

OXIDATIVE ENZYMES IN PARAFFIN SECTIONS

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Introduction

As a recent current of enzyme histochemistry, fresh frozen sections have been widely used in parallel with the development of the "cryostat" technique. Especially for the oxidative enzymes, unfixed freshness has been recommended since Seligman & Rutenburg for succinic dehydrogenase¹⁾ and Farber et al. for DPN and TPN diaphorase systems²⁾. And it has been seemed to be indispensable to use unfixed sections for oxidative enzymes because of their instability. In fact, the cryostat procedure has some merits to give the marked staining. The fresh frozen sections, however, have the disadvantage of the damage of organelle during freezing and thawing, and, moreover, may result in further damage and even diffusion of the enzymes and reaction products during incubation procedure. Therefore, some recommendations were made to fix the fresh sections by acetone and other fixatives. (Pearson³⁾, Mizutani^{4,5)}, Novikoff⁶⁾)

Another disadvantage of cryostat technique is the difficulty to reserve the frozen material. It is desirable to have the material maintained for a long time without damaging enzyme activities and to stain the sections in case of need. Therefore, the most suitable and brief procedure for these demands may be the paraffin embedding if it does not destroy the enzyme activities.

The purpose of the present study is to show the usefulness of the paraffin sections for some oxidative enzyme histochemistry.

Material and Methods

The kidney, liver, heart and skeletal muscles, stomach and adrenal of rats were used. Immediately following sacrifice small thin pieces of tissues were fixed in cold acetone (2°-0°C) for 2-3 hours and embedded in paraffin (m.p. 50°C). The total embedding time did not exceed 1 hour (xylene 30 min., paraffin 30 min.).

The paraffin sections (4-5 μ in thickness) were directly floated on the incubating mixtures described in later for 5 to 30 min., or stored in a refrigerator without

sticking on the slide or cover glass. The sections can be stored more than 4 months maintaining the enzyme activities in a cooled condition.

During the incubation, the initial color of the reactive tissues was violet and by increasing the incubating period blue precipitate was progressively yielded. The tissues with high enzyme activity stained in blue color from the beginning of the reactions and the excessive incubation disturbed the cytological examinations by redundant formazan precipitation. After the proper incubation, the sections were floated on distilled water, stuck on slide glass, dried at 50°C, treated with xylene, and then mounted in Canada balsam. Through the xylene deparaffination, feeble violet color diffused out of the tissues which showed relatively low activity, but no blue diformazan did.

Incubating mixture

a) DPN- and TPN-diaphorase (Pyridine nucleotide-tetrazolium reductase)

0.1% Nitro-BT	0.5 ml
0.1M phosphate buffer (pH 7.2-7.4)	0.5 ml
distilled water	0.5 ml
DPNH or TPNH	5 mg

b) DPN- or TPN-linked dehydrogenase systems

0.1% Nitro-BT	0.5 ml
0.1M phosphate or tris-malate buffer (pH 7.0-7.2)	0.5 ml
0.1M substrate	0.5 ml
DPN or TPN	5 mg

(Substrate: Sodium lactate, Sodium malate, Isocitric acid, β -Hydroxybutyric acid, Glucose-6-phosphate)

The addition of KCN as a ketone fixative in the mixtures might enhance the reactions, but, at the same time, caused the nonspecific coloration of control sections probably due to the reductive action of cyanide for DPN or TPN. It should be used with a minimum concentration (under 0.01 Mol), if needed.

c) Succinic dehydrogenase system

0.1% Nitro BT	1 ml
0.1M Sod. succinate	1 ml
0.1M phosphate buffer (pH 7.2-7.4)	1 ml
0.1% phenazine methosulfate	0.1 ml-0.05 ml

The control staining without any substrate is indispensable for each staining procedure to exclude the "nothing dehydrogenase" (Racker⁷), Zimmerman⁸) and other nonspecific tetrazolium reductions. Especially for succinic dehydrogenase procedure, the excessive amounts of phenazine methosulfate may easily result

in the nonspecific reduction of tetrazolium salt and nonenzymatic coloration of the solution and the sections as well.

Results

The reactions for the dehydrogenase systems tested showed their principal localization on the probable mitochondria in the form of dot- or rod-like deposits of formazan; the latter rod-like deposits are most characteristic in the proximal convolutions of the kidney as described later. The intracellular orientation of the deposits in paraffin sections is clear and well defined. The relative activity of the dehydrogenase systems in some tissues of rat are summarized in Table 1.

