide, phosphate buffer. Cobaltous chloride $6H_2O$ (0.5 M) 0.05 ml. was mixed with the buffer and the resulting precipitate filtered off before adding the other ingredients to the final solution The final pH was adjusted to pH 7.0 by "tris." The incubation with this medium should not exceed 15 minutes in order to prevent precipitate formation.

2. Glucose-6-Phosphate Dehydrogenase.—substrate glucopyranose-6-phosphate disodium salt or calcium salt, TPN, sodium azide or amytal, tris (hydroxymethyl) aminomethane buffer, $CoCl_2 \cdot 6H_2O$ (0.5 M) 0.05 ml., sodium fluoride (0.01 M) 0.05 ml.

B. Using Nitro-BT:

The respiratory inhibitor in each case was sodium cyanide (freshly prepared and neutralized).

1. Isocitric Dehydrogenase.—substrate DL-isocitric acid, adjusted to pH 7.0 by "tris," DPN or TPN, phosphate buffer.

2. Malic Dehydrogenase.—substrate sodium-L-malate or L-malic acid adjusted to pH 7.0 by "tris," DPN or TPN, phosphate buffer.

3. Glutamic Dehydrogenase.—substrate sodium-L-glutamate monohydrate, DPN or TPN, phosphate buffer.

4. α-Glycerophosphate Dehydrogenase.—substrate sodium-DL-α-glycerophosphate (neutralized with 0.1 M-HCl), DPN, "tris" buffer.

5. Alcohol Dehydrogenase.—substrate ethanol, DPN, phosphate buffer.

6. Lactic Dehydrogenase.—substrate sodium-DL-lactate, DPN, phosphate buffer.

Each of the above systems has been tested for its pyridine-nucleotide specificity. Further experiments with specific inhibitors and studies on substrate specificity are described below.

The MTT-Co method could only be used for the demonstration of dehydrogenases which could yield a reaction product that is not markedly inhibitory (acetoacetate in the case of β-hydroxybutyric dehydrogenase and D-δ-gluconolactone-6-phosphate (4) in the case of glucose-6-phosphate dehydrogenase). For demonstrat-

[1] DPN (of highest purity) and glucose-6-phosphate Ca-salt was obtained from C. F. Boehringer & Soehne (Mannheim, Germany). TPN and glucose-6-phosphate disodium salt from Sigma Chemical Co. (St. Louis, Missouri).

ing the other dehydrogenases, the addition of cyanide was found necessary to remove the carbonyl groups formed. In these systems, Nitro-BT was used as an electron acceptor since cyanide, in the presence of cobaltous ions, is capable of spontaneously reducing tetrazolium (see below).

After incubation in MTT-Co–containing media the sections were rapidly washed in 0.1 M-HCl and water and fixed in 10 per cent neutral formalin, and were mounted in glycerin jelly containing $CoCl_2$. To mount Nitro-BT treated sections, Nachlas *et al.* used alcohol dehydration in order to remove the red component of the reaction product (arising from a contaminating monotetrazole). We found that dehydration, even after prolonged formalin fixation of the tissues, resulted in rapid aggregation of the nitro-formazan. Aggregation was avoided by washing the formalin-fixed sections in 10 per cent ethanol for about 10 minutes to dissolve most of the red reaction product without causing appreciable shrinkage to the tissues. The sections, after washing in water, were mounted in glycerin jelly.

Essential Conditions:

Requirements for the accurate mitochondrial localization of pyridinoprotein-diaphorase systems can be listed as follows:

1. Reduced State of the Respiratory Chain Enzymes.—Inhibitors of the type studied by Chance and Williams served to block the respiratory enzymes beyond the level of the flavoproteins and thus render possible an efficient competition of the tetrazole with molecular oxygen. The mechanism of action of the various inhibitors tested has been discussed in the previous paper (35). The results obtained with various inhibitors in dehydrogenase-diaphorase systems correspond to the results with DPN and TPN-diaphorases.

