

THE USE OF TETRANITRO-BLUE  
TETRAZOLIUM FOR THE  
CYTOCHEMICAL LOCALIZATION  
OF SUCCINIC DEHYDROGENASE

Cytochemical and Cytological Studies  
of Sarcoma 37 Ascites Tumor Cells

CHARLES G. ROSA, Ph.D., and KWAN-CHUNG TSOU, Ph.D.

From the Daniel Baugh Institute of Anatomy, Jefferson Medical College of Philadelphia, and  
Central Research Laboratories, Borden Chemical Company, Philadelphia

ABSTRACT

Succinic dehydrogenase (SDH) was localized in the mitochondria of Sarcoma 37 ascites tumor cells by the use of tetranitro-BT (TNBT) and nitro-BT (NBT) in smear preparations. Results with each tetrazolium salt as electron acceptor were evaluated with respect to: (a) size and shape of the formazan precipitate relative to standard mitochondrial morphology; (b) crystallization phenomena of reduced dye; (c) lipid adsorption of formazan. The association of formazan- or iron hematoxylin-stained mitochondria with lipid droplets within the cells was investigated, as was also the influence of formalin fixation, with and without cold acetone pretreatment, on mitochondrial morphology and enzymatic staining. Data from these studies appear to indicate that TNBT is more suitable than NBT for use as a cytochemical reagent in oxidative and/or dehydrogenase enzyme histochemistry and cytochemistry.

INTRODUCTION

In 1956, Tsou *et al.* (21) reported the synthesis and cytochemical evaluation of several tetrazolium salts for use in histochemistry as indicators of certain types of oxidative enzymatic activity. As a result of these studies, 2,2'-di-*p*-nitrophenyl-5,5' - diphenyl - 3,3' - (3,3' - dimethoxy - 4,4'-biphenylene)-ditetrazolium chloride (nitro-BT or NBT) was prepared, and this has since played a prominent role in the localization of enzymes such as succinic dehydrogenase (SDH), DPNH- and TPNH-diaphorases or tetrazolium reductases (5-7). In addition to NBT, 2,2',5,5'-tetra-*p*-nitrophenyl - 3,3' - (3,3' - dimethoxy - 4,4'-

biphenylene)-ditetrazolium chloride (tetranitro-BT or TNBT) was synthesized. No extensive investigations or evaluations of the properties of this dye and its use in cytochemistry have been made until relatively recently in brief reports by Pearse and Hess (13), Rosa and Tsou (14), and Velardo and Rosa (22). The last-named workers indicated by illustrations the cytochemical use of TNBT in part of the female reproductive tract.

Studies by Novikoff *et al.* (10) and others (5) have pointed out certain difficulties in using NBT for staining intracellular structures which are in

close proximity to lipid-aqueous interfaces, and the tendency of this particular dinitroformazan (DNF) to crystallize in histochemical preparations has been recognized for some time (12). The present report is centered mainly about these difficulties with NBT, and an attempt is made to show that TNBT is, in spite of certain peculiar features, a more suitable salt and at least in great measure surmounts the above-mentioned artifacts. Comparable studies of the intracellular localization of SDH and mitochondrial staining by the routine morphological methods are presented in order to demonstrate the strong similarity in appearance of the two types of preparations. Finally, a study based on the investigations of Novikoff *et al.* (9, 10) is reported and concerns the use of acetone extraction as a method for checking mitochondrial staining in the absence of lipid.

#### MATERIALS AND METHODS

Sarcoma 37 ascites tumor cells of mice were obtained from host animals purchased from the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine. At about 7 days following the growth of this culture in adult male Swiss albino mice, transfers were made by injecting 0.2 ml of pooled intraperitoneal washings from 6 animals for which about 3.0 ml of sterile saline per animal had been employed. The preparation of films for the studies described below involved the use of the same cell washings that were employed for transferring the cultures at weekly intervals into normal host animals, thereby serving as a physiological check on the viability of the cultures.

