THE USE OF FORMALIN FIXATION IN THE CYTOCHEMICAL DEMONSTRATION OF SUCCINIC AND DPN- AND TPN-DEPENDENT DEHYDROGENASES IN MITOCHONDRIA

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ABSTRACT

Brief formalin fixation in the cold prior to histochemical assay of rat liver and pancreas for various dehydrogenases has been used successfully to circumvent the structural damage and enzymatic loss to which mitochondria of frozen sections would otherwise be subject. To obtain an optimal result a single set of conditions has been devised, including fixation prior to freezing of minute (finely diced) organ blocks in graded concentrations (0.7 to 2.0 per cent) of formaldehyde in chilled (1–4°C) Hanks' balanced salt solution, freezing at not higher than −70°C, and use of nitro-BT or, preferably, tetranitro-BT. The present histochemical study of hepatic and acinar cells indicates that not only are succinic and D-β-hydroxybutyric dehydrogenases located exclusively in the mitochondria but so are lactic, malic, and the isocitric dehydrogenases.

INTRODUCTION

Brief exposure of small tissue blocks to chilled 8 per cent formaldehyde prior to freezing and cutting has been shown to permit a crisp localization of succinic dehydrogenase in mitochondria, a result not obtainable by application of many other "protective" procedures (14). Localization at the organelle level of resolution of various DPN- and TPN-dependent dehydrogenases represents a problem of more formidable proportions than that of localizing succinic dehydrogenase activity. Of all the coenzyme-linked dehydrogenases, only D-β-hydroxybutyric, like succinic, is known to be firmly bound (3). The unbound dehydrogenases are highly susceptible to loss by leakage through damaged organelles. On the other hand, if mitochondria remain perfectly intact during the assay procedure they are not penetrated readily by ditetrazolium salts and demonstration of dehydrogenase activity is not likely to be achieved. Bendall and deDuve (1) state, "The activities of the enzyme in intact mitochondria towards added substrates are relatively low and can be increased sometimes tenfold or more, by damage to the particles." Therefore, it becomes necessary to obtain conditions for cytochemistry favoring mitochondrial permeability to permit penetration by substrate, coenzyme, and ditetrazolium salt without at the same time permitting leakage of the dehydrogenases.

Although we lack an understanding of the precise mode of action of formaldehyde on mitochondria and appreciate the "narrow margin of safety" with which formaldehyde may be used in tissues to be assayed for oxidative enzymes, we were prompted to study the influence of controlled formalin preservation on other dehydrogenase and

diaphorase reactions in view of the encouraging results obtained in a previous study on succinic dehydrogenase activity in formalin-treated tissues (14).

As our main objective we hoped to define a single procedure for histochemical demonstration of the three types of dehydrogenases according to which an optimal cytochemical result would be obtained consistently, in assays of mammalian tissues.

A second objective was to test the validity of the claim (10) that tetranitro-BT (TNBT) (13) is superior to nitro-BT in the histochemistry of dehydrogenases. In early tests of TNBT no advantage had been noted over nitro-BT in fresh frozen sections although it was noted that the diformazan could not be removed from tissue sections with organic solvents just as with the diformazan of nitro-BT, but that the brownish-grey color was a less desirable pigment for histological study than the brilliant bluish-purple color of reduced nitro-BT (5). In the course of an attempt to demonstrate two dehydrogenases simultaneously in the same cell with two different colored diformazans, a small sample of Dr. K.-C. Tsou's original TNBT was used and later TNBT obtained from the Nutritional Biochemicals Corporation, Cleveland, was used. When properly preserved blocks of tissue are used the mitochondria become so intensely stained that the resulting diformazan color is black, both in the case of nitro-BT and tetranitro-BT. Therefore it did not prove feasible to attempt to differentiate qualitatively different populations of mitochondria within a given cell type on the basis of diformazan color differences. Quite incidentally, however, it was noted that mitochondria in TNBT-stained liver sections were better defined than mitochondria of nitro-BT-stained preparations.

Thereafter Rosa and Tsou (10) published the claim of superiority of TNBT, which unfortunately they did not demonstrate in their low power photomicrographs. This report stimulated us to settle the matter definitely by taking advantage of a method whereby organelle preservation is maintained even in liver sections. Although the details of this study will be the subject of a later communication, the advantage of TNBT over nitro-BT was established only for liver, a claim which we document in a few photomicrographs of hepatic cells shown at high magnification.

MATERIALS AND METHODS

1. *Histologic Material*

Approximately six dozen adult rat livers and pancreases were used in this study. We reasoned that, if a means of circumventing artifacts in histochemical preparations of the liver was found, most other mammalian organs would also react favorably to the preparation procedure. In view of the reported difference (6, 7) in distribution of the TPNH- and DPNH-diaphorases in the pancreas, this organ was selected for control of specificity. In addition, by enveloping clusters of minute liver blocks in a strip of pancreas, we were able to avoid damages due to desiccation or to disruptive mechanical and osmotic factors.

2. *Treatment Prior to Feezing*

Anesthetics were not used. Rats, stunned by a blow on the head, were decapitated. Thereafter, from the exposed peritoneal cavity, a strip of pancreas and one or two thin (1 to 2 mm in thickness) slices of liver were excised. Liver slices were removed with a new razor blade into a shallow paraffin dish containing chilled 0.7 per cent formaldehyde in Hanks' balanced salt solution (BSS)[1]. The finely diced liver fragments together with the pancreas were transferred at 2 to 4 minute intervals, consecutively, through three chilled (1–4°C) Hanks' balanced salt solutions, containing 0.7, 1.3, and 2.0 per cent formaldehyde, respectively. The fixation fluid was quickly decanted, and the liver fragments were wrapped with the pancreas and finally immersed in a freezing solution. The pancreas served to hold the liver fragments together.

3. *Quenching and Sectioning*

Tissues were frozen in isopentane (2-methyl butane) maintained at -70 to -75°C by a mixture of dry ice and acetone or at about -150 to -165°C with liquid nitrogen as originally devised by Hoerr (2) and recently applied to the ditetrazole methods by Monis (4). After a few minutes' exposure to -70°C or 30 seconds at -160°C the frozen blocks were transferred to the cryostat (-23 ± 1°C), mounted, and cut into sections of 2 μ thickness. A short ribbon of sections was lifted from the knife edge to the 11 $\times$ 22 mm coverslip and the latter promptly transferred from the cryostat to the incubation tube. The precautions in methodology stated

[1] Hanks' balanced salt solution contains the following concentrations of ingredients expressed in grams per liter: NaCl, 8.0; KCl, 0.4; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.14; $Na_2HPO_4.2H_2O$, 0.06; KH_2PO_4, 0.06; glucose, 1.0. These may be purchased from Difco Laboratories, Inc., Detroit.

in a previous paper (14) were observed strictly in this study. The relative ease with which ribbons of 2 μ thickness could be obtained proved to be a useful indicator of the degree to which cytologic preservation had been achieved, since properly preserved blocks ribboned easily. Blocks that do not section well should be discarded without further investment of time.

