NONDROPLET ULTRASTRUCTURAL DEMONSTRATION OF CYTOCHROME OXIDASE ACTIVITY WITH A POLYMERIZING OSMIOPHILIC REAGENT, DIAMINOBENZIDINE (DAB)

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ABSTRACT

A new method for demonstrating cytochrome oxidase activity, based upon the oxidative polymerization of 3,3′-diaminobenzidine (DAB) to an osmiophilic reaction product, has improved the localization of this enzyme over methods based upon the Nadi reaction, in both the light and electron microscopes. The reaction product occurs in nondroplet form, which more accurately delineates the localization of cytochrome oxidase in mitochondria of heart, liver, and kidney. In electron microscopic preparations the excess reaction product is found to overflow into the intracristate spaces and into the outer compartment between inner and outer limiting mitochondrial membranes. This finding suggests that the enzymatic activity of cytochrome *c* is located on the inner surface of the intracristate space which is the outer surface of the inner mitochondrial membrane. Succinic dehydrogenase activity has also been located at this site by using an osmiophilic ditetrazolium salt, TC-NBT. Considered together, the sites of reactivity of both parts of the respiratory chain have implications for the chemiosomotic hypothesis of Mitchell who suggests a mechanism of energy conservation during electron transport in the respiratory chain of the mitochondrion.

Cytochrome oxidase activity has been demonstrated histochemically in tissue sections with a variety of Nadi reagents (1, 6, 13). The ultrastructural demonstration of cytochrome oxidase activity was made possible by taking advantage of the osmiophilic properties of the indoanilines obtained with the Nadi reagents, purposefully (6) or inadvertently (18). Although with light microscopy, most of these methods gave satisfactory results in heart muscle, it was disappointing to find that all of the reagents gave droplet localization when more closely examined in the electron microscope (6, 8, 18, 20). As suspected with light microscopy and confirmed with electron microscopy, the deposits were related to mitochondria quite regularly in heart muscle, but less so in kidney, and rarely in liver (20).

Although most of the Nadi reagents recom-

mended for cytochrome oxidase do not work well for the demonstration of horseradish peroxidase, we recently found that *N*-benzyl-*p*-phenylenediamine (20) and 1-naphthol histochemically demonstrate horseradish peroxidase very well.[1] On testing these reagents with horseradish peroxidase by electron microscopy we noted the droplet form of the deposit.[1] Comparison of our results with those obtained with the recently introduced method (4) for horseradish peroxidase with the osmiophilic reagent 3,3′-diaminobenzidine (DAB) revealed that the distribution of the reaction product of DAB occurs in an essentially nondroplet form. We attribute the difference in results to the fact that DAB is polymerized on oxidation to a noncoalescing macromolecule (Fig. 1), which is osmiophilic and amorphous and, therefore, more reliably defines the ultrastructural localization of *horseradish peroxidase* activity than do the presently available Nadi reagents. We then observed not only that, under conditions for performing the *cytochrome oxidase* reaction, DAB gave good pigment production of an osmiophilic product, but that, even under light microscopy conditions, the relation of the pigment to mitochondria in heart, liver. and kidney was obvious, in contrast to results with available Nadi reagents. The promise of superior localization in electron microscopy was borne out when fresh or formaldehyde-fixed blocks of these three tissues were tested.

In this paper, preliminary results are presented illustrating the ultrastructural demonstration of cytochrome oxidase activity with the reagent DAB. It will be shown that oxidation of DAB is located on the outer surface of the inner mitochrondrial membrane and that in heavily stained, fresh or formaldehyde-fixed preparations the pigment fills the intracristate space and the outer compartment between inner and outer limiting mitochondrial membranes and does not overflow into the matrical space. Comparison will be made with the localization of succinic dehydrogenase (SDH) activity as revealed with TC-NBT (21); the implication of these results for Mitchell's chemiosmotic hypothesis (11, 12) for the mechanism of energy conservation during electron transport in the respiratory chain of the mitochondrion will be discussed.

[1] Seligman, A. M., A. Abrams, H. L. Wasserkrug, Y. Morizono, R. E. Plapinger, and J. S. Hanker. Manuscript in preparation.

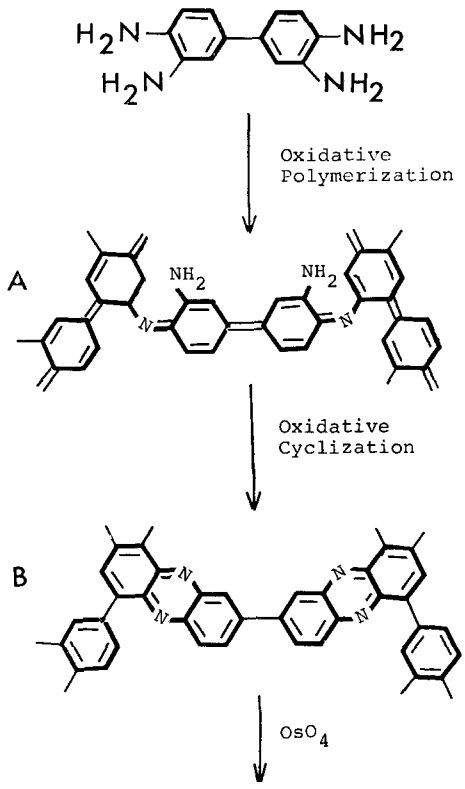


FIGURE 1 Hypothetical formulation of the oxidative polymerization of DAB to an indamine polymer (*A*). This may be followed by further quinoid addition to the primary amine resulting in oxidative cyclization to a phenazine polymer (*B*). This formulation is postulated to explain the absence of primary amino groups in the infrared absorption spectrum of the polymer. That cyclization to a phenazine polymer takes place to a very limited extent is indicated by the strong –NH stretch in the infrared absorption spectrum (see Fig. 2).

METHOD

Preparation of Tissue

Adult Wistar rats and Harvard mice were sacrificed by a blow to the head; the heart, liver, and kidney were obtained from the former and the kidney was obtained from the latter. The tissues were immediately cut with a razor blade into blocks 1 mm^3. Some of the blocks were used fresh, and others were fixed for 50–60 min in cold 4% formaldehyde, prepared by depolymerization of paraformaldehyde with alkali and neutralized to pH 7.4 with alkali or 0.05 M sodium phosphate buffer containing 5% sucrose (15). The fixed blocks were washed for 15 min in 0.22 M

sucrose, 0.05 M with respect to phosphate buffer, at pH 7.4; mouse kidney blocks were washed overnight in cold 5% sucrose, 0.1 M with respect to phosphate buffer at pH 7.4. The fixed, washed blocks or 40-μ chopped sections of mouse kidney were incubated in the medium at room temperature or 37°C (mouse kidney) for 1 hr, and the fresh blocks were incubated for 30 min. For light microscopy fresh-frozen sections were also prepared and incubated in the same medium. In experiments with mouse kidney, catalase, cytochrome *c*, and sucrose were omitted from the medium (Figs. 11–14), and 0.05 M tris-HCl buffer was used instead of sodium phosphate buffer, with equally good results. This observation suggests that cytochrome *c* and catalase may not be able to penetrate a significant distance into blocks or thick sections of tissues or that cytochrome *c* is present in excess. However, added catalase will prevent accumulation of hydrogen peroxide in the incubation medium, and cytochrome *c* may be useful in depleted tissues.

