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Abstract

In an attempt to pursue the relationship of the fine structure of a cell to the biochemical function, the author at first tried to demonstrate cytochemically the actual sites of activity of enzymes in the terminal electron transport system involved in energy production with the use of the electron microscope. Namely, cytochemical reactions were performed by using potassium tellurite, a heavy metal salt, and then the author succeeded in the electron microscopic detection of the enzymes by freezing-drying method and by means of formalin fixation, strong reducing agents and osmium tetroxide fixation. As the results the author has been able to verify that the reactions of the enzymes belonging to the terminal electron transport system are found localizing in the mitochondria being arranged fairly densely and continuously on the critae and partially on the membrane, although some differences in the grade of the activity are found in each mitochondria even in one cell and a marked difference between the mitocndria belonging to the different kinds of cells. Furthermore, it has been clarified that the activity of the endogenous dehydrogenase system (mainly DPNH- or TPNH-dehydrogenase and others) is chiefly strong in cristae, and that the succinoxidase system exists both in cristae and membrane.

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CYTOCHEMICAL DEMONSTRATION OF THE SITES OF ACTIVITY OF THE TERMINAL ELECTRON TRANSPORT SYSTEM WITH THE ELECTRON MICROSCOPE

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For the clarification of the relationship of the fine structure of a cell to the biochemical function it is desirous to investigate directly the correlation of the two at the most microscopic level of cellular structure by applying cytochemical method to the electron microscope. From our investigation of the reduction mechanism of ditetrazolium salts (NT, Nitro-NT, and Nitro-BT) and potassium tellurite by tissue cells and homogenates, we have previously described some new and improved methods for the selective cytochemical demonstration and colorimetric estimation of the activity of each enzyme in the terminal electron transport system by using various substrates and inhibitory agents.[1-3] We have also verified with the aid of the light microscope and microspectrophotometric apparatus that the cytochemical reaction of these enzymes occurs at the sites of mitochondria of various tissue cells and blood cells.[2,4] Now, an attempt has been made to pursue cytochemically the relationship between the fine structure of mitochondria and the sites of activity of these enzymes with the electron microscope. In the present paper a new method of our own device and results of the study are briefly described.

MATERIALS AND METHODS

For the electron microscopic detection of the sites and grade of activity of enzymes in the cell first of all the enzymatic reaction must be of such a nature as it would meet the requirements for cytochemical demonstrations satisfactorily and also it should be such that it would not destroy the fine structure of the cell in the course of the treatment, and the reaction products must be of a high electron-opaque and must remain without changing the position to the very end of the preparation of the specimens for the electron microscopic observation. For this purpose an attempt had been made to combine diformazan, a reduction pro-

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duct of ditetrazolium, with heavy metals, but up to the present no satisfactory electron microscopic picture was obtained[2]. Therefore, with the use of a heary-metal salt, potassium tellurite (K_2TeO_3), an attempt has again been made to apply the histochemical reaction of this substance[5] to the electron microscopic observation. When the solution of this substance is reduced by sodium hydrosulfite, a grey, dark-brown, or black precipitates are obtained according to the degree of reduction, each of these products seems to be an intermediary reduction substance (TeO_2, TeO) or a terminal substance (Te). In the case of the biological reduction this reaction likewise takes place by dehydrogenase or reduction systems in the cells, and the reaction products precipitate in the immediate vicinity of the reaction sites. Moreover, because the atom tellurium (atomic wieght 127.61) is an extremely opaque substance, it is possible to apply its cytochemical reaction to the elcetron microscopic observation.