4. *Selection and Properties of Tetrazolium Salts*

Preliminary experiments were performed with nitro-BT and two samples of TNBT.[2] These tetrazolium salts were reduced by adding a solution of cysteine and sodium carbonate. One sample of highly purified TNBT free of monotetrazole was obtained from Dr. K-C. Tsou. The sample from Nutritional Biochemicals Corporation contained 4 per cent of the red monoformazan which was extracted readily with ethyl acetate. The precipitated diformazans of TNBT, as obtained from the two different sources, looked similar under the microscope. The very fine particles were a gray-blue by transmitted light and the larger particles looked nearly black. The solubility of the diformazans from nitro-BT and TNBT were compared in dimethylformamide. The former was more soluble and gave a purple color. The latter was much less soluble and gave an aquamarine blue color. These observations, therefore, confirm the impression reported by Rosa and Tsou (10, 11) that TNBT yields a diformazan that is less soluble in the lipid of adrenal cortex than that of nitro-BT. Since lipid solubility is apt to lead to crystallization in tissue as noted previously (reference 5, footnote page 421), the lower solubility of the diformazan from TNBT should make this reagent preferable in assays conducted on cell types that contain lipid inclusions. Dr. Tsou's pure sample of TNBT dissolved completely in hot water, whereas the sample from the Nutritional Biochemicals Corporation contained an insoluble residue amounting to 20 per cent. The latter residue on solution in dimethylforamamide gave a red solution, suggesting a monoformazan rather than a diformazan. This mono-reduced product presumably formed on standing and on exposure to light as reported by Rosa and Tsou (11). In spite of the heavy contamination of the TNBT of Nutritional Biochemicals Corporation,[3] the insoluble residue was removed by filtration, and the monotetrazolium salt (6 per cent)[3] contaminating the ditetrazolium salt did not interfere in the histochemical preparations. We noted that a sample of pure monotetrazolium salt, isolated during the preparation of TNBT by Dr. Tsou and sent to us for study, was less readily reduced by alkaline cysteine solution (heat required) than the ditetrazole, TNBT, suggesting that the monotetrazole may not be reduced significantly in the histochemical procedure. This observation was borne out in the histochemical assays conducted on both fresh and formalin-treated specimens of liver.

5. *Incubation Conditions*

Departure from the reaction mixtures as described by Nachlas *et al.* (6, 7) was necessary in order to obtain approximately 0.3 osmolar incubation solutions. The reaction mixtures we used contained buffer, substrate, and cofactors at the following final concentrations:

Buffer, Sorensen's phosphate, pH 7.4, 0.030 M
Substrates, sodium DL-β-hydroxybutyrate and sodium lactate, 0.10 M; sodium malate and sodium succinate, 0.067 M; sodium isocitrate, 0.050 M
Cofactors, DPN and TPN, 0.001 M; $MnCL_2$, 0.0005 M (for TPN-containing media, only)
Ditetrazolium salt, TNBT and nitro-BT, 0.25 mg/ml

Stock solutions of substrates were frozen or kept no longer than 1 week at 5°C; they were added to the reaction mixtures just before each daily use. With but few exceptions, as will be noted, incubations were carried out at 39 $\pm$ 1°C for 20 minutes in the case of liver sections and for 30 minutes in the case of pancreas sections. After incubation, specimens were transferred to a solution of 2 per cent formaldehyde in BSS and, thereafter, mounted in glycerol jelly.

RESULTS

To support our conclusion that a means of circumventing structural and chemical damage to mitochondria of mammalian organs has been

[2] Substrates and cofactors were obtained from California Corporation for Biochemical Research, Inc., Los Angeles; Pabst Chemical Co., Milwaukee, and Sigma Chemical Co., St. Louis, Missouri. Tetranitro-BT (TNBT) was obtained from Dr. K-C. Tsou and Nutritional Biochemicals Corporation, Cleveland. Both nitro-BT and TNBT are available from Dajac Laboratories, Borden Chemical Co., Philadelphia.

[3] Jacob Hanker and Barbara Gorney analyzed 69.4 mg of TNBT obtained from Nutritional Biochemicals Corporation in July, 1962. The following is a summary of that analysis;

Residue	2.70 mg (3.89 per cent)
Monotetrazole	4.26 mg (6.14 per cent)
Ditetrazole	53.25 mg (76.74 per cent)
Reduced TNBT	3.40 mg (4.89 per cent)
Not accounted for	5.69 mg (8.20 per cent)

devised, we will rely upon the photomicrographic demonstration of reactions in the *liver* for succinic (Figs. 1 and 3), D-β-hydroxybutyric (Figs. 9 and 11) and the DPN-linked isocitric (Fig. 8) dehydrogenases and in the *pancreas* for succinic (Figs. 15 to 17), the DPN-linked isocitric (Figs. 12 and 13) D-β-hydroxybutyric (Fig. 10) and malic (Figs. 18 and 19) dehydrogenases. Osmotic damage was minimized by employing gentle fixation prior to quenching. Note that in size and shape the mitochondria, as seen in the illustrations, conform closely to those obtained by the best classical methods. Blocks of liver exposed to 8 per cent formaldehyde prior to freezing yield sections of which only a small proportion are satisfactory (14). In contrast, the result obtained following exposure to graded concentrations of formaldehyde (0.7 to 2.0 per cent in BSS) is uniformly good throughout small blocks of liver. Embedded in the pancreas, the liver was protected against desiccation and would tolerate much longer periods (at least 24 hours) of exposure to the atmosphere of the cyrostat. The pancreas used in this way, *i.e.* as an intimate morphological control, provides a basis for assessing the degree of mitochondrial preservation in various tissues, in which the mitochondrial distribution pattern may be unfamiliar to the investigator.

The reactions obtained in the hepatic and acinar cells by incubation in the DPN-lactate and DPN-malate media were indistinguishable from one another and yet were different in certain particulars from the reactions obtained in these cell types with other incubation media. The diformazan distribution representing succinic, β-hydroxybutyric, and DPN-linked isocitric dehydrogenases was found consistently to be localized in the mitochondria exclusively. Yet, following assays in the DPN-lactate and DPN-malate media, we often observed the deposition of a small proportion

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Most of the specimens illustrated were obtained from blocks that had been fixed successively at 3 minute intervals in chilled balanced salt solutions containing 0.7, 1.3 and 2.0 per cent formaldehyde, respectively, and frozen at -150 to $-165°C$. Liver sections were incubated for 20 minutes and sections of the pancreas were incubated for 30 minutes. Departures from the foregoing conditions will be noted.

Photomicrography was done by Mr. Chester Reather.

FIGURE 1

Even at a magnification low enough to show portions of several hepatic lobules, mitochondria are well defined when selectively stained without disruption. See Fig. 3 for high power. Incubation in the succinate-TNBT medium, 40 minutes. × 300.