Incubation Medium

The following were used as an incubation medium: 3,3′-diaminobenzidine tetrahydrochloride[2] (DAB), 5 mg; sodium phosphate buffer (0.05 M, pH 7.4), 9 ml; catalase[3] (20 μg/ml), 1 ml; cytochrome *c*, type II,[2] 10 mg; sucrose 750 mg. At the end of the incubation period, the blocks of tissue were washed three times for 5 min each in 0.22 M buffered sucrose, 0.05 M with respect to phosphate buffer (pH 7.4) at room temperature. Mouse kidney preparations were incubated in osmium tetroxide–collidine for 1 hr at room temperature as described earlier (4). The other tissues were fixed with osmium tetroxide vapor for 40 min as described earlier (20). They were supported on a stainless steel screen supported in a ½ oz French square vial, screw-capped with a Teflon liner and containing ⅛ g of osmium tetroxide and a few drops of water so that excessive drying of the tissue could be prevented. The vial was placed on its flat side in contact with a sand bath at 55°C. For conservation of osmium tetroxide, the top side of the bottle was cooled with an ice-containing aluminum foil dish before the bottle was opened. The blocks and sections were then dehydrated and embedded in Araldite (17). Ultrathin sections were cut on either a Porter-Blum microtome (MT2) or an LKB Ultratome equipped with a glass knife. Care was taken to ensure inclusion of tissue from the reactive surfaces of the blocks. Some sections, mounted on copper grids, were inspected without staining. Other sections were mounted on gold grids and were treated with the T-O procedure (22), which enhanced contrast of the deposits as well as mitochondrial cristae, dense granules, fat droplets, the myofibrils and sarcotubules of heart muscle, and endoplasmic reticulum of liver and kidney. We studied sections treated with the T-O procedure and untreated sections. Mouse kidney preparations were counterstained with lead citrate (4) (Fig. 12) or were not counterstained at all (Figs. 11, 13, 14). For the T-O procedure, the preparations were exposed to a 1% aqueous solution of thiocarbohydrazide (TCH) at 50°C for 1 hr. The grids were then washed a few times in water at 50°C and OsO_4-fixed for 1 hr in an LKB grid holder cut to fit into a ½ oz French square vial and were OsO_4-fixed as described above, but without addition of water. The excess osmium tetroxide on the grids was allowed to evaporate in a ventilated hood, and the sections were then studied with an RCA EMU3 (model H) electron microscope at 50 or 100 kv with a 35 μ aperture. Mouse kidney was studied with an AE1, EM6B microscope at 30, 40, 60, or 80 kv and a 40 μ aperture.

Control experiments were also performed with the cytochrome oxidase inhibitor, potassium cyanide (6.5 mg, 0.01 M), added to the incubation medium. Although inspection of frozen sections had shown complete inhibition, electron microscopic study of ultrathin sections of Araldite-embedded tissues was also made and showed complete inhibition. In experiments with mouse kidney, sodium azide, when added

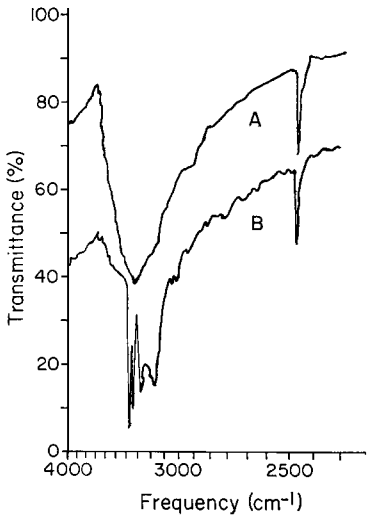


FIGURE 2 Portion of infrared spectra of polymerized DAB (*A*) and DAB free base (*B*) in the —NH stretching region above 3000 cm^{-1}. Comparison of these spectra indicates virtually complete substitution of primary amino groups but preservation of —NH groups on oxidation of DAB. These spectra of samples pressed as KBr wafers (0.5%) were recorded on a Perkin Elmer Model 237 spectrophotometer.

[2] Purchased from Sigma Chemical Co., St. Louis, Mo.

[3] Purchased from Worthington Biochemical Corp., Freehold, N. J.

to the incubation medium (3–6 mM), gave incomplete inhibition, but prior incubation in 6 mM sodium azide overnight gave complete inhibition. Inspection of sections of mouse kidney, incompletely inhibited with sodium azide, revealed deposits of reaction product in the outer compartment or peripheral intramembranous space but absence of deposit in the intramembranous cristate space of the mitochondria (Fig. 14).

In Vitro Preparation of DAB-oxidation Product and Evidence for its Polymeric Character

The ability of aromatic diamines to undergo oxidative polymerization through bis-quinone diimine formation was shown by Willstätter (25, 26). The brown product on oxidation of DAB (Fig. 1) in this histochemical reaction appeared to be polymeric, in contrast to the blue product from benzidine (24), because of the lack of solubility in organic solvents. However, this lack of solubility made unfeasible the characterization of the oxidation product as a polymer by colligative methods. We therefore prepared samples of the oxidation products by three methods in vitro, obtained evidence of their polymeric nature from their extreme insolubility, high melting point, and infrared spectrum, and also demonstrated their osmiophilia by oxidation to an osmium *black* with OsO_4 vapor.

To 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB) dissolved in 10 ml of 0.1 M tris buffer, pH 7.6, was added 0.1 ml of 1% hydrogen peroxide. Upon the addition of 5 mg of horseradish peroxidase, type II, a dark brown precipitate formed immediately which was collected by filtration, digested with trypsin, washed, and dried. Oxidation products were also prepared with cytochrome oxidase (rat heart homogenate) or 4.5 mM potassium ferricyanide. The products failed to melt on heating to 300°C. They were insoluble in alcohol, ethyl acetate, toluene, dimethyl sulfoxide, and dimethyl formamide. Although the infrared spectra of the oxidation products at frequencies below 2000 cm^{-1} differed from one another, they were identical in the region above 3000 cm^{-1}. Comparison of spectra above 3000 cm^{-1} with that of DAB indicated a prevalence of polymerization in the oxidation product due to change in character of the infrared −NH stretching absorption. The spectrum of the DAB monomer had a multiplet attributable to the symmetric and asymmetric modes of the primary amino groups as compared with a singlet in the oxidation product (Fig. 2). This indicated that extensive substitution on the amino groups had occurred. Confirmation of this interpretation was obtained on analysis of the −NH bending modes (about 1600 cm^{-1}) of oxidized and unoxidized DAB. The −NH deformation bands of the DAB monomer are indicative of unsubstituted amine, whereas the deformation bands of the oxidation products indicate a high degree of substitution and, therefore, polymerization. However, sufficient −NH stretching was preserved to indicate that oxidative cyclization to a phenazine polymer was not extensive (Fig. 1).