As for the materials of observations mainly the tissue cells of the heart, liver, kidney and bone marrow of mice were used ; and the enzymatic reactions were made to take place both *in vivo* and *in vitro*. In the case of the *in vivo* reaction, after determining the lethal dose and investigating pathologic changes of poisoning of potassium tellurite in mice, a dose of 0.01-0.0025 mg/g of body weight was injected successively once a day into the abdominal cavity of the test animals for 2 to 16 days, and vital reaction of endogenous dehydrogenase systems was performed. In the macroscopic observations dark stain of the kidney tissue was marked, and also in light microscopic findings it was confirmed that the reaction appeared on the mitochondria in the renal tubular epithelial cells (Fig. 1). Next, histochemical reactions *in vitro* of each of these visceral tissues were made to take place, and light microscopic observations were also carried out at each stage in the course of the preparation of specimens for electron microscopic observations. In order to eliminate the endogenous dehydrogenase reaction fresh tissue blocks were frozen at $-20^\circ C$ to $-40^\circ C$ for 10 minutes before the reaction. As an incubation solution for the succinoxidase system the mixture of equal volume of 0.2 M sodium succinate solution, 0.2% potassium tellurite solution and 0.1 M phosphate buffer solution at pH 7.6 was mostly used at first[4], and later after the report by BARRNETT and PALADE[6] a portion of experiment was conducted according to their method, namely, to 20 ml. of the above mentioned mixed solution 0.5 ml. of 0.6 M sodium bicarbonate, 0.2 ml. of 0.01 M aluminium chloride, and 0.1 ml. of 0.005 M magnesium chloride were added as the activator[6,7]. The osmolar concentration of the incubating medium was

raised to 0.44 M by the addition of sucrose. For the demonstration of the activity of endogenous dehydrogenase systems succinate was omitted from the incubating medium and osmoral concentration was adjusted to 0.44 M by the addition of sucrose. And for the control purpose tellurite was omitted from the incubating medium. Incubation period lasted one to ten hours (usually for two hours).

For the fixation and preparation of the specimens after the incubation the following three methods were tried :

1. *Freezing-drying method :* In order to avoid the intrusion of metal ions and artefact due to fixation, tissue blocks were frozen, dried, and embedded in metacrylate, and ultra-thin sections were prepared for the electron microscopic observation[8].

2. *Fixation by osmium tetroxide :* For the observation of fine structure the osmium tetroxide fixation is the excellent method, but the reduction products of tellurite being re-oxidized and made soluble by this, the fixing time has been shortened to 10 to 15 minutes according to BARNETT and PALADE's method[6]. It has, however, been clarified that even by this technique the reduction products of tellurite, especially the intermediary reduction substances are mostly re-oxidized and dissolved during the fixation and these sustances disappear by dissolution in the processes of immersing in alcohol or of embedding in monomer, as the result only traces of crystals remain. Theoretically the intermediary reduction substances must be all preserved, and for this reason an attempt has been made to replace the reduction products of tellurite by a gold chloride[9]. Although by this means an extremely clear picture was obtained under the light microscope and the reduction products remained withstanding with the above-mentioned processes, this method is not appropriate for the electron microscopic observation as there occurs a non-specific reaction by gold chloride. Therefore, the next method has been devised to overcome this handicap.

3. *Formalin fixation, re-reduction by sodium hyrosulfite, and osmium tetroxide fixation for a short period of time :* After the incubation the tissue blocks were fixed in 10% neutral formalin solution for 2—12 hours, washed in 0.44 M sucrose solution (30 minutes), and were immersed in 0.44 M sucrose solution with an addition of a small amount of sodium hydrosulfite (for 10—20 min.) to reduce the intermediary reduction substances of tellurite completely, and then these were again thoroughly washed by 0.44 M sucrose solution (for about 2 hours); and were fixed in cold 1% osmium tetroxide solution bufferized at pH 7.4 for a short period of time (for 5—15 min.); and finally passing through

alcohol and embedding in monomer, ultra-thin sections were prepared for the electron microscopic observation. By these procedures most of the reaction products were preserved without much loss.

RESULTS

1. *Results of the freezing-drying method* : In the electron microscopic picture of the renal tubular epithelial cells, in which endogenous dehydrogenase reaction was elicited *in vivo* by the tellurite injection, and which was frozen and dried, a strong electron absorption can be observed at the site of mitochondria, especially at the location of the mitochondrial cristae (Fig. 2), but no such an absorption picture can be recognized in the control specimens which did not receive the tellurite injection (Fig. 3). From these observations it is easily understood that the endogenous dehydrogenase activity is strong mainly in mitochondria, especially in the mitochondrial cristae.