2. Protection of the Mitochondria.—Incubation of tissue sections in a hypertonic non-electrolyte medium has been shown to be a prerequisite for cytochemical as opposed to histochemical localization of mitochondrial enzymes (25, 34), and it is absolutely essential for demonstrating soluble or partially soluble enzyme systems. After the onset of mitochondrial swelling in hypotonic incubating media, a gradual diminution in the reactivity of mitochondria was observed together with an increase in size of the dot-like formazan precipitates. The same alteration occurred, to a lesser degree, using isotonic media. These processes are presumably due to diffusion of soluble pyridino-protein enzymes. Since some structural alteration of isolated mitochondria occurs in isotonic sucrose, as suggested by an increase of succinic dehydrogenase activity (36), formation of vesicles, and loss of endogenous potassium and phosphates (1), diffusion of the more soluble dehydrogenases will not be prevented by isotonic media. It can be concluded, therefore, that structurally bound dehydrogenases only are likely to be present in the mitochondrial fraction from homogenates prepared in 0.25 M sucrose. A weak reaction for glutamic dehydrogenase was still obtained in swollen mitochondria after immersion of the tissue section in buffer for 10 minutes at 20°. This treatment resulted in a complete loss of glucose-6-phosphate dehydrogenase activity. Glutamic dehydrogenase, although a soluble enzyme, was found to be largely present in the mitochondrial fraction (14), whereas glucose-6-phosphate dehydrogenase was recovered only from the soluble fraction (11). The diffusion of the latter enzyme system is not even prevented in hypertonic solution since slight mitochondrial swelling induced by starving the tissue, by pre-incubation in buffered 0.88 M sucrose pH 7.0 for 10 minutes at 20°, resulted in a marked decrease in enzymatic activity. Activity was completely lost after pre-incubation in isotonic (0.25 M) sucrose.

For osmotic protection of the tissues, 7.5 per cent polyvinyl pyrrolidone (PVP) was added to the incubating media (34). No improvement in localization of dehydrogenase systems was noted upon the addition to PVP of 0.25 M-sucrose (25).

3. Addition of Co-enzyme.—Despite apparent preservation of morphological integrity, freezing and thawing in the cryostat procedure leads to functional alteration of mitochondria. Freezing of isolated mitochondria in dry ice–acetone was reported to result in release of soluble cofactors, decrease in enzymatic activity, and complete uncoupling of oxidative phosphorylation (31). It is therefore necessary to add high concentrations of pyridine nucleotide to the system in order to ensure a rapid electron transfer by the mitochondria (21) and the need for high substrate concentration may be due to the same cause. An optimal reduction of tetrazolium in our systems was achieved by using stoichiometric amounts of pyridine nucleotide and substrate (32). A sufficiently fast reaction for most purposes, however, was obtained using a ten times smaller concentration (0.01 M) of DPN or TPN.

At a slightly acid pH, DPN is quite stable (19) and can, therefore, be stored in solution at −20°.

That some of the cofactors are still present in fresh frozen tissue sections is suggested by the following experiments. Using the Nitro-BT method, osmotically protected sections showed a slight activity upon the addition either of substrate alone, or of a high concentration of pyridine nucleotide alone. This reaction, which is prevented completely by pre-incubation of the sections in buffer for 10 minutes at room temperature, can be ascribed to the presence of residual endogenous co-enzyme and substrate.

4. Effect of Cyanide.—Sodium cyanide (final concentration 0.01 M) was used in all dehydrogenase-diaphorase systems involving the formation of carbonyl groups. Cyanide was presumed to act both as an efficient carbonyl reagent and as a respiratory inhibitor. No significant reaction could be obtained by substituting hydrazine, hydroxylamine, phenylhydrazine, and

semicarbazide for cyanide. These compounds in fact inhibit dehydrogenases (18) and at concentrations higher than 0.01 M are able spontaneously to reduce tetrazolium salts even at neutral pH. The MTT-cobalt systems cannot be used in the presence of cyanide ions since hexacovalent cobalt (II) cyanide is formed. This complex $(Co(CN)_6)^4$ is unstable and it reduces the tetrazolium salt independently of enzyme activity.

