HISTOCHEMICAL DEMONSTRATION OF THE SITES OF ACTIVITY OF DEHYDROGENASE SYSTEMS WITH THE ELECTRON MICROSCOPE*

By RUSSELL J. BARRNETT‡, M.D., AND GEORGE E. PALADE, M.D.

(From The Rockefeller Institute for Medical Research)

PLATES 180 TO 186

(Received for publication, March 29, 1957)

During an attempt to test current, and develop new, histochemical methods applicable to electron microscopy, early success was obtained with a method for demonstrating the activity of dehydrogenase systems. This method depends on the fact that potassium tellurite (K_2TeO_3) is reduced (hydrogenated) by living tissues to a product of high electron-scattering power. More specifically, this reagent will accept electrons indirectly from respiratory enzyme systems (dehydrogenases and associated enzymes) as metabolites are oxidized by dehydrogenation. The dehydrogenases or transhydrogenases (1) are reversibly oxidized and reduced, whereas the reduced tellurite is stable and insoluble.

Klett (2) was the first to notice the reduction of tellurite to a black, insoluble compound by living bacteria and proposed the reaction as a viability criterion. Subsequent studies used this reagent or selenite on bacteria (3), plant cells (4), seeds (5, 6), and yeasts (7), and Lakon (6) attributed the reaction to the activity of dehydrogenases. Recently, Wachstein (8) revived the use of tellurite as a histochemical reagent for demonstrating the presence of endogenous dehydrogenase systems in fresh animal tissues.

The tellurite reaction has certain features which render it potentially adaptable to electron microscopy. For instance, the final product, reduced tellurite, formed during this reaction, is extremely insoluble and hence, can be expected to precipitate in the immediate vicinity of the reaction sites. This metallic precipitate is opaque to electrons, thus satisfying a basic requirement for any product of a histochemical reaction to be used in electron microscopy. Consequently, the tellurite could be adopted if the preparative procedures could be rendered satisfactory for electron microscopy. The requirements to be met in enzyme histochemistry are numerous and varied. As shown in detail in a recent

* This work was supported in part by a research grant (A-452 C3) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, and in part by a grant from the Milton Fund.

‡ Visiting investigator at The Rockefeller Institute, on leave of absence (1956) from the Department of Anatomy, Harvard Medical School.

review article (9), they concern the preparation of the tissue, the choice of substrates and reagents, the incubation of the tissue in a satisfactory media, and the localization of the final product of reaction. In electron microscopy, the corresponding requirements are less numerous but more exacting, and are primarily concerned with the adequate preservation of fine structure by fixation. Successful adaptation depends largely upon an acceptable compromise among these various requirements. For example, it is impossible to perform the histochemical reaction on tissues already satisfactorily fixed for electron microscopy, since it is well known that the activity of dehydrogenases is sensitive to fixatives, osmium tetroxide included. The choice of the experimental set is then restricted to: (*a*) carrying on the histochemical reaction supravitally, and (*b*) performing the histochemical reaction intravitally with the fixation at the end of the reaction in both cases. The latter condition can be used only for the demonstration of endogeneous enzyme activity, while the former is more amenable to experimentation and can be used for the demonstration of either endogenous or specific dehydrogenase activity. In addition attention must be paid to the penetration of reagents and substrates into tissue and cells, and to the morphological alterations introduced in the tissue by incubation in an abnormal fluid environment for varying periods of time, at a relatively high temperature, in the presence of toxic materials, or in the absence of oxygen. If adequate penetration is achieved and morphological alterations are minimized, the tissue can be fixed at the end of incubation and subsequently prepared for electron microscopy in the usual manner.

The ultimate hope of histochemical investigations at the electron microscope level is to obtain information concerning the relationship of biochemical function to cell components and to their fine structural elements. It is appropriate for such an investigation to begin with certain respiratory enzymes, since much is known about their nature and function, the least of which is that they are bound to well defined cell components, the mitochondria (*cf.* 10–13).

Materials and Methods

The tissues used in these experiments were obtained from freshly killed albino rats and included heart muscle, skeletal muscle, kidney, liver, pancreas, small intestine, ovary, uterus, submaxillary gland, cerebellum, and brown fat. Most of the observations were made on heart muscle and, to a lesser extent, on kidney, and all the control experiments were performed on both these organs.

For the demonstration of the activity of endogenous dehydrogenase systems, fresh tissue blocks, approximately 1 $mm.^3$, were incubated for 10 minutes to 7 hours in a 0.1 per cent solution of potassium tellurite in 0.1 M Sörensen's phosphate buffer, pH 7.6. The osmolar concentration of this incubating solution, as well as all others, was raised to 0.44 M by the addition of sucrose.[1] The endogenous activity was also demonstrated by single or multiple

[1] The osmolar concentration inside the cytoplasm seems to be close to this value as suggested by the fact that at this concentration isolated mitochondria retain their intracellular form (14).

injections of 0.1 to 1.0 per cent solutions of tellurite intraperitoneally or intravenously or by intraluminal installation of the solution into a tied off segment of small intestine, having its blood supply intact, in an anesthetized rat.