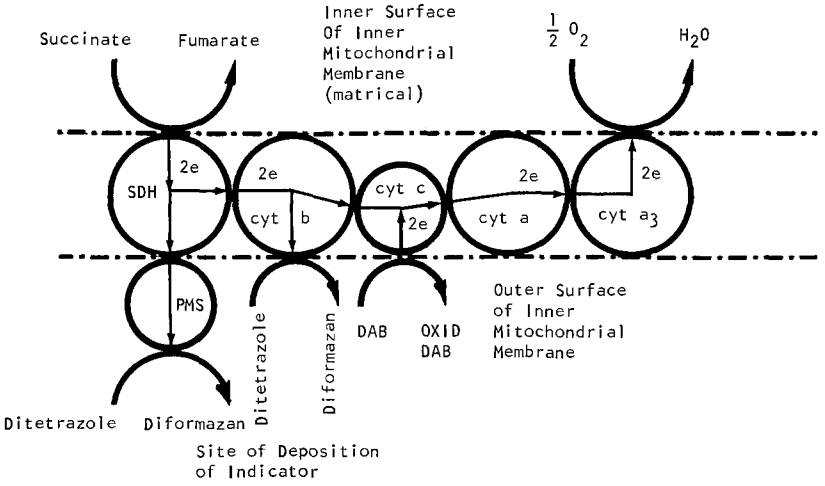
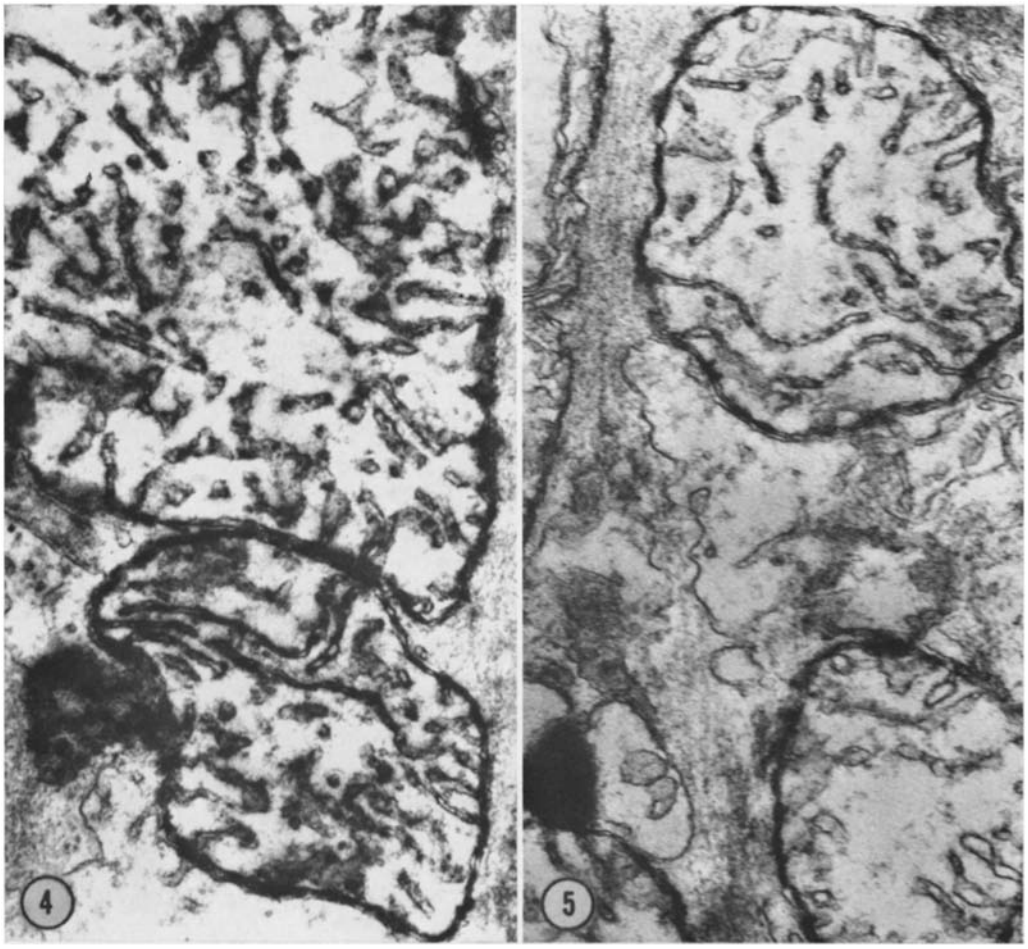


FIGURE 3 Diagrammatic representation of the respiratory chain in the mitochondrial cristate membrane. Note that the reaction product from succinic dehydrogenase activity (SDH) with or without PMS is on the same surface of the membrane as the reaction product of the electron donor (DAB) with cytochrome c. Also note that the indicator for SDH is a hydrogen acceptor whereas the indicator for cytochrome *c* is a hydrogen donor. Whether cytochromes *a* and a_3, which ultimately transfer the electrons *2e* from DAB to oxygen, are on the matrical surface remains to be proved.

Treatment of the weighed oxidation product (horseradish peroxidase) from DAB with osmium tetroxide vapor resulted in a weight gain of 38.3%. This indicated that 0.8 atom of osmium was held by each benzidine unit in the polymer.

RESULTS

Unlike the results with methods based upon the Nadi reaction (20), the results with DAB were similar with heart, liver, and kidney in that the



Figs. 4–14, stained for cytochrome oxidase. Figs. 15–18 stained for succinic dehydrogenase. Figs. 4, 5, 8, 10, fresh blocks of rat tissue incubated in DAB medium for 30 min, fixed OsO_4 vapor for 40 min, dehydrated, embedded in Araldite, cut in ultrathin sections, placed on gold grids, and treated with the T-O procedure (20). Figs. 6, 7, 9, 11–14, formaldehyde-*fixed* rat tissue or mouse kidney (fixed for 50–60-min). Blocks or 40-μ chopped sections incubated in DAB medium for 60 min and treated as indicated above for Figs. 4, 5, 8, 10. Fig. 12 has lead citrate stain; Figs. 11, 13, 14 have no lead counterstain. Figs. 11–14 show tissues incubated without added cytochrome *c* or catalase.

FIGURE 4 *Fresh* rat heart. 50 kv. Note dense deposits in space between inner and outer limiting mitochondrial membranes, and somewhat less dense and less numerous deposits in the intracristate spaces. The deposits in both sites have an interrupted pattern. × 55,000.

FIGURE 5 *Fresh* rat heart. 100 kv. The beaded pattern of the dense deposits in the space between inner and outer limiting membranes is seen. Deposits of limited length are also seen in the intracristate spaces × 55,000.

pigment was confined to what appeared in light microscopic preparations to be mitochondria. This localization was confirmed in electron microscopic preparations. The periods of incubation were chosen to ensure sufficient deposition of pigment so that there would be no doubt about the localization of enzymatic activity. The results with fresh tissue were somewhat different from those with formaldehyde-fixed tissue. In the case of fresh tissue, the reaction was even more intense in the outer compartment than in the intracristate spaces of the mitochondria (Figs. 4, 5, 8, 10). With formaldehyde-fixed tissue the deposits filled the intracristate spaces and formed dense but narrow intracristate deposits. We have assumed that deposit limited to and filling the intracristate spaces arises from the inner surface of the intracristate space which corresponds to the outer surface of the inner mitochondrial membrane. That this localization could be due to inability of the reagents to penetrate the inner membrane might be true in the case of fresh tissue but would be less likely with fixed tissue. The deposits were linear (Figs. 6, 7, 9, 11–13), and they were not droplet in character as was shown previously with the Nadi reagents (6, 8, 18, 20). It was also noted that the staining of cristae in formaldehyde-fixed tissue did not extend to the inner limiting membrane of the mitochondria of all three tissues examined (Figs. 6, 7, 9, 11–13), i.e. the cristate membranes at the periphery of the mitochondria showed minimal or no staining, although deposits in the outer compartment or space between inner and outer limiting membranes were seen. The staining reaction was effectively blocked with cyanide and partially blocked with sodium azide. Sodium azide inhibited activity in the intramembranous cristate spaces, but incompletely in the outer compartment (Fig. 14). Residual activity in the outer compartment due to resistance to sodium azide could be