Smears were prepared on coverslips or glass slides and dried briefly at room temperatures before treating as follows:

1. Cytochemical demonstration of SDH was carried out by the method of Nachlas *et al.* (5) with employment of either NBT or TNBT as tetrazolium salt. All smears were rinsed briefly in cold Tyrode solution (5°C) for the removal of endogenous substrates, with subsequent incubation in either medium for 15 to 30 minutes at 37°C. Fixation for 15 minutes or so in 0.1 M sodium phosphate-buffered (pH 7.4) 4 per cent formaldehyde was carried out at room temperature after rinsing in the buffer solution. In certain instances glycerogel mounting was used after adequate rinsing in distilled water, but most of the preparations were dehydrated in alcohol and mounted in either Canada balsam or synthetic resin. The disposition of stained material showed no difference when prepared with mounting in balsam, resin, or glycerogel.

The formazan obtained by both enzymatic (14)

and dithionite reduction was brownish black and therefore different from the purple-black product obtained initially by Tsou *et al.* (21) with chemical (bisulfite) reduction of TNBT. At least three possible explanations presented themselves:

- a) Shifting in the appearance of the formazan's color (real or apparent) when observed microscopically in tissue sections. This was excluded since precipitates of dithionite-reduced TNBT also appeared brownish black when inspected at magnifications up to 1000 diameters in air, balsam, resin, or glycerogel. Under such conditions of examination the concentration of the formazan was found not to be a factor in determining the color of reduced TNBT.

- b) Contamination of the samples of TNBT, which are difficult to prepare, with other mono- or di-tetrazoles, the color of which when reduced completely masks the purple-black precipitate of tetranitroformazan (TNF). This possibility was especially appealing in view of previously verified claims by others of contaminant(s) in preparations of the related NBT tetrazole. In apparent support of this hypothesis was the observation that a solvent was found which could differentially extract the brownish black formazan in test tube precipitates and tissue sections. This solvent, *N,N*-dimethylformamide, was completely ineffective, however, in solubilizing TNF, and apparently left the purple-black precipitate intact and undisturbed in tissue sections (SDH reduction) and in *in vitro* experiments (dithionite) with TNBT alone. The drastically different solubilities of both formazans in *N,N*-dimethylformamide not only afforded a means for demonstrating pure TNF, but was highly suggestive of the presence of at least two different tetrazolium compounds in the preparations of the agents used.

- c) The simultaneous presence of completely and partially reduced TNBT was a third alternative solution of this problem. This was tested by two approaches. The first of these experiments involved the use of chromatographically pure samples of TNBT. Enzymatic (SDH) or dithionite reduction of such tetrazoles still showed the presence of at least two separate formazans. Alkaline reduction with ascorbic acid according to the method of Pearse and Hess (13) failed to reproduce these results, however, since all such reduced dyes showed the characteristic color of pure tetranitroformazan. Finally, enzymatically reduced pure TNBT in tissue sections and ascites cells containing both brown and purple-black precipitates of formazan was completely transformed to the characteristic color of tetranitroformazan after ascorbic acid treatment. *N,N*-dimethylformamide extraction of these preparations was completely ineffective in altering the pattern of TNF deposition within the cells and tissues. These data would therefore suggest that TNBT, reduced by dithionite or

SDH, results in the production of at least two formazans; one of these is tetranitroformazan, which is extremely resistant to solution by organic solvents. The other compound(s) are highly insoluble in the general solvents used in both histological and electron microscopical preparations, but are quite soluble in *N,N*-dimethylformamide. Therefore, in the present study of cytochemical preparations, where it was desirable to study *only* TNF produced as a result of SDH activity (see Results), the routine dehydration preceding resin or balsam mounting incorporated two 5-minute "rinses" of *N,N*-dimethylformamide extractions interposed between absolute alcohol and a 50:50 absolute ethanol-xylene bath.<sup>1</sup>