For the demonstration of the succinic dehydrogenase system, by necessity, only small blocks of tissue were used. These were either washed in 0.44 M sucrose for 2 hours, or frozen for 5 minutes in a deep freeze at −40°C. The latter procedure was used since it is known that relatively thick, frozen sections show no endogenous activity after thawing (9). It is assumed that this treatment eliminates the interferences of substrates that require pyridine-nucleotide coenzymes by dissociating the enzyme-coenzyme system with resulting inactivation. After freezing and thawing, no activity due to endogenous substrates could be demonstrated in our preparations. The assay system in which dehydrogenase activity was tested contained the following basic ingredients: sodium succinate, potassium tellurite (K_2TeO_3), Sörensen's phosphate buffer, pH 7.6, and sucrose. In certain experiments, designed to increase the activity, either potassium cyanide or the activating ions calcium^{++}, magnesium^{++}, aluminum^{+++}, and bicarbonate^{-} were added singly or in combination to the assay system. The incubations were performed under both aerobic and anaerobic conditions. Essentially, the media and conditions used were those recommended by Seligman and Rutenberg (15), Padykula (16), Rutenberg, Wolman, and Seligman (17), Rosa and Valardo (18), and Goebel and Puchtler (19), in which the tetrazolium salt was replaced by tellurite and to which sucrose was added. A sample of the incubating medium that was used aerobically or anaerobically contained in 20 ml. of 0.1 M Sörensen's phosphate buffer, 0.2 M sodium succinate, activators, and approximately 0.07 M sucrose. The following activators were added from stock solutions: 0.5 ml. of 0.6 M sodium bicarbonate, 0.2 ml. of 0.01 M aluminum chloride, and 0.1 ml. of 0.005 M magnesium chloride. When the activator calcium chloride (0.5 cc. of 0.3 M solution) was also added, the molarity of the phosphate buffer was lowered to 0.05 M to prevent precipitation, and the amount of sucrose was raised correspondingly. When potassium cyanide (0.015 M) was used, it was titrated first to pH 7.6 with 0.1 M monosodium phosphate before adding enough buffer to make a 20 ml. volume. This mixture required a corresponding decrease in the amount of sucrose. Anaerobic conditions were attained by using boiled and cooled incubating solutions placed in a vacuum-desiccator, or covered with mineral oil, or solutions through which oxygen-free nitrogen was bubbled for 1 hour before incubation as well as during incubation.

After an incubation period of usually 2 hours at 37°C., the tissues were fixed for 5 to 15 minutes in cold 1 per cent osmium tetroxide buffered at pH 7.4 with acetate veronal and containing enough sucrose to raise the over-all osmolar concentration to 0.44 M.[2] The fixed tissues were washed briefly in 0.44 M sucrose, dehydrated in increasing concentrations of ethyl alcohol, embedded in *n* butyl methacrylate, and sectioned with a Porter-Blum microtome. Thin sections were mounted on carbon-coated grids and viewed with an RCA (EMU 2b) electron microscope. Thick sections (1 μ) of the same blocks were mounted on glass slides and examined without additional staining with a light microscope. Alternatively, formalin-fixed, frozen sections 10 μ thick were also made of stained blocks for light microscopy.

For control purposes, tissue blocks were: (*1*) immersed into sucrose solution for 5 minutes at 80°C. before incubation; (*2*) fixed for ½ hour in 4 per cent formaldehyde or 80 per cent ethanol before incubation; (*3*) preincubated for 1 hour in a solution of sucrose containing iodoacetate (0.01 M) or N-ethylmaleimide (0.04 M); (*4*) tellurite was omitted from the incubating medium; (*5*) succinate was omitted from the incubating medium; and (*6*) sodium malonate (0.2 M) was added to the incubating medium. The first four controls were used

[2] J. Caulfield was the first to show, in still unpublished work, that increasing the total osmolar concentration of fixing medium by the addition of sucrose results in a better preservation of morphological structure.

for the endogenous system and all six were used for the succinic dehydrogenase system. In addition, other substrates of the tricarboxylic acid cycle, such as sodium malate or citrate, were assayed in place of succinate, without the addition of the pyridine nucleotide coenzymes, on the grounds that a negative result with these substrates would strengthen the position that the initiation of electron transfer, when succinate was used as substrate, was due to succinic dehydrogenase.

RESULTS

General

Endogenous Dehydrogenase.—Small pieces of tissue incubated in the medium described for demonstrating endogenous dehydrogenase activity turned black within a short period of time. Within 10 to 20 minutes, some organs like heart muscle or kidney were noticeably darkened, while others took a longer period. In general, the intensity of the color developed increased with time up to 2 hours incubation and remained stationary or increased only slightly thereafter.

Tellurite was extremely toxic when injected intravenously, the rats dying within a few minutes. The amount of tellurite reduced by the tissues under these conditions appeared to be small. Even when 5 ml. of a 1 per cent solution of tellurite was administered, only the kidney showed a positive reaction grossly. When administered intraperitoneally in large amounts, tellurite was also toxic, the rats usually succumbing in less than 1 hour. By that time, however, the intestines, parts of the liver, and the female reproductive tract contained reduced tellurite. When smaller, daily doses (0.5 cc. of 0.1 per cent solution for 12 days) were administered, some rats survived and the abdominal organs including the kidneys contained reduced tellurite. Intraluminal instillation of a tellurite solution into a tied off loop of the intestine having its blood supply intact proved to be an excellent means of demonstrating the intravital reduction of tellurite. However, in all cases of intravital use, the blackening of the tissue was less intense and less uniform than in supravital tests. Supravitally, all portions of the tissue blocks were stained. With skeletal muscle, blocks of diaphragm reacted negligibly, tongue reacted strongly, and with extraocular muscles, a spotty reaction occurred.