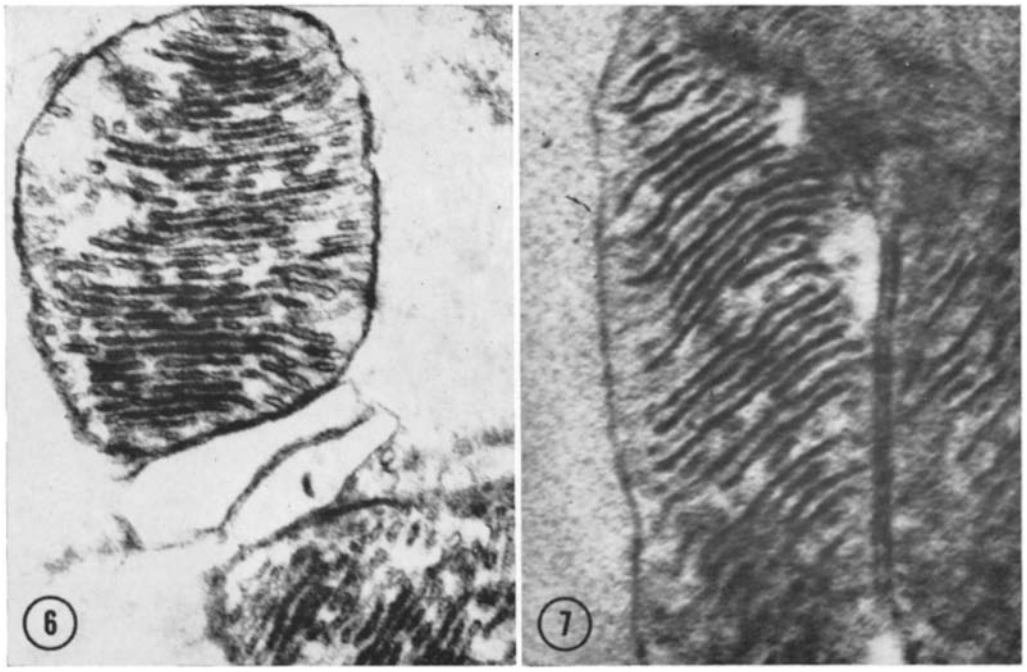


FIGURE 6 *Fixed* rat heart. 50 kv. Note dense deposits in intracristate spaces; the deposits do not extend full length of cristae. These deposits are more striking in fixed tissue, and the deposits between inner and outer limiting membranes of the mitochondria are less striking in fixed rat heart. × 27,000.

FIGURE 7 *Fixed* rat heart. 50 kv. Note the dense deposits in the intracristate spaces. The deposits do not extend to the inner limiting membrane although there is a deposit in the space between inner and outer limiting membranes. × 50,000.

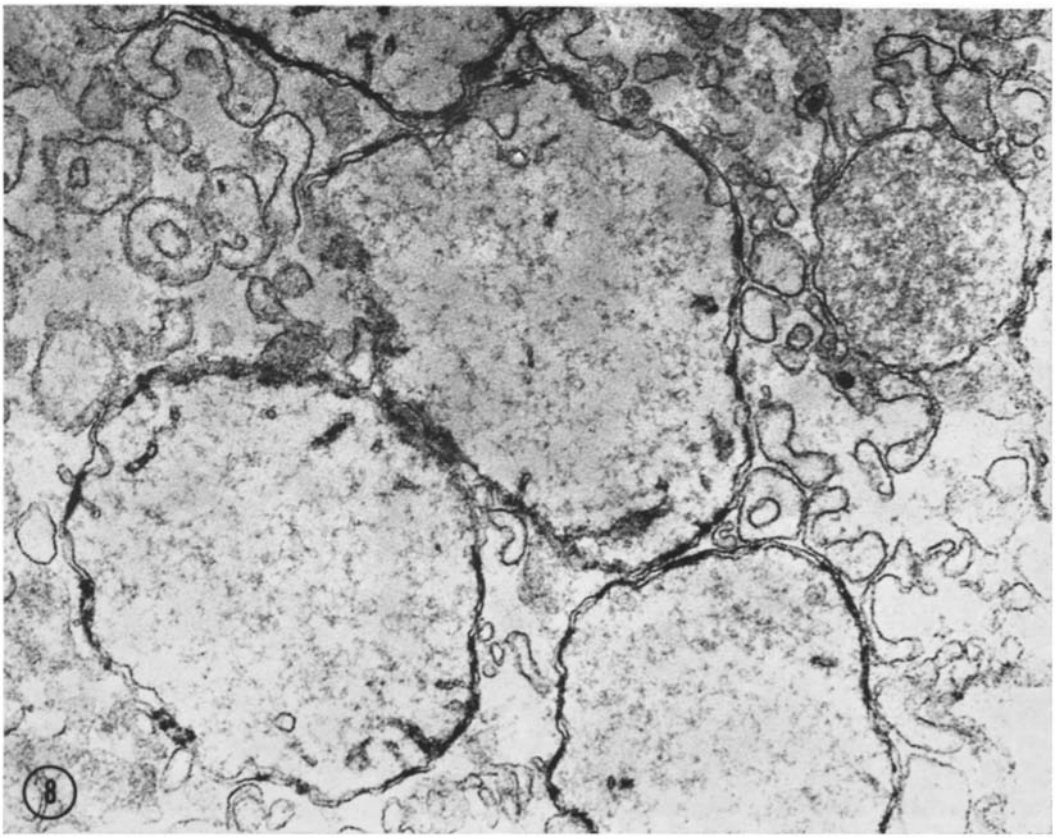


FIGURE 8 *Fresh* rat liver. 50 kv. Note the greater deposit in the space between inner and outer limiting membranes than in the intracristate spaces. The reverse is true with fixed tissue (see Fig. 9). × 55,000.

due to other oxidases such as monamine oxidase (19) or cytochrome b_5 (23) present in the outer membrane.

DISCUSSION

The localization of osmium-containing deposits, owing to oxidation of DAB, within and filling the intracristate spaces of mitochondria as well as in the outer mitochondrial compartment of fresh tissues and of fixed tissue suggests that cytochrome *c* is located on the outer surface of the inner membranes, since electrons are removed from DAB by cytochrome *c* (13) in the first step of the cytochrome oxidase reaction. The outer surface of the inner membrane is, of course, the inner surface of the intracristae space which is formed by invagination of the inner mitochondrial membrane.

It was of interest to determine whether succinic dehydrogenase, which ordinarily donates electrons via cytochrome *b* to nitro-containing tetrazolium salts (14), had a similar or opposite localization for its electron-donating activity as compared to cytochrome oxidase. However, doubt has been cast on our earlier interpretations of the observation of dense, contracted mitochondria in dehydrogenase preparations of fresh rat heart made with an osmiophilic ditetrazolium salt (TC-NBT) (21).[4] Haydon

[4] Material on the market being sold as TC-NBT by Nutritional Biochemicals Corporation, Cleveland, O., is not osmiophilic and melts 30°C lower than our material. Infrared examination of a sample of their material indicated the presence of amide and carboxylic acid carbonyl groups instead of the osmiophilic thiocarbamyl group. This indicates that hydrolysis of the nitrile group, preventing conversion to the thiocarbamyl group, occurred during attempts to isolate the dicyano-ditetrazolium chloride intermediate.

