A SURVEY OF DEHYDROGENASES IN
VARIOUS EPITHELIAL CELLS IN THE RAT

DONALD G. WALKER, Ph.D.

From the Department of Anatomy, The Johns Hopkins University School of Medicine, Baltimore

ABSTRACT

Several different epithelial elements that have intense active transport or protein secretory functions were histochemically assayed in several dehydrogenase media by a recently perfected method. The mitochondria represented the only site of activity, not only when tested in the succinate and D-β-hydroxybutyrate media, but also when tested in the lactate, malate, and isocitrate media. The reaction for D-β-hydroxybutyric dehydrogenase in the mouse kidney was curiously limited to the mitochondria of the distal segment of the proximal convoluted tubule, a finding that most convincingly shows that dehydrogenase activity may be differentiated in certain instances from diaphorase activity by the ditetrazole methods and that D-β-hydroxybutyric dehydrogenase is not present in all mitochondria. Tetranitro-BT is favored over nitro-BT in studies conducted on most organs prepared without fixation and on formalin-fixed tissues that consist of lipid-containing or active transport cells.

INTRODUCTION

Formaldehyde has been used successfully to circumvent the organelle damage to which histologic material is subject during the dehydrogenase and diaphorase assay procedures. The cytochemical result obtained in sections from blocks that had been exposed to an 8 per cent aqueous solution of formaldehyde prior to quenching at $-70°C$ represented a distinct improvement over the result obtained in fresh frozen sections. By further refinement in the use of formaldehyde in conjunction with use of tetranitro blue tetrazolium (TNBT) and more careful control of the quenching temperature, a single procedure has been defined for the cytochemical demonstration of the three types of dehydrogenases according to which an optimal cytochemical result is obtained consistently in assays of mammalian tissues (8).

The present investigation was initiated with the intention of applying the recommended ditetrazole technic to several major epithelial elements besides those of the liver and pancreas in order to determine whether or not (a) glutamic, lactic, malic, and the TPN-dependent[1] isocitric dehydrogenases and the diaphorases consistently are of mitochondrial localization, and (b) mitochondria of the different cell types studied are qualitatively alike in terms of their dehydrogenase activities.

MATERIALS AND METHODS

Histologic Materials

Adult rat cerebellum, cord, duodenum, liver, kidney, pancreas, stomach, submaxillary gland, and

---

[1] Abbreviations: DPN, diphospopyridine nucleotide; DPNH, diphosphopyridine nucleotide, reduced form of; TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide, reduced form of.

trachea as well as adult mouse kidney were assayed in this study. A total of about 150 rats and 9 mice were used. However, usually no more than two organs per animal were prepared for cytochemical study.

Preparation of the Tissues

The duration of exposure to the formaldehyde is so short (6 to 15 minutes) that it is imperative that the various specimens of glands, the cord, and the brain be cut into pieces about 1 mm in all three dimensions and that the fixation fluid be stirred constantly during the fixation period. The hollow viscera should be transferred to the stirred fixative in slices preferably about 1 mm in thickness. Sectioning should be accomplished with as sharp an instrument as possible, and tissue fragments should be washed into the fixing vessel. An organ slice may be diced while submerged in a shallow excavation of a paraffin slab using a new Super-Gillette razor blade; the slab may then be submerged in the fixation bath. In the early phase of this study an 8 per cent aqueous solution of formaldehyde was used. However, a much more uniform result is

obtainable using a graded series of formaldehyde, 0.7, 1.3, and 2 per cent, respectively, in balanced salt solution (8) or simply 1 per cent formaldehyde in balanced salt solution. Intestinal villi and delicate linings, such as respiratory epithelium, should be exposed to the fixative no longer than 6 to 8 minutes. The solid organs, including samples from the central nervous system, should be exposed to the fixative for 12 to 15 minutes. Tolerance to the fixative also varies with the dehydrogenase and diaphorase. The TPN-linked system has the least tolerance, lactic and malic dehydrogenases have the most tolerance, and succinic, D-β-hydroxybutyric, and the DPN-linked glutamic and isocitric dehydrogenases show a tolerance intermediate between these extremes. The preservation of glutamic dehydrogenase activity is perhaps most critically dependent upon adequate fixation.

In anticipation of freezing the fixation, fluid is decanted and the tissue fragments are drained on filter paper and gently rolled into balls about 1 cm in diameter and dropped into isopentane chilled to about −150°C by liquid nitrogen. At this temperature the isopentane is somewhat viscous. To

FIGURE 1

The cerebellar cortex is horizontally bisected by a single layer of large neurons, the Purkinje cells. The molecular layer occupying the upper half of this figure has been stained more uniformly than the granular layer seen in the lower half. The extensive unstained patches in the granular layer are occupied by closely packed nuclei which, like nuclei in general, lack oxidative enzymatic activity. Fixed in aqueous 8 per cent HCHO for 4 minutes, quenched at −70°C, incubated in the malate-DPN-TNBT medium for 30 minutes. × 400.

FIGURE 2

The framed portion of Fig. 1 is illustrated here at a magnification high enough to resolve the mitochondria which seem to be present in high concentration throughout the cytoplasm of nerve cells. The activity is most highly concentrated in the glomeruli (G) which represent extensive synaptic arborizations of the granular layer of cerebellum. P, Purkinje cells; C, capillary. × 1500.

FIGURE 3

The large cell bodies illustrated here were located in a section of the submandibular gland and are believed to represent perikaryons of submandibular ganglion cells. The cytoplasm possesses a high concentration of rod-shaped mitochondria. A bright but unstained nucleolus is seen near the center of the illustration. Fixed in aqueous 8 per cent HCHO for 5 minutes, quenched at −70°C, incubated in the malate-DPN-TNBT medium for 30 minutes. × 1500.

FIGURE 4

The pattern of diformazan distribution obtained in cerebellar cortex after incubation in the isocitrate-DPN-TNBT medium closely resembles that obtained in the malate DPN-TNBT medium shown in Fig. 2. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BBS for 5 minutes, quenched at −165°C, incubated in the isocitrate-DPN-TNBT medium for 30 minutes. × 1000.
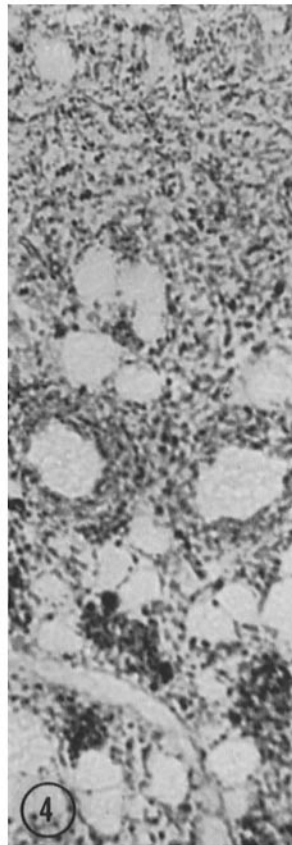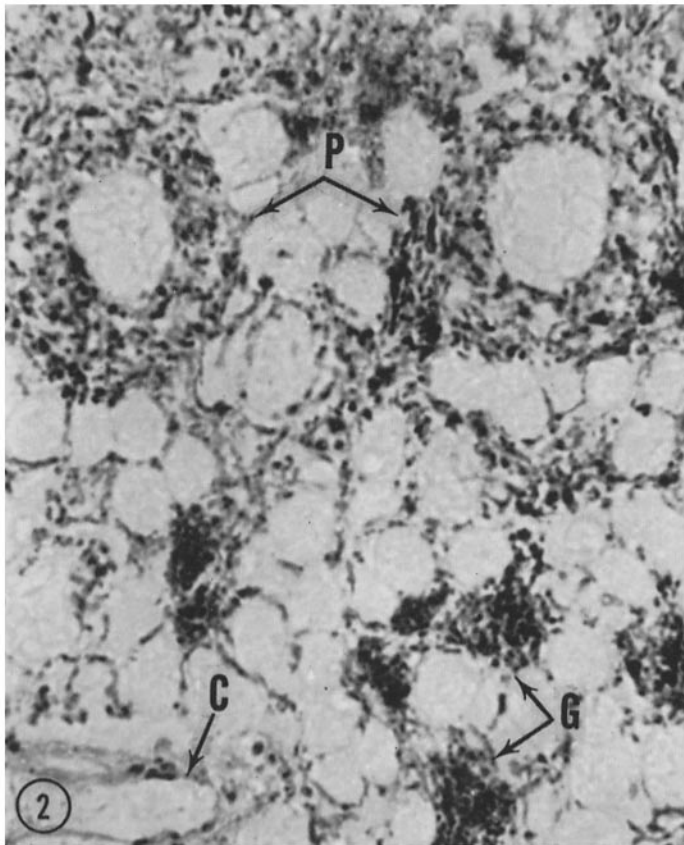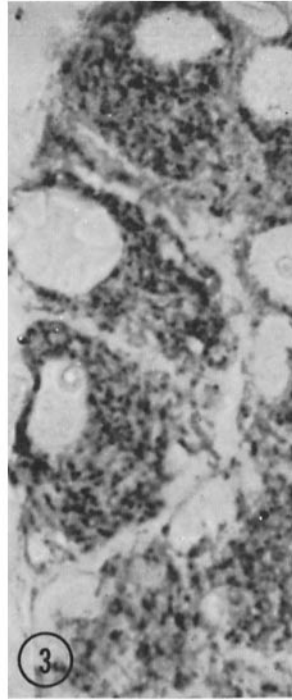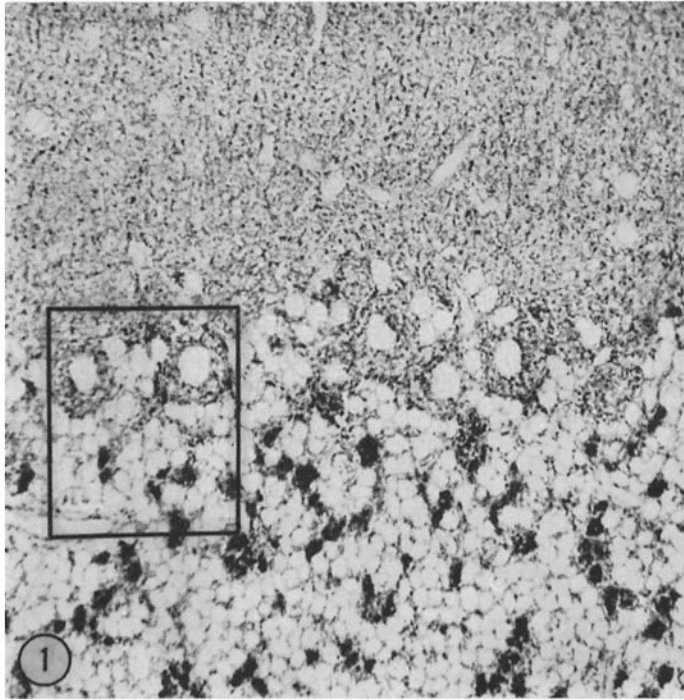
avoid adherence of the tissue block to the quenching vessel, the isopentane may be stirred or the block repeatedly moved. After about 45 to 60 seconds' exposure at −150°C the block is transferred to the cryostat (Harris Refrigeration Co., Cambridge, Massachusetts) maintained at −23°C. Contrary to expectations, the blocks of finely diced organs cut more easily than undiced fresh frozen blocks. It was a rare block that did not ribbon well when sectioned at a thickness of 2μ (using a Minot rotary microtome). It is fortunate and curious that the tissue fragments, rather than tending to fall out of the section or to render the block brittle, are so compactly fused together that it is impossible to detect the lines of fusion with the eye. The blocks of diced tissue fragments will yield uniformly good sections throughout the unthawed portion of the block and may be kept for a day. However, desiccation gradually renders the block useless thereafter. In a matter of 15 to 30 seconds a ribbon of sections may be cut, transferred to a pair of 11 × 22 mm coverslips (Corning Glass Co., no. 1 thickness), and the latter, oriented "back to back," transferred to shell vials containing 3 ml of ditetrazole reagent. To avoid desiccation, 2-μ-thick sections should be removed promptly from the cryostat.