5. Effect of pH.—Incubation at a neutral or slightly acid pH is essential for maintaining mitochondrial structure in tissue sections (34). An alkaline pH both increases the possibility of non-enzymatic reduction of tetrazolium and of inactivation of oxidized pyridine nucleotide (19). Tetrazolium methods, therefore, are not suitable for demonstrating dehydrogenases or oxidases having a narrow pH-optimum in the alkaline range, as for example the aldehyde dehydrogenases (32, 33).

EXPERIMENTAL RESULTS

The reactions for each of the dehydrogenase systems tested resulted in mitochondrial localization in the form of rows of dot-like deposits of formazan, the dots having an average diameter of 0.2 to 0.3 μ. This pattern corresponds to the localization of the succinic dehydrogenase (28) and diaphorase systems respectively (35).

The relative activity of the dehydrogenase systems investigated in the different parts of rat stomach, kidney and salivary gland are summarized in Tables I and II. It is clearly shown that every system studied exhibits a characteristic distribution pattern and this represents only part of the complete diaphorase reactions. Especially significant are the marked differences between DPN and TPN-linked systems. The most important properties of some of the dehydrogenases investigated are as follows:

Isocitric Dehydrogenase:

The DPN-linked enzyme in animal mitochondria has only recently been described by Plaut and

TABLE I

Sites of Activity of DPN-Linked Enzymes

0 — 4 = graded visible reaction indicating relative degree of enzymatic activity. Incubation times: diaphorase reaction 15 minutes; dehydrogenase reactions 30 minutes, in order to demonstrate weak activities.

Organ	Structure	Diaphorase	Dehydrogenases						
			Isocitric	Malic	α-Glycerophosphate	Glutamic	β-Hydroxybutyric	Alcohol	Lactic
Stomach (parietal)	Surface epithelium	1	3	3	2	1	2	2	4
	Parietal cells (neck)	3	2	2	3	1	4	2	3
	Parietal cells (deep)	4	3	2	3	1	2	4	4
	Zymogen cells	1	1	1	1	1	1	0	2
	Smooth muscle, vessels	4	3	3	3	3	0	1	4
	Autonomic plexus	2	2	2	2	2	0	0	2
Stomach (squamous)	Malpighian layer	3	3	3	3	2	3	2	3
Kidney	Glomerular endothelium	1	1	1	1	1	0	1	1
	Proximal tubules	2	3	3	3	3	1	3	3
	Descending limbs	4	3	3	4	4	4	3	4
	Thin limbs	1	1	1	1	1	0	1	1
	Ascending limbs	2	2	2	2	3	2	2	3
	Distal tubules	2	2	2	2	2	3	2	2
	Collecting ducts (papilla)	2	2	2	2	2	0	2	2
Salivary gland	Serous cells	2	2	1	2	1	2	1	1
	Mucous cells	1	1	1	1	1	1	1	1
	Intercalated ducts	2	2	1	2	2	2	2	2
	Excretory ducts	4	3	3	4	4	4	4	4

TABLE II

Sites of Activity of TPN-Linked Enzymes

Organ	Structure	Diaphorase	Dehydrogenases			
			Glucose-6-phosphate	Isocitric	Glutamic	Malic
Stomach (parietal)	Surface epithelium	1	1	3	0	1
	Parietal cells (neck)	2	1	3	0	1
	Parietal cells (deep)	2	2	3	1	2
	Zymogen cells	1	1	2	0	1
	Smooth muscle, vessels	4	2	4	0	2
	Autonomic plexus	4	4	4	0	1
Stomach (squamous)	Malpighian layer	3	3	3	0	1
Kidney	Glomerular endothelium	0	0	0	0	0
	Proximal tubules	2	0	2	1	1
	Descending limbs	3	1	4	0	1
	Thin limbs	1	0	1	0	0
	Ascending limbs	2	1	1	0	1
	Distal tubules	2	3	1	0	0
	Collecting ducts (papilla)	1	0*	1	0	0
Salivary gland	Serous cells	2	0	2	0	1
	Mucous cells	1	0	1	0	0
	Intercalated ducts	2	1	2	1	1
	Excretory ducts	3	2	4	1	2

* Macula densa.