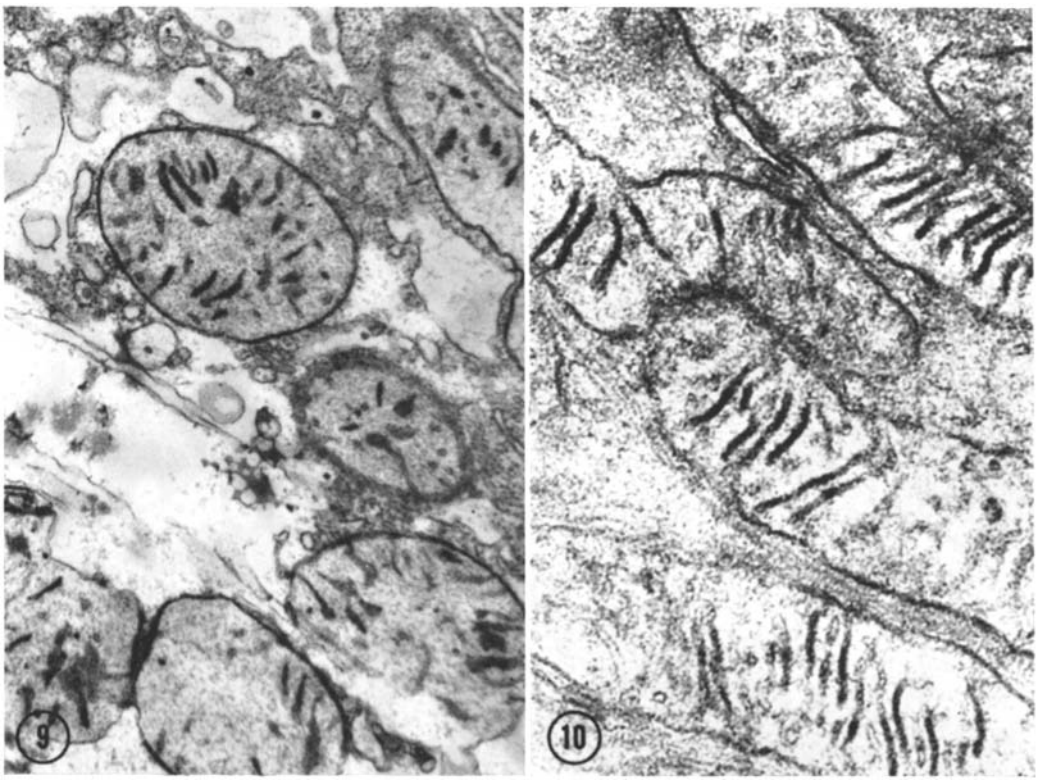
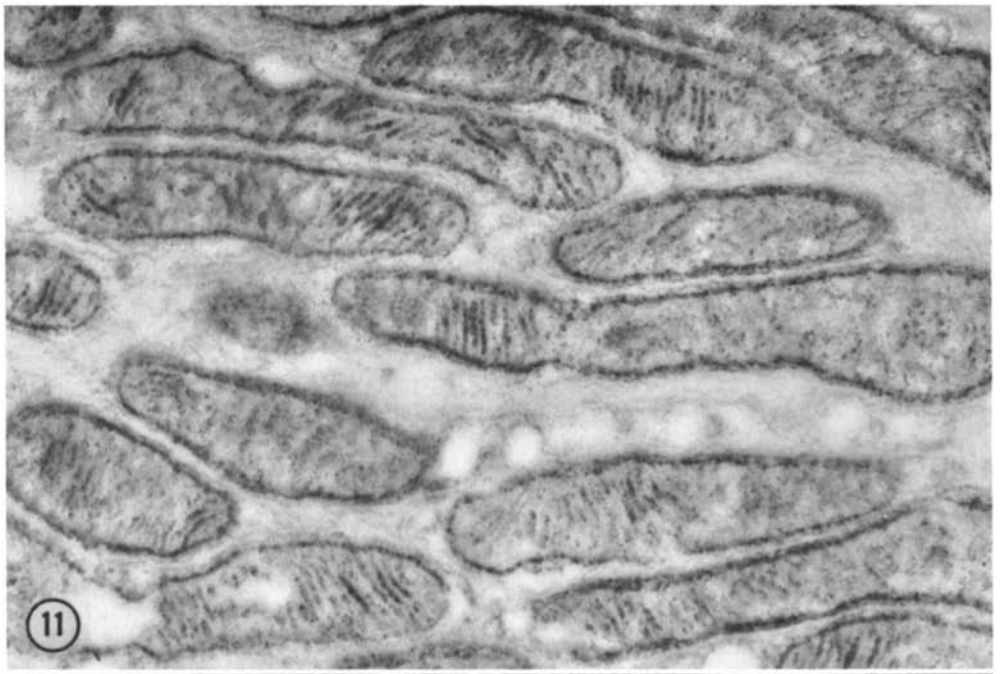


FIGURE 9 *Fixed* rat liver. 50 kv. Note that dense deposits in intracristate spaces are even more prominent than the deposits between inner and outer limiting mitochondrial membranes. The reverse is true for fresh tissue (see Fig. 8). × 27,000.

FIGURE 10 *Fresh* rat kidney. 100 kv. Note that dense deposits in the intracristate spaces are most prominent in the center of the mitochondria, and less so at the periphery. Some deposit is also seen in the space between inner and outer limiting membranes, but less prominently than in heart and liver. × 55,000.

et al. (7) observed that similarly dense, contracted mitochondria could be found in a zone within 30 μ of the surface of blocks of fresh rat heart in the absence of the ditetrazolium salt, and that the number of such dense mitochondria was increased when succinate or adenosine diphosphate (ADP) was added to the incubation medium which included phosphate buffer. These findings were consistent with Hackenbrock's demonstration (5) that isolated rat liver mitochondria are reversibly converted to a contracted, dense form (condensed matrix) by stimulating phosphorylation with substrate, ADP, and oxygen. We have therefore undertaken a reevaluation of the demonstration of dehydrogenase activity with TC-NBT. We now find that TC-NBT penetrates less well into fresh uninjured mitochondria than we at first suspected. Freezing and thawing, fixation, or greatly prolonged incubation increases penetration. From observations on such material, it is now apparent that succinic dehydrogenase-cytochrome *b* activity is located on the same surface of the inner mitochondrial membrane as noted above for cytochrome *c* activity. This is also true for SDH when phenazine methosulfate (PMS) is used as an intermediate electron donor (14) to TC-NBT. Although details of the method and results will be published later, examples are shown of the overstaining due to succinic dehydrogenase activity in the outer compartment and in intracristate spaces of mitochondria of fresh rat heart incubated for 1 (Fig. 15) and 2 hr (Fig. 16). The longer incubation resulted in better penetration of the doubly charged TC-NBT probably owing to further deterioration of mem-