The incubation solution (8) contained a buffer, a substrate, a ditetrazole, and a coenzyme (except when succinate was used as substrate or when the coenzyme was omitted for control purposes). To obtain an approximately isosmolar solution the reagents were used at the following final concentrations:

Buffer, Sorensen's phosphate, pH 7.4, 0.030 M
Substrates, sodium DL-β-hydroxybutyrate, sodium glutamate, 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; MnCl₂, 0.0005 M (for TPN-containing media only)
Ditetrazolium salt, TNBT and nitro-BT, 0.50 mg/ml

Henceforth, in the text and in the lengends to the figures, the incubation solution will be identified by the substrate, coenzyme, and ditetrazole used, e.g., the incubation solution used to demonstrate malic dehydrogenase activity will be referred to as the malate-DPN-TNBT medium.

The duration of incubation was 30 minutes in most instances (exceptions will be noted). Following incubation the specimens were fixed in 2 per cent formaldehyde–balanced salt solution (HCHO-BSS) for 5 minutes and thereafter mounted in glycerin jelly.

RESULTS

The rate of the histochemical reaction varies with the substrate used. The DPN-linked isocitric, lactic, malic, and succinic dehydrogenases were distributed coextensively. The reaction rate for a given cell type in the DPN-lactate and DPN-malate media was usually about twice that observed in the DPN-isocitrate and succinate media. The histologic distribution *patterns* were alike in the case of these four systems. The reactions for DPN-linked glutamic, D-β-hydroxybutyric, and the TPN-linked isocitric dehydrogenases varied from negative to very intense depending both upon the cell type assayed (Table I) and upon the degree of fixation. To illustrate how very critical the duration of exposure to the fixative may be, assay of liver for glutamic dehydrogenase may be cited

---

FIGURE 5

A very high concentration of mitochondria is characteristic of cells devoted intensively to active transport functions. As shown here, the duodenal absorptive cells have a very high concentration of mitochondria which are spheroidal in shape infranuclearly and filamentous supranuclearly. N, nucleus. Fixed in aqueous 8 per cent HCHO for 5 minutes, quenched at −70°C, incubated in the succinate-TNBT medium for 30 minutes. × 2000.

FIGURE 6

Although somewhat overstained, the mitochondria appear to be the only site of activity of this TPN-dependent reaction. Absorptive cells of the villous epithelium of the duodenum which was fixed in 8 per cent aqueous HCHO for 9 minutes, quenched at −70°C, and incubated in the isocitrate-malate-TPN-nitro-BT medium for 30 minutes. × 2000.

FIGURE 7

A cross-section of a duodenal crypt as seen in a field from the same specimen as that shown in Fig. 5. N, nucleus; L, lumen of crypt. × 2000.
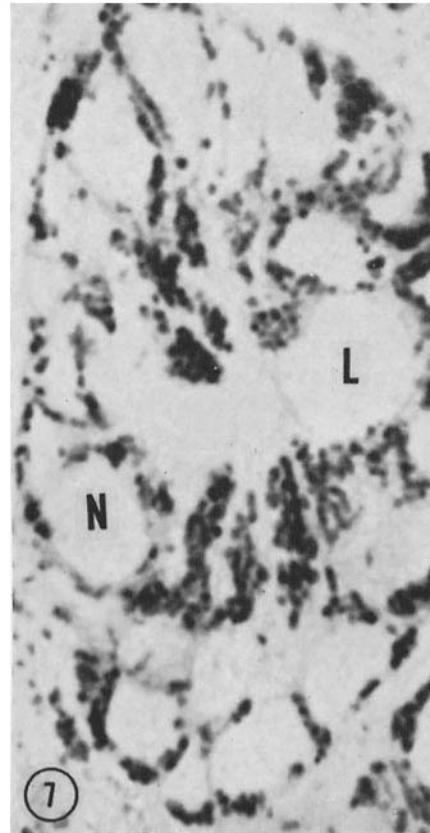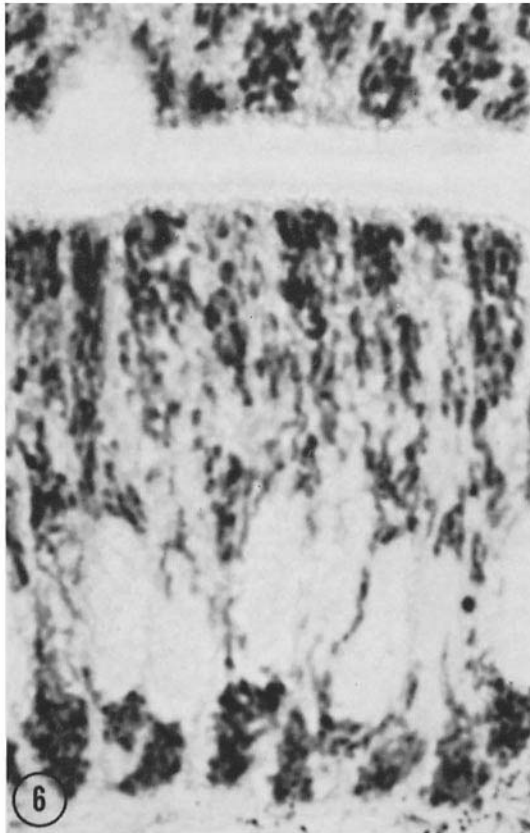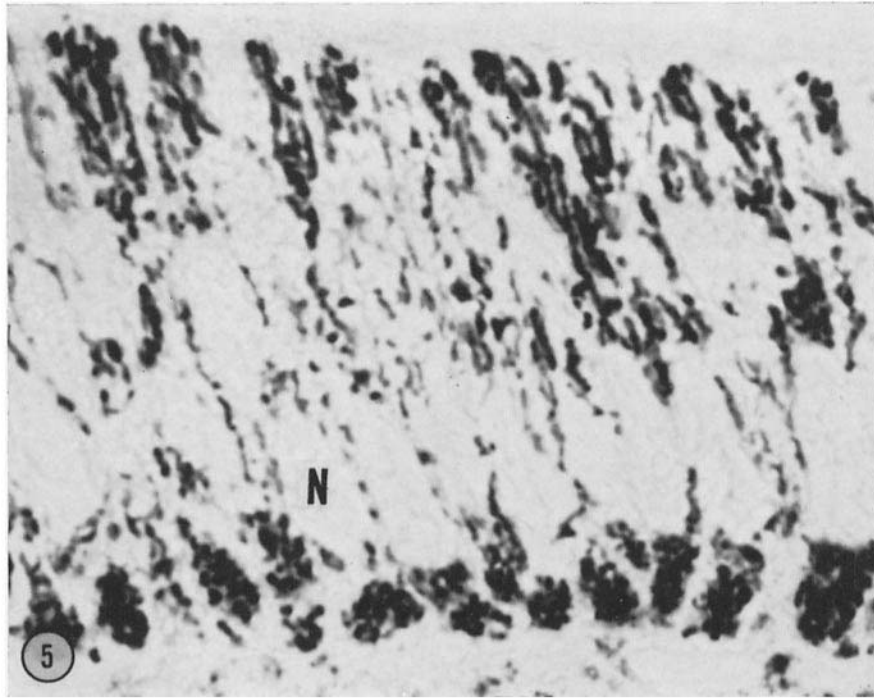
TABLE I

