MITOCHONDRIAL LOCALIZATION OF OXIDATIVE ENZYMES: STAINING RESULTS WITH TWO TETRAZOLIUM SALTS

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ABSTRACT

A comparison is made of the staining results obtained with Nitro-BT and MTT-Co^{++} as acceptors when DPNH is the substrate in frozen sections of cold formol-calcium-fixed rat kidney (normal and following ligation of the blood vessels) and human liver containing lipofuscin granules. The kidney results are evaluated in terms of mitochondrial morphology seen after classical mitochondrial stains and in electron micrographs. It is concluded that, except for formazan deposition on lipid-aqueous interfaces, Nitro-BT staining indicates the intracellular localization of oxidative enzymes, at least at the level of light microscopy. In contrast, the use of MTT-Co^{++} is not reliable for such intracellular localizations. The deposition of the formazan of MTT-Co^{++} is determined in large part by physicochemical factors other than enzyme localization. Despite marked abnormalities of the mitochondria in cells of the ligated kidney, MTT-Co^{++} formazan is generally deposited in the same dotlike fashion as in cells of normal kidney.

In 1957 Pearse (23) introduced the use of 3-(4,5-dimethylthiazolyl - 2) - 2,5 - diphenyl tetrazolium (MTT) for the localization of oxidative enzymes in tissue sections. Without addition of chelating ions, clusters of formazan crystals rapidly accumulated in the tissue. Cobaltous ions were effective in preventing formation of these needle-like crystals; the formazan remained as small dots. Describing the use of cobaltous ions, Pearse (25) writes, "Thus a capture reaction has been established which prevents crystallization and which presumably also prevents diffusion of the free formazan."

From observations with the MTT-Co^{++} procedure, all with unfixed cryostat-cut sections, Pearse and his colleagues were led to far-reaching conclusions. These included the following: (*a*) that the cristae were arranged within mitochondria not in the fashion described by electron microscopists but as "a single continuous spiral" (26); see also (28); (*b*) that "if a single mitochondrion in an organ, or at least in a section of that organ, is abnormal one can demonstrate this fact and determine to some extent the metabolic direction of this abnormality" (24); and (*c*) that this technique provided "a simple and reliable method for demonstrating the mitochondrial population of mammalian, invertebrate and plant cells. Nobody should further consider for an instant the employment of older staining techniques for these organelles" (24).

By proper choice of material it is possible to put such claims for intramitochondrial localizations to critical test. The cells in the distal and proximal convolutions of the renal tubules in the rat are ideal for unequivocal identification of mitochondria. The mitochondria are long thick

threads which may be converted to spherical form by simple surgical procedures. In frozen sections of tissue fixed in cold formol-calcium, mitochondrial form is well preserved, in marked contrast to their generally poor preservation in unfixed cryostat-cut sections. The tetrazolium salt, 2,2′ - di - *p* - nitrophenyl - 5,5′ - diphenyl - 3,3′ - (3,3′ dimethoxy - 4,4′ - biphenylene) ditetrazolium dichloride (Nitro-BT or NBT), of Tsou *et al.* (30) and Nachlas *et al.* (10) provides an excellent standard of comparison since with this salt the formazan apparently forms within the mitochondria, thus "staining" them to make their forms evident (20, 16). Frozen sections of cold formol-calcium-fixed tissue still show reduced diphosphopyridine nucleotide (DPNH) - tetrazolium reductase and reduced triphosphopyridine nucleotide (TPNH)-tetrazolium reductase activities (21, 20).[1] Since there is relatively little ergastoplasm in the cells of the distal and proximal convolutions, the formazan-stained mitochondria are not obscured by nonmitochondrial formazan (17, 18).

MATERIALS AND METHODS

Kidneys were rapidly removed from decapitated 150 to 250 gm. Sprague-Dawley rats of either sex. Slices were fixed overnight in cold formol-calcium (1). In some instances, the capsule was stripped from the left kidney and its artery and vein ligated. Rats were killed at 30 minutes, 2 hours, 7 hours, and 12 hours after surgery. Both ligated and unligated kidneys were fixed in cold formol-calcium and parallel frozen sections were incubated in the DPNH-Nitro-BT and DPNH-MTT-Co^{++} media. In experiments performed for other purposes, sections were prepared from rats killed from 15 minutes to 7 days after surgery and were incubated in the DPNH-Nitro-BT medium.

Sections, usually 10 μ, were cut into cold formol-calcium with the Bausch and Lomb freezing microtome. They were rinsed in cold water and incubated for 15 and 30 minutes in the Nitro-BT medium and 30 and 60 minutes in the MTT-Co^{++} medium.

DPNH-Nitro-BT Medium: 4 mg. DPNH (Sigma Chemical Company, St. Louis); 1.2 ml. H_2O; 0.8 ml. 0.1 M phosphate buffer, pH 7.4; and 1 ml. Nitro-BT, 1 mg./ml.

The Nitro-BT used in these experiments were commercial samples purchased from Dajac Laboratories, Philadelphia, and a purified sample generously given us by Dr. A. M. Seligman. Identical results were obtained with both.

DPNH-MTT-Co^{++} Medium: 7 mg. DPNH (Sigma Chemical Company), 8 mg.; 0.85 ml. H_2O; 0.15 ml. 0.5 M cobaltous chloride; 2.5 ml. 0.1 M "tris" buffer, pH 7.2, and 1.5 ml. MTT, 1 mg./ml.

The MTT was a generous gift of Dr. A. G. E. Pearse.

Mitochondria were isolated from polyvinyl pyrollidone (PVP)-sucrose homogenates (13) of rat liver and kidney. They were observed with the phase-contrast microscope immediately after mixing with each of the following media: (*a*) DPNH-MTT-Co^{++}, (*b*) DPNH-Nitro-BT, (*c*) as (*a*), with succinate (0.05 M) substituted for DPNH, and (*d*) as (*b*), with succinate substituted for DPNH.