FIGURE 2

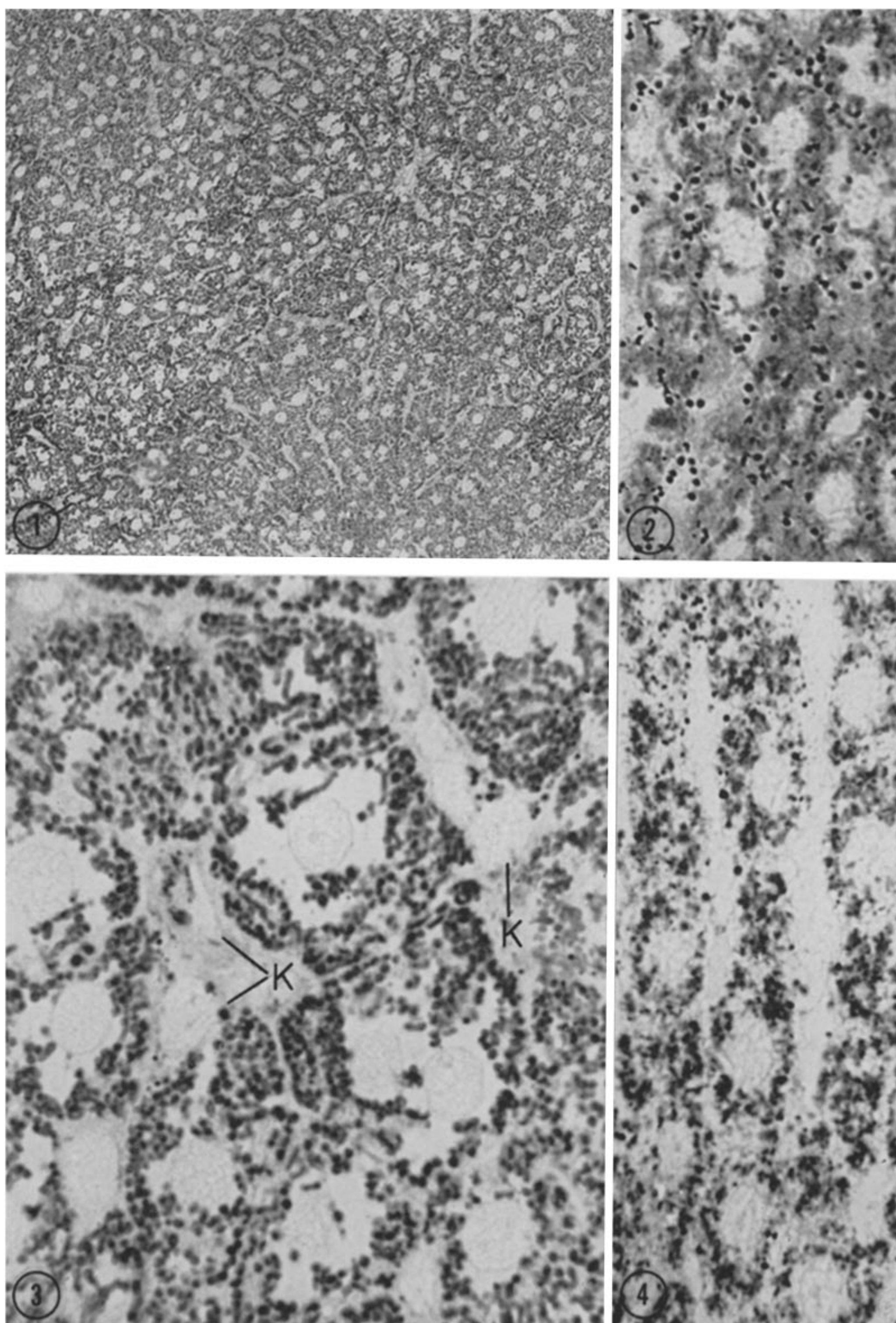
When histochemical assays are attempted on liver without the use of formalin, not only is the degree of cytologic damage severe, as seen here, but also there is considerable loss of the "soluble" dehydrogenases. Sections prepared from blocks frozen fresh at $-160°C$, incubated in the succinate-TNBT medium for 40 minutes. × 1000.

FIGURE 3

Variations in form and size of mitochondria are readily apparent when the liver section illustrated in Fig. 1 is examined with the oil-immersion system. The white perinuclear patches probably represent sites that were occupied by glycogen in hepatic cells. In the small, attenuated Kupffer cells, the nuclei of which are indicated by (K), activity is represented as small juxtanuclear clusters of diformazan particles. × 1500.

FIGURE 4

Surprisingly enough, when nitro-BT is used in place of TNBT for assays conducted on liver sections prepared by the recommended method, resolution of mitochondria is imperfect. On the other hand, in the pancreas, with nitro-BT (Fig. 20), definition was excellent. Excellent localization was also obtained with liver and nitro-BT (Fig. 16 reference 14). Succinate-Nitro-BT medium. × 1250.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of diformazan in the cytoplasm extramitochondrially. The extramitochondrial diformazan component, although conspicuously less intense than that of the mitochondria, imparts to the hepatic cell cytoplasm a dark, homogeneous cast (Figs. 5 and 6), and in acinar cells the distribution of the extramitochondrial diformazan seems to coincide with that of the chromidial substance (Fig. 25). The presence of extramitochondrial diformazan representing lactic dehydrogenase and malic dehydrogenase activities was correlated with the size of the organ block and, therefore, adequacy of fixation. Blocks of liver, the largest dimension of which was no more than 3 mm at the time of fixation, yielded sections the activities of which were exclusively localized in the mitochondria (Fig. 7). To obtain adequate fixation of the rat pancreas, strips were cut no more than 2 mm in thickness (Figs. 25 and 24).

The reaction obtained in the pancreas with the TPN-isocitrate medium was singularly bolder in the islets than in acinar cells (Figs. 20 and 21). Irrespective of the reaction mixture used, and in striking contrast to the mitochondrial reaction of the neighboring acinar cells, the reaction in most of the islets cells was more or less diffuse in character and, therefore, apparently not confined to the mitochondria.

The use of appropriate controls to rule out the possibility of activity attributable to the presence of endogenous substrates and coenzymes is imperative in ditetrazole assays, especially in specimens with intact organelles. Sections of liver over 6 μ thick will develop a stain of moderate intensity when incubated for 30 minutes in media containing lactate or malate but lacking exogenous DPN or in a medium containing DPN but lacking exogenous substrate. Therefore, to minimize reactions attributable to endogenous coenzyme (especially DPN) and substrate, sections no thicker than 2 μ should be used. In our experience blocks tend to ribbon more easily when cut at 2 μ and, of course, resolution of mitochondria in sections over 2 μ in thickness is imperfectly achieved, especially in cells like the hepatic cells with a high concentration of mitochondria.