Sung (30). Fig. 10 shows the presumably mitochondrial localization of this enzyme system in a parietal cell of gastric mucosa. Fig. 4 shows its distribution pattern in the kidney. TPN-linked isocitric dehydrogenase is only partially recoverable from the mitochondrial fraction of homogenates (13). Nevertheless, it showed a strong activity (Fig. 5) and a mitochondrial pattern in tissue sections.

Malic Dehydrogenase:

Substituting TPN for DPN resulted in a marked decrease of enzymatic activity (33) without change in distribution pattern. Clear evidence of "malic enzyme" activity (26) could not be obtained.

α-Glycerophosphate Dehydrogenase:

Apart from the organs studied (Fig. 2), the high activity of this enzyme in striated muscle (2) (Fig. 8) has been confirmed. β-glycerophosphate could not be substituted for α-glycerophosphate.

Glutamic Dehydrogenase:

Replacing DPN by TPN resulted in a very weak reaction (33). The DPN-linked activity (Fig. 6) was inhibited by the addition of glutamine.

β-Hydroxybutyric Dehydrogenase (Figs. 1 and 9):

No reaction was obtained with both α-hydroxybutyrate and L(+)-β-hydroxybutyrate as substrates. The enzymic reaction responsible for the reduction of tetrazolium in the presence of DPN was found to involve only the dehydrogenation of D(−)-β-hydroxybutyrate to acetoacetate. This reaction could not be inhibited by dicoumarol since only the further oxidation of acetoacetate is dependent on phosphorylation (3). The racemic mixture of β-hydroxybutyrate could substitute for the D-isomer because there is no competitive antagonism between the two steric forms (20).

Glucose-6-Phosphate Dehydrogenase:

The high activity of this enzyme system in the macula densa of the distal convoluted tubule (Fig. 7), in ganglionic cells and fibres of the peripheral autonomous nervous tissue (Fig. 3) is of great interest. The localization of glucose-6-phosphate dehydrogenase in mitochondria by the tetrazolium method, which is at variance with the results of biochemical studies on homogenates in isotonic media (11) is of considerable interest when one considers the role of this enzyme system in the direct oxidative pathway of glucose (7).

The addition of phosphate to our system, or prolonged freezing of the tissues, proved to be inhibitory. The reaction could be enhanced by blocking the glycolytic pathway by fluoride. The addition of 0.005 M-adenosine triphosphate to the incubating medium caused a marked inhibition of the histochemical reaction obtained with glucose-6-phosphate as a substrate. The effect is probably due to the participation of oxidative decarboxylation of 6-phosphogluconate. This TPN-dependent enzymatic step (17) which follows the TPN-linked dehydrogenation of glucose-6-phosphate to D-δ-gluconolactone-6-phosphate is competitively inhibited by adenosinetriphosphate (11).

DISCUSSION

The results of the experiments reported here demonstrate that the activity of individual dehydrogenases can be shown in a system in which mitochondrial diaphorase is implicated in the electron transfer to tetrazolium salts. Although diaphorase activity is present wherever dehydrogenases occur, the distribution pattern of various dehydrogenases is different from and usually more restricted than the distribution of the diaphorase using reduced pyridine nucleotide as a substrate. Thus, by utilizing appropriate cofactors and substrates all the sites of co-enzyme activity demonstrated in the previous communication (35) can be resolved into sites of individual enzyme activity. These findings are at variance with the results of Farber and coworkers and fully corroborate the views already expressed by Nachlas *et al.* Furthermore, the distribution of TPN-linked systems is quite different from the localization ascribed by Farber *et al.* to TPN-diaphorase. This fact is probably due to the much greater sensitivity of the new tetrazolium methods. To some extent our results differ with respect to the distribution of dehydrogenase-diaphorase systems from those reported by Nachlas *et al.* These disparities may be due to the use by these authors of a hypotonic medium containing only a very small amount of co-enzyme.