Several approaches were tried to learn the significance of the deposition on lipids of the MTT-Co^{++} and Nitro-BT formazans, using frozen sections of formol-calcium-fixed unligated and ligated kidneys and of Novikoff hepatoma (the cells of which contain many lipid droplets (12, 19)); and smears of the Novikoff ascites hepatoma cells (4) (which also contain numerous lipid droplets), both unfixed and fixed for 2 minutes in cold formol-calcium. The following procedures were used: (*a*) the usual one in which sections and smears were incubated in the tetrazolium medium, following rinsing in water, and were mounted in glycerogel, following rinsing in water; (*b*) the sections and smears were first treated with acetone (2-4°C.) for 15 minutes (15), incubated, and mounted in glycerogel; (*c*) the sections and smears were first incubated, then dehydrated in graded alcohols, treated with acetone for 5 or 15 minutes at room temperature[2], and mounted either in permount, after clearing in xylol, or in glycerogel, after treatment with 50 per cent acetone and water; (*d*) staining of unincubated and incubated sections and smears, with and without pretreatment or after treatment with acetone as indicated above, with oil red O in triethyl phosphate, according to Gomori (8); (*e*) kidney sections and ascites tumor smears were heated in boiling water for 5 minutes and then incubated in DPNH-Nitro-BT medium; (*f*) heat-inactivated sections and smears were flooded with either DPNH-cytochrome c reductase from pig heart[3] or bacterial

[1] Cytochemical localizations have been described in the cells of different organs, using frozen sections of formol-calcium-fixed tissue, Nitro-BT as acceptor, and DPNH and TPNH as substrates (14–19, 3).

[2] Karmarkar *et al.* (9) used treatment of sections for an hour with alcohol-xylol to remove the red monoformazan from sections incubated with Nitro-BT. The work of Glenner *et al.* (7) suggested the use of acetone for this purpose.

[3] We are indebted to Dr. Sasha Englard for a gift of this reductase.

diaphorase (Sigma Chemical Company) for 5 minutes, then rinsed in water, and incubated in the DPNH-Nitro-BT medium; and (*g*) heat-inactivated smears were flooded with the DPNH-Nitro-BT medium and a small quantity of DPNH-cytochrome c reductase was added to produce Nitro-BT-formazan. In addition, lipid was separated from the Novikoff ascites hepatoma[4] and tested both as dried smears and in test tubes, for DPNH-Nitro-BT reductase activity and for its ability to adsorb either pig heart DPNH-cytochrome c reductase or Nitro-BT-formazan produced by addition of reductase to the DPNH-Nitro-BT medium.

Classical mitochondrial preparations were made from Helly-fixed tissue by staining with iron-hematoxylin. For electron microscopy, the tissue was fixed in cold buffered OsO_4-sucrose (6), dehydrated and embedded in a 5:1 mixture of *n*-butyl-methyl methacrylate. Thin sections were cut in the Servall Porter-Blum or the LKB Ultratome. They were studied and photographed with the RCA EMU-3B microscope.

RESULTS

Figs. 1 and 2 show the elongated mitochondria in the proximal and distal convolutions of the normal kidney and the spherical ones of the ligated kidney, as seen in iron-hematoxylin preparations. The fine structure of these mitochondria in the normal kidney is that described by Rhodin (27) and others. In the cells of both distal and proximal convolutions the cristae are short and many appear to traverse the width of mitochondria. In the ligated kidneys 2 hours after surgery (Fig. 3) the mitochondria are roughly circular in outline. Their cristae are less numerous than in the normal mitochondria. Some appear disrupted, but some at least seem intact and are much longer than in the normal organelles. Although they show variability in details, all mitochondria examined are markedly abnormal. Interesting changes in other organelles are mentioned in the description of Fig. 3; they will be described more fully elsewhere, as will be the mitochondria of kidneys ligated for shorter and longer time intervals.

Figs. 4 and 5 show the formazan deposits resulting from incubation of sections with DPNH-MTT-Co^{++} 2 hours after surgery. They are in the form of small dots in both normal and ligated kidneys. Knowing the location and shapes of the mitochondria in the normal kidney, one may select formazan dots that appear to be oriented along the length of the mitochondria (Fig. 4). However, this is a subjective matter and in many instances one may consider the same dot in different linear arrays, some perpendicular to one another (Fig. 4, arrows). The high degree of uncertainty concerning deductions about mitochondrial form from DPNH-MTT-Co^{++} preparations is emphasized by the ligated kidney sections. Although the mitochondria have rounded out (Fig. 2) and are very abnormal (Fig. 3), the formazan dots show no essential change from normal (Fig. 5). It is no more difficult to find the dots in apparent linear arrays in cells with spherical mitochondria as in cells with elongated mitochondria. At 7 hours following surgery, a few mitochondria may be found which do not show formazan dots in their vicinity; instead they appear to be "stained." Such stained mitochondria are more numerous at 12 hours after surgery, but the sections still show an abundance of formazan dots such as are present in normal kidney sections.

The marked superiority of the DPNH-Nitro-BT preparations for demonstrating the mitochondria is illustrated by Figs. 6 and 7. In the cells of the proximal and distal convolutions of the normal kidney the mitochondria are seen as thick elongated threads (Fig. 6). Two hours after renal vessel ligation they are all roughly spherical (Fig. 7).

As has been noted in Seligman's laboratory, sections incubated in DPNH-Nitro-BT contain a red monoformazan as well as a blue diformazan (30, 11, 9). Our observations, like their earlier ones, create the impression that at least some of the monoformazan in the fixed sections results from relatively slow enzymatic reduction since the mitochondria are distinctly purple in the cells of the proximal convolutions. This contrasts with the blue color in the cells of arterioles, glomeruli, and thick limbs of Henle's loops and distal convolutions; the rate of tetrazolium reduction appears to be higher in these cells than in those of the proximal convolutions. However, Nachlas *et al.* (11) have shown that this is more likely to be due to the presence in the Nitro-BT of a monotetrazolium contaminant which is extremely difficult

[4] The tumor cells, separated from the ascitic fluid by slow-speed centrifugation, were homogenized in distilled water. When centrifuged at 25,000 *g* for 20 minutes in the multispeed attachment of the International Refrigerated Centrifuge, lipid floats to the top of the tube. The lipid was pipetted off and dispersed in distilled water by homogenization.

to remove completely (30, 9). The situation thus resembles that described for neotetrazolium by Burtner *et al.* (5).

When DPNH is oxidized in either Nitro-BT or MTT-Co^{++} medium, formazan deposition occurs at the interfaces between aqueous cytoplasm and lipid droplets. This is seen in the interstitial cells of normal kidneys as well as kidneys of operated animals (Fig. 10) and at the base of the cells in the proximal convolutions (Fig. 11) and other regions of the renal tubule in kidneys of operated animals, and in both solid and ascites tumor cells (Figs. 12 and 13). In the tumor cells it is evident in all but the very small droplets—particularly from the preparations stained in oil red O after incubation—that the formazan is deposited at the edge of the droplet, on or near its surface (Figs. 10, 11, 13). No such deposits form if the lipid is first removed by cold acetone treatment and then the section or smear is incubated in the DPNH-tetrazolium media (Fig. 14). It should be noted that such acetone treatment results in over-all reduction in the intensity of staining in the rest of the cell. In such acetone-treated preparations incubated in the DPNH-Nitro-BT medium, the red monoformazan, removable by acetone, is more evident than in preparations not treated with acetone.