2. *Results of the osmium tetroxide fixation* : The fine structure is quite well preserved in the tissue specimens, which underwent enzymatic reaction by the presence of tellurite and immediately fixed in osmium, but as has already been explained even by shortening the fixation period to 10—15 minutes a greater part of the reduction products of tellurite has disappeared leaving only traces of them. Figs. 4 and 5 show the heart muscle specimens fixed in osmium for 15 minutes immediately after two hours' succinoxidase reaction of the tissue previously frozen to eliminate the endogenous dehydrogenase reaction. These figures show depositions of electron opaque granules on mitochondrial cristae and on mitochondrial membrane scatteringly. These are assumed to be products of enzymatic reduction by tellurite. Fig. 6 shows the specimen of a rat liver cell in which the same reaction was made to take place for 10 hours, and in this the structure of mitochondria is broken down to a large extent, but the deposition of reaction products is marked on both the mitochondrial cristae and membrane.

3. *Results of formalin fixation, re-reduction by hydrosulfite, and osmium tetroxide fixation* : By this method although intracellular fine structure presents a picture lacking somewhat in clarity, the major portion of the reaction products are well preserved as shown in Figs . 8, 9, and 10. Fig. 7 is the control specimen treated identically with those in Figs. 8, 9, and 10 with the incubating solution without tellurite, presenting swollen mitochondria of erythroblasts but no abnormally strong absorption of electron. Figs. 8 and 9 show the tellurite reduction of reticulocytes by the activities of the succinoxidase system and endogenous

dehydrogenase systems with succinate as substrate. A strong absorption can be seen at the sites of mitochondria. This seems to be a cytochemical proof that mitochondria are contained in reticulocytes. Fig. 10 shows the activities of the succinoxidase system and endogenous dehydrogenase in mitochondria of a bone marrow cell also with succinate as the substrate. A quite clear absorption can be observed at the sites of mitochondrial cristae, and there is no doubt that this is due to the deposition of tellurite reduction products. The reaction is limited to mitochondria, and can not be observed in any other site. Moreover, it is quite interesting to note that there exist reaction-positive mitochondria and reaction-negative one in a same cell, reflecting the functional conditions of mitochondria. In addition, the reaction in this specimen is especially strong in cristae but weak in the membrane. The reaction is not at random but appears continuously along cristae.

DISCUSSION

With the purpose to scrutinize the nature of the enzymatic reduction of tellurite and the elcetron-microgram, the author analyzed the mechanism of tellurite reduction with small tissue pieces and tissue homogenates by the same method as done in the case of ditetrazolium slats[1,2], but the mechanism was more complex. In other words, since endogenous dehydrogenase reaction in living tissue is quite strong and also tellurite itself inhibits the succinic dehydrogenase activity to some extent, it is difficult to increase to so great extent the reaction even using succinate as the substrate when compared with endogenous dehydrogenase reaction. From the results of investigation about inhibitory effects of malonate, antimysin A and cyaninde, the tellurite reduction by the succinoxidase system does not appear to take place directly conjugating to succinic dehydrogenase itself, but indirectly conjugating at each step of the same system in the presence of some soluble auxilliary factors. Endogenous dehydrogenase reaction seems to be chiefly the reactions conjugating to flavoproteins of DPNH- or TPNH-dehydrogenase systems existing in mitochodria and the reactions by other reduction series ; but in the case of the living tissue a slight reduction of tellurite sometimes also occurs due to the reduction series in the sites other than mitochondria. From these reasons it is very difficult to demonstrate quite selectively the activity of each enzyme belonging to the terminal electron transport system by the use of the electron microscope. However, on the whole there is no room for doubt that these reactions indicated here are mainly the reactions by the

terminal electron transport system. Moreover, it is believed that the activity of the endogenous dehydrogenase system (mainly DPNH- or TPNH-dehydrogenase or other dehydrogenating system) is strong in mitochondrial cristae and the activity of the succinoxidase systme exists both in mitochondrial membrane and cristae.

SUMMARY AND CONCLUSIONS

In an attempt to pursue the relationship of the fine structure of a cell to the biochemical function, the author at first tried to demonstrate cytochemically the actual sites of activity of enzymes in the terminal electron transport system involved in energy production with the use of the electron microscope. Namely, cytochemical reactions were performed by using potassium tellurite, a heavy metal salt, and then the author succeeded in the electron microscopic detection of the enzymes by freezing-drying method and by means of formalin fixation, strong reducing agents and osmium tetroxide fixation. As the results the author has been able to verify that the reactions of the enzymes belonging to the terminal electron transport system are found localizing in the mitochondria being arranged fairly densely and continuously on the critae and partially on the membrane, although some differences in the grade of the activity are found in each mitochondria even in one cell and a marked difference between the mitocndria belonging to the different kinds of cells. Furthermore, it has been clarified that the activity of the endogenous dehydrogenase system (mainly DPNH- or TPNH-dehydrogenase and others) is chiefly strong in cristae, and that the succinoxidase system exists both in cristae and membrane.