FIGURE 5

The unstained nuclei of the hepatic cells stand out in bold relief against the blackened cytoplasm. The periportal stromal elements are free of the artifacts of compression and collapse to which such delicate connective tissues are subject. Lactate-DPN medium. Artery, *A*; vein, *V*. $\times$ 300.

FIGURE 6

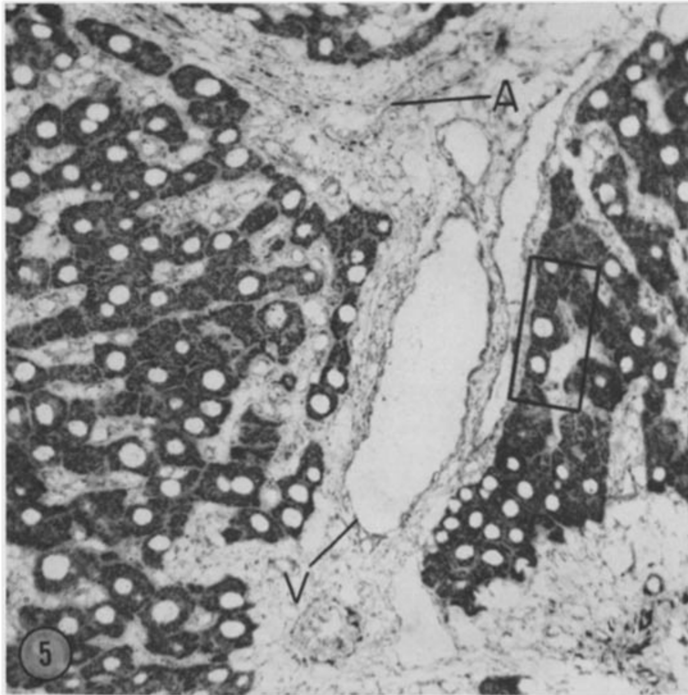
An enlargement of an area to the right of center in Fig. 5 facilitates the identification of the organelles representing sites of the activity. In the hepatic cells a small component of activity is represented in the extramitochondrial compartment. We believe that the latter represents activity of soluble lactic dehydrogenase that escaped from mitochondria that were imperfectly fixed. The dimensions of the section illustrated here is about 10 $\times$ 9 $\times$ 6 mm. To obtain a uniformly good result in the liver, we suggest dicing the liver into blocks no more than 3 mm in any one dimension. $\times$ 1500.

FIGURE 7

An exclusively mitochondrial localization of lactic dehydrogenase activity is obtained in properly fixed specimens of liver. This specimen was obtained from a 2 $\times$ 3 $\times$ 3 mm block of liver. Several minute blocks may be held in a cluster by means of a strip of pancreas and frozen together. The latter maneuver facilitates handling of minute tissue blocks and protects them against desiccation. Lactate-DPN-TNBT medium. $\times$ 1500.

FIGURE 8

The DPN-linked isocitric dehydrogenase of liver sections seemed to be less susceptible to loss through damaged mitochondria than lactic, malic, or the TPN-linked isocitric dehydrogenases. Isocitrate-DPN-TNBT medium. $\times$ 1000.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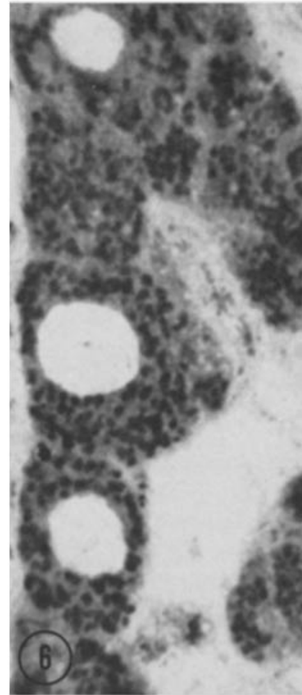
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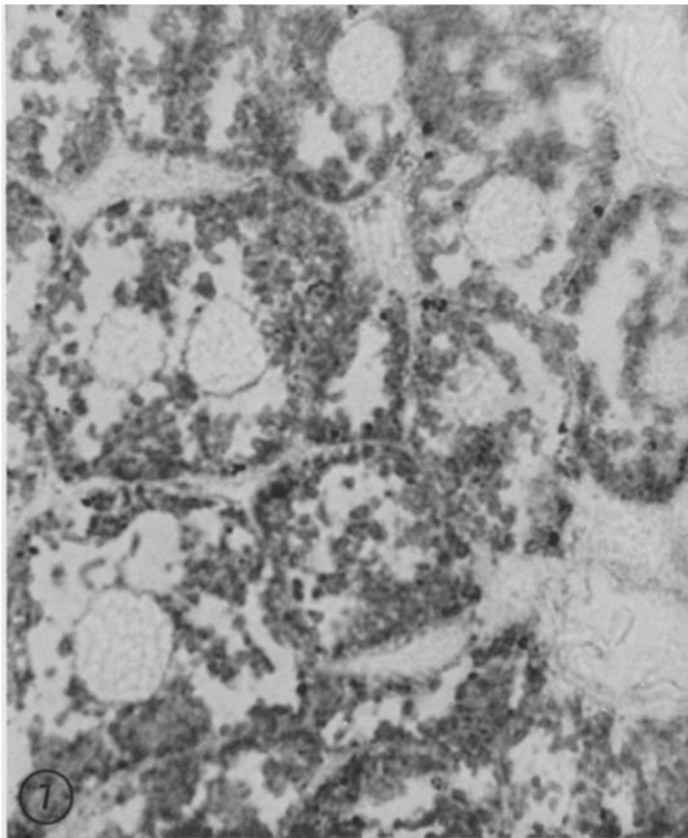
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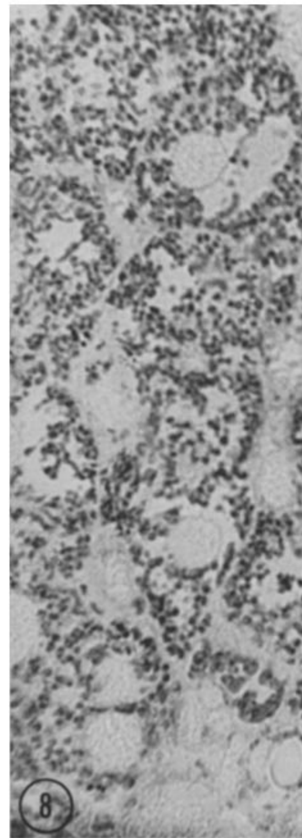
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A painstaking evaluation was conducted with formalin-fixed blocks of liver and pancreas to determine the relative merits of TNBT as compared with nitro-BT as a cytochemical reagent for dehydrogenase assays and of quenching at −150 to −165°C as compared with −70 to −75°C. The cytochemical result in the liver obtained with TNBT (Fig. 3) was superior to that obtained with nitro-BT (Fig. 4). The result obtained in the acinar cells of the pancreas, on the other hand, was excellent with either TNBT (Fig. 24) or nitro-BT (Fig. 23).