The formazan deposits on lipid, resulting from Nitro-BT reduction, have the deep blue color characteristic of the Nitro-BT diformazan. When incubated sections or smears are treated with acetone, the lipid is removed but not the formazan which had deposited on it (Fig. 15). In striking contrast, when sections or smears incubated in DPNH-MTT-Co^{++} medium are similarly treated with acetone all trace of formazan is removed.

Diformazan deposition on lipid surfaces occurs in a similar fashion in ascites smears incubated without fixation as after formol-calcium fixation.

The lipid isolated from the ascites tumor gives no evidence of DPNH-Nitro-BT reductase activity, either in the test tube or as dried smears. Neither lipid fraction nor boiled kidney sections or tumor smears show evidence of nonenzymatic reduction of Nitro-BT.

Our limited tests give no indication of adsorption of either pig heart reductase or bacterial diaphorase by lipid droplets of kidney or tumor. Nor do they give unequivocal evidence that the droplets will adsorb Nitro-BT formazan. When the isolated lipid fraction is dispersed in the DPNH-Nitro-BT medium and pig heart reductase is added to produce Nitro-BT formazan, the lipid droplets remain unstained. If inactivated tumor smears are treated in similar fashion, the lipid droplets are stained only slightly, much less than in the usual active preparations.

A significant difference in behavior of MTT-Co^{++} and Nitro-BT formazans is seen in the lipofuscin-containing liver sections. A few deep blue Nitro-BT diformazan crystals are deposited on the lipofuscin granules (insert, Fig. 8), but there is no appreciable effect on formazan deposition elsewhere in the cells (Fig. 8). With MTT-Co^{++}, clusters of needle-like deposits of formazan are

Explanation of Figures 1 to 7

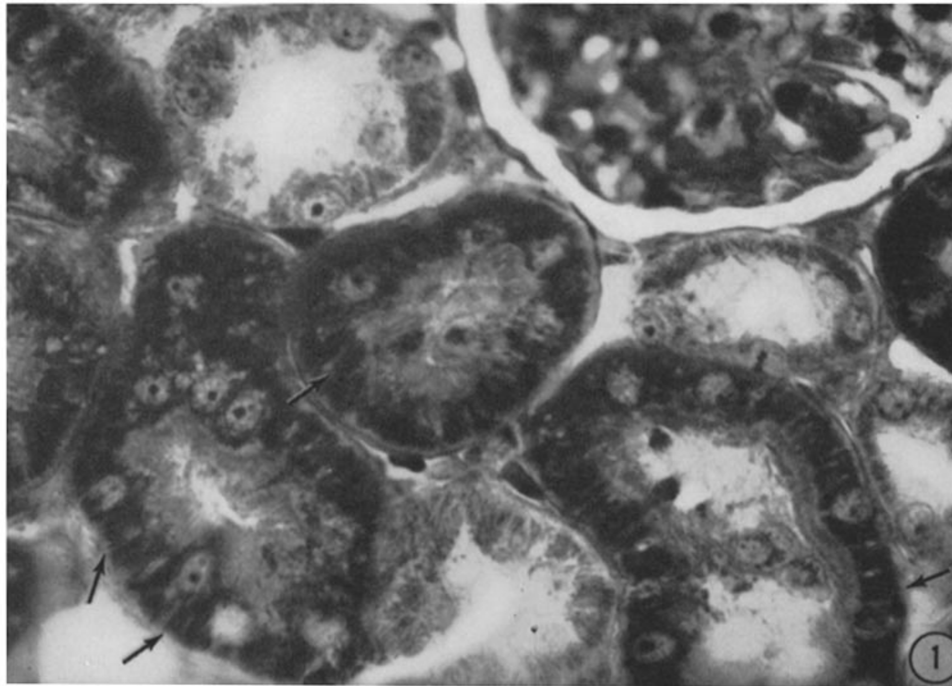
Figs. 1 to 7 show sections of the unoperated and ligated kidneys from one rat, removed 2 hours after unilateral ligation of the renal artery and vein. Figs. 1, 4, and 6 are from parallel sections of the unoperated kidney and Figs. 2, 5, and 7 are from parallel sections of the ligated kidney.

FIGURE 1

Helly-fixed, hematoxylin-stained section of unoperated kidney. × 880. The elongated mitochondria are more readily distinguished in the more lightly stained distal convolutions. They may also be seen in favorable areas of the proximal convolutions (arrows).

FIGURE 2

Helly-fixed, hematoxylin-stained section of ligated kidney. × 880. Note the rounding of all mitochondria in the tubules. The erythrocytes in the peritubular capillaries are stained black. Below the glomerular tuft and within Bowman's space is a portion of proximal convolution (*pc*), as described by Wachstein and Meisel (31).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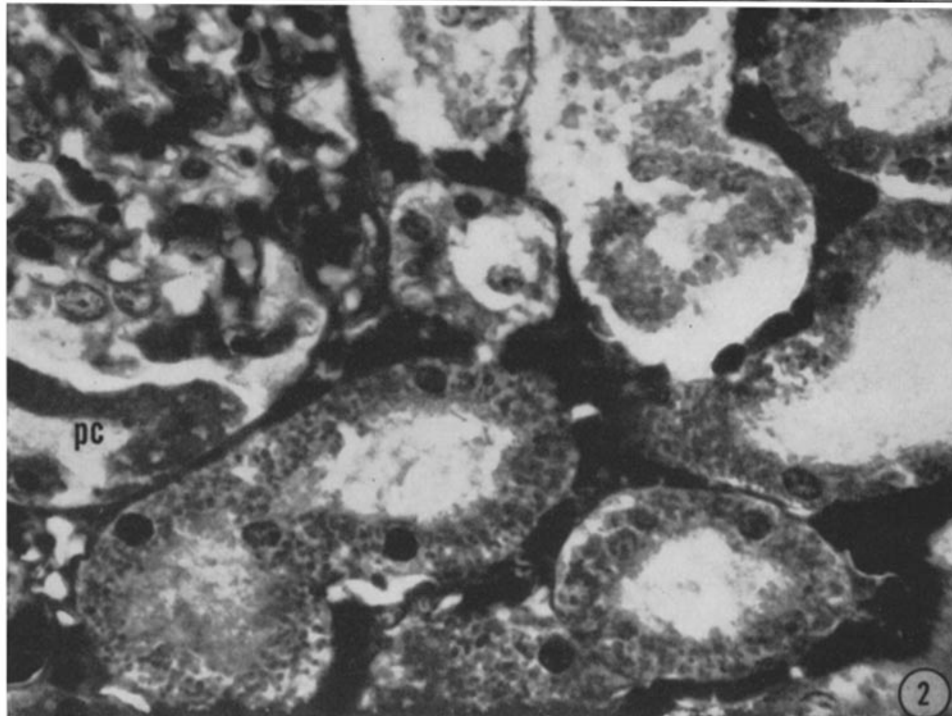
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present in lipofuscin-containing cells. These clusters are surrounded by zones free of formazan. In cells without visible lipofuscin, there are the usual formazan dots (Fig. 9). Presumably, formazan was produced in all cells but that near lipofuscin granules diffused to foci of precipitation.