*Relative Intensity of the Diformazan Reaction in Several Different Epithelial Elements after Thirty Minutes' Incubation in Various Dehydrogenase Reagents**

Epithelial element assessed	Flavoproteins TPNH diaph.	Flavoproteins DPNH diaph.	SDH	Dehydrogenases TPN-linked Isocitric	DPN-linked β-OHB	DPN-linked Isocit.	DPN-linked Lact.	DPN-linked Malic	Controls No coenzyme With malate	No coenzyme With isocit.	No substrate With DPN	No substrate With TPN
Cerebellar:												
Purkinje cells	+	6+	3+	+	+ or 0	4+	6+	6+	0	0	0	0
Duodenal:												
Absorptive cells	4+	6+	3+	4+	+ or 0	3+	6+	6+	+ or 0	0	0	0
Gastric:												
Surface epith.	2+	4+	2+	2+	+	2+	4+	4+	0	0	0	0
Mucous neck cells	2+	2+	+	2+	+	+	2+	2+	0	0	0	0
Parietal cells	2+	6+	5+	2+	+ or 0	3+	6+	6+	+	0	0	0
Chief cells	+	2+	+	+	0	+	2+	2+	0	0	0	0
Hepatic	4+	6+	2+	4+	4+	3+	6+	6+	+	0	+ or 0	0
Pancreatic:												
Acinar	+	3+	2+	+	+	+	3+	3+	0	0	0	0
Renal:												
Glomerulus	++	2+	+	+	0	+	2+	2+	0	0	0	0
Prox. conv. tub.	4+	6+	4+	3+	0 to 4+	2+	6+	6+	+ or 0	0	0	0
Thin limbs	++	+	+	+	0	+	+	+	0	0	0	0
Dist. conv. tub.	3+	6+	4+	3+	0	+	6+	6+	+ or 0	0	0	0
Collecting ducts	+	+ to 4+	+	+	0	+	+ to 4+	+ to 4+	0	0	0	0
Submaxillary:												
Serous cells	++	2+	++	++	0	++	2+	2+	0	0	0	0
Mucous cells	++	+	++	++	0	++	+	+	0	0	0	0
Saliv. ducts	2+	6+	3+	2+	+	++	6+	6+	+ or 0	0	0	0
Tracheal:												
Ciliated epith.	++	4+	++	++	+	++	4+	4+	0	0	0	0
Serous cells	++	2+	++	++	0	++	2+	2+	0	0	0	0

* The intensity of the enzymatic reaction as indicated by the concentration of diformazan in the cytoplasm estimated after 30 minutes' incubation at 39°C varied from negative, 0, to barely discernible, +, through increasing concentrations, 2+, 3+, etc., to one of maximum intensity, 6+.

(Figs. 33 to 35). The liver was diced and fixed for 15 minutes in BSS containing 1 per cent formaldehyde. After 1 hour of incubation, the non-uniformity of distribution pattern was very apparent at low magnification (Fig. 33). Examination at high magnification of areas corresponding to the core of each small cube of liver revealed signs of mitochondrial damage, such as swollen, over-stained, and clumped organelles (Fig. 34). Areas corresponding to the superficial zone of the small cube of liver were free of such artifacts (Fig. 35).

A most interesting dehydrogenase distribution pattern was demonstrable in mouse kidney sections that had been incubated in the D-β-hydroxybutyrate-DPN medium (Fig. 14). At the margins of the active zone in the mouse kidney sections one finds piebald tubules, the lining of which is made up of cells, some of which show intense β-hydroxybutyric dehydrogenase activity, and the remainder of which are negative (Figs. 13 and 15). The curiously restricted distribution pattern of D-β-hydroxybutyric dehydrogenase represents the most convincing piece of evidence supporting the rationale according to which dehydrogenase distribution may be differentiated from diaphorase distribution in histochemical preparations. The DPNH-flavoprotein is demonstrable in *all* cells of the mouse kidney when DPNH is used as a hydrogen donor in the histochemical reaction. Therefore, the finding that a characteristically restricted pattern of diformazan was obtained in the mouse kidney when D-β-hydroxybutyrate was used as the hydrogen source indicates that the restricted pattern represents the distribution of D-β-hydroxybutyric dehydrogenase activity. That the latter reaction is reproducible and localized exclusively in mitochondria (Figs. 13, 15, and 16) helps to exclude the possibilities that the restricted pattern might have been due to either selective extraction or inhibition.

That a mitochondrial pattern of distribution was obtained in specimens assayed in the TPN-isocitrate-malate medium (Figs. 6, 17, and 18) is contradictory to expectations based upon biochemical (2, 3) and histochemical (6, 7) studies and, therefore, is not acceptable without substantial confirmation.

The cell types devoted primarily to protein secretion have approximately the same concentration of mitochondria, which represents but a small fraction of the concentration found in the cell types performing active transport functions (Table II).

Protein secretory cell types illustrated in this study include the islet (Fig. 37) and acinar cells of the pancreas (Fig. 36), the mucous cells of the gastric surface epithelium (Fig. 17) and gland necks (Fig. 19) and the chief cells (Figs. 21, 23, and 24), serous cells of the tracheal glands (Fig. 22), and mucous cells (Fig. 30) and serous cells (Figs. 30 and 32) of the submandibular gland. Hepatic cells and neurons not only maintain vital active transport functions,

TABLE II

Cytoplasmic Mitochondrial Concentrations of Several Major Epithelial Elements*

Epithelial element	Mitochondrion per μ^3 of cytoplasm‡
Cerebellar Purkinje cell	0.76 ± 0.30
Duodenal absorptive cell	0.54 ± 0.11
Gastric chief cell	0.14 ± 0.05
Gastric parietal cell	0.62 ± 0.13
Gastric surface epithelium	0.66 ± 0.26
Hepatic cell	0.55 ± 0.12
Pancreatic acinar cell	0.19 ± 0.04
Proximal convoluted tubule	0.55 ± 0.16
Salivary duct epithelium	0.90 ± 0.27
Submandibular mucous cell	0.21 ± 0.06
Submandibular serous cell	0.25 ± 0.07

* The mitochondrial counts were made on photomicrographs of the highest resolution as follows: Using frosted acetate sheeting (2.932 × 10⁶ u^2 per mg), a histogram of each cytoplasmic area sampled was prepared. The weight of each histogram was measured and expressed in units of cytoplasmic area (μ^2) with due allowance for the magnification at which each photomicrograph was made. Since the sections were 2μ in thickness and undehydrated, the mitochondrial concentration was equal to the number of mitochondria per histogram divided by twice the area of the histogram.
‡ Mean value and standard deviation.

but also produce proteins. However, since active transport is by far the more energy-demanding role, it is not surprising that these cells have the high concentration of mitochondria characteristic of active transport protoplasm.

In view of the variety of cells and dehydrogenase activities embraced in this study, the fact that all reactions appeared to be localized in mitochondria exclusively is highly revealing. In the absence of qualitative activity differences, the intensity of the staining reaction obtained in a particular section and substrate, as indicated in Table II, was a

function of the mitochondrial concentration. Thus, the most intensely stained elements were those that maintain intense active transport functions, including neurons (Figs. 1 to 4 and 38), parietal cells (Figs. 18 to 21, 23, and 24), absorptive cells (Figs. 5 to 7), the salivary duct epithelium (Figs. 28, 29, 31, and 32), and the convoluted tubules and thick limbs of the nephron (Figs. 8 to 12 and 15). In all these, as well as hepatic cells (Figs. 33 to 35), the concentration of mitochondria is characteristically high, an observation that has been made repeatedly in both light and electron microscopic preparations. It is assumed that the energy requirement of a cell maintaining a major active transport function is many times greater than that needed to maintain protein production in a cell.

Any one of the epithelial cells (including those of the trachea, Fig. 22) will serve to illustrate the fact that nitro-ditetrazole cytochemistry has been freed largely of one major limitation, namely, artifacts attributable to disruption of mitochondria. Maintenance of mitochondrial integrity throughout the cytochemical procedure was achieved consistently by, (a) the judicious use of formaldehyde, (b) quenching well below the eutectic temperature of ice, and (c) the use of TNBT in the incubation solution (8). Susceptibility to cytologic damage varies with the organ or tissue assayed. In this study the liver and submandibular gland represented the extremes (high and low, respectively) of susceptibility to damage. Therefore, a fairly good result may be obtained in assays of fresh frozen sections of the submandibular gland (Fig. 25) but not so using fresh frozen sections of the liver (see Fig. 2 of Reference 8). The importance of the choice of the ditetrazole also depends in

FIGURE 8

The basal half of the convoluted tubular epithelium is characterized by the presence of rod-shaped mitochondria compactly arranged end to end in parallel rows. Each row usually contains three to eight mitochondria and is oriented perpendicular to the basement membrane. A factor tending to maintain this orderly mitochondrial disposition is the compartmentalization afforded by deep infoldings of the cell membrane basally. Fixed in aqueous 8 per cent HCHO for 5 minutes, quenched at $-70°C$, incubated in the succinate-TNBT medium for 30 minutes. $\times$ 1500.