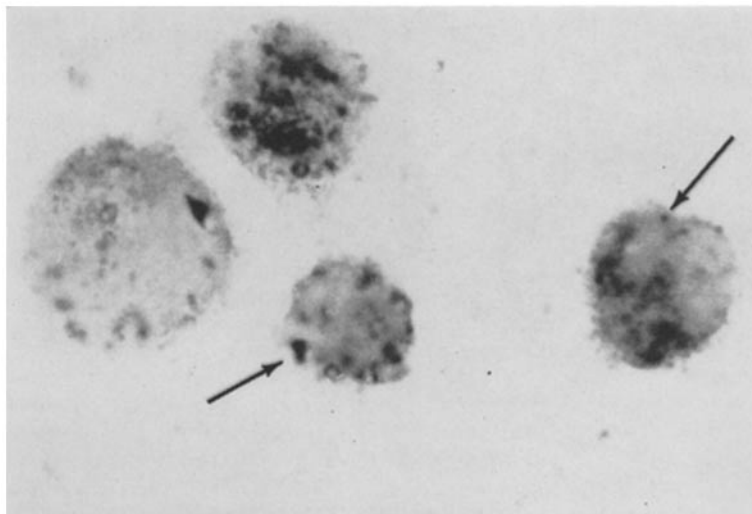


FIGURE 1

Cells containing mitochondria stained with iron hematoxylin. Note variable sizes of these organelles from large (left arrow) to extremely fine (right arrow). The central cells show mitochondria arranged circularly around central small spaces which are probably lipid (right arrow; see Fig. 10).  $\times 1200$ .

2. Sites of intracellular lipid were revealed by either the alcoholic-sudan IV or the triethyl phosphate-oil red O method of Gomori after formalin fixation of fresh smears (2). In addition, lipid and either SDH or mitochondria were demonstrated in the same preparation by subsequent superimposition of the oil red O technique upon the formazan- or iron hematoxylin-stained mitochondria at a stage just prior to mounting in glycerogel.

<sup>1</sup>Migration of the formazan in TNBT-stained, formalin-fixed rat kidney sections some time after mounting in glycerogel has been reported to us (A. B. Novikoff, personal communication). We believe that the *N,N*-dimethylformamide "rinses" with resin mounting subdue this migration in normal kidney.

3. Routine demonstration of mitochondria was attempted through the use of Regaud's iron hematoxylin methods as outlined by Lillie (4). The shortened and/or low temperature versions of the technique were found to be generally satisfactory. The newer method of Bharadwaj and Love (1) was completely unsatisfactory with these tumor cells, with or without any of the procedures recommended for the removal of background staining.

4. For the purpose of testing the influence of cold 4 per cent formaldehyde overnight with and without a subsequent 15-minute cold (5°C) acetone extraction, smears were treated according to Novikoff's recommendations (10) followed by each of the

methods described above. The formalin fixation method of Walker and Seligman (23), which preserved some SDH in mitochondria, was also used in an attempt to demonstrate these organelles morphologically. Cold 4 per cent formaldehyde fixation for 5 minutes was used prior to Regaud's fixation or cold acetone extraction followed by Regaud's fixation.

## RESULTS

### *Localization of SDH in Fresh Smears of Ascites Tumor Cells*

The staining method of Nachlas *et al.* (5) was employed for demonstrating SDH without the use of prior cold formalin fixation because the cells showed diminished (23) or no SDH activity

(10) after exposure to formaldehyde (see last paragraph under Results). When NBT was incorporated as the constituent tetrazole, there was a strong reduction in unfixed cells to the corresponding dinitroformazan (DNF) in certain regions some of which corresponded to the locali-

zation of mitochondria (Figs. 1 to 3). Precise localization of formazan, however, showed two general patterns, the first of which included the localization of dye in or at mitochondrial sites. In many of the cells, however, there were very large aggregates of formazan which exceeded