1) *The Liver*

The hepatic cells showed the marked activities of the various oxidative enzymes tested except isocitric and G-6-P dehydrogenase systems. The reactions showed the almost equal distribution through the hepatic cords, and sometimes stronger in the central area. Succinic dehydrogenase, however, showed rather peripheral localization with the marked formazan deposition in the peripheral hepatic cells near by Glisson's sheath. As a characteristic finding, the paraffin sections sometimes showed the biased intracellular localization in the hepatic cells, especially at the external area of the specimens. This is apparently an artificial result caused by the dislocation of the enzyme through the acetone fixation.

Isocitric acid dehydrogenase system exceptionally showed no activity in the liver where it was sufficiently detected by cryostat procedure (with acetone fixation), although moderately in other tissues of paraffin sections. It may be due to the heat sensitivity of the hepatic enzyme. On the contrary β -hydroxybutyric acid dehydrogenase system is markedly reactive in spite of its weakness in the kidney and other organs.

2) *The Kidney*

Generally, most of the dehydrogenase procedures revealed the marked formazan deposition in the kidney. Especially prominent were the reactions of DPN-D, TPN-D and succinic dehydrogenase. In the proximal and distal convolutions and ascending limb of the Henle, the intracellular localization was most distinctive and characteristic. In these cells, the blue rod-shaped formazan deposits caused a closely packed perpendicular striation of their basal part, reaching the level of the nucleus, where parallel rod-like mitochondria might occupy. Moreover, dot-shaped deposits were sometimes seen in the inner half of the cells but not so prominent. The brush border covering the free surface

of the cells were always completely negative. Other tubuli and vascular wall were also reactive in various grades.

In the glomeruli, the moderate formazan deposition as DPN- and TPN-diaphorase activities localized in the "visceral epithelium" covering the surface of the glomerulus adhere closely in a continuous layer to the capillary loop. The "parietal epithelial cells" were also weakly positive. Succinic dehydrogenase, on the other hand, was completely negative in the glomeruli.

Table 1

	DPNH	lactate	malate	isocitric acid	β -hydroxy-butyric acid	TPNH	G-6-P	succinate.
liver	###	##	+	—	##	##	—	##
kidney								
glomerular epithelium	+	+	—~±	—	—	+	—	—
capsular epithelium	+~±	+~±	?	—	—	+	—	—
proximal convolution	##	##	##	+	+~±	##	—	##
descending convolution (thin)	±~+	±	±	±~—	—	+	—	+
ascending convolution (thick)	##	##	##	##	##	##	—	##
distal convolution	##	##	##	+	±~—	+	—	##
collecting tubule	+	±	±	+	—	—	—	—
papillar epithelium	+	+	+	—	—	—	—	—
heart	##	+	##	+	—	+	—	##
skeletal muscle	##	##~##	##~##	##~##	—	+	—	—
stomach								
superficial epithelium	±	+	+~±	—	—	±	—	±
parietal cell (neck)	##	##	##	##	+~##	##	—	##
parietal cell (deep)	##	##	+	+	—	##	—	##
chief cell	+~±	+	±	—	—	+	—	+~±
muscle	+	+~±	±	—	—	+	—	±
adrenal cortex	##	##	##	—	—	##	##	+
intestine	##	+	+	±	±	+	—	±

The other enzyme systems tested have revealed the various activities as shown in Table 1. G-6-P-dehydrogenase system has completely lost its activity through the paraffin embedding.

3) Other Organs

The heart muscle showed the marked activities in sarcosomal localization with fine dot-like deposits of formazan perpendicular to myofibril-direction and paralleled with striation. The perinuclear concentration of the staining was also characteristic.

The skeletal muscle can be divided in two types; the relative smaller muscle bundle in cross section showed the marked activities, especially in its peripheral one third. The other larger bundle was completely negative. In the longitudinal sections, the blue formazan deposits accumulated around the nuclei, especially at their ends and extended linearly along the sarcoplasm. These localizations in paraffin sections were precise and distinctive.