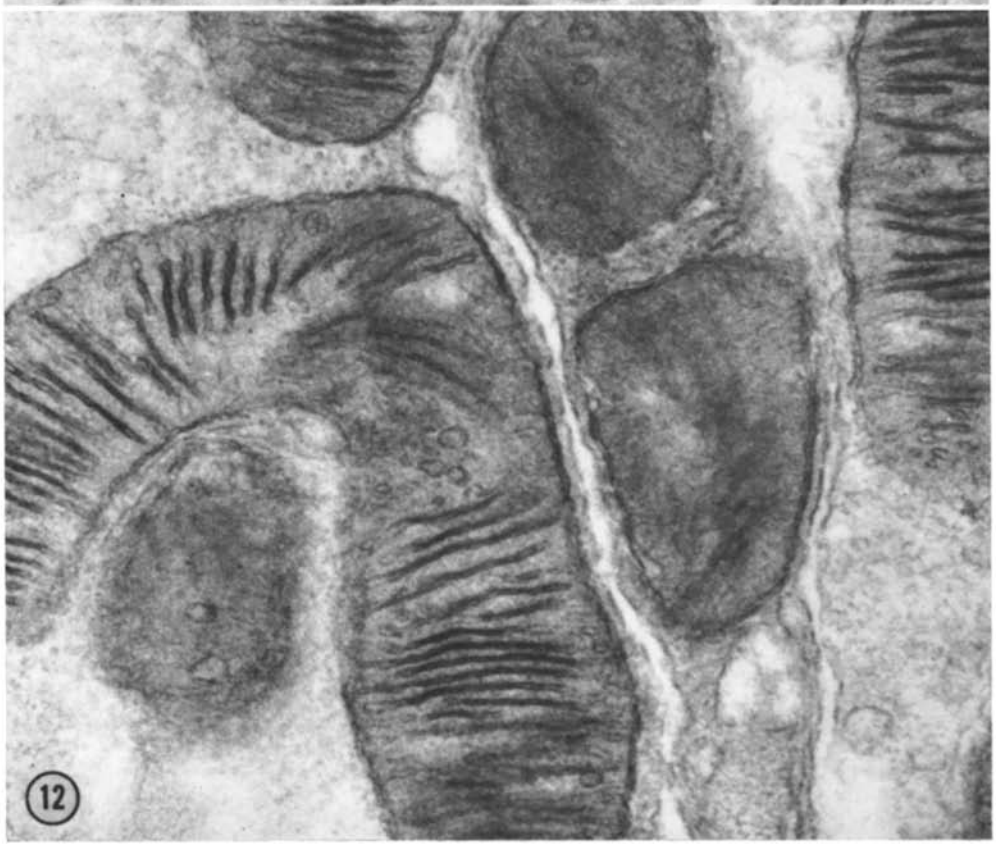


FIGURE 11 *Fixed* mouse kidney. 40 kv. The beaded character of the deposits in the intracristate spaces and in the space between the peripheral mitochondrial membranes is apparent. The cristate membranes at the periphery of the mitochondria show minimal-to-no staining. × 30,000.

FIGURE 12 *Fixed* mouse kidney. 80 kv. Stained with lead citrate. The deposits are not beaded in this preparation and are most striking in the intracristate spaces of the central portions of the mitochondria. × 60,000.

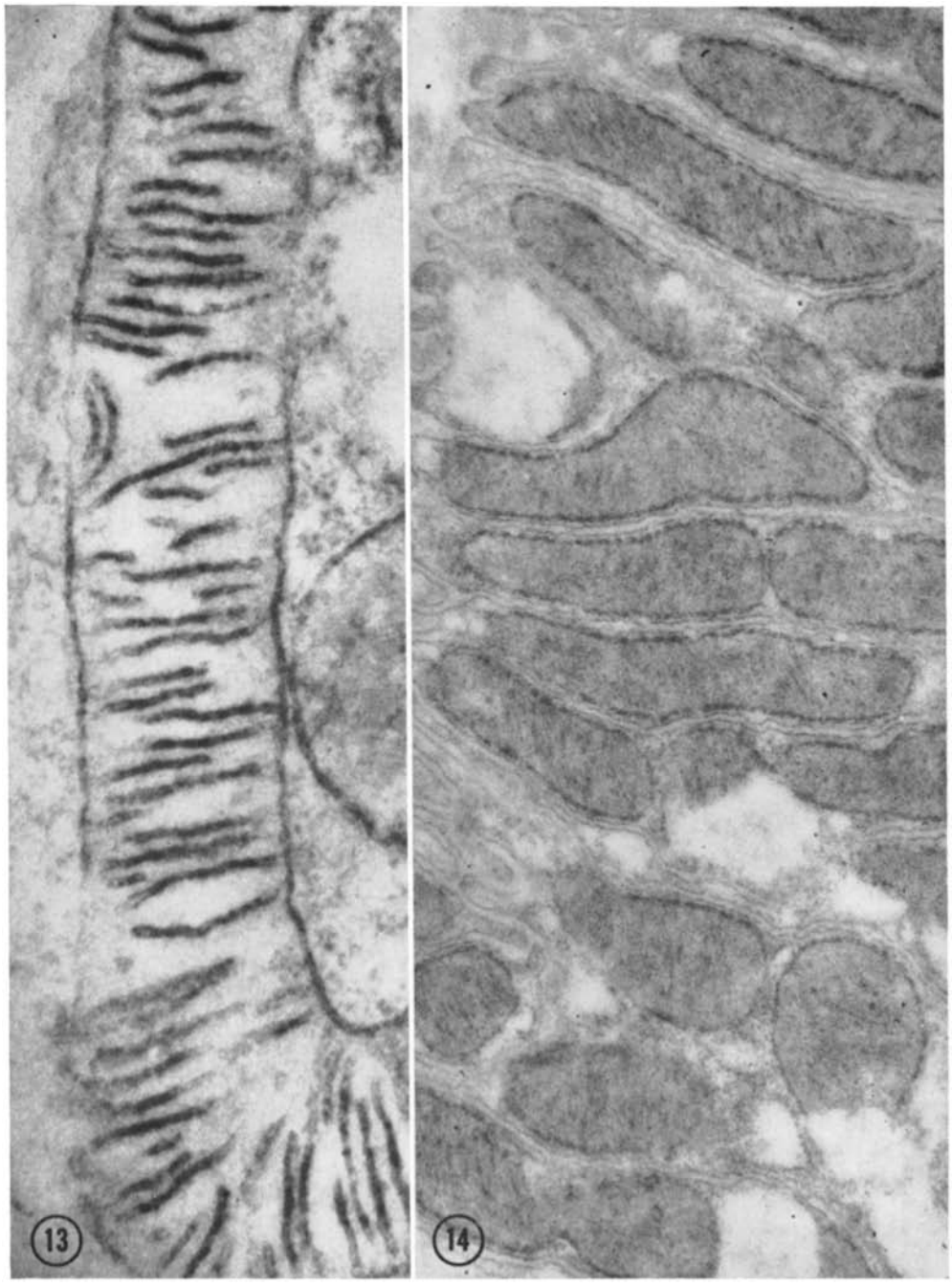
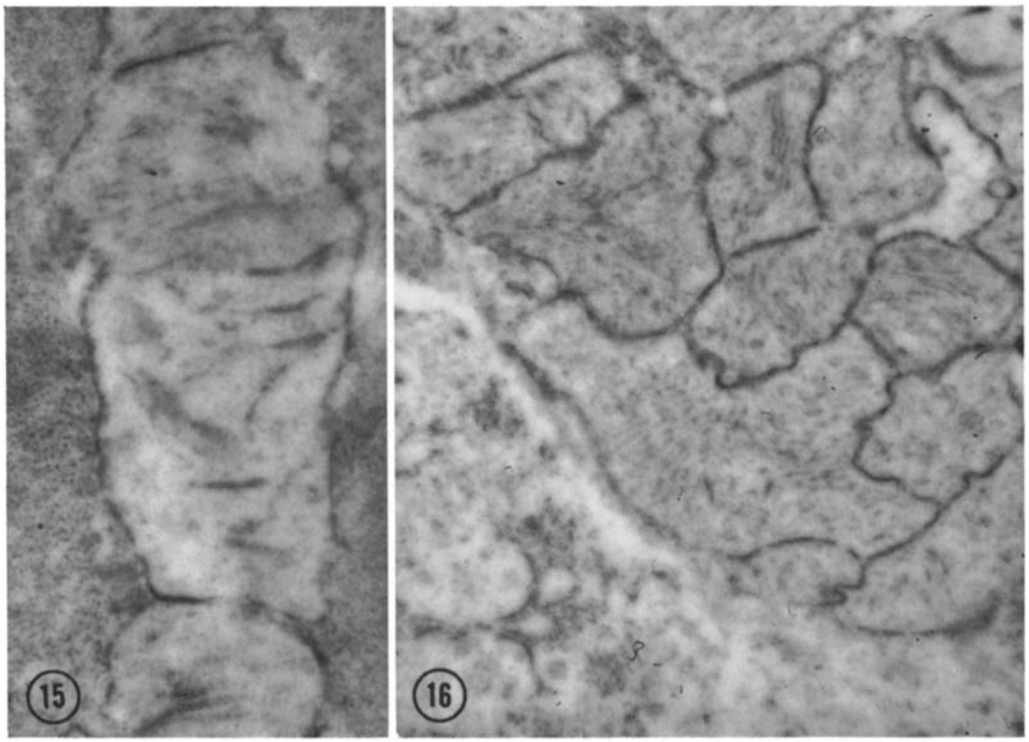