Different results are also encountered when isolated mitochondria are exposed to MTT-Co^{++} and Nitro-BT media, using either succinate or DPNH as substrate. With Nitro-BT, both liver and kidney mitochondria are stained internally, without evidence of particulate formazan visible with phase-contrast microscopy (20). With MTT-Co^{++}, small formazan spheres are formed on the surface of the mitochondria.

DISCUSSION

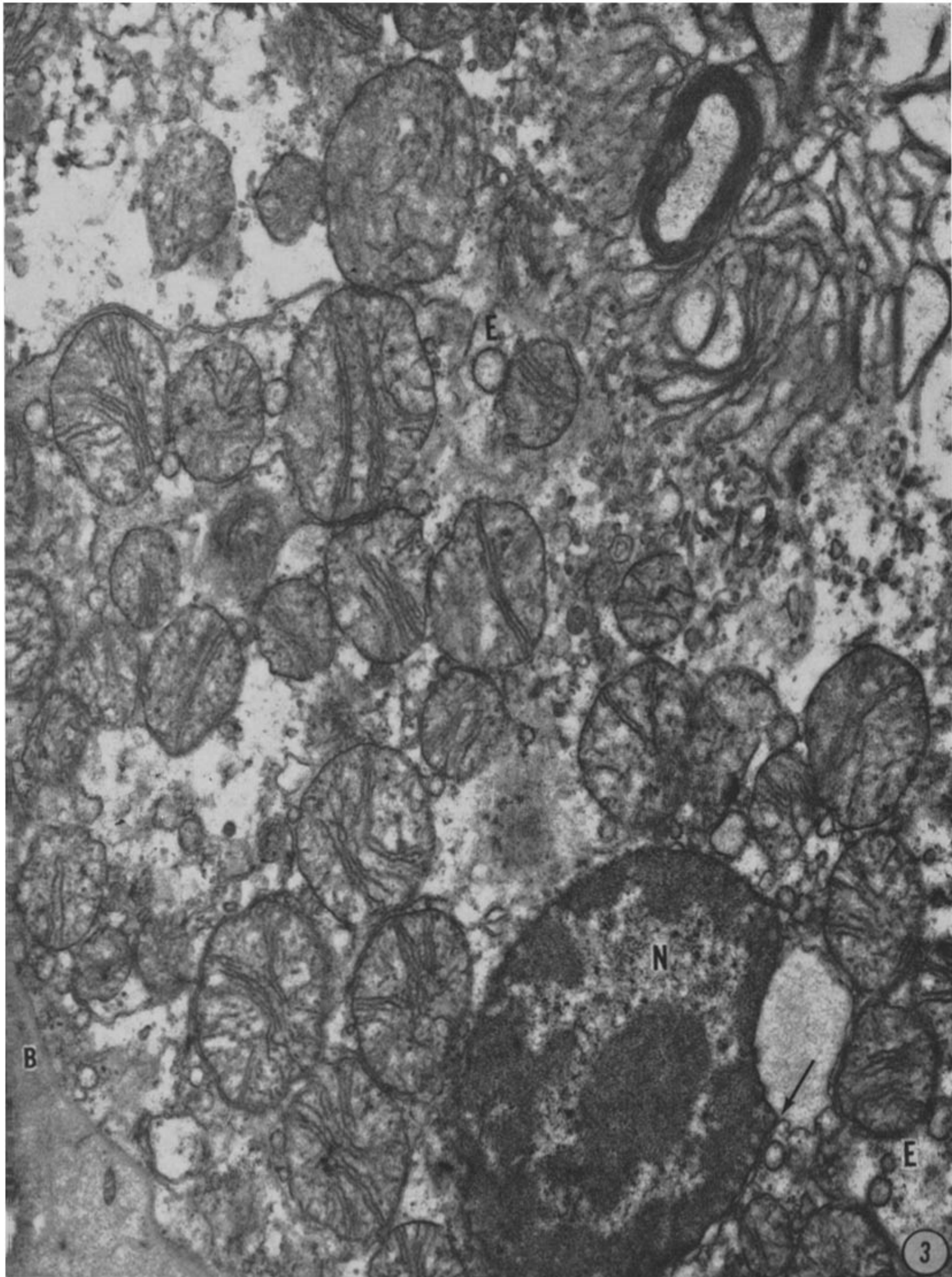
The results presented indicate the hazards in deducing the form of mitochondria from the formazan deposits in MTT-Co^{++} preparations. In the experiments here reported, the subjective element has been eliminated from assessment of mitochondrial morphology. This was achieved by fixing the tissue so as to preserve the organelles in their original shapes, as elongated thick threads in the normal cells of distal and proximal convolutions of the rat kidney and as spheres in cells of kidneys in which the blood vessels were ligated for 2 hours. Irrespective of mitochondrial shape, elongated or spherical, the formazan appears as small dots which may sometimes seem to be arranged in linear fashion. The same dots may appear to be in two different linear arrays perpendicular to each other. Manifestly both arrays can not be mitochondria if the dots are intramitochondrial, and it is reasonable to conclude that the dots are not in the mitochondria at all but are above them. In discussing the hazards in deducing acid phosphatase localization from the variable distribution of lead phosphate precipitate, Pearse (25) suggests that the variability of precipitate is dependent "on physicochemical conditions rather than on enzyme distribution." This appears to apply to MTT-Co^{++} formazan deposition. As dramatic as the unchanged appearance of the dots despite the change in mitochondrial shape is the observation of apparent diffusion of formazan toward the lipofuscin granules in the cells of human liver, resulting in clusters of formazan crystals surrounded by areas without visible formazan.