The stomach was also one of the organs that showed the characteristic distribution of the oxidative enzymes. In its mucosa, the parietal cells rich in mitochondria revealed the marked activities, especially at the neck part. The fine granular precipitation was not observed along the intracellular canaliculi. In some cases, the chief cells and superficial epithelium also stained in various grades. The smooth muscle, vessels, autonomic plexus and squamous epithelium (Malphigian layer) also showed the formazan deposition, but the Malphigian layer gave the similar reaction even by the control mixture without any substrate suggesting the nonenzymatic tetrazolium reduction by a sulfhydryl group. It should be excluded from the positive results, although true enzymatic reactions might take place in some parts as the formazan formation at the layer.

The intestinal mucosa was also moderately or weakly positive without the formazan deposition in the goblet cells. The columnar cells gave the precipitation in the area between the striated border and the nuclei.

The reactions of the adrenal cortex also showed the characteristic staining patterns. Generally, the tetrazolium reactions of fresh adrenal sections had an inevitable disadvantage to lose the precise enzyme localization owing to the high lipid content. The use of Nitro-BT also can not be out of this weak point. In fact, it is not so rare during the incubating procedure to give rise to the blue clouding into the reaction mixture from the immersed fresh adrenal tissues in spite of mitochondrial protection by 7.5% polyvinyl pyrrolidone (PVP). While, the present procedures using acetone fixed paraffin sections from which lipids were sufficiently removed could avoid such a disadvantage and may give the distinct distribution patterns with various substrates. And, moreover, the preservation of marked activity of G-6-PD system in the adrenal cortex would be emphasized, in spite of almost disappearance of the reaction in other organs such as the liver and kidney. In the adrenal cortex, zona reticularis was somewhat stronger, and zona glomerulosa weaker or negative for the dehydrogenase reactions tested.

Then, it should be noticed that the sections stored in a refrigerator for 4 months sufficiently maintained their activities such as diaphorase, succinic dehydrogenase and others.

Discussion

The present results have shown that the activities of some oxidative enzymes could be maintained and revealed in paraffin sections through the proper embedding and adequate incubating procedures. Before the present studies, there might be no successful report about the histochemical detection of oxidative enzymes in paraffin section.

It seems most likely that the success of the present experiments might be due to two factors; the first is the use of Nitro-BT, of which formazan is insoluble to organic solvents, as the hydrogen acceptor, and the second is the floating of sections on the incubation mixture without deparaffination. As to the Nitro-BT, there will be no need to discuss here about its superiority to the early tetrazolium salts such as TTC and NT. The Nitro-BT has extensively employed for the morphologic histochemical studies of oxidative enzymes because of its excellent properties on high sensitivity and accurate intracellular localization. (Tsou, et al.⁹⁾) At the present studies, the insolubility of Nitro-BT formazan in organic solvents has made it possible to mount the sections in Canada balsam (after staining) through the deparaffination by xylene. The feeble violet-coloration of balsam by diffusion of incomplete formazan out of the sections did not disturb the observation.

Then, the inability to reveal the oxidative enzymes in paraffin sections might have been partly due to the deparaffination procedure by which the enzymes would have been carried away. In fact, the present brief test showed that the enzymes which maintained their ability to reduce tetrazolium salts through acetone fixation and paraffin embedding have almost completely lost it by deparaffination.

And then, although the fresh frozen or fixed frozen sections contain the high enzyme activities, it may be inevitable that the enzymes diffuse out into the incubating mixture in various grades, especially "soluble enzymes" such as lactic dehydrogenase to produce the reduced pyridine nucleotides which act as substrates for the diaphorases. It should also be taken into consideration that the actual reduction of tetrazolium salts in incubation mixture is not brought about by their interaction with the pyridine nucleotide linked dehydrogenases, but by the later process of reduced pyridine nucleotide oxidation. Therefore, there is no difficulty to expect the mitochondrial staining, irrespective of the true localization of the dehydrogenases. The strict intracellular localization of "soluble dehydrogenases" may be out of discussion by the light microscopy under the present methods and instruments, even if mitochondrial protection is attempted by addition of PVP (Scarpelli & Pearse¹⁰⁾) and others. From the stand point of the prevention of enzyme diffusion, the present procedure

will be most reasonable, although there would be no precise evidence that the enzymes did not fuse out of their intracellular sites during the procedure. The authors prefer to conclude that pyridine nucleotide diaphorase (reduced pyridine nucleotide-tetrazolium reductases) and insoluble succinic dehydrogenase mainly localize in or on the mitochondria and other DPN- or TPN-linked dehydrogenases probably take the similar localization but not certain. The complete protection of the mitochondria such as "cyclophorase preparation" (Green et al.¹¹) may be useful in biochemical studies. In the histochemical procedure, however, by the use of tetrazolium reduction, the complete mitochondrial protective process would cause the nonspecific formazan formation through the so-called "endogenous activity", of which separation from any specific oxidative enzyme reaction might be almost impossible under that condition. And here, it should be reminded again that the tetrazolium reduction could not occur as a hydrogen acceptor of the specific dehydrogenase action but did in later pathway.