FIGURE 13 *Fixed* mouse kidney. 60 kv. Unstained with lead or OTO methods. Note deposits in cristate spaces and in space between inner and outer limiting membranes of mitochondria. Deposits have an interrupted pattern. Minimal-to-no deposits may be noted in cristate membranes at the periphery of the mitochondria. × 70,000.

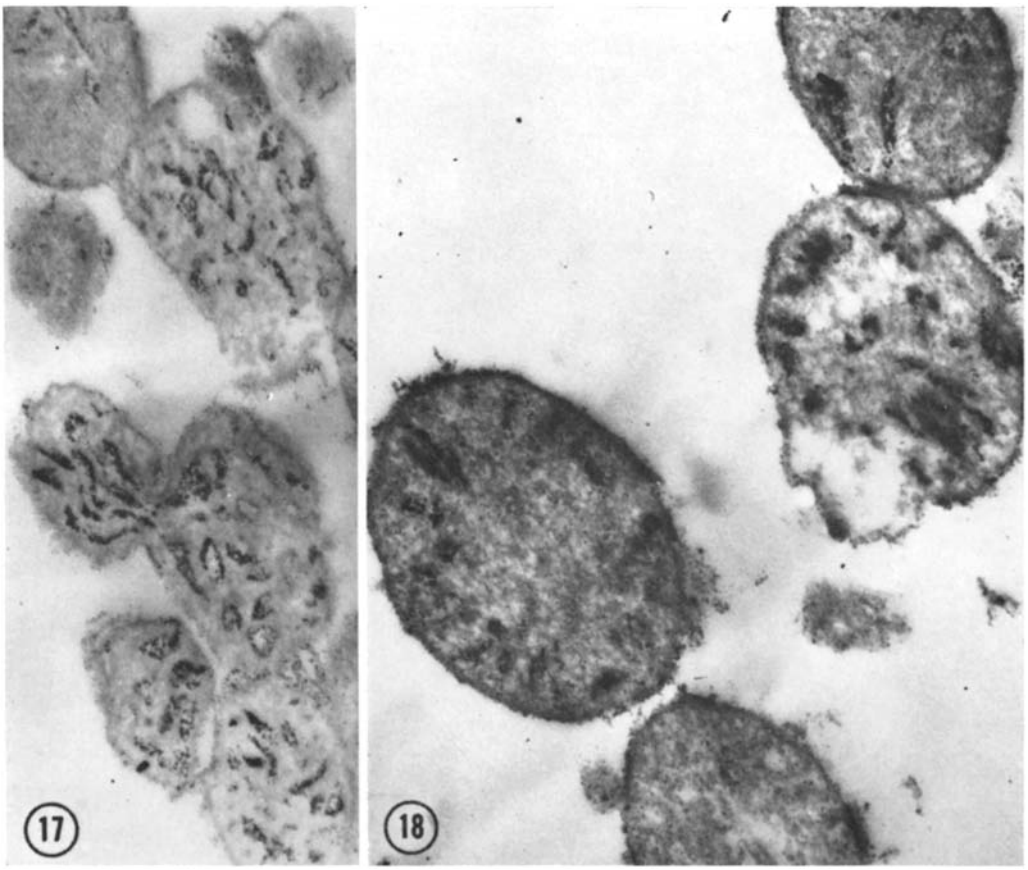
FIGURE 14 *Fixed* mouse kidney. 30 kv. Cytochrome oxidase reaction partially inhibited with sodium azide. Note absence of deposits in intracristate spaces, but persistence of reaction in the space between inner and outer limiting mitochondrial membranes. × 29,000.

brane integrity. In Figs. 17 and 18 may be seen the intracristate staining for succinic dehydrogenase activity of isolated rat liver mitochondria, fixed in cold 4% formaldehyde (15) for 3 min and stained with TC-NBT for succinic dehydrogenase activity after a 30-min incubation. Comparison with Fig. 9 reveals the strikingly similar localization of cytochrome *c* in fixed rat liver. Thus it appears that the respiratory chain from flavoprotein to cytochrome *c* manifests its activity histochemically on the outer surface of the mitochondrial inner membrane, as illustrated diagrammatically in Fig. 3.

The question naturally arose whether these results could throw any light on the controversial subject of the mechanism of energy conservation during electron transport in the respiratory chain of the mitochondrion. The issue involves two radically different hypotheses: the classical chemical-coupling hypothesis and the chemiosmotic hypothesis (10). The former postulates that energy coupling occurs through the device of a chemical intermediate of high energy nature, generated by electron transport and then used as a direct precursor in the formation of adenosine triphosphate (ATP) (10). Although the oxidative phosphorylation process can be resolved by fractionation of mitochondria and then reconstituted, Lehninger has pointed out that there is no successful reconstitution that does not require or produce reasonably



FIGURES 15 and 16 Fresh rat heart stained for *succinic dehydrogenase* activity with TC-NBT according to the method described earlier (21), but with tris buffer substituted for phosphate buffer to discourage phosphorylation. Tissue incubated for 1 hr in Fig. 15 and for 2 hr in Fig. 16. No counterstaining procedures were used. The deposits are due to *osmium black* alone and are most prominently seen in the outer mitochondrial compartment as an interrupted linear deposit after 1 hr and as a confluent deposit after 2 hr. Deposit is also seen in the intracristate spaces, more regularly after 2 hr than 1 hr, and more regularly in the center of the mitochondria than near the periphery. These features and localizations are similar to those noted with the present method for cytochrome oxidase. The long incubation required for fresh tissue, which is longer than the incubation required for fixed or frozen tissue, is due to the difficulty of the doubly charged TC-NBT to penetrate intact mitochondrial membranes. This difficulty is much less noticeable with DAB, which does not have quaternized nitrogen. Fig. 15 × 41,000; Fig. 16, × 20,000.