It may be suggested that considerations arising from our observations are irrelevant to the claims of Pearse and colleagues regarding intramitochondrial localizations, since their contentions are based on the behavior of unfixed rather than fixed tissue. There is, however, little other than formal merit to support this suggestion. The photographs of normal kidney tubule cells published in numerous publications from the Pearse laboratory show essentially the same distribution of formazan dots as we have found; the subjective element is decisive in deducing where the mitochondria lie. No critical tests such as that described in this paper have been performed by Pearse and colleagues. There has been no direct demonstration of the mitochondrial morphology in the cryostat-cut sections against which the formazan deposits could be assessed. Furthermore, our studies with mitochondria isolated from PVP-sucrose homogenates of liver and kidney

FIGURE 3

Portion of a cell in the proximal convolution of the ligated kidney. × 20,500. In the normal kidney, the mitochondria in the proximal convolutions are long and narrow. Their numerous cristae are short and many appear to traverse the mitochondria (27). In contrast, the mitochondria in this ligated kidney appear roughly circular in outline. Their cristae are less numerous and far more variable than in normal mitochondria. Some appear disrupted. Some, however, appear much longer than those of normal mitochondria. The possible implications of this finding will be discussed elsewhere.

Other changes of interest include: (*a*) the formation of lamellated structures ("myelin forms") from the brush border and associated membranes (upper right hand corner of figure); (*b*) a separation of the two nuclear membranes (arrow) by a material of low electron opacity which has the same appearance as the material in small vesicles, apparently swollen granular endoplasmic reticulum or ergastoplasm (*E*); and (*c*) the condensation of chromatin in the nucleus (*N*). A small part of the basement membrane, cut tangentially, is seen at *B*.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demonstrate tnat the MTT-Co^{++} formazan resulting from succinate or DPNH oxidation is deposited on the outside of the mitochondria. The results resemble those with earlier tetrazolium salts, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) (20), 2,2′,5, 5′-tetraphenyl-3,3′-(*p*-biphenylene) ditetrazolium dichloride (NT) and others (29), and differ from Nitro-BT where the mitochondria are stained internally without formation of crystals visible at the light microscope level (20). It may be contended that the difference in formazan deposition with MTT-Co^{++} and Nitro-BT in these isolated mitochondria and in sections and smears signifies a difference in number of reactive sites along the mitochondrion, the dots reflecting the presence of relatively few and separate sites that are active with MTT-Co^{++} and the uniform deposition of Nitro-BT formazan resulting from the abundance of closely spaced reactive sites with Nitro-BT. There is, however, no evidence for this contention. Evidence against it may be seen in the observations that: (*a*) the MTT-Co^{++} formazan is outside the mitochondria whether the mitochondria are isolated or inside cells; (*b*) the disposition of the MTT-Co^{++} formazan is essentially unchanged in the greatly altered mitochondria of the ligated kidney; (*c*) the MTT-Co^{++} formazan apparently diffuses toward the lipofuscin granules; and (*d*) the MTT-Co^{++} formazan is readily soluble in lipid solvents like acetone. In contrast to its ineffectiveness in removing the diformazan of Nitro-BT, all traces of MTT-Co^{++} formazan are removed by acetone. This emphasizes the absence of "substantivity" of the MTT-Co^{++} formazan, and may help explain the ease with which this formazan changes its distribution.

Pearse and colleagues have made many observations of what are considered "abnormal" or unusually "sensitive" mitochondria. It is, however, probable that "the truly remarkable sensitivity of mitochondria to apparently trivial alterations in their environment" (25) reflects changed physicochemical conditions affecting the nature of formazan deposits. Perhaps with more drastic change in the mitochondria, such as occur 7 and 12 hours or more after ligation, these conditions are altered enough so that some mitochondria no longer form formazan dots and instead appear stained by the formazan.

It is clear that the Nitro-BT diformazan faithfully delineates the mitochondria in both normal and ligated kidneys. Our studies, however, have revealed a source of artifact, its deposition on lipid-aqueous interfaces. Although it does not form large clusters in or near the lipofuscin granules of the liver cells, some formazan is deposited on or near these granules (insert, Fig. 8). It is also deposited on the lipid droplets seen in the rat kidney, those present normally in the interstitial cells of the medulla and those which appear at the base of the tubules following surgery (Figs. 10 and 11), and on the lipid droplets of tumor cells (Fig. 13), and many other cells (19, 15). Pearse (25) is of the opinion that Nitro-BT formazan "appears to be completely insoluble in those lipids normally found in the tissues." He suggests that when Nitro-BT formazan deposits do occur in lipid, it "is due to reduction of absorbed tetrazolium by the autoxidation of unsaturated fats." This explanation appears to be excluded by our observation that formazan does not appear in control sections incubated without substrate. Unless enzymatically produced formazan is present, these lipid structures do not possess formazan deposits.

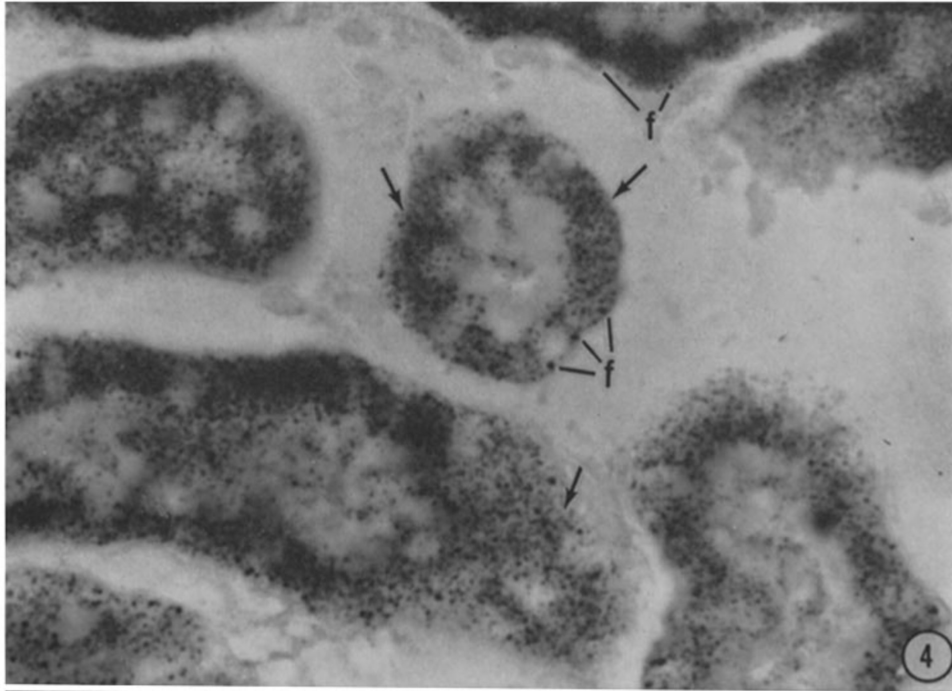
It is possible that formazan deposits on lipid-

Figure 4

Frozen section of formol-calcium-fixed unoperated kidney incubated in DPNH-MTT-Co^{++} for 30 minutes. × 880. Note the formazan deposition as small dots. Apparent linear arrays of the dots may be seen in many places. Frequently, the same dots may be thought of in two linear arrays perpendicular to one another (arrows). Formazan on lipid droplets is seen at *f*.