Succinic Dehydrogenase.—For the demonstration of succinic dehydrogenase, both frozen blocks of tissue and blocks washed for 2 hours in sucrose were satisfactory, since no endogenous activity could be demonstrated in either case. The former treatment was preferred as a matter of convenience as well as for the fact that specimens so treated appeared better preserved for electron microscopy. The control experiments indicated that the reduction of tellurite was on an enzymatic basis. Immersion of the blocks of tissue into a hot (80° C.) sucrose solution for 5 minutes, fixation in formalin or ethyl alcohol for 1 hour, or pretreatment with N-ethyl maleimide or iodoacetate before incubation prevented the reduction of tellurite as indicated by the absence of visible blackening and the absence of the reduced product microscopically. In addition, no reaction occurred if sodium malonate was added to the incubating medium

containing succinate, or if malate or citrate were substituted for succinate. No reaction product could be visualized with either the light or electron microscope if either the succinate or tellurite were omitted from the incubating medium (Figs. 8, 11). When succinate and tellurite were used, reduced tellurite was present throughout the small blocks of tissue. It can be assumed, therefore, that the penetration of both reagents into the tissues tested was satisfactory.

The addition of activators, especially bicarbonate and calcium, definitely increased the rate of the blackening of the tissues. In most cases, the addition of potassium cyanide, or to a lesser extent the use of anaerobic conditions for incubation, seemed to improve the rate of blackening of tissues over that seen with the activators alone. There were no differences in the sites of deposition of the product with any of the combinations of reagents tested. Formalin-fixed, frozen sections (10 μ) of heart and kidney examined with the light microscope showed that the deposition of the black final product was restricted to spherical and rod-like bodies which were tentatively identified as mitochondria. The sections of methacrylate-embedded material (1 μ) were not suitable for light microscopy presumably because of the small amount and fine dispersion of the final product.

Electron Microscopy

Dense deposits representing the final product of a positive reaction, were observed in all the tissues tested. The best results were obtained with heart muscle which incurred less damage than other tissues during the relatively long periods of supravital incubation. In addition this tissue showed a more intense and more sharply localized reaction. For these reasons, the heart was the material most extensively used for electron microscopy.

General Morphology of Incubated Specimens.—The morphology of heart muscle that was subjected to the incubating procedures mentioned was remarkably well preserved for the history of the specimens. The best preservation was found in tissue blocks incubated for endogenous dehydrogenase activity, without any remarkable differences caused by the duration of incubation up to 2 hours (Fig. 1). Specimens incubated for 4 hours and especially for 7 hours appeared somewhat extracted, but the sarcolemma, myofibrils, nuclei, and mitochondria were still recognizable. Specimens from which the endogenous substrates were removed by washing showed the poorest general preservation; the mitochondria were particularly affected by swelling and extraction. Preservation of the material that was frozen before incubation was good, with no obvious evidence of damage from ice crystal formation. In such specimens, mitochondria usually contained scattered vacuoles. Regardless of the length of incubation, frozen and thawed specimens were better preserved than those in which the endogenous substrates were removed by washing. The peripheral zone of all incubated blocks showed extensive extraction and disorganization of the tissue.

In blocks taken from other organs and incubated for the demonstration of the endogenous activity or activity of succinic dehydrogenase systems, the fine structure of the cells was less well preserved than in the heart. The swelling of the endoplasmic reticulum was more pronounced, mitochondria showed more extensive extraction and swelling, and the cristae seemed fewer in number. Membranous structures in general showed varied degrees of disruption and disorganization, while the cytoplasmic matrix was to a large extent extracted.

Preservation of the material in which tellurite was used intravitally, especially the intestine, was excellent, and showed little or no difference from control pieces of tissue taken for routine electron microscopy.

The Fine Structure of Incubated Heart Muscle Fibers.—In all incubated specimens the myofibrils were found contracted with recognizable Z bands and with myofilaments still in regular alignment (Fig. 1). The myofilaments appeared finer and their orderly disposition was lost to a varied extent after prolonged incubation. The nuclei showed clearly recognizable nuclear envelopes (Fig. 2) of usual structure (20) and were characterized by a curious clumping of relatively dense particles that occurred in the nucleoplasm. These particles appeared after incubation irrespective of the presence or absence of tellurite in the incubating medium. The irregular mass formed by these clumped particles was surrounded by lighter material of finer grain. The elements of the sarcoplasmic reticulum were found in their usual location in the interfibrillar sarcoplasm (21) and as a rule appeared swollen (Figs. 4 and 5).

The innumerable mitochondria of heart muscle are conspicuous round or oval bodies which occur either in rows, in between the myofibrils (Fig. 1), or in clusters in the sarcoplasm, often located in close proximity to the poles of the nuclei (Fig. 2). When in rows, they sometimes occur one to a sarcomere. Contrary to the findings of Harman (22), and in agreement with previous descriptions (23), the mitochondria are biwalled structures which contain numerous cristae that extend internally. Most of the cristae appear to end as blind loops rather than extending across the full length of the mitochondrion. In the incubated specimens, these cristae were sometimes dilated, especially at their blind ends (Fig. 4). The vacuolar transformation of the cristae was more pronounced after incubation in the absence of substrate, or in the presence of potassium cyanide or in anaerobiosis. As already mentioned, similar vacuoles were encountered in frozen and thawed material. The mitochondrial matrix showed after incubation small and irregular areas of low density (Figs. 1 and 4) supposedly the result of an uneven, spotty extraction. The dense intramitochondrial granules usually found in the matrix were, as a rule, absent from incubated specimens.