FIGURE 9

Mitochondria appear as a swarm of dots in areas where a convoluted tubule has been transected near its base as illustrated here. The large white oval bodies are nuclei. This photomicrograph is from the same section as that shown in Fig. 8. $\times$ 1500.

FIGURE 10

Two overlapping photomicrographs were used to make this illustration of the renal corpuscle. A proximal convoluted tubule at origin and macula densa are seen in opposite corners of the figure. In all the various elements of this specimen, the activity seems to be localized strictly in mitochondria. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BSS for 20 minutes, quenched at $-165°C$, incubated in the malate-DPN-TNBT medium for 30 minutes. $\times$ 1000.

FIGURE 11

Even in fresh frozen sections assayed for a "soluble" dehydrogenase, such as lactic dehydrogenase, one *occasionally* finds a tubule in which mitochondria are discernible. This result was obtained in certain instances when TNBT was used but not when nitro-BT (Fig. 12) was used. Fresh specimen of rat kidney, quenched at $-165°C$, incubated in the lactate-DPN-TNBT medium for 20 minutes. $\times$ 1000.

FIGURE 12

Mitochondria are not individually delineated in this fresh frozen section of kidney that was assayed for succinic dehydrogenase in the presence of nitro-BT. Quenched at $-165°C$, incubated for 20 minutes. $\times$ 1000.
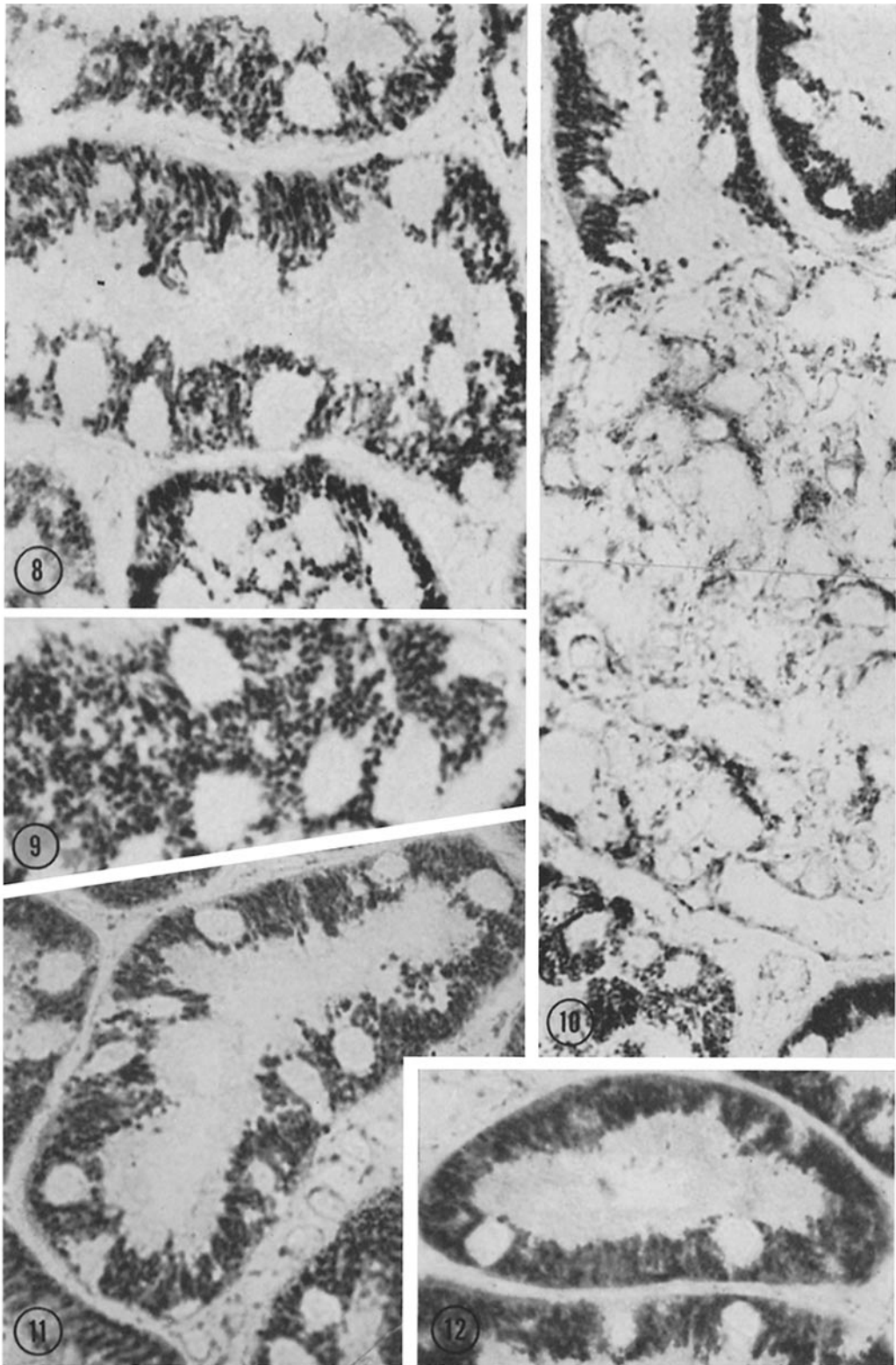
part upon the tissue to be assayed and whether or not use of formalin is contemplated. Although one may obtain routinely an excellent result when nitro-BT is used for assays conducted on formalin-fixed salivary (Fig. 26) or pancreatic acini, TNBT yields results superior to those obtained with nitro-BT in assays conducted either on fresh tissues or on formalin-fixed active transport cells (compare Fig. 25 with Fig. 27, Fig. 32 with Fig. 28, and Fig. 11 with Fig. 12, respectively).

DISCUSSION

The cytochemical reactions on the formalin-treated tissues indicate that the mitochondria represent the exclusive site of activity for the dehydrogenases and diaphorases in the cell types tested. That selective inactivation of non-mitochondrial sites took place during the exposure to formaldehyde very possibly accounts in part for the results obtained. It must be admitted that the intensity of the extramitochondrial reaction in protein-producing cells, especially, seems to vary inversely with the degree of mitochondrial preservation. However, as an alternative to selective in-

activation, it is suggested that the extramitochondrial component may represent activity that had escaped from damaged mitochondria. As a matter of fact, according to various recent biochemical studies conducted on cell fractions, high lactic, malic, and isocitric dehydrogenase activities in the postmitochondrial fractions and supernatant fluid indicate extensive damage to the mitochondria (1, 9). At the same time, little or no lactic and isocitric dehydrogenase activity can be elicited from mitochondria which are maintained intact throughout preparation and assay. Therefore, to obtain valid quantitative data on mitochondrial dehydrogenase assays, one must take every precaution to maintain mitochondrial integrity during preparation of the cell fractions, and just before conducting the assay by sonic or other means of disruption, one must be certain to release all the dehydrogenase into the assay medium (9). Histochemically, by a judicious use of formaldehyde, mitochondria may be rendered impermeable to dehydrogenases and diaphorases, but still permeable to coenzymes, ditetrazolium ions and substrates.

Figure 13

Renal tubules of the outer cortex of the mouse kidney as seen framed near the upper left corner of Fig. 14 are shown at high magnification. Although all the cells lining the tubules shown have a high concentration of mitochondria, less than half of them are positively stained for D-β-hydroxybutyric dehydrogenase activity. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BSS for 9 minutes, quenched at $-70°C$, incubated in the β-hydroxybutyrate-TNBT medium for 40 minutes. $\times$ 1200.

Figure 14

At low power the "sawtooth-like" distribution pattern of D-β-hydroxybutyric dehydrogenase is boldly displayed. The activity seems to be located in the *distal* segment of the *proximal* convoluted tubule, which essentially is confined to the inner cortex. The reaction obtained by incubation of adjacent sections in the malate-DPN medium is intense throughout the specimen, as demonstrated in a previous report (5) and confirmed in the present study. $\times$ 100.

Figure 15

A little below and to the left of center in Fig. 14 is a second framed area which is shown at higher magnification here. At this level of the cortex all the tubular elements are intensely stained, and close scrutiny at high resolution leaves no doubt that the activity is localized exclusively in the mitochondria. $\times$ 1200.

