

FORMALIN FIXATION IN THE CYTOCHEMICAL DEMONSTRATION OF SUCCINIC DEHYDROGENASE OF MITOCHONDRIA

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ABSTRACT

A variety of established methods for protecting mitochondria were tested on rat duodenal epithelium during the histochemical assay for succinic dehydrogenase. The use of sucrose at isotonic or hypertonic concentrations, 7.5 per cent polyvinylpyrrolidone, divalent cations, physiological salt solutions, phenazine methosulfate, coenzyme Q_{10} , and menadione failed to improve the quality of the histochemical preparation once fresh frozen sections were prepared. However, preservation of mitochondrial integrity with little diminution in succinic dehydrogenase activity was obtained by fixing tissue slices (less than 1 mm. in thickness) in 8 per cent unneutralized, aqueous formaldehyde from 8 to 16 minutes at from 5° to 10°C. prior to freezing. To offset the inhibition of enzymatic activity it was necessary to extend the incubation period by 10 to 15 minutes. Two-micron-thick sections were easily obtained from the frozen blocks of such fixed tissue and incubated in the unmodified Nitro-BT-succinate medium. Once the optimum conditions for fixation of intestinal epithelium were determined, many other tissues were subjected to the same procedure. From the morphological standpoint the appearance of the mitochondria in these histochemical preparations compares favorably with the results obtained using the classical Regaud iron-hematoxylin staining procedure. With most tissues, the results are superior to those with fresh frozen sections. However, results with muscle, sperm, and kidney tubular epithelium are not as strikingly improved as with gut and liver.

INTRODUCTION

Application of the highly sensitive methods developed for cytochemical study of certain oxidative enzymes has been limited by the difficulty in maintaining the morphological integrity of the organelles in frozen-sectioned material. Many procedures thought to "protect" the mitochondria have been proposed. Especially enthusiastic are those who have urged the application in cytochemical studies of those procedures used successfully in cell fractionation chemistry. The use of sucrose as a suspension medium introduced by Hogeboom, Schneider, and Palade (5) in

1948 is still used routinely in the preparation of cell fractions. According to certain investigators who have examined their preparations under the electron microscope, sucrose at hypertonic (0.44–1.2 M) concentrations more effectively preserves the fine structure of the mitochondria than at isotonic (0.25 M) concentration (21, 25). The swelling and shrinking reactions of mitochondria in suspension has been most thoroughly studied by Lehninger (9), who concludes, "mitochondrial swelling initiated by the agents *L*-thyroxine, Ca^{++} , inorganic phosphate, arsenate, hypotonic conditions, *p*-chloromercuribenzoate,

phlorizin, U factor, and that occurring spontaneously may in each case be reversed by the addition of adenosine triphosphate (ATP). and that the reversal of various types of mitochondrial swelling by ATP + Mg⁺⁺ is inhibited by (0.3 M) sucrose." According to Lehninger (9) the mitochondrial swelling as elicited under certain experimental conditions is reversible through a common ATP-driven enzyme mechanism. Scarpelli and Pearse (20) report that not only ATP, but ADP (adenosine diphosphate) and even AMP (adenosine monophosphate) may be used to inhibit mitochondrial swelling for a limited period (10 to 15 minutes) during the histochemical reaction for succinic dehydrogenase. A rationale to explain how the energy-poor adenine nucleotides might inhibit mitochondrial swelling was not offered.

However much is known about the behavior of mitochondria in cell fractions and how well defined may be the conditions for maintaining the ultrastructural integrity of the mitochondria in such suspensions, this information cannot be used to prevent the damage sustained by tissue subjected to the freeze-thaw treatment. The only methods for circumventing this destructive influence has been to carry out the enzymatic procedure for succinic dehydrogenase on minute blocks of fresh tissue in the presence of protecting agents such as sucrose, and then fixing the blocks with formalin prior to producing frozen sections as was used by Goddard and Seligman (3), or fixing the stained blocks with osmium tetroxide followed by preparation of the tissue for electron microscopy as used by Karmarkar, Barnett, Nachlas, and Seligman (8).

Having more important bearing on the problem confronting the histochemist is the finding that many, if not most enzymes, as demonstrated in histologic sections, will tolerate a limited exposure to certain fixatives (16, 22). Up to the present time, the employment of fixatives prior to the quenching step in cytochemical assays for oxidative enzymes has *not* been satisfactorily explored. Novikoff and Masek (15) have demonstrated lactic dehydrogenase and diphosphopyridine nucleotide diaphorase (DPND) activities in tissues that had been exposed to cold calcium-formol either prior to or after the preparation of frozen sections. These authors claimed to have thus secured sections "histologically and cytologically superior to any published figures." This statement is not supported by their illustrations, which include two

photomicrographs, one showing kidney cortex at $\times 125$ magnification, the other, heart muscle which though highly magnified ($\times 1750$), demonstrates discretely not mitochondria but myofibrillar (presumably anisotropic) bands (2). But curiously enough, reference is made to "intramitochondrial" staining. Others also have used the latter term, referring to a row of two to five formazan particles thought to represent the sites of DPND activity within a single mitochondrion (17, 19). Estimates indicate there may be several thousand individual equidistantly spaced sites of DPND activity in a mitochondrion of average size (rat liver) (10). The presence of cristae positively identifies the mitochondrion and should be demonstrable before one may convincingly identify histochemical reaction products at sites of enzyme activity within the organelle. Therefore, we would like to suggest that the expression "intramitochondrial localization" be reserved for the cytochemist who has at his disposal the higher resolving power of the electron microscope.