Morphology and Localization of the Reaction Product in Heart Muscle.—In the myocardium, the final product of the histochemical reaction for demonstrating endogenous or succinic dehydrogenase activity was found almost exclusively localized in the mitochondria (Figs. 1, 4 to 7, and 9). It occurred either as rela-

tively large needles or plates 100 to 200 A in thickness by at least 1000 A in length (Figs. 1, 4, and 5), or as small, extremely dense particles 50 to 100 A in size (Figs. 1, 3 to 5).

The needles were assumed to be crystals because of their regular shape, straightness, and sharp angles. They occurred either singly or in small sheaves in the mitochondrial matrix sometimes oriented parallel to the cristae and apposed to their membranes (Figs. 1 and 10). Occasionally, however, similar crystals occurred on the outer limiting membrane of the mitochondria (Figs. 3 and 6). Only rarely were similar needles found in the sarcoplasm in the close proximity of, or at a small distance (less than 0.1 μ) from, a mitochondrial profile (Fig. 6). Almost always, all other structures and components in the heart muscle fiber, *i.e.*, myofibrils, sarcoplasmic reticulum, sarcoplasm, and nucleus, were free of crystals. Exception to this localization was the very rare occurrence of crystals apparently free in the sarcoplasm (Fig. 3) or applied to the outer membrane of the nuclear envelope (Fig. 2). However, since mitochondria usually occur in close proximity to the sites mentioned, the crystals may have been associated with mitochondrial membranes just out of the plane of section.

The very small, extremely dense particles occurred exclusively in the mitochondrial matrix, again in close relationship with the cristae. They were not evenly distributed throughout the mitochondria, but tended to occur in groups or clusters (Figs. 1, 3 to 5). It is difficult to decide whether the small particles are located in the matrix, in between the cristae, within their membranes or closely applied to the latter. Most micrographs suggest that they lie in the matrix in close relationship to the membrane outlining the cristae (Figs. 3 to 5). These tiny particles varied in number from one preparation to another.

In specimens demonstrating succinic dehydrogenase activity, the crystals were in general smaller and less numerous (Figs. 7 and 9). They were located either inside or on the mitochondria, and as a rule, extramitochondrial deposits were not encountered. Small particles were rare, or absent, but fine rods of slightly larger dimensions (Figs. 7 and 9) were rather frequent and occurred either individually or in small clusters in the mitochondrial matrix. These rods as well as the larger crystals showed occasionally discontinuities or "holes" in their substance (Figs. 9 and 10).

Observations on Other Tissues.—Intramitochondrial deposition of reduced tellurite particles occurred also in the cells of other organs especially in intestinal epithelia when the tellurite was used intravitally. In supravital tests, however, intramitochondrial localization of small particles was not common for either the endogenous or the succinic dehydrogenase system, but relatively large crystals occurred inside the mitochondria. More frequently, the deposits were in the form of large crystals that overlaid the outer mitochondrial membranes. In fact, often the crystals were curved so as to fit closely to the rounded surface of the membranes. Extramitochondrial localization of crystals occurred more

frequently in tissues other than heart, especially when the activity of endogenous dehydrogenase systems was demonstrated. As in the heart, the reduced tellurite occurred on the outer membrane of the nuclear envelope and in addition on other membranous structures in the cytoplasm (endoplasmic reticulum).

Influence of Fixation upon the Final Product.—Finally, in all materials tested, the crystals and fine particulate deposits were absent in the disrupted zone at the periphery of the block as well as in a narrow subjacent zone of well preserved tissue; they could be demonstrated in the rest of the specimen only after a short fixation with osmium tetroxide. As a rule, the length of fixation could not exceed 15 minutes; if extended to 1 hour, no evidence of reduced tellurite was found in the tissues. Apparently long exposure to the fixative resulted in the dissolution of the crystals and small particulate deposits.

Formalin was an adequate fixative for the preservation of the final product, but it was not used beyond the preliminary experiments. Although crystals were clearly visible in or on mitochondria in preparations so fixed, the relationship of these deposits to mitochondrial structure could not be studied because the mitochondrial membranes were not satisfactorily preserved.

DISCUSSION

This paper presents evidence of a successful histochemical method for the demonstration of the activity of endogenous and succinic dehydrogenase systems with the electron microscope. The method used potassium tellurite as the hydrogen or electron acceptor with the reduced tellurite as a final product, opaque to electrons. We have referred to this product as reduced tellurite instead of tellurium since we did not know the composition of the final product by analysis. Both tellurium (Te) and tellurium monoxide (TeO) are black and insoluble in aqueous solutions, and the native metal turns silver on standing (24). Although tellurium is readily produced from a solution of tellurite by the action of reducing agents, it cannot be stated with assurance that all deposits of final product observed in our tests are of the same nature. This possibility is supported by the existence both of finely particulate deposits and relatively large crystals in the tested specimens. The deposits[3] could be tellurium monoxide, which is "amorphous," whereas the crystals may be tellurium.

Although the present experiments utilizing succinate as substrate indicate specificity of the enzyme which initiates electron transfer, we have used the term "system" in relation to the activity demonstrated rather than the specific enzyme. Experiments on isolated mitochrondria and a "purified" succinoxidase system indicate that the electrons are not directly transferred from the dehydrogenase to tellurite and that the reaction requires a number of cofactors. The same is probably true of the endogenous dehydrogenase system. This subject

[3] Another possibility under investigation is that the fine particulate deposits represent the final product of a different enzymatic reaction. This possibility ensues since the deposits were observed in the preparations tested for endogenous activity.

will be covered in another communication (made in collaboration with E. G. Ball), as will the use of tellurite in demonstrating the activity of dehydrogenase systems requiring pyridine nucleotide coenzymes (25).