Although an intramitochondrial localization was obtained with the dehydrogenase systems tested, it was not possible to demonstrate any extramitochondrial oxidative pathway (6, 9) taking part in the reduction of tetrazolium salts.

The extramitochondrial electron transfer, supposedly localized in microsomes, is mediated by a cytochrome-*c*-reductase, which, in contrast to its intramitochondrial counterpart, is not sensitive to both blocking agents and hypotonic treatment (6). Microsomal respiratory enzymes might be responsible for the weak diffuse formazan staining occasionally observed in the cytoplasm. In addition, the slow reduction of tetrazolium occurring in the substrate media about 1 hour after incubation of the tissues may likewise be due to eluted microsomal enzymes.

Ernster (8) has shown that DPNH, generated by intramitochondrial dehydrogenases, reacts preferentially with the intramitochondrial DPNH-cytochrome-*c* reductase which is sensitive to respiratory inhibitors. This electron transfer occurs even in aged mitochondria which are dependent on added DPN. The findings of Ernster are supported by our observations on frozen and thawed mitochondria in tissue sections. The diaphorase activity responsible for the intramitochondrial localization of formazan, is entirely dependent on reduction of added pyridine nucleotide by intramitochondrial dehydrogenases.

In view of the present results it seems doubtful that the granular or diffuse pattern of TPN-diaphorase activity observed by Nachlas *et al.* (24) can be interpreted as indicating a microsomal activity. It is rather suggested that their granules are related to the dot-like localization of diaphorase activity in mitochondria. This view is further supported by the high activity of TPNH-cytochrome *c* reductase found by de Duve *et al.* (6) in heavy mitochondrial fractions from rat liver homogenates.

The present discrete histochemical methods have the disadvantage of yielding only qualitative results. The quantitative differential centrifugation experiments on the other hand are greatly limited in being essentially of a statistical nature (5). The cytochemical demonstration of a given enzyme system in a tissue section overcomes this drawback in visualizing differences between various cell types or even in distinguishing between different functional states of the same cell type.

The marked differences we observed in distribution of the various dehydrogenase and diaphorase systems strongly suggest that mitochondria are truly heterogeneous in enzyme activity. These observations lend further support to the same assumption made in an earlier paper (29) and based on a comparison of succinic dehydrogenase and DPN-diaphorase activities in various mammalian and insect tissues.

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Explanation of Plates

Plate 382

Fig. 1. Rat stomach (parietal). Localization of β-hydroxybutyric dehydrogenase mainly in parietal cells near the neck region of the glands. × 140.

Fig. 2. Rat stomach (parietal). α-glycerophosphate dehydrogenase localized mainly in parietal cells of the middle parts of the glands. Strong activity is exhibited by smooth muscle cells and by bacteria adjacent to the surface epithelium. × 132.

Fig. 3. Rat stomach (squamous). Strong activity of glucose-6-phosphate dehydrogenase in the Malpighian layer and in elements of the autonomous plexus. Smooth muscle cells show moderate activity. × 120.

Fig. 4. Rat kidney. DPN-linked isocitric dehydrogenase in descending and ascending limbs of Henle's loop and in collecting ducts of the papilla. × 180.

Fig. 5. Rat kidney. TPN-linked isocitric dehydrogenase strongly active in descending limbs of Henle's loop, very weak in collecting ducts of the papilla. × 180.

Fig. 6. Rat salivary gland. Localization of glutamic dehydrogenase in secretory and intercalated ducts. × 395