Figure 5

Frozen section of formol-calcium-fixed ligated kidney incubated in DPNH-MTT-Co^{++} for 60 minutes. × 880. Note similarity of formazan deposition in this and Fig. 4. Because the sections of some tubules are thinner in this kidney, the formazan dots are more readily seen. Note (arrows) apparently linear arrays of dots. As in Fig. 4, the same dots may often be considered in two arrays perpendicular to one another.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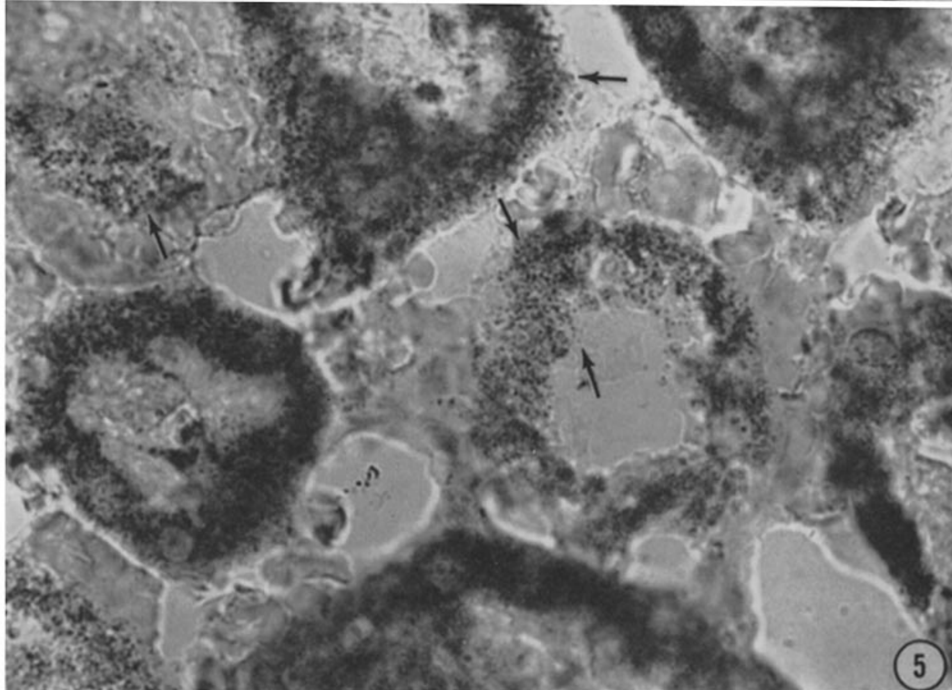
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water interfaces result from enzyme localized in the lipid. However, this seems unlikely from our observation that lipid droplets isolated from the tumor cells show no DPNH-Nitro-BT reductase activity. The possibility that the lipid droplets may adsorb enzyme, particularly in cryostat-cut unfixed tissue, needs to be entertained; our inability to show adsorption of pig heart reductase or bacterial diaphorase under our conditions does not exclude this possibility. At present it seems most likely that the interphases between lipid droplets and surrounding cytoplasm act as foci of precipitation for the Nitro-BT diformazan in the same fashion as the lipofuscin granules do. This would account for the surface localization of formazan, clearly evident in all lipid droplets studied with the exception of the smallest droplets in tumor and kidney cells. It might also account for the inability of the isolated tumor lipid to adsorb formazan. Our inability to obtain striking formazan adsorption in the tumor cells may be due to suboptimal rates of formazan diffusion into the cells.

Other effects of physicochemical conditions on the Nitro-BT deposits, if there be any, are not apparent at the level of light microscopy. Deductions concerning intracellular localization of oxidative enzymes are thus possible, if the organelles are well preserved (as in formol-calcium-fixed tissue) and if the enzymes involved are not free to diffuse from their sites in the cell. Elsewhere (20, 15–17, 19), we consider the problems created by such diffusion in the case of many DPN- and TPN-dependent dehydrogenases.

For electron microscopy, the requirements with regard to morphological integrity and diffusion of either enzyme or formazan are more rigid. Encouraging beginnings have been made with cardiac muscle by incubating small blocks of unfixed tissue (2), but even with this tissue the extent to which formazan deposits coincide with intramitochondrial loci of enzyme is not yet clear. The usefulness of Nitro-BT (2), and possibly its iodo derivatives (9), for establishing intramitochondrial localizations may be seriously limited by the readiness with which the diformazan apparently moves to lipid-aqueous interfaces. Such attraction to interfaces may prove as restrictive for electron microscopy as formazan solubility in lipid was for light microscopy with the early tetrazolium salts.

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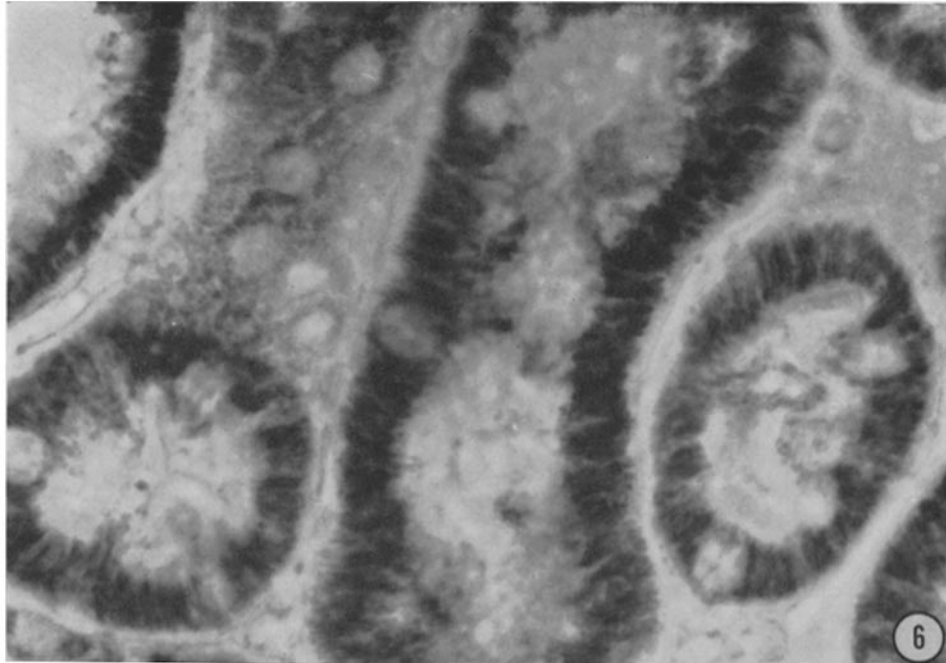