The essential part of this report was delivered at the 19th Congress of Japanese Hematological Society on April 4, 1957 and at the Symposium of the Society for Celluar Chemistry on October 26, 1957.

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EXPLANATION OF FIGURES

Fig. 1. Light microscopic picture of the endogenous dehydrogenase reactions *in vivo* in the kidney tissue of a mouse by the intraperitoneal injection with potassium tellurite, counterstained with hematoxylin-eosin. Deposition of the reduction proucts of tellurite in the form of distinct black granules in the cytoplasm of the convoluted tubular epithelial cells.

Fig. 2. Electron microscopic picture of a tubular epithelial cell of the kidney by freezing drying after endogenous dehydrogenase reaction was performed *in vivo* by potassium tellurite injection. This specimen is the same as that in Fig. 1. Mitochondria (M) show high density of electron absorption.

Fig. 3. Electron microscopic picture of a tubular epithelial cell of the kidney by freezing drying, without the injection of potassium tellurite. A control specimen for that in Fig. 2. Mitochondria do not show particulary high densty of electron absorption.

Figs. 4 and 5. Electron microscopic pictures of the mitochondria of the cardiac muscle by osmium tetraoxide fixation (15 minutes) after the succinoxidase system reaction *in vitro* with potassium tellurite. Granules of high density of electron absorption, likely the reduction products of tellurite (t), are found scatteringly on both mitochondrial cristae and membrane.

Fig. 6. Electron microscopic picture of the mitochondria of a liver cell by osmium tetraoxide fixation (15 mimutes) after the succinoxidase system reaction *in vitro* (10 hours) with potassium tellurite. Although mitochondria have been considerably destroyed, abundant depositions of reduced tellurite are found on both mitochondrial cristae and membranes.

Fig. 7. Electron microscopic picture of a erythroblast treated similarly as the specimen of next figures but without tellurite in the incubation medium. A control specimen. Mitochondria are swollen and no particularly high density is recognizable in them.

Figs. 8 and 9. Electron microscopic picture of a reticulocyte, performed the succinoxidase system and endogenous dehydrogenase reaction with potassium tellurite (2 hours) and treated identically as the specimen in Fig. 10. Mitochondria show very high desity of electron absorption, which is considered to be due to the reduced tellurite deposited on or in mitochondria.

Fig. 10. Electron microscopic picture of a bone marrow cell performed succinoxidase system and endogenous dehydrogenase reaction with potassium tellurite, fixed by 10 per cent formalin solution (12 hours), washed with 0.44 M sucrose solution (30 minutes), treated with sodium hydrosulfite solution added sucrose to 0.44 M (10 minutes), washed with 0.44 M sucrose solution (2 hours), fixed with bufferized 1 per cent osmium tetroxide solution (15 minutes). High density of electron absorption due to the reduced tellutite deposited on cristae mitochondriales is clearly demonstrated. Some differences in the grade of the depositions are found in each mitochondria and some mitochondria have no depositions. No such high density are recognizable in the part other than the mitochondria. The sites where this deposition took place considered to be the sites of the activities of succinoxidase system and endogenous dehydrogenase.