The purpose of the present investigation was to find a procedure for treating tissues which would enable them to tolerate the freeze-thaw sequence with minimal losses of morphological integrity as well as oxidative enzymatic activity. All of the so called protective media were re-evaluated but effort was focused particularly on the usefulness of formalin fixation prior to freezing. Since Novikoff and Masek (15) have claimed that calcium-formol fixation effected complete loss of succinic dehydrogenase activity, we selected this enzyme as a basis for exploration and evaluation rather than one of the more formalin-resistant enzymes such as DPND.

MATERIALS AND METHODS

1. *Histological Material.* The duodenal epithelium was selected as the assay material since these cells have a high concentration of filamentous mitochondria and are not supported by virtue of being located in compartments separated by membranous septa or by fibrillar structures. Kidney and striated muscle were also used, but it was noted that the cells of the convoluted tubular epithelium and muscle tolerate more abuse before signs of mitochondrial damage are obvious than do cells of the intestinal lining. Once the optimum conditions for fixation of intestinal epithelium were determined, the following organs were tested accordingly: kidney, striated

muscle, liver, stomach, submaxillary gland, pancreas, developing bone, and testis.

2. *Incubation Conditions:* Except when otherwise stated, the reaction mixture was that originally described by Nachlas *et al.* (11). The Nitro-BT (23) was obtained from the Dajac Laboratories, Division of the Borden Company, Philadelphia, Pennsylvania. The product now marketed by Nutritional Biochemicals Corporation contains considerable quantities of a monotetrazolium contaminant.

The incubation time used, in general, was about one and a half times longer (30 minutes, usually) than that originally recommended.

The incubation medium was modified in order to test the ability of certain reagents to protect mitochondria from morphological damage while the histochemical reaction is taking place. These protective reagents are listed below in the final concentration at which each was used:

- (a) Polyvinylpyrrolidone, (PVP) 7.5 per cent.
- (b) sucrose, 0.25 M, 0.44 M, 0.88 M, respectively.
- (c) PVP, 7.5 per cent in 0.25 M sucrose.
- (d) 0.1 M ATP and 0.1 M MgCl₂.
- (e) 0.1 M acetate buffer, pH 6.7 in place of the phosphate buffer.

Phenazine methosulfate (PMS), menadione, and coenzyme Q₁₀¹ were also tested since these reagents have been found to promote the rate of reaction between succinic dehydrogenase and Nitro-BT (12, 13, 24). At a concentration of 1 mg. per ml., stock solutions were prepared: PMS in water, menadione in absolute ethanol, and coenzyme Q₁₀ in absolute acetone. One part of the PMS and menadione stock solutions were added, respectively, to nine parts of the incubation solution just before the sections were introduced. These reaction mixtures were not used more than once. One drop of the coenzyme Q₁₀ stock was dried on the surface of a clean coverslip and subsequently sections were mounted according to the procedure of Wattenberg and Leong (24).

3. *Treatment Prior to Freezing:* Blocks of tissues and organs 1 millimeter or less in thickness were quickly excised from the ether-anesthetized animal and transferred to the fixative.² Formaldehyde over a wide

¹ Coenzyme Q₁₀ was obtained from Merck, Sharp and Dohme through the kindness of Dr. Karl Folkers.

² The use of intra-arterial formalin injection for fixing brain and retina was tested. Rats were ether-anesthetized and the thorax was opened. The right ventricle was opened and exanguination allowed to proceed. Saline was injected into the left ventricle, until clear fluid appeared in the venous drainage. Formalin (20 per cent) at 15°C. was injected gently and perfusion was continued with syringe and needle for not more than 5 minutes. The tissues were removed and directly thereafter frozen at -70°C. and imme-

range of concentrations, at varying pH values and in combination with many different cations as well as alone or with polyvinylpyrrolidone (7.5 per cent) and sucrose (0.25 M, 0.44 M, and 0.88 M) was tested. The influence of temperature and duration of exposure to the fixation solutions were also assessed.

4. *Quenching and Sectioning:* The tissue blocks were quenched in isopentane at -70°C. for 1 or 2 minutes and thereafter transferred to the cryostat, the temperature within which was maintained at -25°C. Within 4 hours after their removal from the animal, the blocks were sectioned at a thickness of 2 microns. The sections were not permitted to thaw in the cryostat, but were immediately placed on 11 × 22 mm. thin coverslips, and removed from the cryostat to the incubation solution.