The results described appear to have some practical value for working out preparatory procedures satisfactory for both electron microscopy and histochemistry. In this respect, certain methodological findings in our experiments may be helpful in the adaptation of other histochemical methods to electron microscopy. It is evident that histochemical techniques can be used intravitally or supravitally and that the substrates and reagents we used can easily penetrate intact cells *in situ* or in small blocks of tissue. The penetration is obviously a function of the dimensions of the tissue blocks and of the character of the reagents used, and as such, our findings may not apply to other compounds and other methods (26, 27). It also appears that reasonable conditions of incubation for histochemistry are compatible with unexpectedly good preservation of fine structure in certain tissues. Even hours of incubation at 37° C., in the presence of toxic compounds at abnormal pH, and in the absence of oxygen, do not necessarily alter beyond recognition the fine structure of certain cells. It is also clear, however, that some cell types are more seriously affected in this regard than others. It follows that judicious choice of tissue and careful investigation of the conditions of incubation can facilitate the production of satisfactory results for the development of histochemical tests that can be used in electron microscopy. In this regard, heart muscle was found to be a more rugged tissue than kidney cortex and liver, while brown fat gave poor results in all instances, mostly because of extensive cell disintegration. Furthermore, regardless of cell type tested, incubating media that contained sucrose resulted in the best preservation of structure without any detectable change in the localization of the histochemical product. It should also be pointed out that brief fixation in buffered osmium tetroxide, after the histochemical reactions are completed, is a procedure that ensures the preservation of the final histochemical product as well as the preservation of the fine structures of the cells, and thus allows the study of their interrelations.

The final point to be discussed concerns the relationship between the actual localization of the enzyme tested and the site of the deposition of the product. In our case, the system tested has a number of favorable features, namely: the insoluble character of the respiratory enzymes and the fact that they are structurally bound within or to the mitochondria as indicated by numerous cytochemical studies (*cf.* 10–13). In addition, the insolubility of reduced tellurite and the high rate of electron transfer (which presumably leads to a correspondingly high rate of tellurite reduction and rapid saturation of the immediate surroundings of the enzymatic site) would favor a minimal distance between enzyme and precipitated product. Our results bear out this expectation because the deposits and crystals of dense material are as a rule located within, or on, the surface of mitochondria. More rarely, crystals are found in close

proximity to these organelles. In this respect it should be pointed out, however, that the use of thin sections practically reduces topographic information to only two dimensions in electron microscopy. A crystal lying at a relatively large distance from a mitochondrion in the plane of the section may be close or adjacent to another mitochondrion above or below this plane. Such geometrical complications induced us to look for a material in which a favorable distribution of cell structure occurs. The heart muscle, with its relatively large mitochondria-free spaces, served this purpose satisfactorily. In its case, the deposits and crystals occurred almost exclusively within, or on, mitochondria, in close association with mitochondrial membranes. They were not found in the nucleoplasm or in the myofibrils. In view of these results we can assume that in heart muscle fibers the distance between the site of reduction and the site of deposition is in general not greater than 0.1 μ.

It should also be pointed out that below this level the occurrence of the final product as crystals minimized the accuracy of localization of the site of enzyme activity. On the one hand, the occurrence of such crystals in close relationship to mitochondrial membranes may be heartening. On the other, the large size of the crystals may overspread the site by several hundred A. With this condition of large crystals and the fact that crystal formation requires supersaturation which is more likely to occur at the sites of highest enzymatic activity, it can be assumed that a very small portion of a crystal is in close proximity to the enzyme site.

It has been repeatedly suggested (28, 29) that the insoluble succinoxidase complex is closely associated with, or built in, the mitochondrial membranes. The recent work of Watson and Siekevitz (30, 31), which integrates biochemical results and electron microscope studies on isolated mitochondria, provides conclusive evidence in favor of this suggestion. Our results represent visual evidence obtained on mitochondria *in situ* in favor of the same conclusion.

The combination of histochemistry and electron microscopy makes possible an integrating step in cytology and can lead towards the clarification of the relationship of structure to biochemical function in many cases. Further studies combining histochemistry and electron microscopy are now in progress (25, 27, 32).

SUMMARY

In the present study a histochemical method demonstrating the activity of dehydrogenase systems was developed for electron microscopy, utilizing potassium tellurite as the hydrogen or electron acceptor. This reagent was used intravitally (intravenously, intraperitoneally, or intraluminally in hollow organs) or supravitally on small blocks of tissue for the demonstration of endogenous dehydrogenase activity. Blocks of tissue which had been frozen and thawed or which had been washed in 0.44 M sucrose to prevent endogenous activity, were used to demonstrate the activity of the succinic dehydrogenase

system. In the latter case, the incubating medium contained tellurite, succinate, phosphate buffer, sucrose, and activators. The incubation was as performed either aerobically (with or without the addition of potassium cyanide) or anaerobically. The specificity and the enzymatic nature of the reactions were ascertained by appropriate control experiments.