FIGURES 17 and 18 Isolated and fixed rat liver mitochondria stained for *succinic dehydrogenase*. Freshly isolated mitochondria were fixed for 3 min in cold 4% formaldehyde (15) containing 5 mM $MgCl_2$, washed with 5% sucrose containing 5 mM $MgCl_2$ and, after resuspension, were incubated in TC-NBT-succinate-tris buffer medium (21) at 37°C for 30 min. The resuspended mitochondria were washed several times with cold 5% sucrose containing 5 mM $MgCl_2$, and fixed for 40 min at 55°C in unbuffered 4% osmium tetroxide solution. They were then centrifuged, washed, dehydrated, and embedded in Araldite. Ultrathin sections were counterstained with the OTO method (22) only in Fig. 18. Note deposits due to *succinic dehydrogenase* in the intracristate spaces or on the inner surfaces of the intracristate spaces. The stained outer compartments have peeled away and were numerous in other parts of the pellet. Compare with similar localization of cytochrome oxidase in Fig. 9. Fig. 17, × 41,000; Fig. 18, × 61,000.

intact membrane structure (10). The apparent requirement for a membrane is completely predictable by the chemiosmotic hypothesis (11, 12), which postulates that there are no high energy chemical intermediates generated during electron transport as direct precursors of ATP and that the membrane is a necessary element in energy coupling (10). In 1950, Davies and Ogston (3) suggested mechanisms by which electron transport in a membrane could drive secretion of gastric HCl. Mitchell in 1961 (11) first formulated the chemiosmotic hypothesis for oxidative phosphorylation in the mitochondria, and more recently he has elaborated and refined it (12).

The chemiosmotic view of the coupling mechanism makes a new departure in that the chemical reactions by which ATP is synthesized are assumed to be spatially (and chemically) separate from the energy-yielding oxidoreductions. Electron transport by the respiratory chain enzymes in the membrane generates a proton gradient across the mitochondrial membrane so that the external

phase becomes more acid and the internal phase more alkaline (12). This gradient is the driving force for ATP synthesis by a vectorial membrane-linked ATPase. Recent observations that mitochondria can bring about energy-linked ion movements strongly suggest that the respiratory chain can serve as a proton pump, i.e., it can make the surrounding medium more acid (10). As Lehninger (9) has pointed out, these directional properties of the respiratory assemblies provide some basis for Mitchell's concept of anistropy of location of respiratory enzymes in the mitochondrial cristate membrane and for the concept of vectorial enzyme action in membranes as distinguished from the scalar or nondirectional action of soluble enzymes in solution. Since it is unlikely that the large insoluble molecular end products of the histochemical reactions for cytochrome *c* and for succinic dehydrogenase are able to traverse the membrane, the location of these products on the surface of the inner membrane is considered to be consistent with Mitchell's chemiosmotic hypothesis. Fig. 3 diagrammatically summarizes these considerations both with and without PMS. It should be emphasized that the histochemical reagents for these enzymes serve different functions, i.e. in the case of SDH the histochemical reagent is a hydrogen acceptor and in the case of cytochrome oxidase the histochemical reagent is a hydrogen donor. SDH-cytochrome *b* or SDH-PMS and cytochrome oxidase receive and discharge their electrons on opposite sides of the cristate membrane. To this extent the results appear consistent with Mitchell's chemiosmotic hypothesis. Further confirmation by histochemical means must await development of independent methods for cytochromes *a* and a_3. Opposition to the hypothesis has also been published (2).

The nondroplet character of the deposits of the oxidized DAB compared to the droplets given with the Nadi reagents (18, 20), reveals the superiority for electron microscopy of methods that yield polymeric end products over those methods that do not. This is so even if the end products of the histochemical reaction are osmiophilic, since the precise localization of enzyme activity can be no better than that provided by the organic end products of the reaction.

We have recently prepared a bis-p-phenylene diamine (BAXD) (16) which, like DAB, is oxidized to an osmiophilic polymer by cytochrome oxidase, horseradish peroxidase, or potassium ferricyanide and yields nondroplet deposits on demonstrating cytochrome oxidase with the electron microscope.

These same principles are being utilized in the development of better methods for the localization of hydrolytic and oxidative enzymes, based on the formation of polymeric, osmiophilic, end products of the histochemical reaction.

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REFERENCES

1. BURSTONE, M . S. 1959. *J. Histochem. Cytochem.* **7**:112.
2. CHANCE, B., L. CHUAN-PU, and L. MELA. 1967. *Federation Proc.* **26**:1341.
3. DAVIES, R. E., and A. G. OSGTON. 1950. *Biochem. J.* **46**:325.
4. GRAHAM, R. C., and M. L. KARNOVSKY. 1966. *J. Histochem. Cytochem.* **14**:291.
5. HACKENBROCK, C. 1966. *J. Cell Biol.* **30**:269.
6. HANKER, J. S., A. R. SEAMAN, L. P. WEISS, H. UENO, R. A. BERGMAN, and A. M. SELIGMAN. 1964. *Science.* **146**:1039.
7. HAYDON, G. B., S. O. SMITH, and A. M. SELIGMAN. 1967. *J. Histochem. Cytochem.* **15**:752.
8. KERPEL-FRONIUS, S., and F. HAJÓS. 1967. *Histochemie.* **10**:216.
9. LEHNINGER, A. L. 1964. The Mitochondrion. W. A. Benjamin, Inc., New York.
10. LEHNINGER, A. L. 1967. *Federation Proc.* **26**:1333.
11. MITCHELL, P. 1961. *Nature.* **191**:144.
12. MITCHELL, P. 1967. *Federation Proc.* **26**:1370.
13. NACHLAS, M. M., D. T. CRAWFORD, T. P. GOLDSTEIN, and A. M. SELIGMAN. 1958. *J. Histochem. Cytochem.* **6**:445.
14. NACHLAS, M. M., S. I. MARGULIES, and A. M. SELIGMAN. 1960. *J. Biol. Chem.* **235**:2739.
15. PEASE, D. C. 1964. Histological Techniques for

Electron Microscopy. Academic Press Inc., New York. 2nd edition.
16. PLAPINGER, R. E., S. L. LINUS, T. KAWASHIMA, C. DEB, and A. M. SELIGMAN. 1968. *Histochemie.* **14**:1.
17. RICHARDSON, K. C., L. JARETT, and E. H. FINKE. 1960. *Stain Technol.* **35**:313.
18. SABATINI, D. C., K. BENSCH, and R. J. BARRNETT. *J. Cell Biol.* **17**:19.
19. SCHNAITMAN, C., V. G. ERWIN, and J. W. GREENAWALT. 1967. *J. Cell Biol.* **32**:719.
20. SELIGMAN, A. M., R. E. PLAPINGER, H. L. WASSERKRUG, C. DEB, and J. S. HANKER, 1967. *J. Cell Biol.* **34**:787.
21. SELIGMAN, A. M., H. UENO, Y. MORIZONO, H. L. WASSERKRUG, L. KATZOFF, and J. S. HANKER. 1967. *J. Histochem. Cytochem.* **15**:1.
22. SELIGMAN, A. M., H. L. WASSERKRUG, and J. S. HANKER. 1966. *J. Cell Biol.* **30**:424.
23. SOTTOCASA, G. L., B. KUYLENSTIERNA, L. ERNSTER, and A. BERGSTRAND. 1967. *J. Cell Biol.* **32**:415.
24. STRAUS, W. 1964. *J. Histochem. Cytochem.* **12**:462.
25. WILLSTÄTTER, R. 1909. *Ber. Deut. Chem. Ges.* **42**:4123.
26. WILLSTÄTTER, R., and I. KALB. 1906. *Ber. Deut. Chem. Ges.* **39**:3476.