RESULTS

No treatment introduced after the quenching step was found to reverse the morphological damage sustained by the tissue at the time of freezing. The only consistently effective treatment introduced prior to the quenching step was the 8 to 16 minute exposure to unneutralized (pH, 4.0) 8 per cent formaldehyde (20 per cent formalin) at 5 to 10°C. Adjustment of the pH of the formalin solution to a value of 2.0, 5.5, 7.0, and 9.0, respectively, with or without a buffer did not significantly alter the morphological result. At a pH of 2.0 inactivation of succinic dehydrogenase was somewhat hastened. The concentration of formalin within a range of from 5 to 50 per cent did not appear to be of critical importance as long as the temperature was maintained at 5-10°C. and the fixation time was between 8 to 16 minutes. As expected, the fixation time was optimum only within narrow limits. The latter point was graphically demonstrated in sections cut from thick blocks of tissue (more than 1 millimeter in all three dimensions) and fixed for more than 20 minutes. When such sections were examined at the end of the incubation period it was evident that the fixative had failed to reach the core of the block and at the same time had inhibited en-

diately cut in the cryostat at 2 μ. The results were similar to those with immersion fixation. The perfusion technique had the advantage of providing the entire central nervous and sense organs for study, and the disadvantage of limitation to the experimental animal and that of increasing the risk of enzymatic activity loss since the fixative will continue to act after perfusion until sections are introduced into incubation solution.

zymatic activity at the margins of the block. The portion of the block in between these two extremes appeared to be satisfactory both from a morphological and enzymatic standpoint. Thus, if a uniformly good histochemical result is to be obtained, the blocks must be trimmed so that at least one dimension is not more than 1 mm. thick at the time of fixation.

Addition to the formalin solution of 1 per cent CaCl_2 or an equimolar amount of MgCl_2 , $\text{Co}(\text{NO}_3)_2$, MnCl_2 , respectively, had little effect as long as temperature and fixation time were not altered. Because the use of calcium with formalin introduced by Baker (1) has become very popular, the possible merit as a fixative of calcium-formol over formalin alone was repeatedly tested on a variety of tissues. However, there was no evidence that indicated any advantage was gained by the addition to the formalin of 1 per cent calcium chloride.

The following points deserve to be stressed since they were found to influence profoundly the

quality of the histochemical preparation finally obtained:

- (a) Rapidity and uniformity of fixation could only be obtained by exposing thin (1 mm. or less) shavings of the tissues to the formalin solution at the temperature and concentration and for the length of time stated above.
- (b) Resolution of sites of enzymatic activity is determined by the thinness of the section. If a microtome knife edge is properly maintained, no difficulty should be encountered in obtaining 2-micron-thick frozen sections of any tissue, including undecalcified bone of newborn rats.
- (c) Storage for longer than a few hours in the cryostat should be avoided since growth of ice crystals and desiccation promotes both morphological deterioration and enzymatic inactivation. It is most important to avoid freezing and thawing the tissue more than once throughout the entire procedure. Additional freeze-thawing will cause morphological disruption.

Legends for Figures

The mitochondria of normal adult rat tissues as revealed after formalin fixation by the succinic dehydrogenase-Nitro-BT and Regaud's hematoxylin methods. The histochemical preparations were made by 30 minutes' incubation in the succinate medium and were mounted in glycerin jelly; the Regaud preparations, in permount.

FIGURE 1

Duodenal villus in longitudinal section. The sampling of surface epithelium is large enough to give one an impression of the degree of uniformity of morphological and enzymatic preservation. $\times 600$.