Figure 16

The third framed area, located near the bottom of Fig. 14, is shown at high magnification here. The line separating active from inactive cells is sharp, and corresponds to the corticomedullary line or juncture. $\times$ 1200.
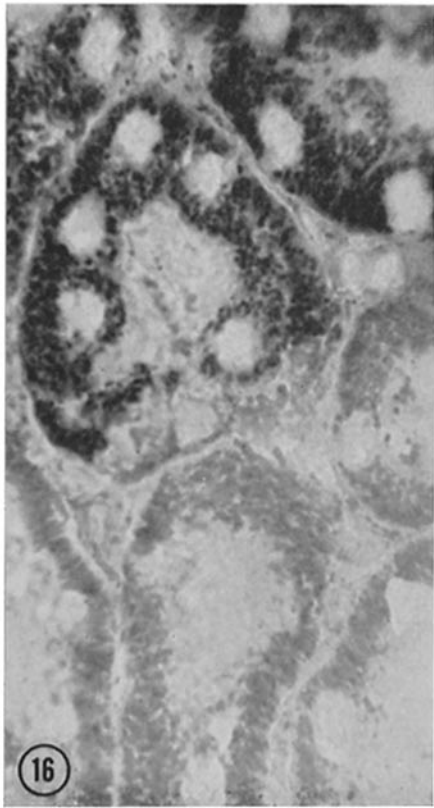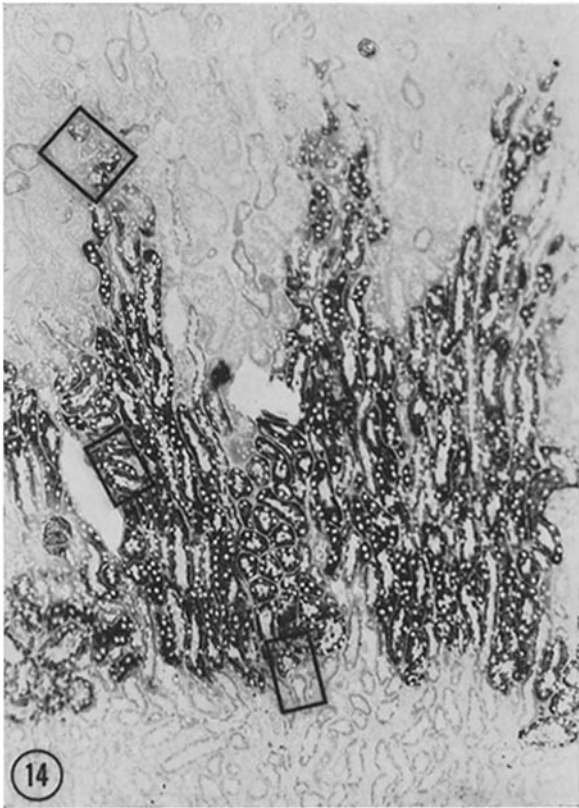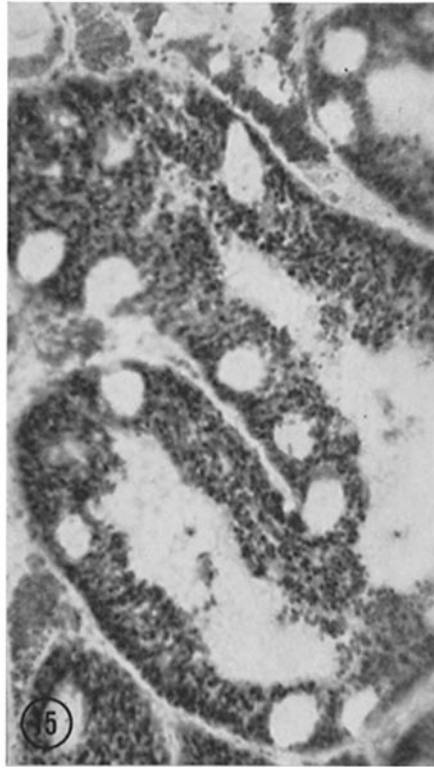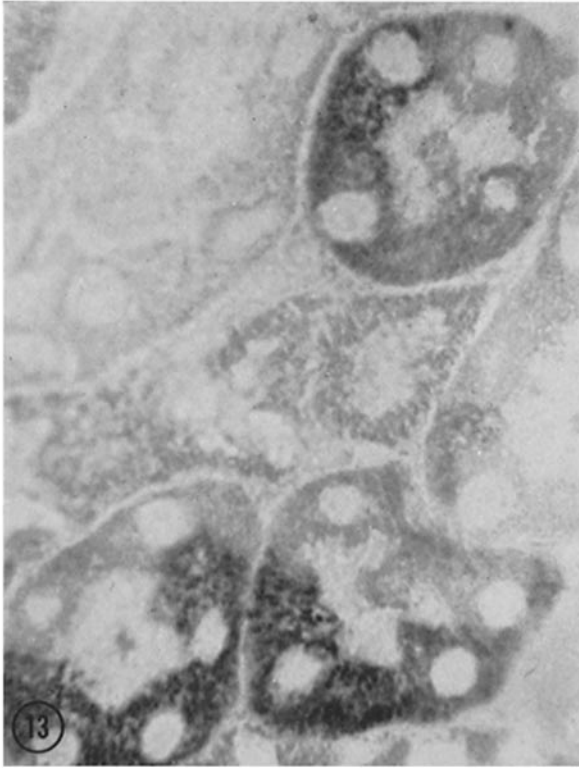
That succinic and D-β-hydroxybutyric de-
hydrogenases are located exclusively in the mito-
chondria is generally accepted. These enzymes are
"built into" the mitochondrial membrane (4). The
restricted renal distribution pattern obtained in
the histochemical assay for D-β-hydroxybutyric
dehydrogenase would indicate that this enzyme is
not ubiquitous as it has been considered to be (4).

FIGURE 17

The mucus-producing cells of the surface epithelium of the stomach have small spheri-
cal mitochondria which are most highly concentrated in the supranuclear cytoplasm.
It is noteworthy that a mitochondrial localization was obtained in the gastric mucosa
by virtue of TPN-dependent enzymatic activity. Fixed in an aqueous solution of 8
per cent HCHO for 5 minutes, quenched at −70°C, incubated in the malate-isoci-
trate-TPN-TNBT medium for 30 minutes. × 1500.

FIGURE 18

Parietal cells of the gastric glands as seen in another field of the specimen illustrated
in Fig. 17. The mitochondria, although well defined, appear to be slightly swollen
and overstained, changes attributable to the high concentration (8 per cent) of for-
maldehyde used. × 1500.

FIGURE 19

Several gastric glands transected at the neck are illustrated. The high concentration
of mitochondria seen in the cells illustrated here and in Fig. 17 (excluding the parietal
cells) suggests that, perhaps in addition to mucus production, these elements maintain
some important active transport (absorptive?) function. Fixed in 0.7 to 2.0 per cent
HCHO-BSS for 6 minutes, quenched at −165°C, incubated for 30 minutes in the
lactate-DPN-nitro-BT medium. × 1000.

FIGURE 20

A gastric gland, transected at the level of the neck, is shown lined by three parietal
cells and two or three mucous cells. The average diameter of the mitochondria of the
parietal cells shown in this preparation is about half that of the mitochondria in the
parietal cells of Fig. 18. Perhaps the fixation solution was responsible for this mito-
chondrial size difference. Fixed with 0.7 to 2.0 per cent HCHO for 9 minutes, quenched
at −165°C, incubated in the malate-DPN medium for 30 minutes. × 1500.
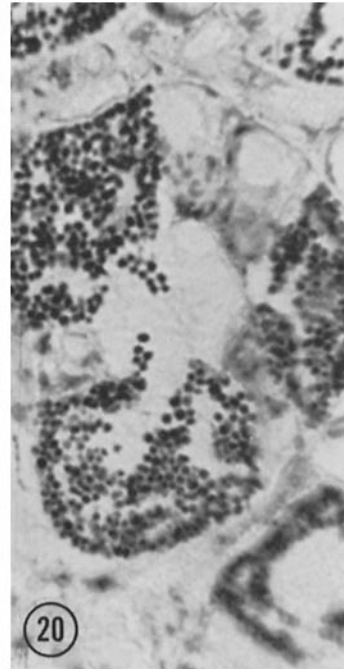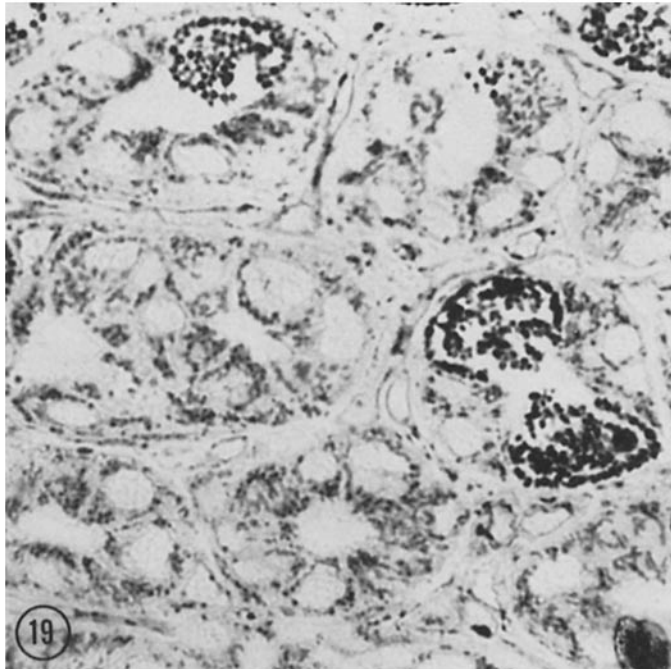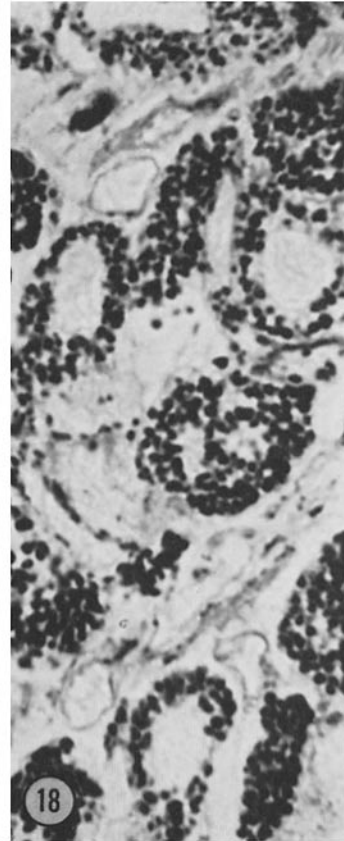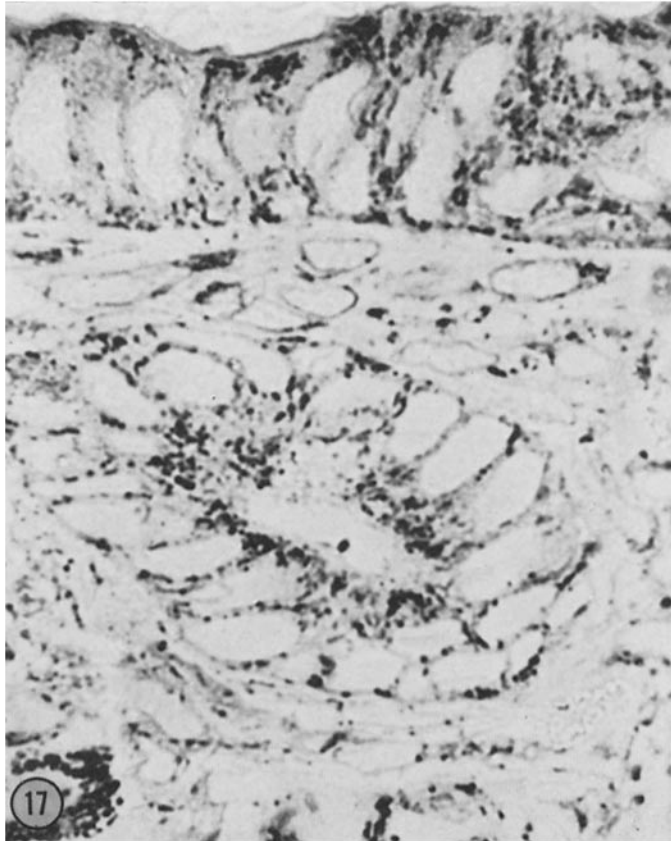
REFERENCES