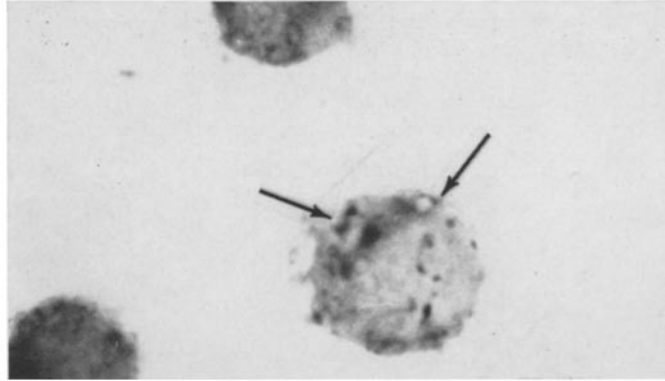


FIGURE 2

Same preparation as Fig. 1. The cell in the center of the photograph contains mitochondria on either side of a clear central space (right arrow). The mitochondrion to the left of the space is large and out of focus, while that to the right is very small. Other mitochondria of variable sizes are in a cluster about a clear space in the upper left of this cell (left arrow).  $\times 1200$ .

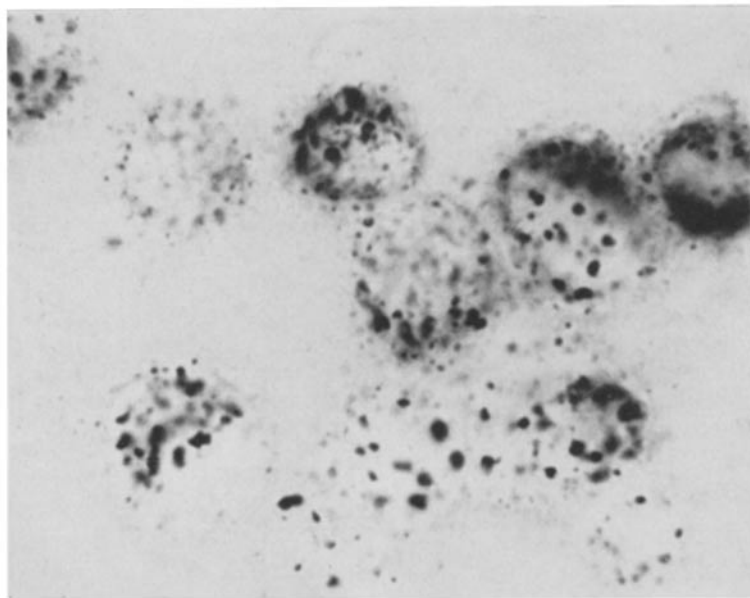


FIGURE 3

Tumor cells stained for SDH with NBT according to Nachlas *et al.* (5). Mitochondria are not situated around any clear central zones. Very large particulate staining is often present as a result of crystal formation of the DNF.  $\times 1200$ .

the general size of mitochondrial bodies and were localized in the close vicinity of lipid droplets. This displacement of formazan due to the adsorption of DNF to lipid-aqueous surfaces was very similar to that reported recently by Novikoff *et al.* (10) and was confirmed in our own experimental results with the use of appropriate counterstaining procedures such as