FIGURE 2

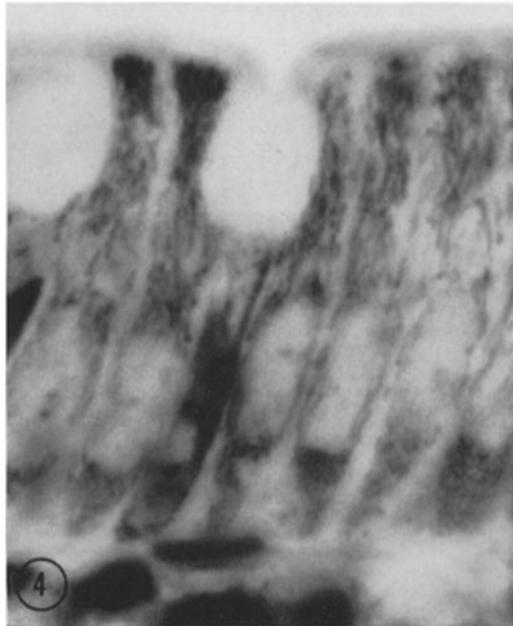
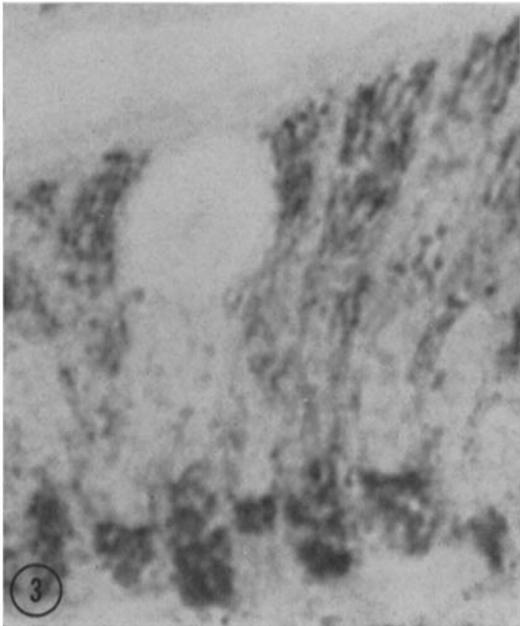
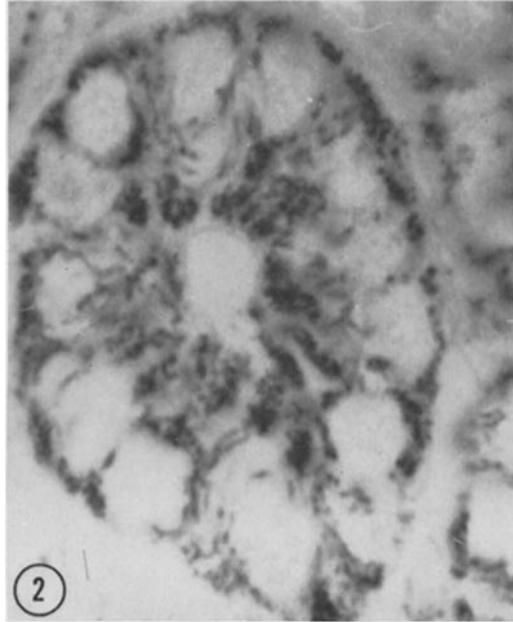
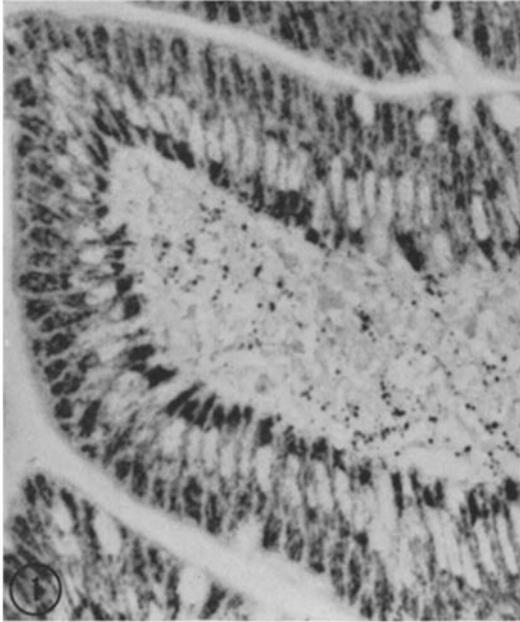
Duodenal crypt in cross-section. Most of the mitochondria are rod-shaped and concentrated in the supranuclear portion of these cuneiform cells. $\times 600$.

FIGURE 3

Surface epithelium of small portion of duodenal villus in longitudinal section. Filamentous and rod-shaped mitochondria may be discerned on the apical side of the nucleus and aggregations of round mitochondria are present in the basal region of the absorptive cells. Although unstained, the striated border, one goblet of mucous, and a row of the epithelial cell nuclei are seen in outline. $\times 1500$.

FIGURE 4

Surface epithelium of a small portion of a duodenal villus. Compare the mitochondria of the absorptive cells as shown here with those of Fig. 3. Regaud's hematoxylin. $\times 2000$.



(d) Tissue incubated for longer than 30 minutes is apt to show overstaining artifacts.

Phenazine methosulfate (PMS) was found to accelerate the rate of the histochemical reaction in *fresh* frozen sections by a factor of from two to five, without improving the quality of cytologic detail. However, when sections from formalin-fixed blocks were tested, no succinic dehydrogenase activity could be detected in the presence of PMS, even though adjacent sections incubated without PMS stained properly. We must conclude that since PMS, itself, inhibits succinic dehydrogenase activity in sections that have been exposed to formalin, this reagent cannot be used to recover any activity lost during the exposure to formalin. Menadione and coenzyme Q₁₀ accelerated the reaction in fresh frozen sections by a factor of about 1.5 to 2. Coenzyme Q₁₀ had no effect on the reaction of formalin-fixed material. Menadione promoted the rate of the reaction slightly even after formalin fixation. Neither coenzyme Q₁₀ nor menadione were able to counteract the inhibitory effect of PMS on the succinic dehydrogenase activity of formalin-fixed tissues.