1. BENDALL, D. S., and DE DUVE, C., Tissue fractionation studies, *Biochem. J.*, 1960, **74**, 444.
2. ERNSTER, L., and NAVAZIO, F., Studies on TPN-linked oxidations. I. Pathways of isocitrate oxidation in rat liver mitochondria, *Biochim. et Biophysica Acta* 1957, **26**, 408.
3. HOGEBOOM, G. H., and SCHNEIDER, W. C., Cytochemical studies of mammalian tissues. III. Isocitric dehydrogenase and triphosphopyridine nucleotide cytochrome c reductase of mouse liver, *J. Biol. Chem.*, 1950, **186**, 417.
4. LEHNINGER, A. L., SUDDUTH, H. C., and WISE, J. B., D-β-Hydroxybutyric dehydrogenase, *J. Biol. Chem.*, 1960, **235**, 2450.
5. NACHLAS, M. M., WALKER, D. G., and SELIGMAN, A. M., A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 29.

6. NACHLAS, M. M., WALKER, D. G., and SELIGman, A. M., The histochemical localization of triphosphopyridine nucleotide diaphorase, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 467.
7. WALKER, D. G., NACHLAS, M. M., and SELIGman, A. M., Differences in the intracellulai localization of DPN and TPN diaphorases as demonstrated with nitro-BT, *J. Histochem. and Cytochem.*, 1958, **6**, 395.
8. WALKER, D. G., and SELIGMAN, A. M., The use of formalin fixation in the cytochemical demonstration of succinic and DPN- and TPN-dependent dehydrogenases in mitochondria, *J. Cell Biol.*, 1963, **16**, 455.
9. ZIEGLER, D. M., and LINNANE, A. W., Studies on the electron transport system. XIII. Mitochondrial structure and dehydrogenase activity in isolated mitochondria, *Biochim. et Biophysica Acta*, 1958, **30**, 53.

FIGURE 21

In cross-sections of gastric glands deep in the fundic mucosa, chief and parietal cells command attention. The parietal cell is replete with boldly stained spheroidal mitochondria. The chief cells possess delicate filamentous mitochondria. The diformazan is localized exclusively in the mitochondria. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BSS for 9 minutes, quenched at −165°C, incubated in the isocitrate-DPN-TNBT medium for 30 minutes. × 1500.
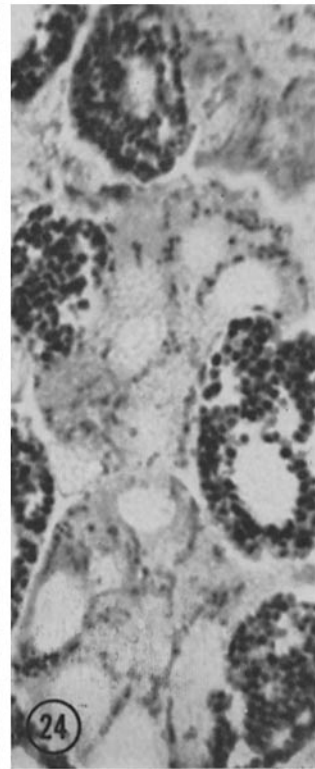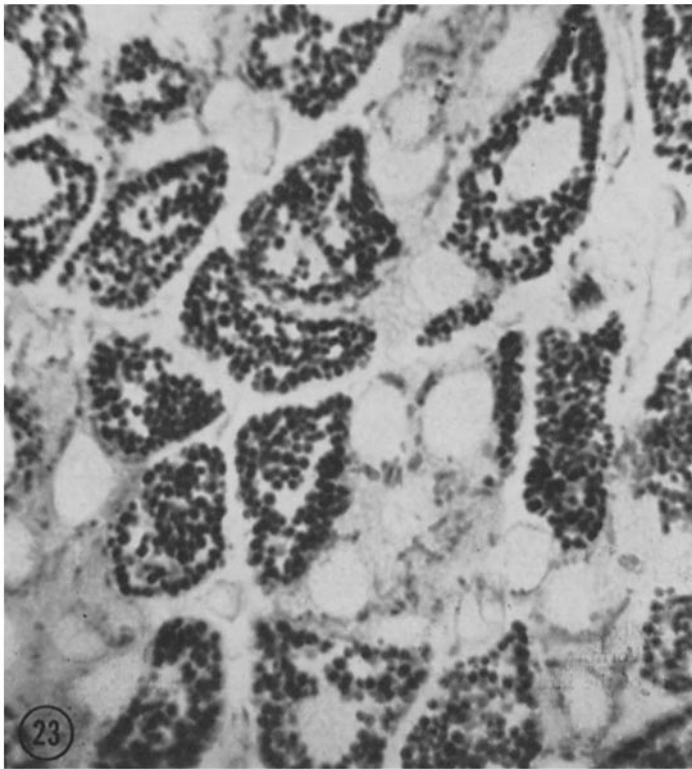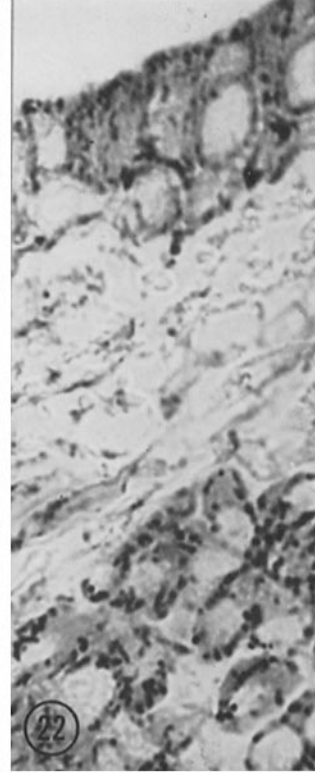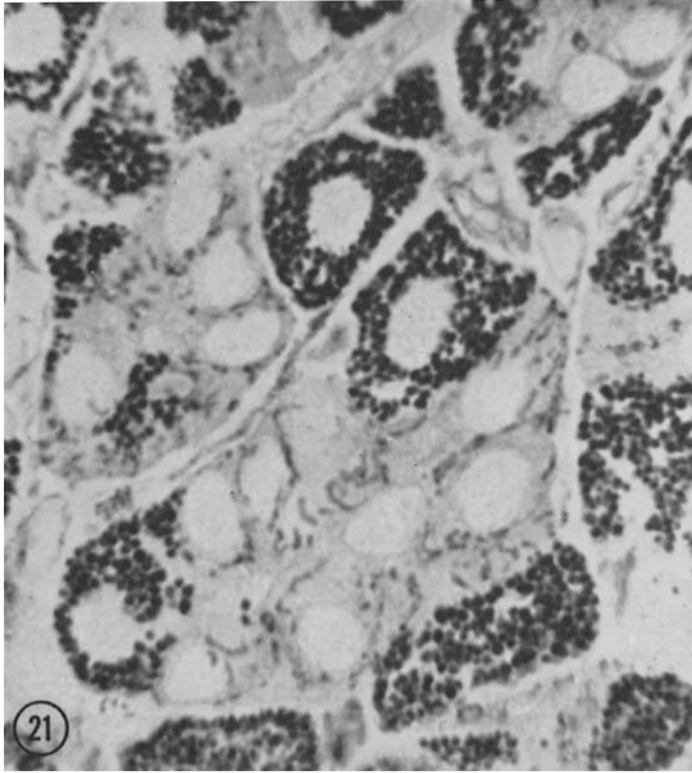
FIGURE 22

A small portion of the rat tracheal mucosa is shown. Although cilia are not discernible in this illustration, the mitochondria of the lining epithelium are easily identified. The respiratory epithelium as seen here resembles the gastric surface epithelium as seen in Fig. 17. The serous glandular elements seen in the lower third of this figure closely resemble other zymogenic cells. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BSS for 9 minutes, quenched at −165°C, incubated in the malate-DPN-TNBT medium for 40 minutes. × 1500.