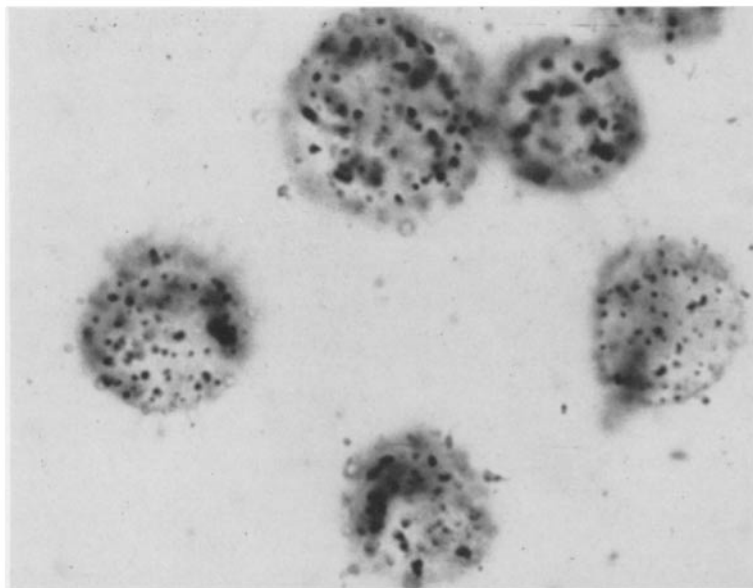


FIGURE 4

Tumor cells treated as above but counterstained with Sudan IV. Dense clumps of DNF are embedded in gray regions which represent lipid. In the cell to the right, and others, note how small mitochondria are retained especially in "clear" regions of the cell where there is no lipid. Excessive crystallization artifact is apparent in areas of moderate to high sudan IV staining.  $\times 1200$ .

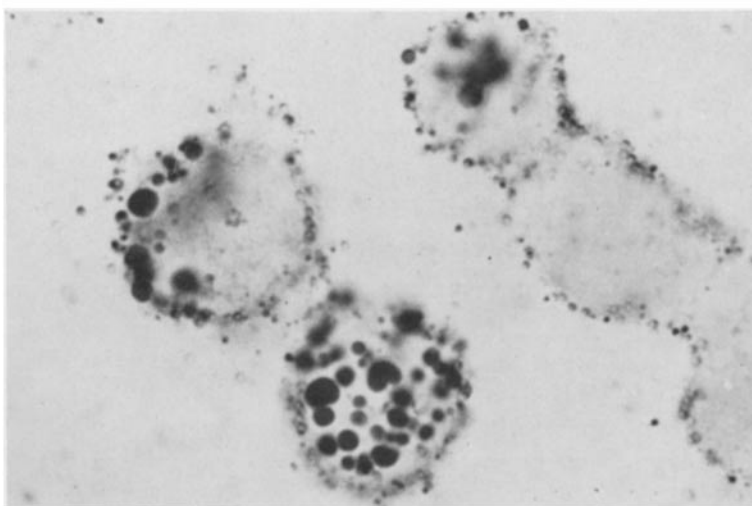


FIGURE 5

Localization of lipid droplets in Sarcoma 37 ascites cells with oil red O. Note great variation in amount and size of lipid spheres in the different cells.  $\times 1200$ .

sudan IV or oil red O. In addition to the above adsorption of formazan, giant crystals of dinitroformazan were found in many instances *within* large lipid droplets. It should be noted that in

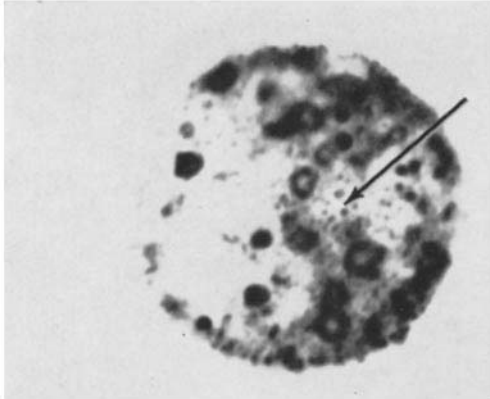


FIGURE 6

A giant tumor cell stained for SDH with TNBT. Note dissimilarity in the sizes of the stained mitochondria, which are in many instances arranged around clear spaces. Very small mitochondria are seen best near the center of the cell (arrow).  $\times 1200$ .

spite of the occurrences of both of the above artifacts (in many cases within the same cell) some precipitated DNF could be found which approximated the size and forms of mitochondria. In such instances, these stained structures (presumably mitochondria or small aggregates of dye) were *never* found in the vicinity of large accumulations of lipid (Figs. 4 and 5).