In order to illustrate the superior results obtained by exposing tissues to the formalin solution prior to freezing we have selected an assortment of organs. Since it is generally accepted that succinic dehydrogenase forms an integral part of the mitochondrial membrane, we have pre-

sented as a morphological basis for comparison, specimens stained for mitochondria by the classical method of Regaud (Figs. 4, 7, and 15). The following descriptive notes are made in reference to the histochemical reactions for succinic dehydrogenase in the formalin-fixed tissues:

Intestinal epithelial (absorptive) cells as seen covering the villi possess a high concentration of mitochondria which in the supranuclear portion of the cytoplasm are filamentous or rod-shaped and oriented lengthwise and in the infranuclear portion of the cytoplasm are spherical and even more compactly arranged (Figs. 1 to 3).

Kidney tubular epithelium possesses an exceptionally high concentration of short rod-shaped mitochondria, arranged in rows, oriented perpendicular to the cell bases and, for the most part, confined to the cytoplasm on the basal half of the cell (Figs. 5 and 6). Although the striated border is readily appreciated in thicker sections, mitochondria are not likely to be individually discernible in preparations more than 3 microns in thickness. Although the mitochondrial preservation may be satisfactory in occasional tubular elements of fresh kidney sections, a uniformly good result may be obtained throughout the section following formalin fixation.

Submaxillary gland's excretory duct (Figs. 8 to 10) is lined by an epithelium in which the mitochondria are arranged to form basal striations which closely resemble those seen in the kidney

FIGURE 5

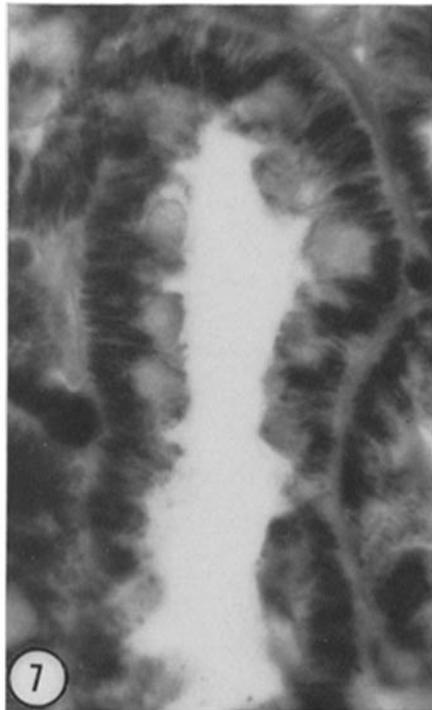
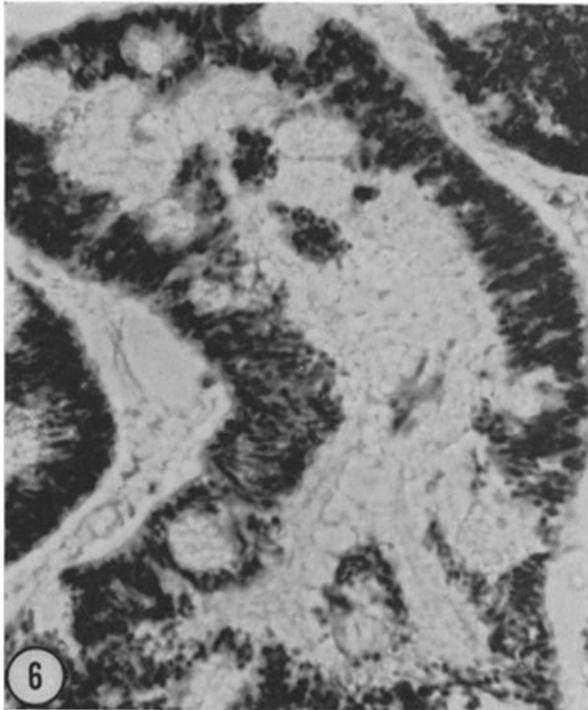
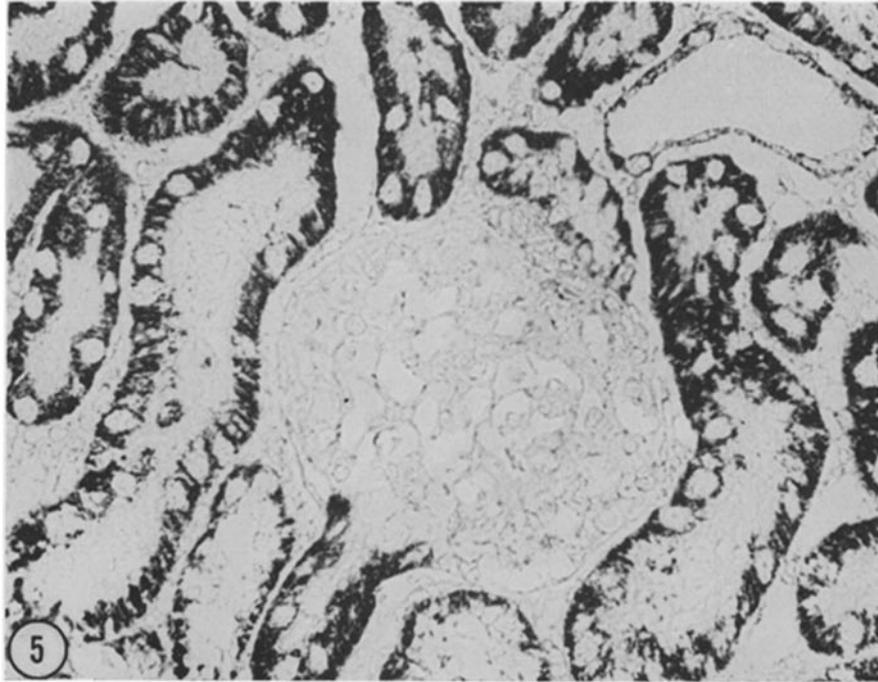
Kidney cortex. At opposite poles of the unstained renal corpuscle the ostium of a proximal convoluted tubule and the macula densa of a distal convoluted tubule may be seen. The dark basal striations representing the mitochondria are seen in all of the tubules of this field except the thin-walled, wide lumined collecting duct located in the corner nearest the macula densa. $\times 500$.