Reduced tellurite, the end product of this histochemical reaction, could be visualized in thin sections of osmium tetroxide-fixed, methacrylate-embedded tissues as crystals or fine particulate deposits of high density, localized on, or in close relationship to mitochondrial membranes. The results of these experiments are demonstrated, utilizing heart muscle (rat) as the source of the enzyme systems.

BIBLIOGRAPHY

1. Hoffman-Ostenhof, O., *Advances Enzymol.*, 1953, **14,** 219.
2. Klett, A., *Z. Hyg. u. Infectionskrankh.*, 1900, **33,** 137.
3. Conradi, H., and Troch, P., *Münch. med. Woch.*, 1912, **53,** 1652.
4. Schreiner, O., and Sullivan, M. X., *Bot. Gaz.*, 1911, **51,** 121.
5. Hasegawa, K., *Japan. J. Bot.*, 1936, **8,** 1.
6. Lakon, G., *Ber. bot. Ges.*, 1939, **57,** 191.
7. Kuhn, R., and Jerchel, D., *Ber. chem. Ges.*, 1941, **74B,** 949.
8. Wachstein, M., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72,** 175.
9. Deane, H. W., Barrnett, R. J., and Seligman, A. M., Histochemical methods for the demonstration of enzymatic activity, *in* Handbuch der Histochemie, (W. Graumann and K. Neumann, editors), Stuttgart, Gustaf Fischer, 1957, **6,** in press.
10. Lindberg, O., and Ernster, L., Chemistry and physiology of mitochondria and microsomes, Protoplasmatologia, Handbuch Protoplasmaforsch., **3A,** Wien, Springer, 1954, 4.
11. De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, 1955, **60,** 604.
12. Hogeboom, G. H., and Schneider, W. C., The Cytoplasm, *in* The Nucleic Acids, (E. Chargaff and J. N. Davidson, editors), New York, Academic Press Inc., 1955, **2,** 199.
13. Goddard, J. W., and Seligman, A. M., *Anat. Rec.*, 1952, **112,** 543.
14. Witter, R. F., Watson, M. L., and Cottone, M. A., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 127.
15. Seligman, A. M., and Rutenburg, A. M., *Science*, 1951, **113,** 317.
16. Padykula, H. A., *Am. J. Anat.*, 1952, **19,** 107.
17. Rutenburg, A. M., Wolman, M., and Seligman, A. M., *J. Histochem. and Cytochem.*, 1953, **1,** 66.
18. Rosa, C. G., and Velardo, J. T., *J. Histochem. and Cytochem.*, 1954, **2,** 110.
19. Goebel, A., and Puchtler, H., *Virchows Arch. path. Anat.*, 1955, **326,** 312.
20. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 257.
21. Porter, K. R., and Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 269.
22. Harman, J. W., *Internat. Rev. Cytol.*, 1956, **5,** 89.
23. Palade, G. E., *J. Histochem. and Cytochem.*, 1953, **1,** 188.

24. Mellor, J. W., A Comprehensive Treatise on Inorganic and Theoretical Chemistry, New York, Longmans Green and Co., 1931, **11**, 1.
25. Barrnett, R. J., *Anat. Rec.*, 1957, **127**, 395.
26. Novikoff, A. B., Burnett, F., and Glickman, M., *J. Histochem. and Cytochem.*, 1956, **4**, 416.
27. Barrnett, R. J., Palade, G. E., Goldstein, T., and Seligman, A. M., *J. Histochem. and Cytochem.*, 1957, in press.
28. Palade, G. E., *Anat. Rec.*, 1952, **114**, 427.
29. Cleland, K. W., and Slater, E. C., *Biochem. J.*, 1953, **53**, 547.
30. Watson, M. L., and Siekevitz, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 639.
31. Siekevitz, P., and Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 653.
32. Barrnett, R. J., *J. Histochem. and Cytochem.*, 1957, in press.

EXPLANATION OF PLATES

PLATE 180

FIG. 1. *Rat Myocardium.* After excision from a living animal, the specimen was incubated *in vitro* aerobically for 2 hours at 37°C. in a medium containing potassium tellurite, succinate, activators, phosphate buffer (pH 7.6), and sucrose. At the end of the incubation period, the specimen was fixed for 15 minutes in 1 per cent OsO_4 in acetate veronal buffer, pH 7.6 (total osmolar concentration raised to 0.44 M with sucrose). The preparation demonstrates both endogenous and succinic dehydrogenase activity.

The micrograph shows a relatively large area in the cytoplasm of a sectioned heart muscle fiber. Myofibrils, marked *mf*, cross the field diagonally and appear to branch and join together as they usually do in the myocardium. The muscle is contracted as indicated by the absence of I bands and by the presence of relatively wide and dense Z bands (*z*).