Localization of SDH with TNBT resulted in the formation of TNF and sufficient quantities of the presumably partially reduced form of TNBT (see Materials and Methods) This imparted to the stained structures a brownish black coloration which completely masked the underlying blue-black precipitate of TNF. The latter was revealed, however, through the use of appropriate DMF extraction procedures (see above), in which case the localization patterns of these forms of reduced TNBT could be compared. They appeared to be similar. Staining of cellular components with this SDH technique resulted in the coloration of structures no larger than those revealed with the conventional iron hematoxylin methods for mitochondria (Figs. 1, 2, 6, and 7). Furthermore, there was a complete

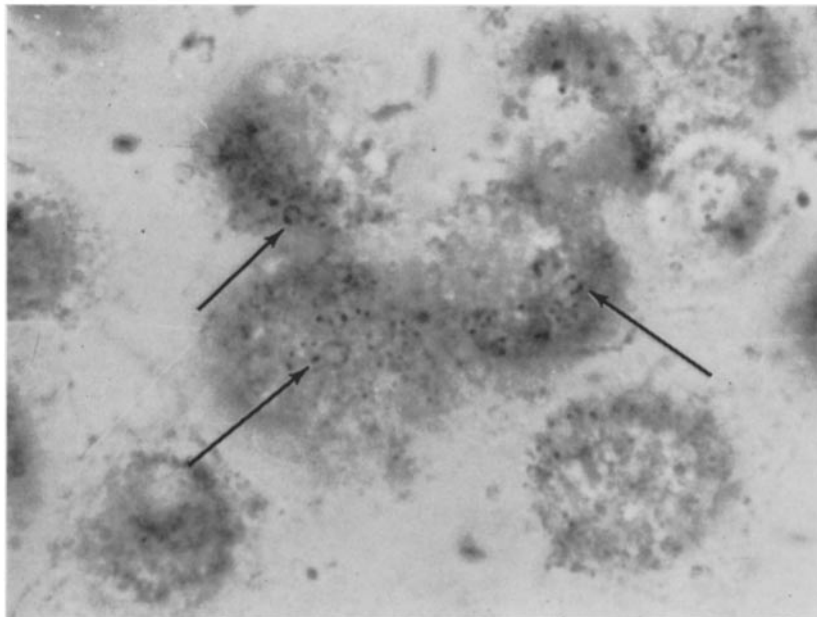


FIGURE 7

Tumor cells fixed in cold 4 per cent formaldehyde and stained for SDH with TNBT. Large and very small mitochondria are present in various cells and some can be found around clear zones (arrows). There is a considerable reduction of enzymatic activity after the fixation procedure of Walker and Seligman (23).  $\times 1200$ .

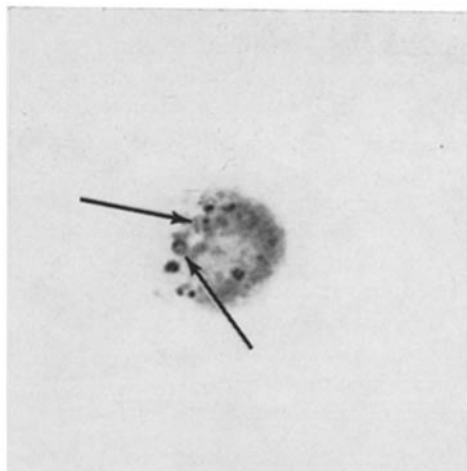


FIGURE 8

Tumor cell stained for SDH with TNBT and counterstained with sudan IV. Note that mitochondria are in close association with a lipid droplet (circular gray zones, arrows).  $\times 1200$ .

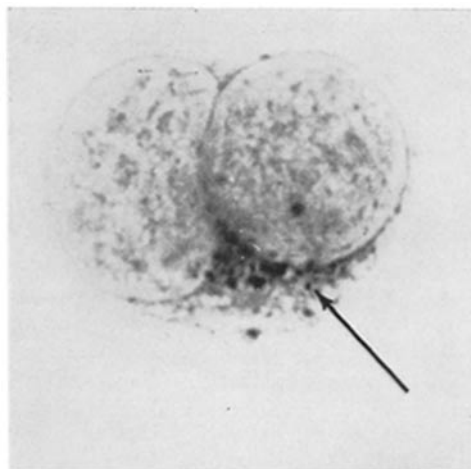


FIGURE 9

Large binucleate tumor cell stained for SDH with TNBT and counterstained with sudan IV. The lower part of the cytoplasm (arrow) contains densely stained mitochondria of very small and moderate dimensions in a region of considerable lipid staining. Note the fineness of the precipitated formazan retained in this lipid zone, a phenomenon never seen within comparable regions in NBT-lipid-stained preparations (see Fig. 4).  $\times 1200$ .