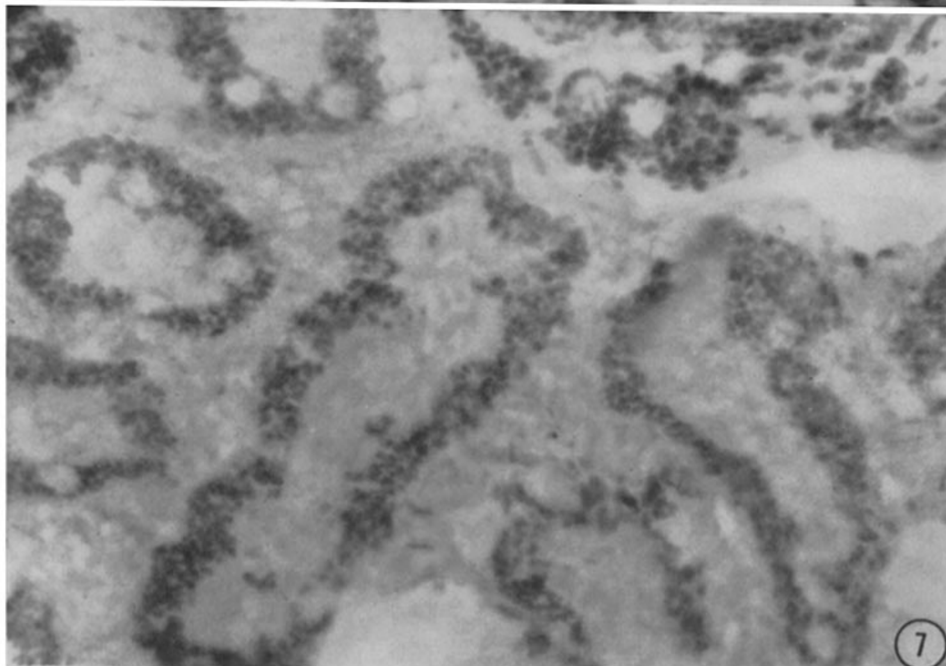

FIGURE 6

Frozen section of formol-calcium-fixed unoperated kidney incubated in DPNH-Nitro-BT for 15 minutes. × 880. At the upper left part of the photograph is a section of a distal convolution; the other sections are of proximal convolutions. Note the elongated mitochondria in both proximal and distal convolutions. Compare with Fig. 1.

FIGURE 7

Frozen section of formol-calcium-fixed ligated kidney incubated in DPNH-Nitro-BT for 15 minutes. × 880. The sections at upper right and left of the photograph are of distal convolutions; the others are of proximal convolutions. Note the rounded mitochondria. Compare with Fig. 2.

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Figure 8

Frozen section of formol-calcium-fixed human liver incubated in DPNH-Nitro-BT for 30 minutes. × 490. Because of the dark formazan deposits in these cells, the lipofuscin granules are not readily seen except in places (arrows).

Insert, cells from another specimen of human liver incubated in DPNH-Nitro-BT for 30 minutes. × 1,250. Note formazan deposits in the lipofuscin granules (arrows).

Figure 9

Parallel section to Fig. 8, incubated in DPNH-MTT-Co^{++} for 60 minutes. × 490. Note the large needle-like clusters of formazan. These are surrounded by areas from which the dotlike deposits seen elsewhere are absent.

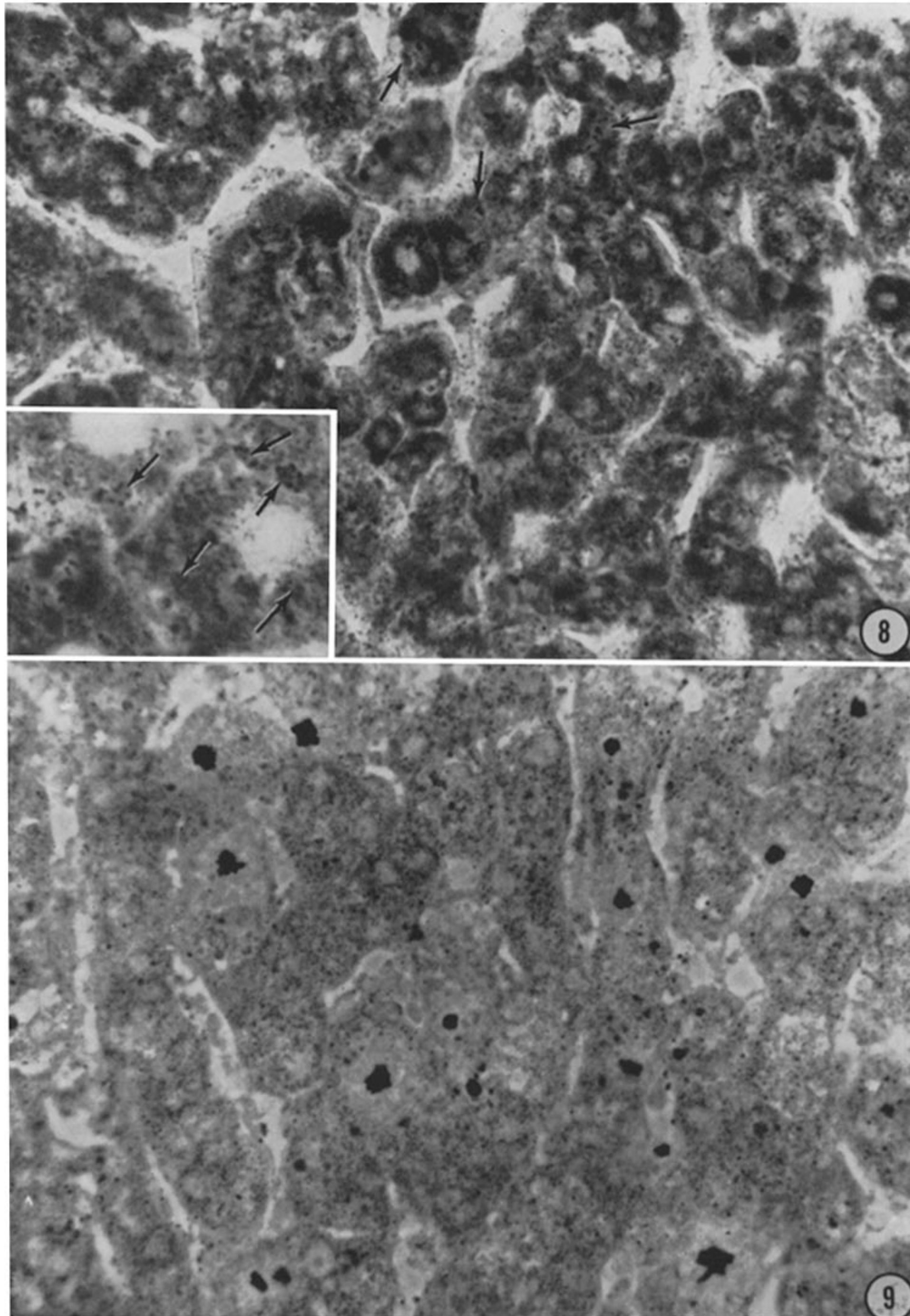
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Figure 10