FIGURE 6

A proximal convoluted tubule. Depending upon the plane in which they are sectioned, mitochondria appear dot-like in form (transversely cut) as in the cytoplasm adjacent to the lower margin and in the right upper corner of the figure, or as rows of short rods as seen in most cells elsewhere in the illustration. $\times 1500$.

FIGURE 7

A distal convoluted tubule. The mitochondria of the convoluted tubules have essentially the same appearance whether stained for succinic dehydrogenase as in Fig. 6 or by Regaud's hematoxylin method as shown here. $\times 1500$.



tubular epithelium (Figs. 5 and 6). The secretory (acinar) cells have fewer lightly stained, randomly distributed, rod-shaped, and filamentous mitochondria (Fig. 11).

The *pancreatic acinar* cells present a mitochondrial distribution pattern resembling that of the salivary acinar cells. The epithelial cells lining the excretory ducts however, unlike those seen in the salivary glands possess only a few small mitochondria (Fig. 12).

The acinar (chief or zymogenic) cells of the *fundic gland of stomach* resemble the previously mentioned salivary and pancreatic acinar cells. The cytoplasm of parietal cells is densely packed with rather bulky ovoid mitochondria, which are uniform in size (Figs. 13 and 14).

In the *hepatic cells* the mitochondria, though more nearly spherical in shape, resemble those of the parietal cells in appearance (Fig. 16). Compare the excellent preservation of the mitochondria of hepatic cells shown here with Fig. 9 in reference 11, using unfixated liver.

In *testis*, as has been reported (11), an intense reaction for succinic dehydrogenase was demonstrable in the neck piece of spermatozoa but not in other elements of the seminiferous epithelium. No improvement in degree of localization was obtained by fixing with formalin prior to sectioning.

The *osteoclast*, unlike any other cellular elements of the connective tissues, shows very intense succinic dehydrogenase activity. The prominent round or ovoid forms, though less uniform in size, resemble the mitochondria seen in the parietal and hepatic cells (Fig. 17).

Striated muscle cells are singular in that the succinic dehydrogenase activity is localized not only in mitochondria (interfibrillar and perinuclear) but in transversely disposed lines or bands, presumably represented by the Z lines or telophragma and A bands (2). No striking improvement was noted in skeletal and cardiac muscle by fixation with formalin over the good results obtained with fresh frozen sections.

DISCUSSION

The results of the present investigation indicate that to demonstrate succinic dehydrogenase activity histochemically in any type of tissue the most satisfactory result may be obtained by exposing thin blocks of tissue to an unmodified formalin solution prior to freezing. The findings in the present study are at odds with the following claims that have appeared in recent publications: (a) the succinic dehydrogenase activity (as detected histochemically) will not tolerate a limited exposure to formaldehyde (15), (b) that formalde-

FIGURE 8

Submaxillary gland. The junction of intercalated and salivary ducts is seen in longitudinal section. The taller epithelial cells that line the salivary ducts possess mitochondria which in size, shape, concentration, and disposition closely resemble those of the convoluted tubules of the kidney. The mitochondria of the intercalated ducts and of the terminal portions of the gland (seen above and below the duct junction) are decidedly less intensely stained than those of the salivary duct. $\times 1500$.