absence of large crystals such as those seen with NBT staining and found to be localized within lipid droplets. Finally, in many instances certain structures which were stained appeared to be in intimate association (contact) with lipid droplets of varying sizes (Figs. 5, 7 to 10). These entities, although showing considerable variation in size and in form, nevertheless closely corresponded to similar structures (mitochondria) seen with the iron hematoxylin methods and phase contrast microscopy. Staining procedures in the former case involved lipid localizations which were superimposed upon iron hematoxylin-stained preparations with subsequent mounting in glycerogel.

#### *Mitochondrial Morphology after Formalin Fixation Without or Following Cold Acetone Extraction*

Ascites cells which were fixed in cold formalin for short periods (10 minutes) or for periods ranging up to overnight, and then processed in the fixatives, mordants, etc., of any of the iron hematoxylin methods, showed in most cases a decreased staining capacity of the mitochondria. In all instances, control studies included films from the same batch of cells and were processed simultaneously through routine procedures for

mitochondria. This lack of staining capacity did not always occur, although in certain cases a distinct decrease in the density of staining and/or absence of a considerable number of stained mitochondria was noted. When acetone extraction (Novikoff's extraction procedure) was interposed between formalin fixation and fixation required for mitochondrial preservation and staining, there was a distinct absence of any mitochondrial staining. Estimates of the completeness of fat extraction were approximated through the use of sudan IV and oil red O staining techniques, by which none of the cells were colored. Our experience in staining such preparations for SDH was similar to that found with the conventional procedures for mitochondria when using Novikoff's procedure. This, unfortunately, precluded the possibility of demonstrating SDH within fixed mitochondria in the absence of intracellular lipids. The technique of Walker and Seligman profitably demonstrated that some SDH is retained after short periods of formaldehyde fixation in spite of the inactivation of considerable enzyme. Acetone extraction after

such fixation, however, also eliminated the possibility of showing stained mitochondria with either TNBT or iron hematoxylin.

#### DISCUSSION

For some time various authors have supported the original claim that NBT can be used as an indicator dye for the discrete intracellular localization of certain oxidative enzymes. Most recently, Novikoff *et al.* have shown that such claims are not necessarily applicable in every instance (10). The use of NBT for the localization of DPNH-tetrazolium reductase (diaphorase) has been shown to produce considerable staining

(SDH) within cells. Furthermore, the solubility properties of TNF are very similar to those of DNF, thereby making feasible its use with the standard reagents involved in resin mounting of specimens or those necessary for methacrylate or Epon 812 embedding procedures.

The experiments with extended cold formalin fixation (overnight), in which the iron hematoxylin staining capacity of mitochondria was found to be absent or decreased, show that this procedure is not compatible with retention of these structures or their stainability in the case of Sarcoma 37 ascites tumor cells. In addition, the adverse influence of formaldehyde on SDH activity has

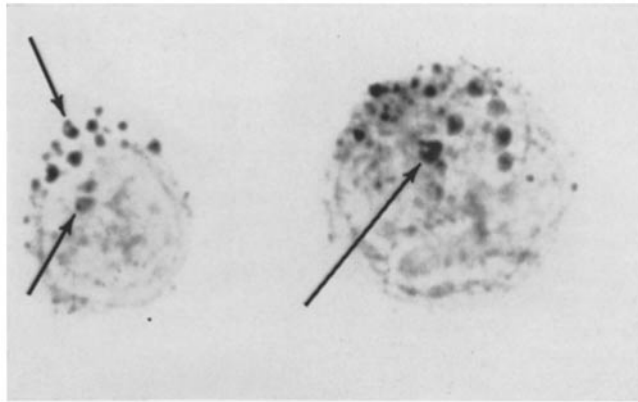


FIGURE 10

Same staining as Fig. 9. In both cells large and small mitochondria (arrows) can be seen in association with lipid-stained spheres (gray).  $\times 1200$ .