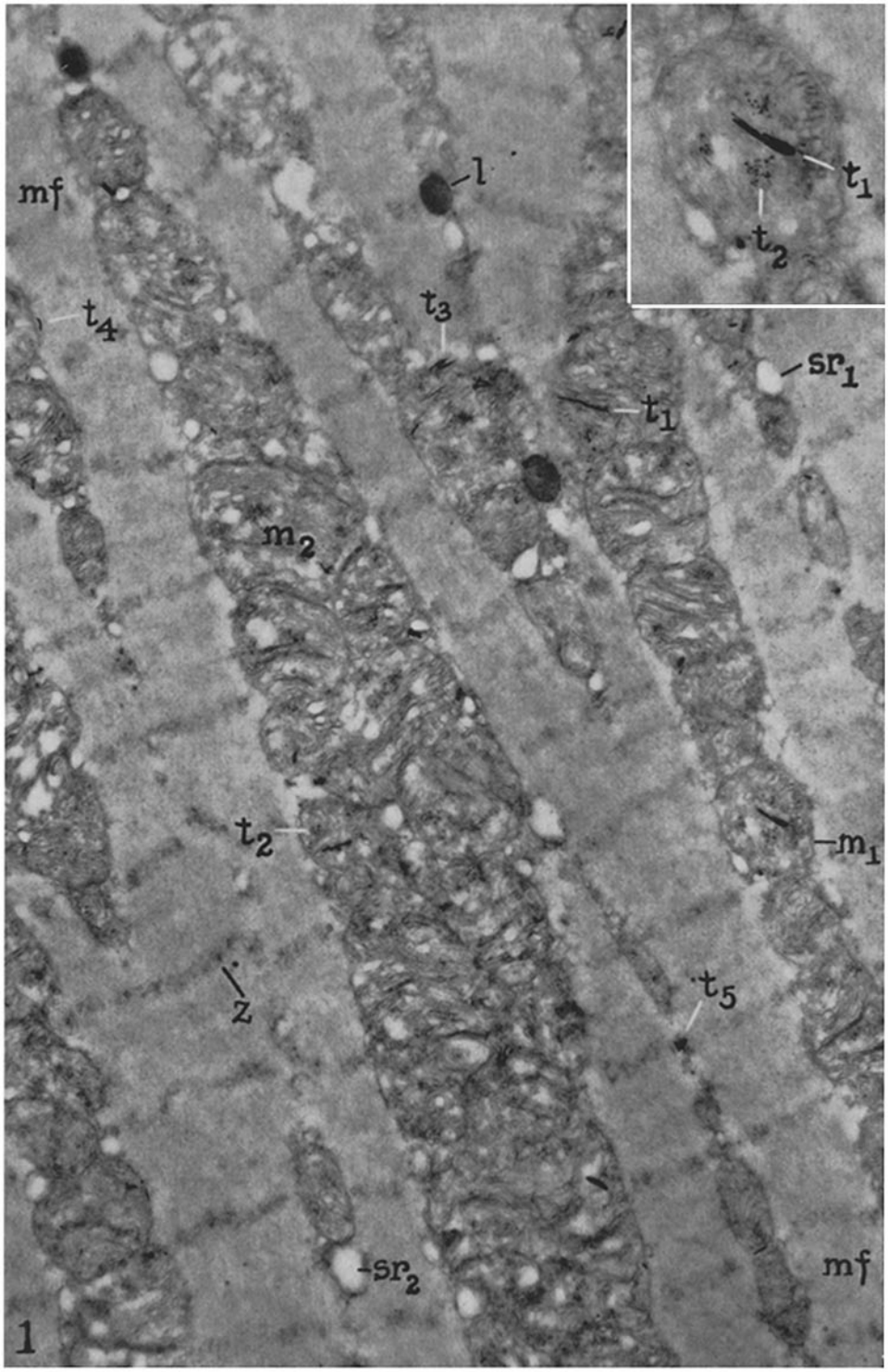
Numerous mitochondria (m_1, m_2) can be seen disposed longitudinally in rows in the interfibrillar sarcoplasm. Elements of the sarcoplasmic reticulum (sr_1, sr_2) and lipide inclusions (*l*) occur in close proximity to mitochondria.

Small needles (t_1) and fine particulate deposits (t_2) of high density can be seen inside the mitochondria. They represent reduction products of tellurite. The fine particulate deposits might be tellurium monoxide (TeO) and the needles, crystals of tellurium metal.

Note that the crystals and deposits are preferentially located inside the mitochondria. Crystals are rarely found outside in close apposition to (t_3), or in the immediate vicinity (t_4) of, the limiting mitochondrial membrane. Only occasionally (t_5) they occur in the sarcoplasm away from any mitochondrion in the plane of the section. They may be, however, close to organelles located above or below this plane. The myofibrils appear entirely free of both crystals and fine deposits.

Note that although the specimen was fixed after 2 hours incubation in a foreign medium, the cytoplasmic components retain most of their structural features. The most obvious changes are the spotty vacuolization of mitochondria (m_2) and the swelling of the sarcoplasmic reticulum (sr_2). Magnification, 26,000.

Inset. The inset shows, at a higher magnification, the mitochondrial profile marked m_1 in Fig. 1. A sheaf of needles appears at t_1 and one cluster of fine particles of high density at t_2. Magnification, 50,000.



(Barrnett and Palade: Activity of dehydrogenase systems)

FIG. 2. Rat myocardium incubated for 2 hours in a mixture containing tellurite phosphate buffer (pH 7.6) and sucrose to demonstrate endogenous dehydrogenase activity. The micrograph shows part of a nucleus and the surrounding cytoplasm in a heart muscle fiber.

The nuclear envelope shows its two membranes at *e* and its pores at the arrows. The nucleoplasm is clearly segregated into two types of materials: dense and relatively coarse granules form irregular clumps (*nc*), mainly in the central regions of the nucleus, and appear surrounded by a lighter matrix of finer grain (*lm*). The appearance is characteristic for the nuclei of incubated specimens irrespective of the presence or absence of tellurite in the incubation medium.

The nucleus is surrounded by numerous mitochondria (*m*), and by contracted myofibrils. A Z band can be seen at *z*. There is little vacuolization inside the mitochondria but the cavities of the adjacent sarcoplasmic reticulum (*sr*) appear distended.