Regarding the importance of the temperature of the freezing solution, we observed no improvement in the cytochemical result by virtue of having frozen minute blocks of liver and pancreas at −150 to −165°C as compared with that obtained in blocks that had been frozen at − 70 to −75°C. The critical factor was the use of formalin, not the temperature (within the −70 to −165°C range) of the quenching solution. We never obtained satisfactory preparations from blocks of liver and pancreas that had been fresh frozen at −150 to −165°C (Figs. 2 and 24).

Quite unsatisfactory, too, were the sections obtained from fixed blocks quenched at temperatures *higher* than −60°C, an observation consistent with the observations of Hoerr (2), and Monis (4). In regard to preparation of frozen material for cytologic study we observed most of the precautions and methodology as suggested by Hoerr (2). In our experience a higher yield of good sections was obtained if we attempted to prepare no more than one or two blocks during any single assay run. However, if several blocks were to be sampled in one run, it was possible to stagger the fixation so as to permit quenching of only one block at a time, and to be sure that the temperature of the isopentane remained well below −70°C, we used liquid nitrogen. The size of the block obviously influences the speed of freezing. We used a stainless steel crucible for quenching. The maximum dimensions of the blocks were 2 × 5 × 10 mm.

FIGURE 9

The reaction for D-β-hydroxybutyric dehydrogenase in liver is of singular intensity and of strictly mitochondrial localization, observations that are supported by the quantitative biochemical studies of Lehninger, Sudduth, and Wise (4). D-β-hydroxybutyrate-DPN-TNBT medium. × 400.

FIGURE 10

Rod-shaped mitochondria in moderately high concentration are seen randomly distributed throughout the cytoplasm of the pancreatic acinar cells. After 1 hour's incubation in the D-β-hydroxybutyrate-DPN-TNBT medium. × 1000.

FIGURE 11

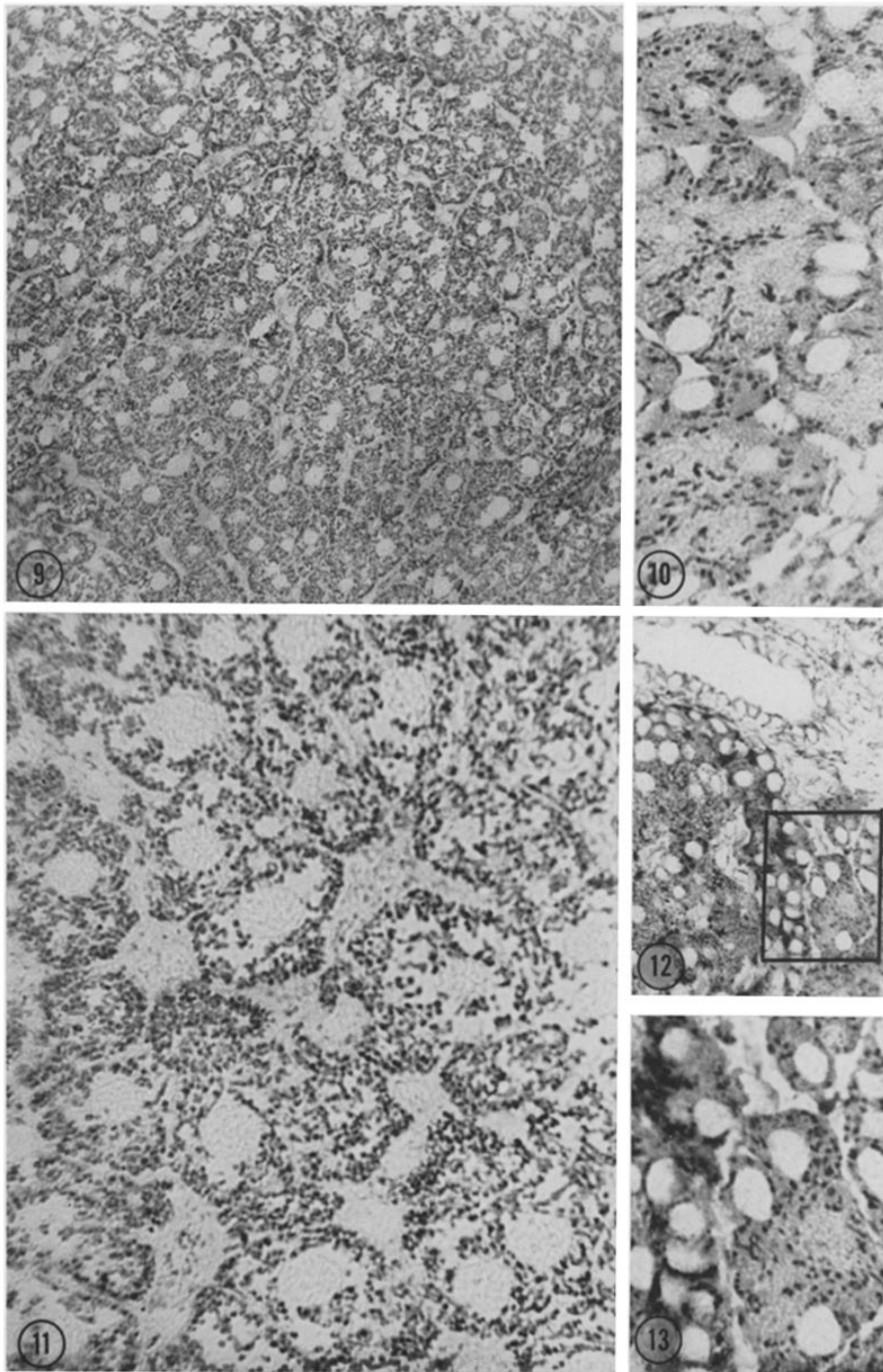
An area of the specimen illustrated in Fig. 9 as seen with the oil immersion lens system. Swollen and overstained organelles are of exceptional occurrence in this preparation. × 1000.

FIGURE 12

Midway along the border of this illustration a pancreatic duct extends to the right. In the area below the duct an islet "capped" with a double row of intensely stained cells is seen. On the basis of their distribution and concentration the latter are tentatively identified as alpha cells. Acinar tissue occupies the right upper and lower corners of the figure. Isocitrate-DPN-TNBT medium. × 300.