FIGURE 23

Gastric glands of a section from the same block as that shown in Fig. 21 as seen stained for succinic dehydrogenase. The result approximates that obtained in assay for the DPN-dependent isocitric dehydrogenase. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BSS for 9 minutes, quenched at −165°C, incubated in the succinate-TNBT medium for 30 minutes. × 1500.

FIGURE 24

Gastric glands of a section from the same block as the sections illustrated in Figs. 21 and 23. It is noteworthy that the distribution pattern of lactic dehydrogenase, a soluble dehydrogenase, is strictly a mitochondrial one, as in the case of the other reactions cited. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BSS for 9 minutes, quenched at −165°C, incubated in the lactate-DPN medium for 30 minutes. × 1500.

FIGURE 25

The delicate filamentous mitochondria seen in the serous cells illustrate once more that even in fresh frozen sections assayed for a soluble dehydrogenase, discrete localization of activity is obtained *occasionally*. The mitochondria of the salivary duct epithelium, though intensely stained, are poorly defined. Rat submandibular gland quenched without fixation at $-160°C$ and incubated for 25 minutes in the lactate-DPN-TNBT medium. $\times$ 1000.

FIGURE 26

Serous acini of the submandibular gland as seen after 25 minutes' incubation in the lactate-DPN-nitro-BT medium. Fixed with 0.7 to 2.0 per cent HCHO in Hanks' BSS for 9 minutes. $\times$ 1000.
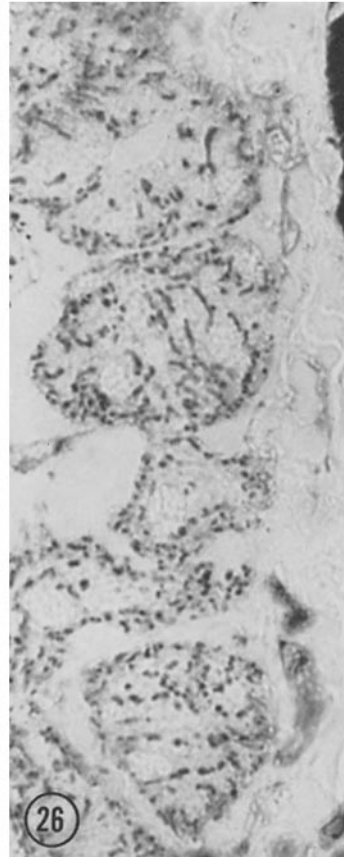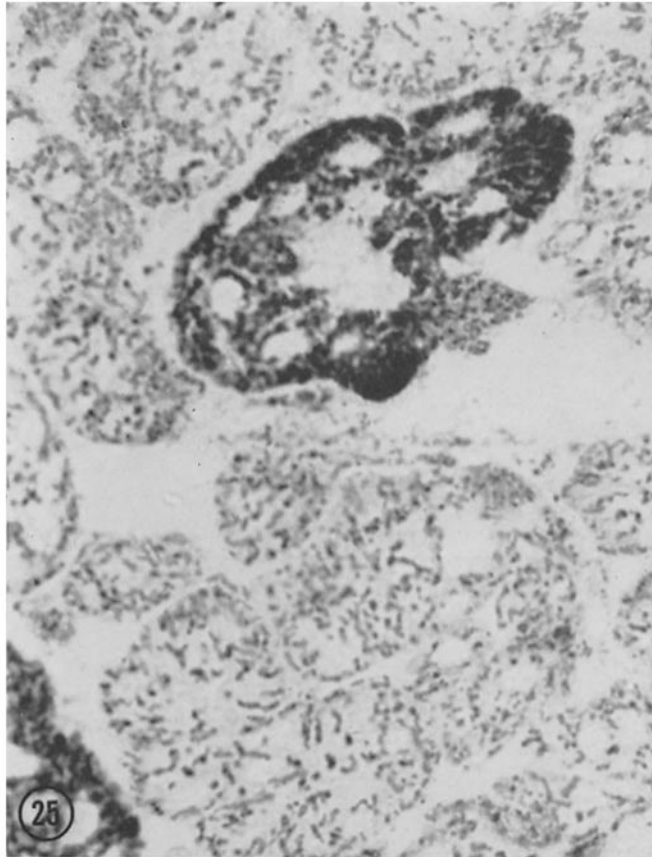
FIGURE 27

This reaction, representing succinic dehydrogenase activity in the submandibular serous acini, cytologically does not compare well with that illustrated in Fig. 25 representing lactic dehydrogenase activity. Both specimens were quenched without fixation at $-160°C$. However, whereas TNBT was used to obtain the result illustrated in Fig. 25, nitro-BT was used to make the specimen illustrated in Fig. 27. Therefore, TNBT is recommended over nitro-BT in assays conducted on *fresh* frozen sections. $\times$ 1000.

FIGURE 28

The preservation of mitochondria in this duct of the submandibular gland was obtained as a result of using the recommended fixation procedure. In areas where mitochondria are heavily concentrated, as along the basal margins of the salivary duct epithelium, the diformazan of nitro-BT usually forms aggregates large enough to obscure the form of the individual mitochondria. As can be seen in Fig. 32, TNBT does not tend to form diformazan aggregations. Figs. 26 and 28 represent halves of a single photomicrograph. $\times$ 1000.
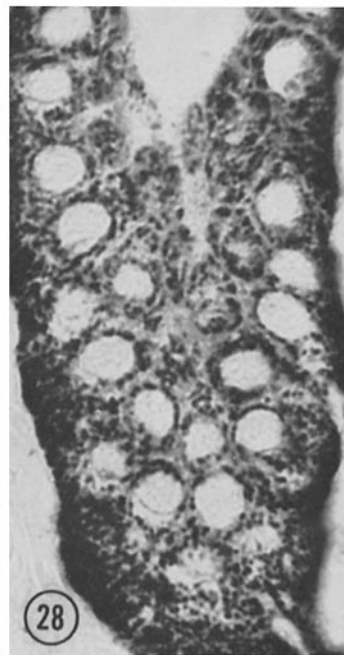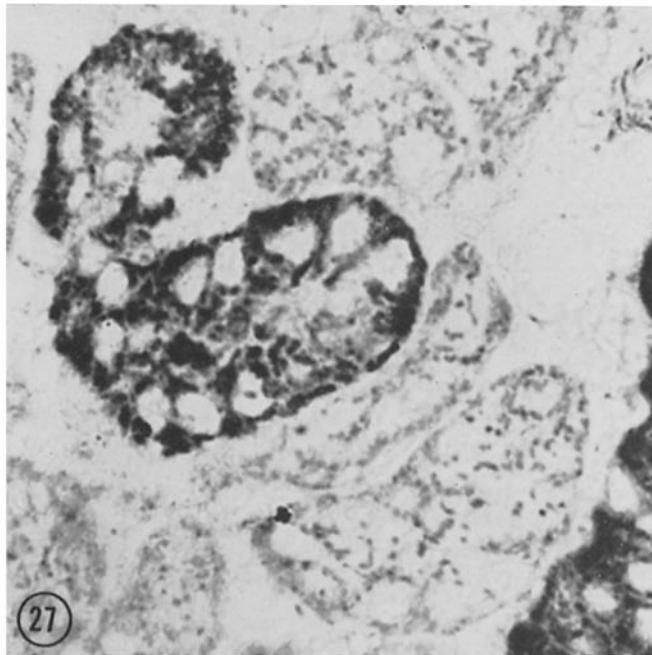
FIGURE 29

Bisecting the figure diagonally is a salivary duct, the margins of which have a darkly striated appearance. The more weakly stained secretory portion of the gland is delicately mottled, an appearance attributable to the fact that it is a mixed gland, consisting of both serous acini (the gray patches) and mucous acini (the white patches). Fixed with an aqueous 8 per cent solution of HCHO for 5 minutes, quenched at −70°C, incubated in the malate-DPN-TNBT medium for 30 minutes. × 400.
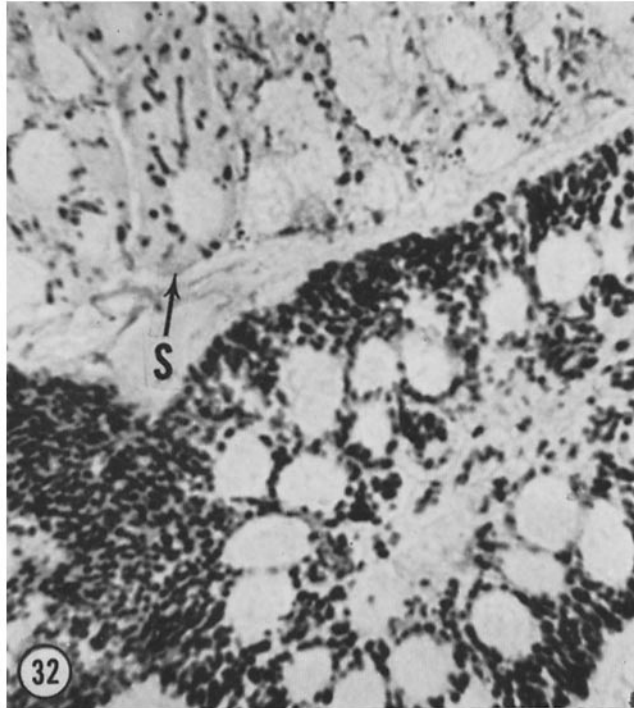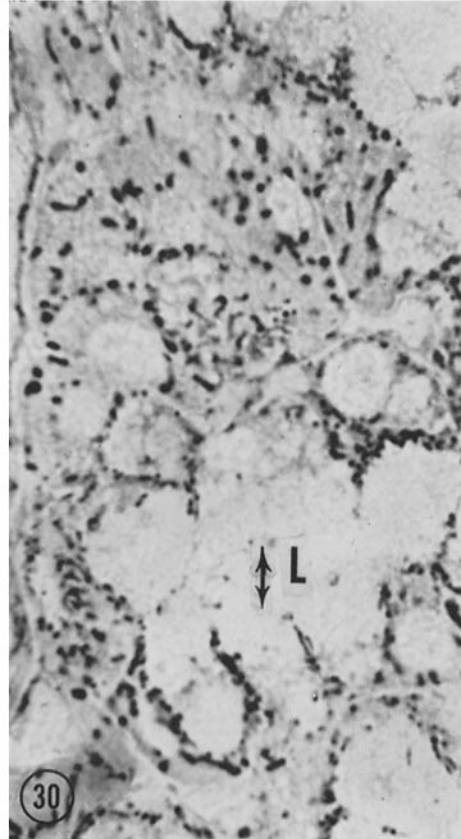
FIGURE 30