The present results have shown the sufficient preservation of DPN- and TPN-diaphorases and succinic dehydrogenase activities in all tissues tested. And other pyridine nucleotide linked dehydrogenases changed the degree of tetrazolium reduction and generally diminished their activities comparing those of fresh or fixed frozen sections. Especially, glucose-6-phosphate dehydrogenase system has almost completely lost its activity except in the adrenal cortex. The present procedure will be sufficiently recommended for the tetrazolium reaction of the adrenal cortex to avoid the diffusion of diformazan to lipid-aqueous interfaces.

Then, following the routine substrate mixture, succinic dehydrogenase system in paraffin sections reacted feebly or not at all to the tetrazolium salts, and phenazine methosulfate was recommended as an intermediary of the electron pathway (Farber¹²). Pearson and his coworkers⁴ have reported that lipids of cryostat sections could be removed by pretreatment of the sections in a 1:1 n-butanol and ether mixture at -65°C and this has not destroyed the enzyme. The fact should be noticed that the addition of phenazine methosulfate have made it possible to demonstrate succinic dehydrogenase activity in the defatted tissues. More recently, Wattenberg and Leong¹³ have shown that Coenzyme Q_{10} and menadione enhanced succinic dehydrogenase activity and they also indicated that the two quinons did not simply act as nonspecific intermediate electron carriers between dehydrogenase system and tetrazolium salt, but that they enhanced enzyme activity by a more specific interaction with some component of the enzyme system. The authors have also investigated the effects of naphthoquinons and other diketones as the intermediary to succinic dehydrogenase

activity in paraffin sections¹⁴⁾. The present study has used only phenazine methosulfate. Pearson and his coworkers¹²⁾ have used the reagent with 0.016 mg/ml in final concentration. But such a concentration, as the authors indicated, may cause the nonenzymatic coloration of the control sections without any substrate. It is preferable to use more diluted reagent (about 0.01-0.005 mg/ml). Thus, the present study is characterized as follows.

1. Some oxidative enzymes can be demonstrated in paraffin sections by means of floating them on the incubating mixture which contains Nitro-BT as a hydrogen acceptor.
2. The procedures on DPN- and TPN-diaphorases and succinic dehydrogenase system give the excellent results for the precise enzyme localization. Other enzyme systems can be also demonstrated but are not so sensitive.
3. The present procedure can practically avoid of diffusion of the formazan by lipid contents and is most reasonable to avoid the transition of enzyme localization.

Summary

The usefulness of paraffin sections was shown for the histochemical detection of some oxidative enzymes. The acetone fixed paraffin sections were floated on the incubating mixture containing Nitro-BT, substrates and others without sticking on the slide or cover glass, or stored in a refrigerator until use. DPN- and TPN-diaphorase (DPND, TPND), DPN- and TPN-linked dehydrogenase systems and succinic dehydrogenase system were examined. The diaphorases and succinic dehydrogenase have shown their marked activities and other dehydrogenases in various grades but not so prominent. The present procedures will give the sufficient results for the detection of oxidative enzymes in the lipid-rich tissues such as the adrenal cortex.

It seems most likely that the success of the present studies might be due to the use of Nitro-BT for the hydrogen acceptor and to floating the paraffin sections directly on the incubating mixtures.

Thus, the present procedures made it possible to stock the tissues maintaining the oxidative enzyme activities and to avoid or minimize the diffusion of the enzymes and produced formazan deposits.

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Explanation of the plates

All plates showed the staining of paraffin sections of rat.