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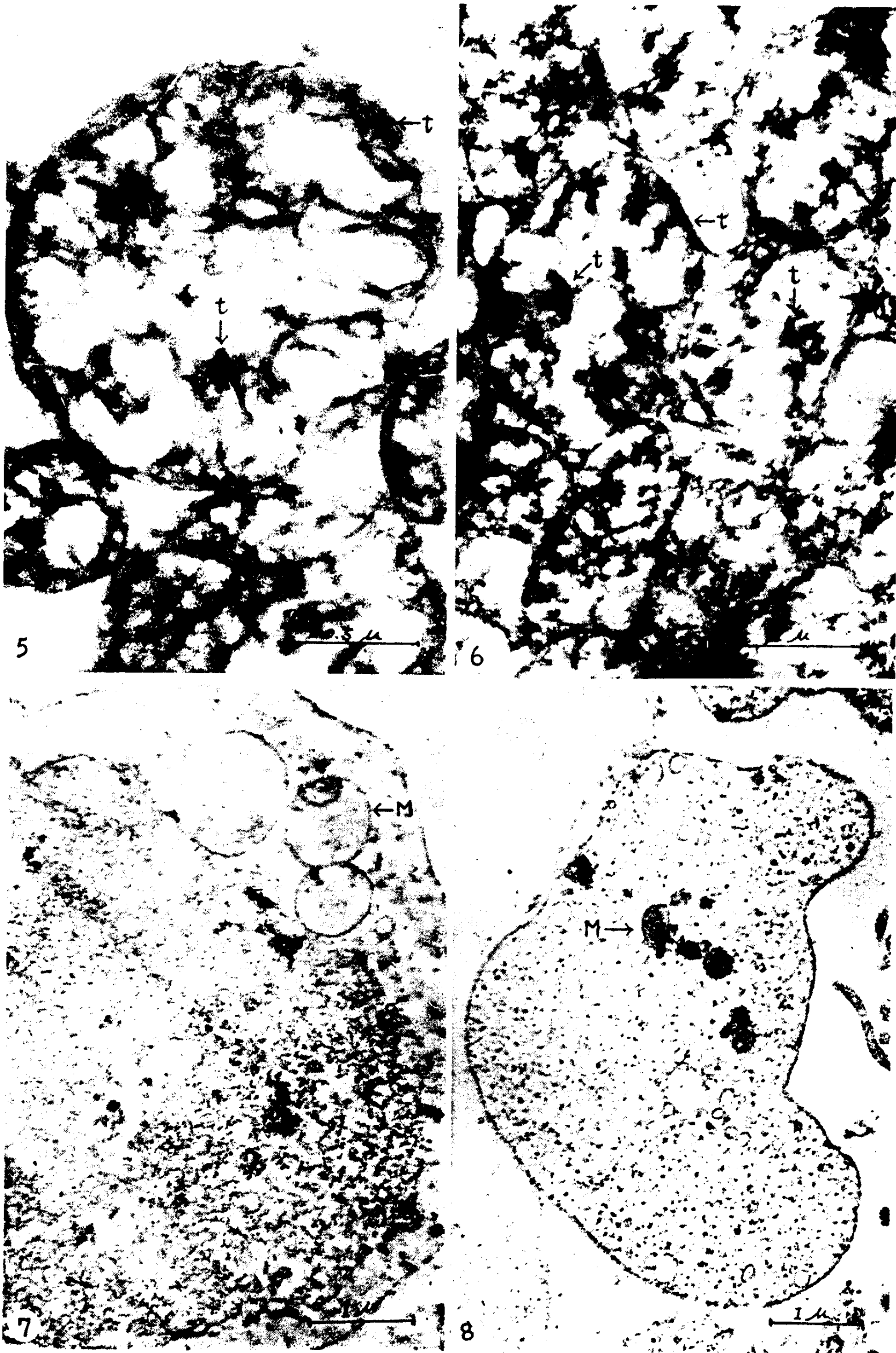
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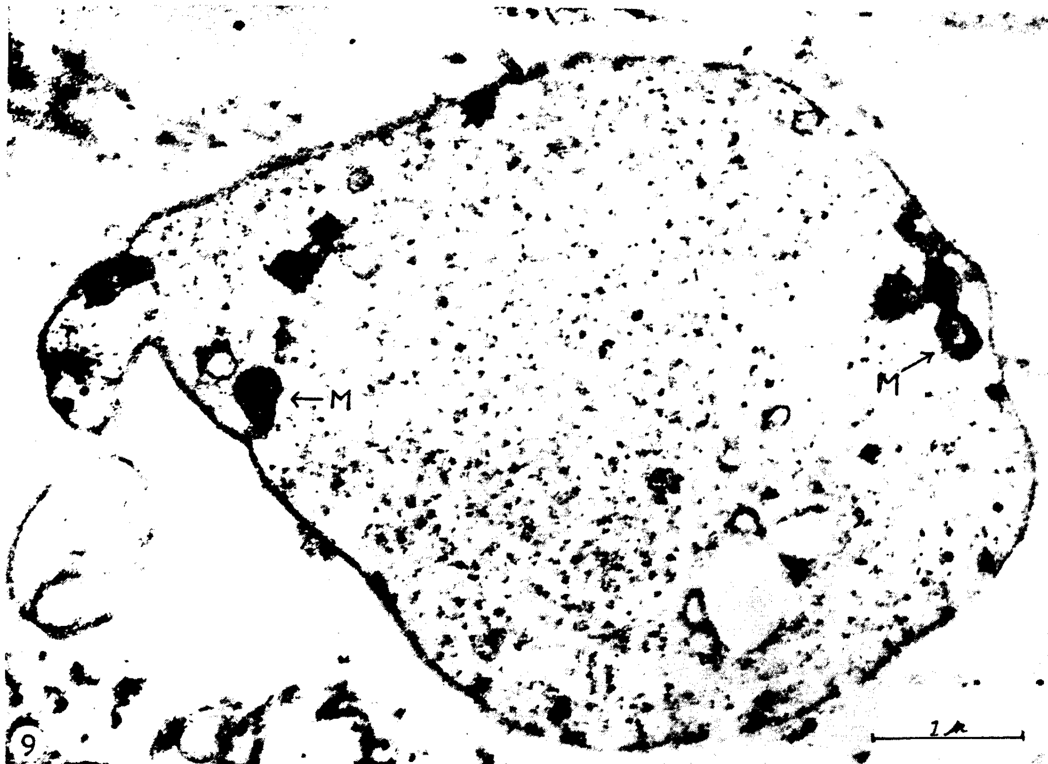