The two types of secretory units, serous and mucous, may be compared in this enlargement of the area framed in the upper right corner of Fig. 29. The cells of the serous acinus produce a watery secretion rich in a hydrolytic enzyme (ptyalin). Mucus is a highly viscous product, passage of which to the exterior requires that the duct lumen (L) be wide and patent even at its origin. The mitochondria of the mucous cells are crowded toward the periphery, whereas in serous cells the mitochondria are distributed in a random fashion throughout the cytoplasm. Nuclei appear as unstained, oval bodies located in the lower half of the cell. The secretory granules occupy most of the apical region. × 1500.

FIGURE 31

Several salivary ducts converging upon one another are seen in this small area obtained from the same specimen as that illustrated in Fig. 29. Mitochondria are clearly defined even at this low magnification. × 400.

FIGURE 32

An enlargement of an area framed near the left center of Fig. 29. An especially well delineated serous cell is seen above and to the left of center (S). A good example of a filamentous mitochondrion is seen above the unstained oval nucleus of the indicated cell. The cells of the salivary ducts do not participate in protein synthesis, yet their concentration of mitochondria is far more impressive than that of either the serous or the mucous cell. The disposition of the mitochondria, in every detail including relationship to infoldings of the cell membrane in the salivary ducts, approximates that seen in the distal convoluted tubules of the nephron, which, of course, subserves important active transport functions. The functional implication of this structural likeness begs experimental intervention. × 1500.

The patchy character of the pattern representing the distribution of glutamic dehydrogenase activity in this specimen of rat liver is a reflection of the penetration rate of formaldehyde. The liver fragments were no larger than 1 mm^3 in size during the 15-minute exposure to 1 per cent formaldehyde in BSS. Incubated for 60 minutes in the glutamate-DPN-TNBT medium. X 50.
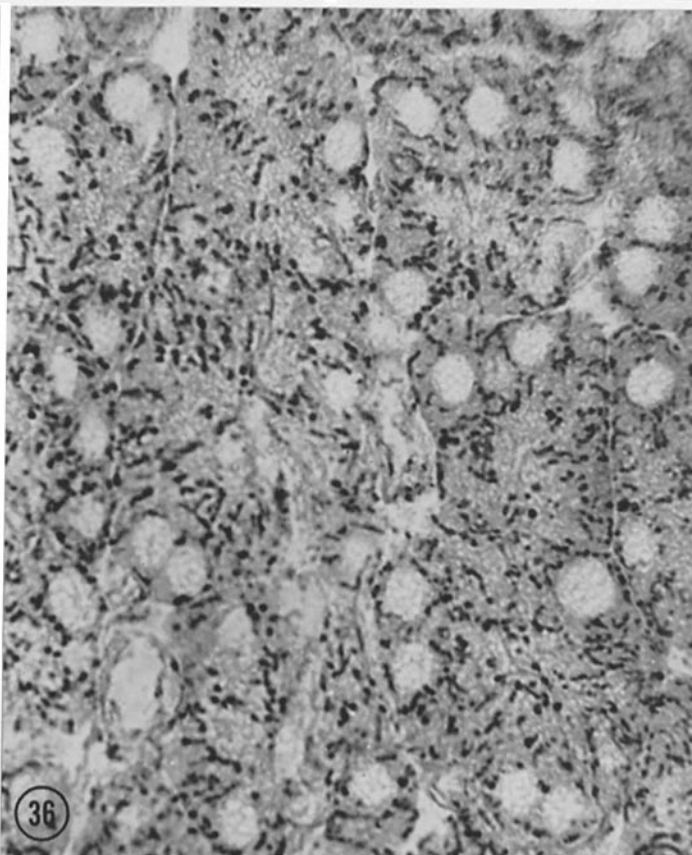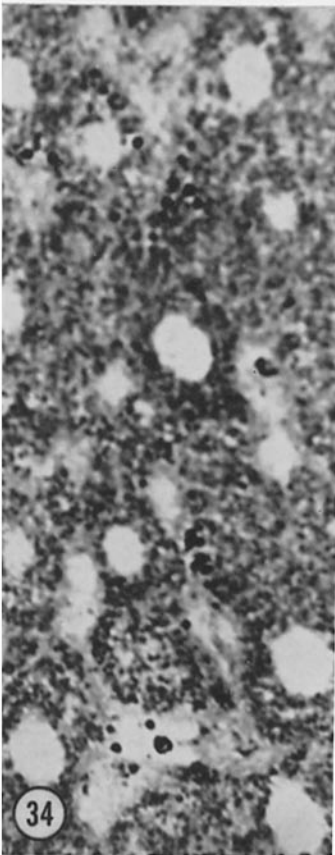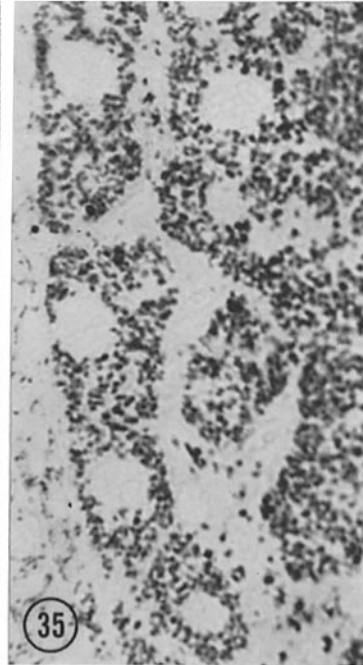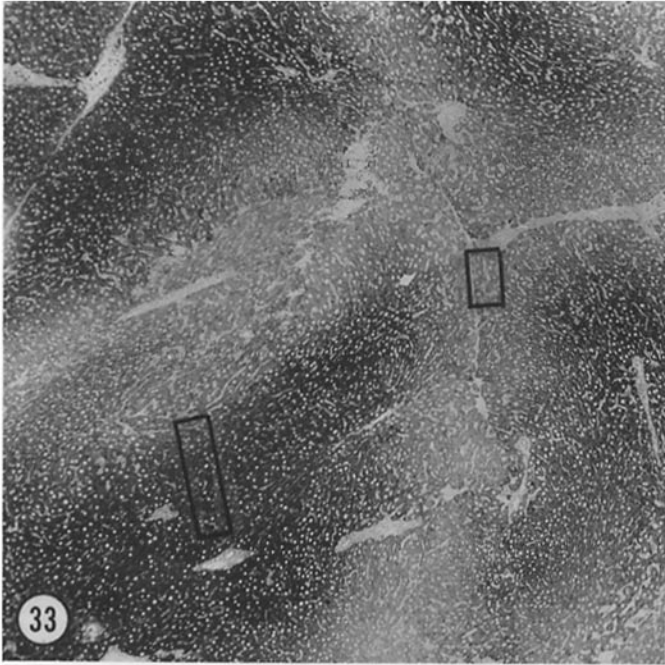
FIGURE 34

The area framed toward the left side of Fig. 33, corresponding to the core of one of the diced liver fragments, is illustrated at a magnification high enough to resolve evidences of mitochondrial damage, including swelling, over- and understaining, and clumping of organelles. A longer exposure to the fixative would have prevented such damage. However, at the periphery of the diced fragments (Fig. 35) signs of enzymatic inhibition may then appear. The duration of exposure to formaldehyde necessary to preserve GDH activity in sections of the liver and pancreatic islet is especially critical. X 1000.

FIGURE 35

The area framed at the right of Fig. 33, corresponding to the periphery of one of the diced liver fragments, has been enlarged to show that the mitochondria are intact. X 1000.

FIGURE 36

Exocrine pancreas after 1 hour in the glutamate-DPN-TNBT medium. X 1000.

FIGURE 37

The mitochondria of the pancreatic islet cells are much more easily damaged than those of acinar cells. This difference in susceptibility to osmotic damage may lead one to believe that a given dehydrogenase of mitochondrial localization in acinar cells is extramitochondrially situated in islet cells. Dicing of the organ into fragments of less than 1 mm³ and agitation during the exposure to chilled 1 per cent formaldehyde in BSS are prerequisites for excellent histochemical dehydrogenase localization. Incubated for 30 minutes in the lactate-DPN-TNBT medium. A, acinar cell; α, alpha cell, β, beta cell. $\times$ 1000.

FIGURE 38

Ventral horn cells of a cervical level of the rat spinal cord are illustrated. The gray matter of the central nervous system is curiously homogeneous in properly prepared TNBT preparations. Since the cell boundaries are not delineated, the presence of the four soma of ventral horn cells is known by virtue of the presence of the unstained nuclei (N). Incubation for 60 minutes in the malate-DPN medium. $\times$ 1000.