FIGURE 13

An area near the center of Fig. 12 is shown at higher magnification. In the pancreatic acinar cells occupying the right half of this illustration distinct mitochondria are evident. Elsewhere in the islet, organelle form is less well defined. The elements referred to as alpha cells possess prominent juxtanuclear aggregations of diformazan. × 1000.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DISCUSSION

The results of the present investigation indicate that the dehydrogenase and diaphorase activities of liver and pancreas are monopolized by the mitochondria to an extent even greater than that suggested by former biochemical (12) and histochemical (7, 8, 9) assays. Any damage sustained by cells during preparation (especially freezing and fractionation) or incubation would tend to promote loss of dehydrogenase activity from the mitochondrial compartment. This effect would be less noticeable with the bound dehydrogenases than with the more easily mobilized TPN-linked isocitric dehydrogenase or lactic dehydrogenase (Figs. 6 and 14). In hepatic cells, mitochondria derive no support from infoldings of the cell membrane or from fibrillar elements. Furthermore, hepatic cells of frozen sections exposed to aqueous incubation solutions are subject to the disruptive influence attending solubilization of massive glycogen inclusions. Therefore, unless proven otherwise, we believe any extramitochondrial reaction for lactic, malic, or TPN-linked isocitric dehydrogenases observed in rat hepatic and acinar cells represents activities displaced from damaged mitochondria. Displacement of the bound enzymes, succinic and D-β-hydroxybutyric dehydrogenases, would occur only if the mitochondria were severely disrupted. However, lactic dehydrogenase became displaced after slight damage to mitochondria as illustrated by the gray cellular matrix of hepatic and pancreatic cells in Figs. 6 and 25. If we can be confident that DPNH-diaphorase like the two dehydrogenases is exclusively an integral part of mitochondrial membranes, then we have no alternative but to regard extramitochondrial activity as artifact. However, question has been raised whether mitochondrial localization of soluble dehydrogenases is valid when noted, or whether this should be considered primarily due to localization of the diaphorase. It appears unlikely to us than an uncompartmentalized and unbound soluble dehydrogenase of a 2 μ thick section after uniform distribution in the medium is capable of maintaining a concentration of DPNH high enough to account for the intense reactions obtained after 20 minutes.

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FIGURE 14

The crisp localization in hepatic cell mitochondria of succinic and D-β-hydroxybutyric and the DPN-linked isocitric dehydrogenases was not obtained in assays for the TPN-linked isocitric dehydrogenase. Though swollen and overstained mitochondria (arrow) were frequently encountered, there was never any doubt but that the mitochondria were the only site of activity. $\times$ 1000.

FIGURE 15

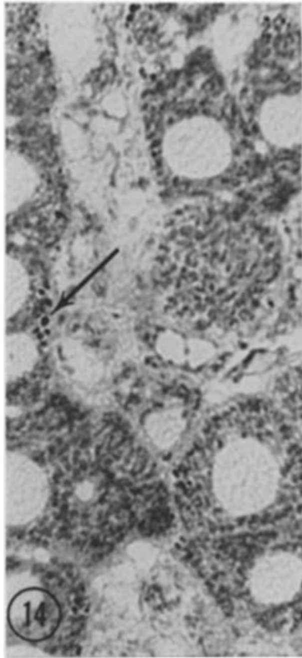
The shape of mitochondria in hepatic cells is characteristically ellipsoidal (Fig. 3) but in acinar cells many filamentous and rod-shaped organelles are seen. In histochemical specimens of pancreas prepared without use of formalin, succinic dehydrogenase activity is weak and diffusely distributed. Succinate-TNBT medium. $\times$ 1500.

FIGURE 16

An area framed in Fig. 17 is shown as seen under the oil immersion lens. All but the lower margin of the field is occupied by poorly stained islet elements. Distinct black mitochondria are seen in the acinar cells only. Succinate-TNBT-medium. $\times$ 1500.

FIGURE 17

In the center of this illustration a pale islet of Langerhans is seen well delineated from the surrounding distinctly granulated acinar tissue. The few intensely stained elements of the islet may be alpha cells. Succinate-TNBT medium. $\times$ 300.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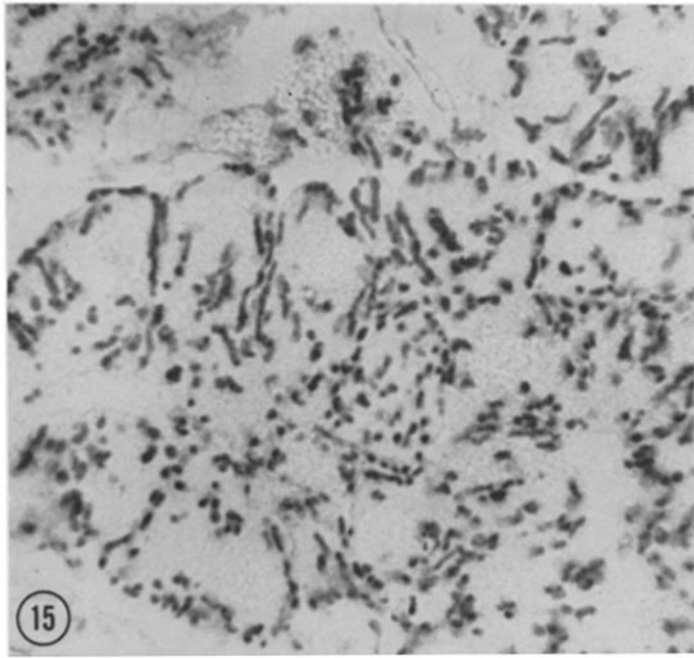
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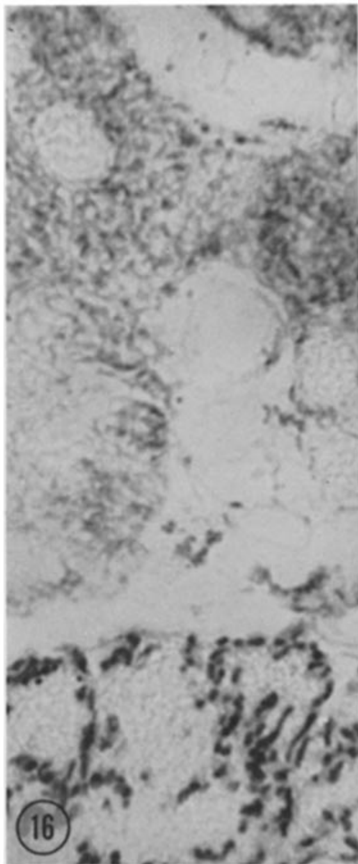
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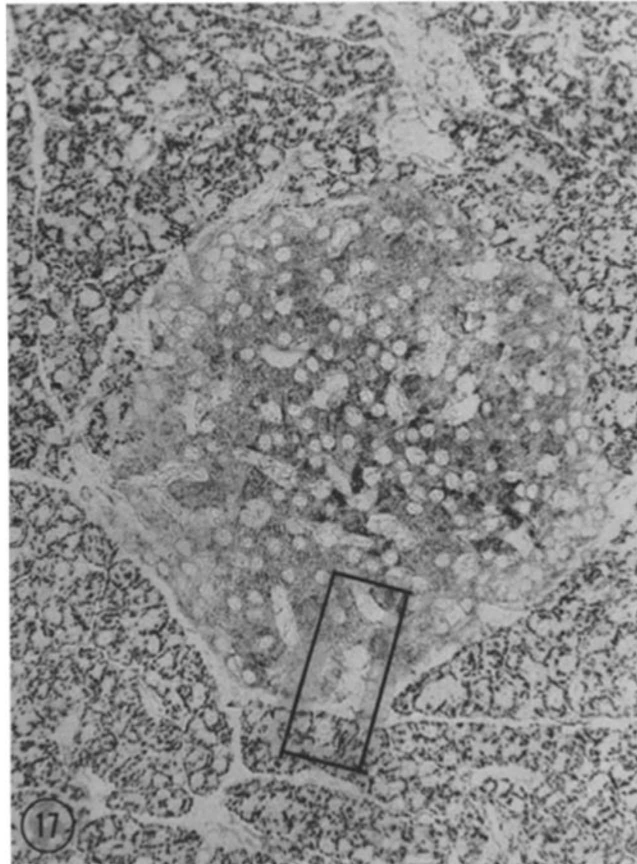
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Figure 18