artifacts, phenomena referable to gross crystallization in the presence or relative absence (14) of large amounts of lipid. This occurs most strikingly in certain structures such as the zona fasciculata of the adrenal, where an intense adsorption of DNF occurs at the surface of intracellular lipid deposits (8). In view of such circumstances, Sedar and Rosa (18) have pointed out the probability of artifact with employment of NBT in ultrastructural studies where supra- or intramitochondrial deposits of DNF were shown as a result of SDH activity in rat myocardium and other tissues. The data presented in the present report and elsewhere (14, 16, 19, 20) indicate that TNBT used at the light and electron microscopic levels of study appears to eliminate some of the technical barriers imposed by the use of NBT for localizing oxidative enzymatic activity

been repeatedly reported, and a brief study has recently appeared on the kinetics of this enzymatic inhibition (17). It would appear, therefore, that such conditions of prior cold formalin fixation are from an enzymatic and morphological point of view not generally applicable without adequate testing of study materials (23).

Cold acetone extraction after formalin fixation according to Novikoff's method was found to eradicate completely any remnants of iron hematoxylin staining in the tumor cells of the present study. Though this prevents comparison of localizations with NBT and TNBT in the absence of fat, it nevertheless indicates again that such treatment readily alters mitochondrial morphology (staining) in certain instances. The basic tenet in this study is that the varied forms visualized with iron hematoxylin procedures



represent mitochondria; and the data with TNBT staining and phase microscopy lend support to this idea.

SDH staining of mitochondria with TNBT in the absence of lipid is nevertheless an objective which must remain foremost in the attempt to divorce the true mitochondrial SDH patterns from those of possible adsorption artifacts which might be a potential source of dye deposit in the vicinity of lipid. The data presented, however, indicate strongly that mitochondria can and do in many instances maintain positions in close proximity to lipid droplets, a situation also reportedly seen in the normal adrenal by Sabatini and De Robertis (15), or in certain situations of starvation in the pancreas (11). The SDH staining pattern of mitochondria as seen in the present study, therefore, confirms enzymatically what is seen morphologically with iron hematoxylin (tumor cells) or ultrastructurally with the electron microscope (adrenal, pancreas). Thus, there can exist certain regular situations in which formazan (TNF) staining in close association with lipid might represent a real relationship rather than one of artifact. Preliminary studies in our laboratory on mouse and rat adrenals have completely confirmed the electron microscope studies of Sabatini and De Robertis (15) by light microscopic observations of iron hematoxylin- and TNF-stained (SDH) mitochondria which are in intimate association with lipid (oil red O).

It would appear, therefore, that TNBT should be especially useful in studies which involve the localization of enzymes in cells containing considerable amounts of lipid, whether they are solid or ascitic tumor cells such as those considered in the present report, or normal elements such as those found in the adrenal gland, gonads, or adipose tissues. It should be recalled that certain tumors possess considerable quantities of lipid within their cellular mass, thereby necessitating the use of a tetrazole such as TNBT for

accurate localization of enzymes. Especially inviting is the possibility of localizing within specific endocrine cells certain steroid dehydrogenases (3, 24) under conditions of normally changing or induced hormonal environments, or perhaps even during certain pathologic states. Finally, the possibility exists that mitochondrial oxidative enzymes and those associated with the endoplasmic reticulum might be localized with the electron microscope in the absence of relatively gross adsorption artifacts which are associated with the use of NBT as demonstrated in the present report and in the accounts of Novikoff *et al.* (8, 10) and the more recent studies of tetrazole substantivity by Pearse and Hess (13). We are taking these factors into consideration in our current experiments with TNBT (16, 19, 20), by which we hope to elucidate more accurately the localization of oxidative enzymes within cells and cellular organelles.

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