FIGURE 9

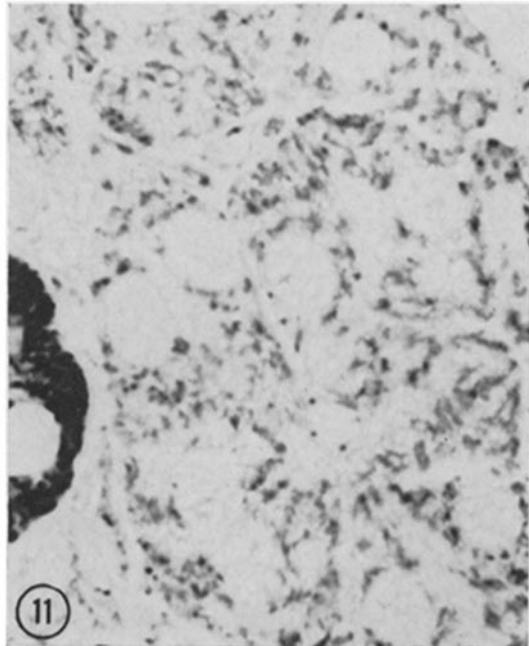
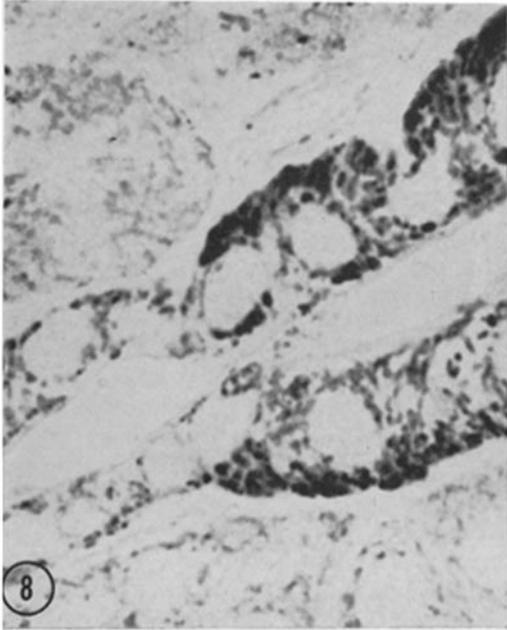
Submaxillary gland. Compare the demonstration of the mitochondria of salivary duct shown here with the proximal convoluted tubule in Fig. 6. $\times 1500$.

FIGURE 10

Submaxillary gland. A salivary duct cut in cross-section. $\times 1500$.

FIGURE 11

Submaxillary gland. In the serous terminal portions as shown here in cross-section, delicate filamentous mitochondria are seen randomly distributed throughout the cytoplasm. $\times 1500$.



hyde fixation is improved by the addition of cations (1, 15), (c) that the use of hypertonic, non-electrolyte solutions are a prerequisite for cytochemical localization of mitochondrial enzymes (6, 7, 14, 18, 20), (d) and that any modification of the incubation conditions can offset or reverse the damage caused by the freezing of unfixed tissue blocks (18).

One may, on the other hand, introduce with impunity many agents that will not alter the beneficial effects of formalin fixation. However, this should not be done under the illusion that such additional agents improve the results. Not all tissues are equally improved by the addition of formalin fixation. It is interesting to note that in several instances where mitochondria are located in compartments separated by membranous, septa, or fibrillar structures, such as in spermatozoa, portions of the nephron and in striated muscle, the improvement due to fixation was less or absent.

It may be worth noting that the mitochondria demonstrated with Nitro-BT, exhibit the localization of only part of the succinoxidase system. Recent work of Nachlas, Margulies, and Seligman

(12, 13) has shown that electrons are passed to tetrazolium salts having a 3-N-*p*-nitro phenyl group from the succinic dehydrogenase-cytochrome *b* complex or from succinic dehydrogenase-phenazine methosulfate.

CONCLUSIONS

The use of formaldehyde prior to freezing is recommended for histochemical studies of succinic dehydrogenase activity. An exposure for 8 to 16 minutes in a chilled (5°–10°C.) unneutralized aqueous solution of formaldehyde (8 per cent) afforded morphological preservation to many different types of tissue without causing significant loss of enzymatic activity that could not readily be compensated for by prolonging the incubation period to 30 minutes. From the morphological standpoint the appearance of the mitochondria in the histochemical preparations compares favorably with that seen in the classical Regaud stain.

The duration of fixation and temperature is of critical importance. The concentration of formaldehyde and the pH at which the latter is used

FIGURE 12

Pancreas. The cells of the pancreatic acinus, three of which are shown in cross-section here, closely resemble the serous cells of the salivary glands as shown in Fig. 11.