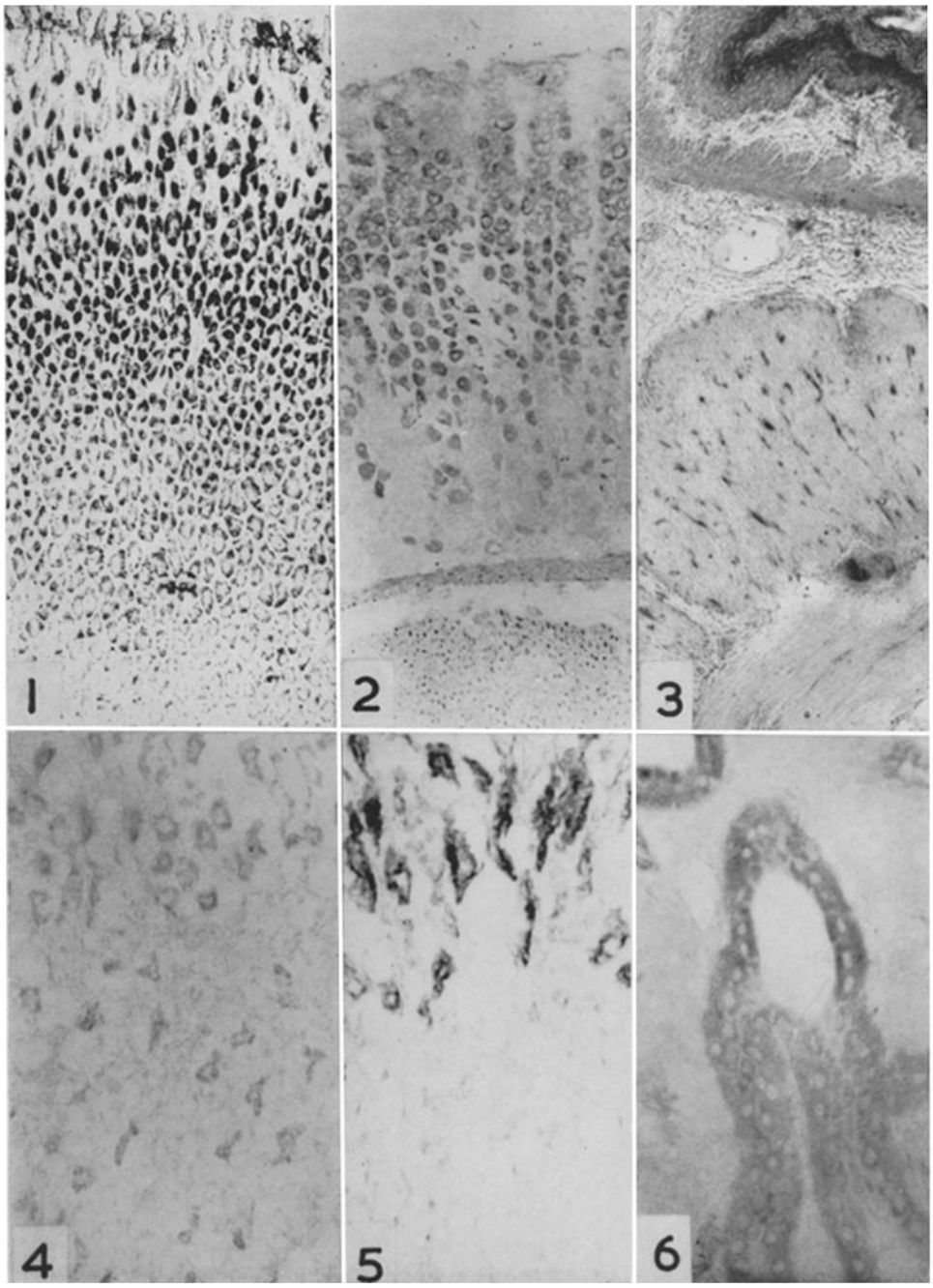

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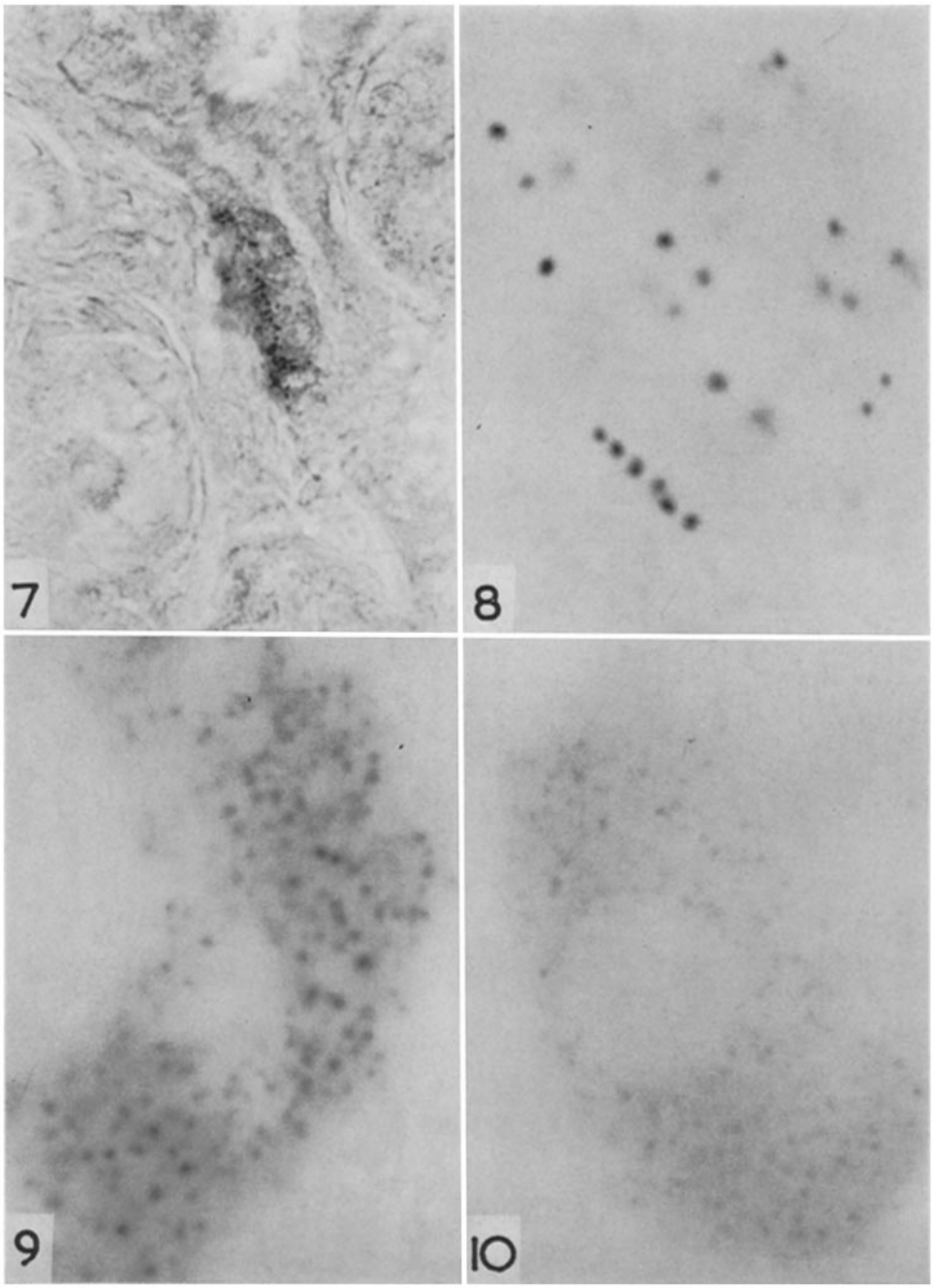
PLATE 383

FIG. 7. Rat kidney. Localization of glucose-6-phosphate dehydrogenase in the macula densa of a distal tubule adjacent to a glomerulus. × 1320.

FIG. 8. Rat psoas muscle. Localization of α-glycerophosphate dehydrogenase in sarcosomes (Nitro-BT method). × 4400.

FIG. 9. Rat kidney. Cells of descending limb of Henle's loop showing intramitochondrial localization of β-hydroxybutyric dehydrogenase (MTT-Co method). × 4400.

FIG. 10. Rat stomach. Parietal cell showing mitochondrial localization of DPN-linked isocitric dehydrogenase (Nitro-BT method). × 4400.



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