Frozen section of formol-calcium-fixed unoperated kidney, 7 hours after unilateral ligation of the renal artery and vein, incubated in the DPNH-Nitro-BT medium for 15 minutes and stained with oil red O. Inner medulla. × 650. The blue diformazan deposits appear black in the photograph. They are deposited on the lipid droplets, mostly in the interstitial cells (18). The blue diformazan is readily distinguishable from the red-stained lipid in the preparation but in the photograph it is difficult to distinguish the gray lipid areas and black diformazan, even at arrows.

Insert, an area containing the lipid droplet indicated by the arrow with the letter *i*; both lipid (lighter) and diformazan (darker) are visible. × 1,300.

Figure 11

Same preparation as in Fig. 10. Cortex. × 650. See Fig. 10 for comments on lipid and diformazan, and for explanation of insert.

Figure 12

Formol-calcium-fixed smear of Novikoff ascites hepatoma, stained with oil red O. × 570. Note the variability in the size of lipid droplets. This preparation had more of the larger droplets than usually.

Figure 13

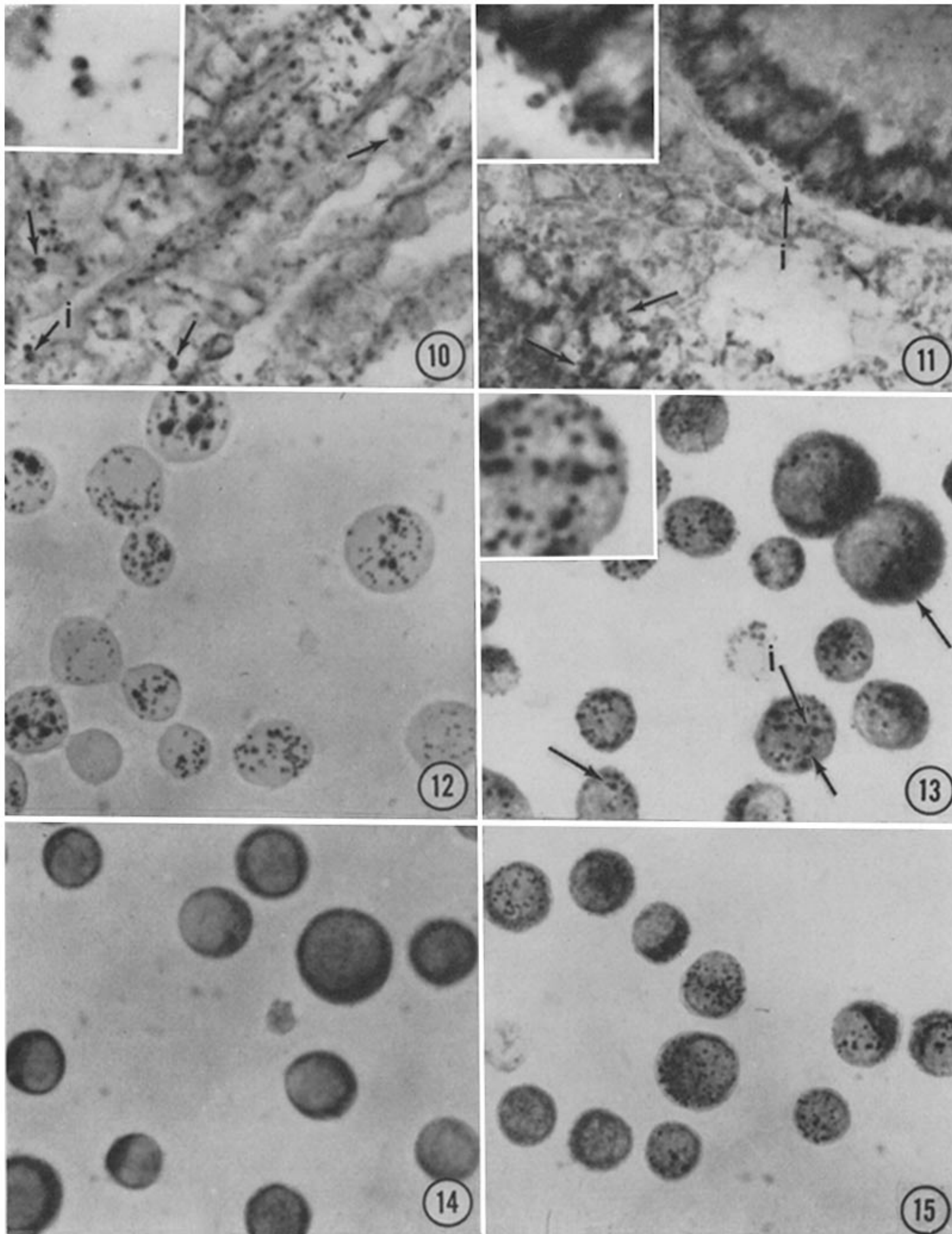
Formol-calcium-fixed smear of Novikoff ascites hepatoma, incubated in the DPNH-Nitro-BT medium for 15 minutes and stained with oil red O. × 570. See Fig. 10 for comments on lipid and diformazan, and for explanation of insert, the magnification of which is × 1,400.

Figure 14

Formol-calcium-fixed smear of Novikoff ascites hepatoma, treated in cold acetone prior to incubation for 15 minutes in the DPNH-Nitro-BT medium. × 570. With the removal of lipid from the cells, the formazan is no longer concentrated locally; instead, it has a fairly uniform distribution in the cytoplasm.

Figure 15

Formol-calcium-fixed smear of Novikoff ascites hepatoma, incubated in the DPNH-Nitro-BT medium for 15 minutes, then dehydrated in alcohols, treated with acetone and cleared in xylol. × 570. The blue deposits of diformazan, black in the photograph, have remained despite the removal of the lipid droplets.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