FIGURE 13

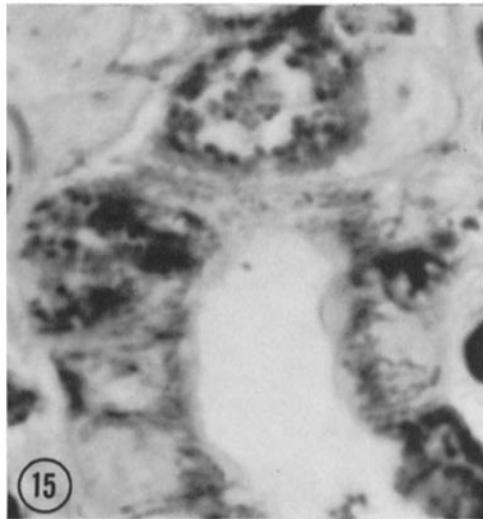
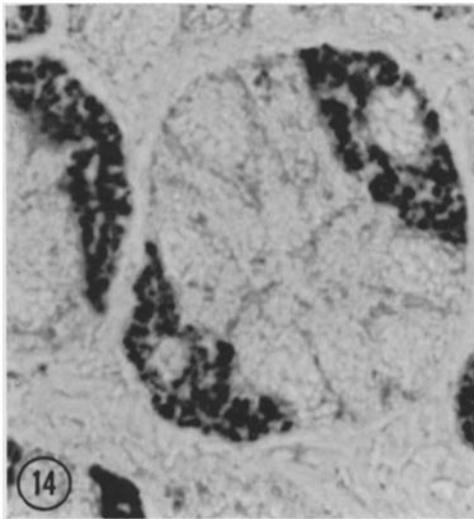
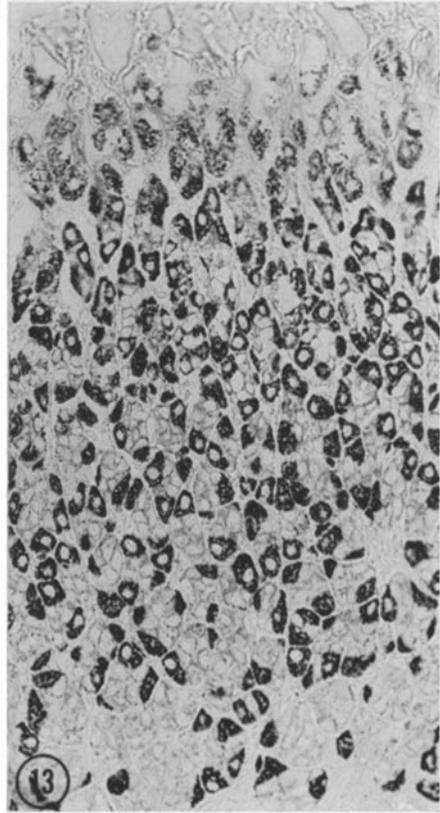
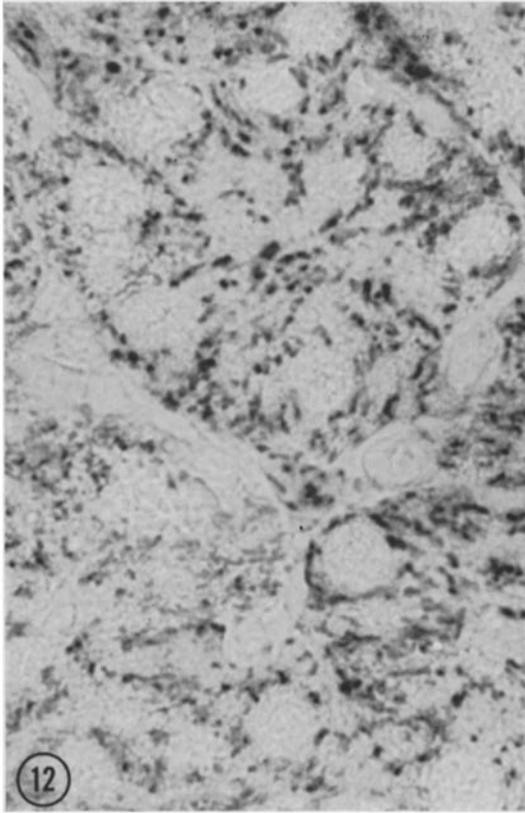
The mucosa of the fundic portion of the stomach. The parietal cells stand out boldly by virtue of their intense histochemical reaction. The chief cells that are most numerous in the basal third of the gastric glands resemble the other two types of zymogenic cells illustrated above (Figs. 11 and 12). The mucous neck cells and surface epithelium are unstained. $\times 250$.

FIGURE 14

A gastric gland cut in cross-section. The darkly stained cells are parietal cells; the other, chief cells. The mitochondria are shown as round or elliptical bodies. $\times 1500$.

FIGURE 15

A gastric gland obliquely sectioned at the level of the neck. The parietal cell centered in the upper portion of the illustration is to be compared with those of Fig. 14. The mitochondria are round or elliptical bodies about 0.5 to 1.0 micron in diameter. Regaud's hematoxylin. $\times 2000$.



need not be rigorously controlled. The use of hypertonic sucrose, polyvinylpyrrolidone, divalent cations, or any of the various physiological salt solutions failed to improve the quality of preparation. Not all tissues were equally improved by fixation.

Phenazine methosulfate was found to accelerate up to fivefold the rate of succinic dehydrogenase activity in fresh frozen sections but completely

inhibited the reaction when tested on formalin-fixed tissues.

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FIGURE 16

Liver. The mitochondria, though larger in diameter than when demonstrated in the dehydrated specimens prepared by Regaud's method, are discrete and unfragmented. To obtain a result as uniform as illustrated here, it is especially important to use very thin blocks of the organ. $\times 1500$.

FIGURE 17

Developing bone. By virtue of its intense reaction for succinic dehydrogenase, the osteoclast stands out as conspicuously in a section of developing bone as the parietal cell in a section of the stomach. In shape, size, and concentration, the mitochondria seen in the osteoclast shown here resembles those of the parietal cells shown in Fig. 14. $\times 1500$.