- Fig. 1. Kidney. Substrate; DPNH. The marked staining of convoluted tubuli and weak or moderate staining of glomeruli are noticed. ×100
- Fig. 2. Stomach. Substrate; DPNH. The parietal cells are markedly positive and other cells also stained. ×100
- Fig. 3. Adrenal cortex. Substrate; DHNH. Zona glomerulosa was almost negative. ×100
- Fig. 4. Kidney. Substrate; DPNH. The rod-like perpendicular precipitations of formazan are suggestive of mitochondrial localization (arrow). ×900
- Fig. 5. Heart muscle. Substrate; Sod. lactate. The formazan deposition coincides with the striation. ×900
- Fig. 6. Skeletal muscle. Substrate; Sod. lactate. The perinuclear concentration is noticed. ×200
- Fig. 7. Kidney. Substrate; Sod. succinate. Glomeruli are completely negative. Compare with Fig. 1. ×100



Fig. 1

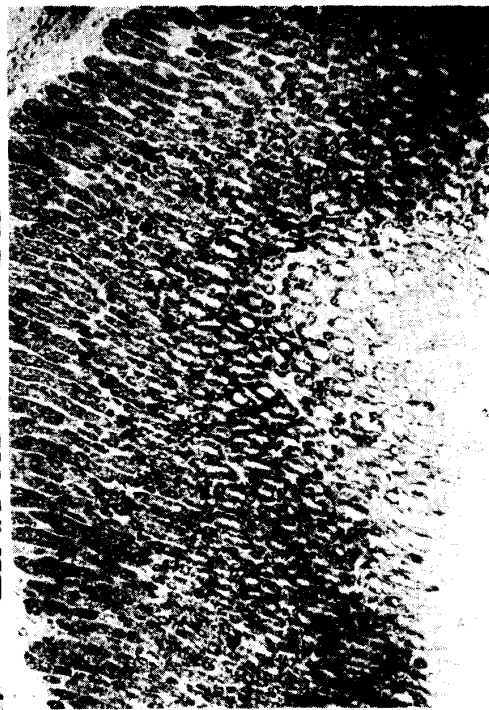


Fig. 2



Fig. 3



Fig. 4

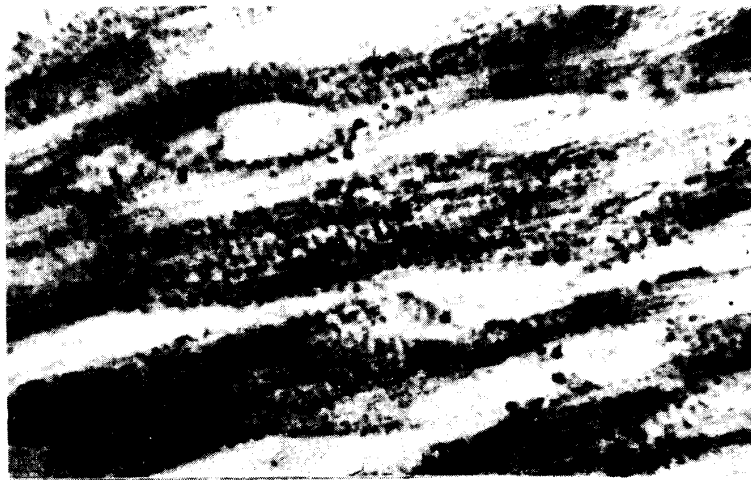


Fig. 5



Fig. 6

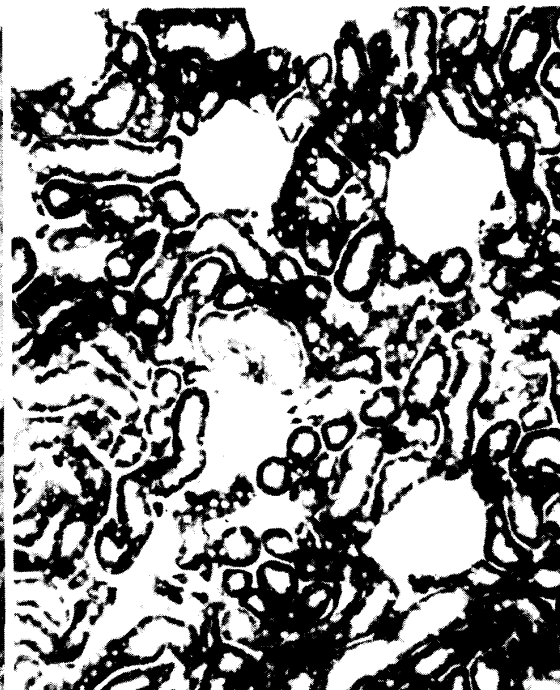


Fig. 7