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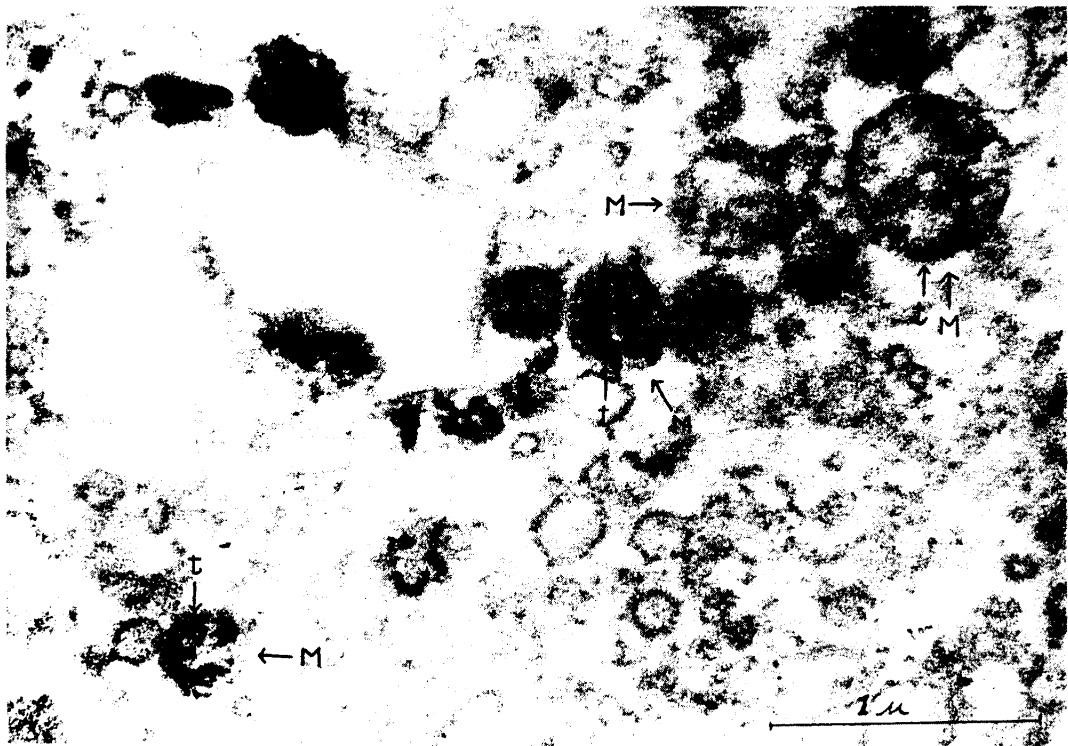
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