The over-all intensity of the reaction is alike in islet (lower half of field) and acini (upper half of field). The acinar cells are speckled with black organelles; the appearance of the islet is mottled owing to the presence of numerous capillaries (irregular white patches) and a few intensely stained epithelial elements (alpha cells?). Malate-DPN-TNBT medium. × 300.

Figure 19

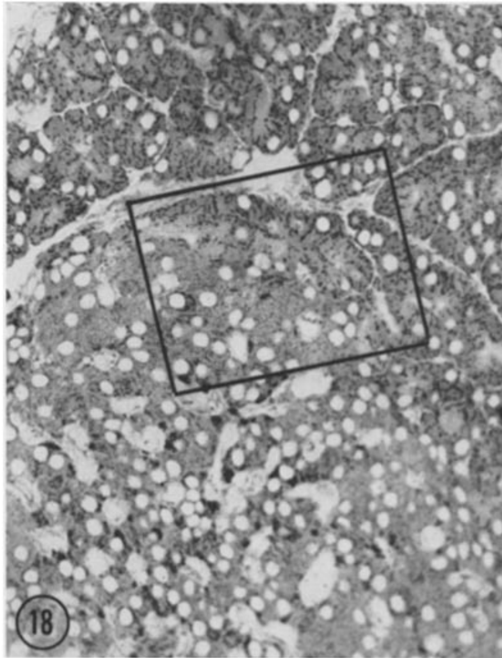
An enlargement of the area framed in Fig. 18. × 1000.

Figure 20

The reaction observed in the pancreas after incubation in the isocitrate-TPN medium is almost the counterpart of that observed in the assays for succinic dehydrogenase (See Figs. 16 and 17). The islet is more intensely stained than the acinar tissue. Isocitrate-TPN-TNBT medium (Incubated for 40 minutes). × 200.

Figure 21

As in the case of each of the other dehydrogenase reactions obtained in acinar cells, that representing the TPN-linked isocitric dehydrogenase was localized exclusively in mitochondria. In striking contrast is the bold diffuse cytoplasmic reaction in islet cells. The singularly faint reaction for succinic dehydrogenase and intense reaction for the TPN-linked isocitric dehydrogenase together with the apparently non-mitochondrial localization, may represent important clues as to a basic specialization of oxidative metabolism in the islet elements. Isocitrate-TPN-TNBT medium after 40 minutes of incubation. × 1250.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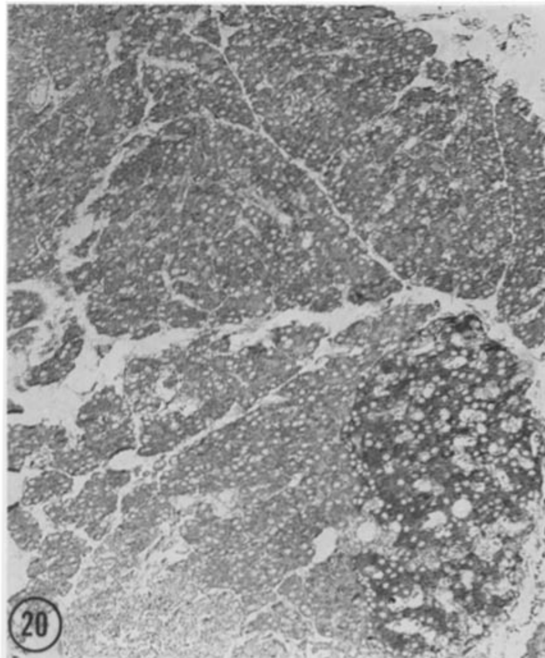
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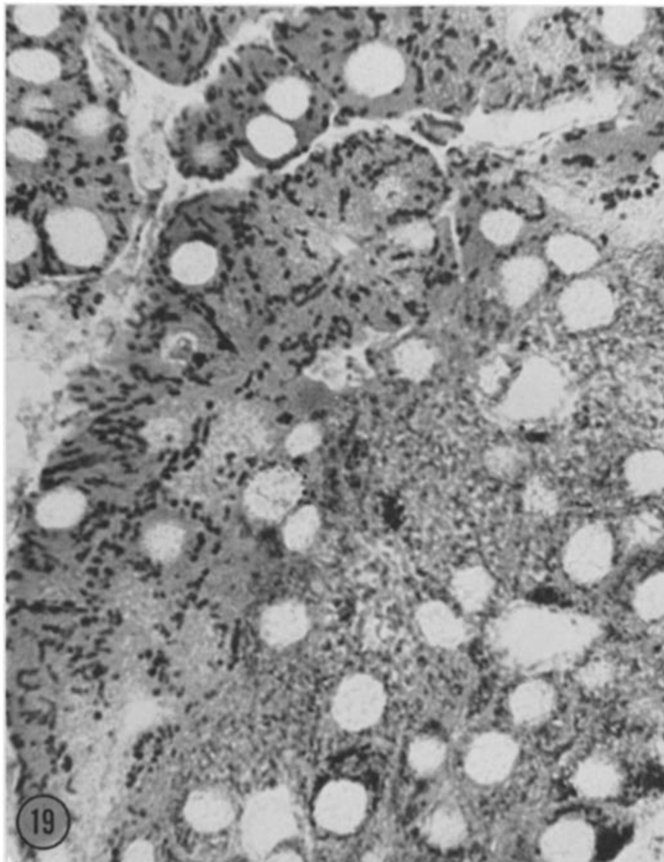
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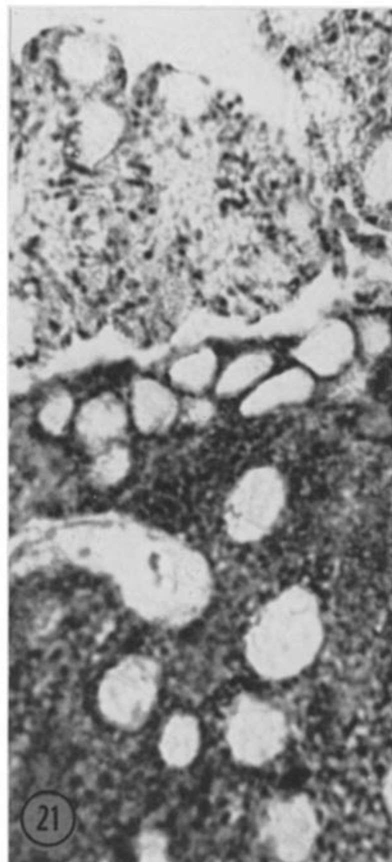
21

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FIGURE 22

A satisfactory cytochemical reaction, even for lactic dehydrogenase, a "soluble" dehydrogenase, may be obtained in small areas of a pancreatic section obtained from a fresh-frozen block. Such areas of fairly good localization are more often encountered when TNBT is used rather than nitro-BT. No exposure to formalin, frozen at $-160°C$, incubated in the lactate-DPN-TNBT medium for 20 minutes.

FIGURE 23

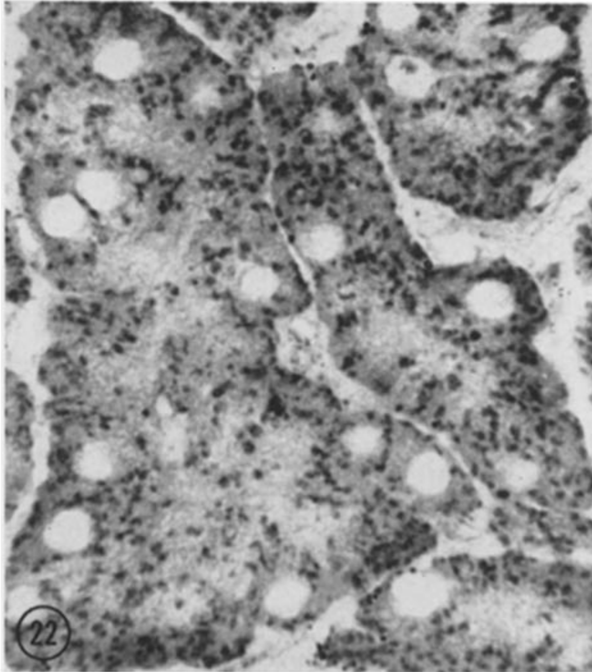
Cytochemical preparations of the pancreas after chilled formalin-BSS fixation are uniformly of high quality regardless of which ditetrazole is used. Lactate-DPN-nitro-BT medium. $\times$ 1000.

FIGURE 24

If the pancreas is cut into strips of 1 or 2 mm thickness prior to fixation an exclusively mitochondrial localization of lactic dehydrogenase activity is obtained. Lactate-DPN-TNBT medium. $\times$ 1000.

FIGURE 25

The darkening of areas corresponding in distribution to the chromidial substance is interpreted as activity that escaped from damaged mitochondria. This specimen of pancreas was fixed whole. Compare with the result obtained in Fig. 24. Lactate-DPN-TNBT. $\times$ 1000.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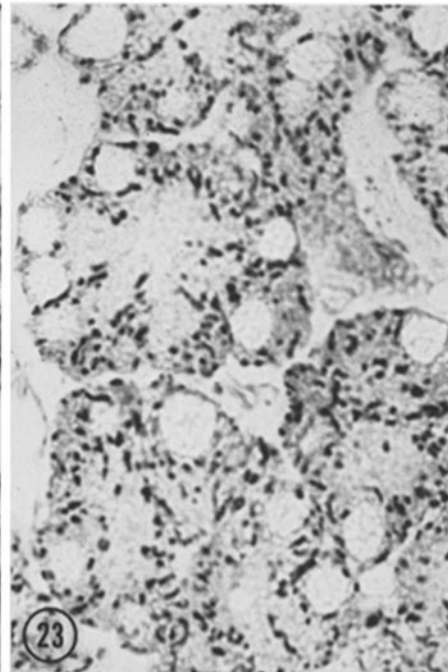
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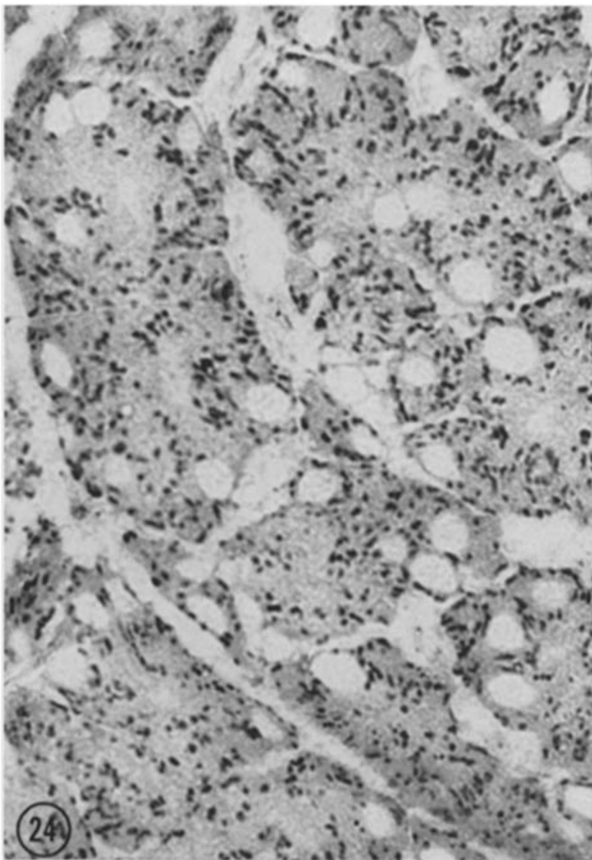
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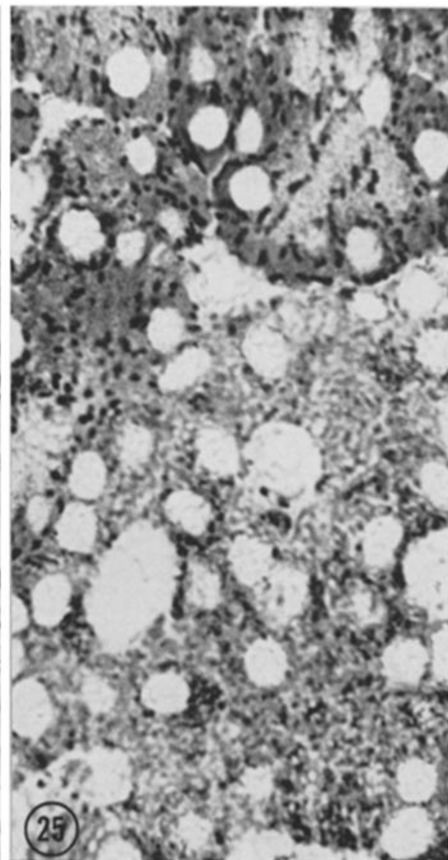
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