Small crystals and fine particles of high density can be seen in a number of places inside the mitochondria (t_1) or on their surface (t_2). They are absent in the myofibrils and in the nucleoplasm. A small deposit of similarly high density is found, however, on the nuclear envelope (t_3). Magnification, 24,000.

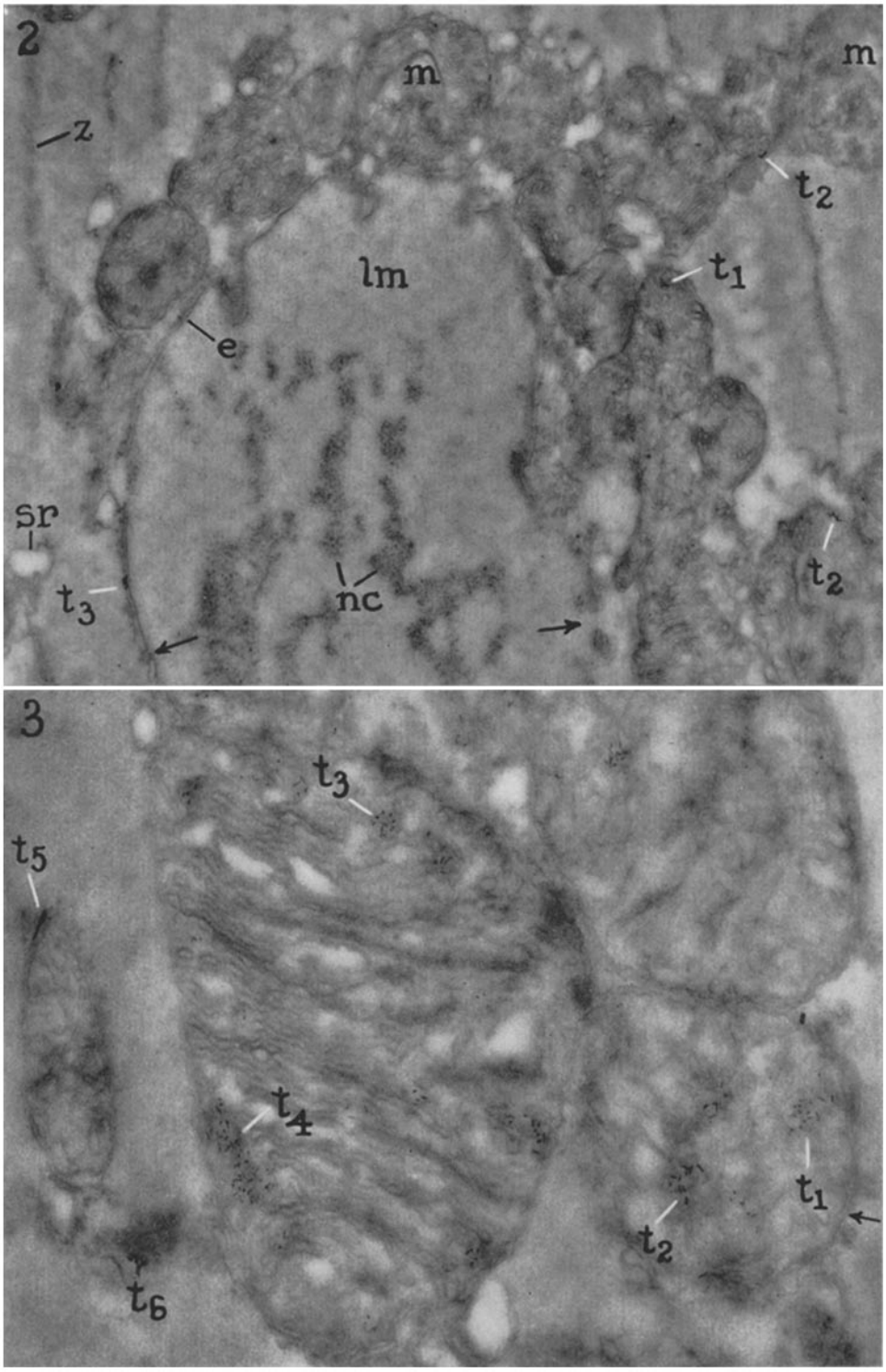
FIG. 3. Four mitochondrial profiles of heart specimen treated as the one in Fig. 2.

Although the mitochondria show a certain amount of vacuolization and spotty removal of matrix with subsequent irregularity in the arrangement of the cristae, characteristic structural details are still recognizable. The two mitochondrial membranes can be seen at the periphery of the right lower profile (arrow), and numerous, almost normally sectioned cristae appear in the large middle profile. In the two profiles to the right the cristae do not show distinctly because of the obliquity of their section.

The micrograph illustrates the fine particulate deposits found inside the mitochondria incubated in the presence of tellurite. These particles appear in sections as fine granules (t_1) or rods (t_2). They occur in clusters (t_1, t_2, t_3, t_4) and are characterized by their high density. Most of the clusters are located in relatively dense intramitochondrial areas which may represent cristae seen in oblique view or condensations of mitochondrial matrix. Within the clusters the particles are randomly oriented and only occasionally they form circular patterns like the one at t_3.

A dense needle (t_5) can be seen at the surface of the small mitochondrial profile to the left, and a deposit of dense material lies apparently free in the sarcoplasm at t_6.

Magnification, 60,000.



(Barrnett and Palade: Activity of dehydrogenase systems)

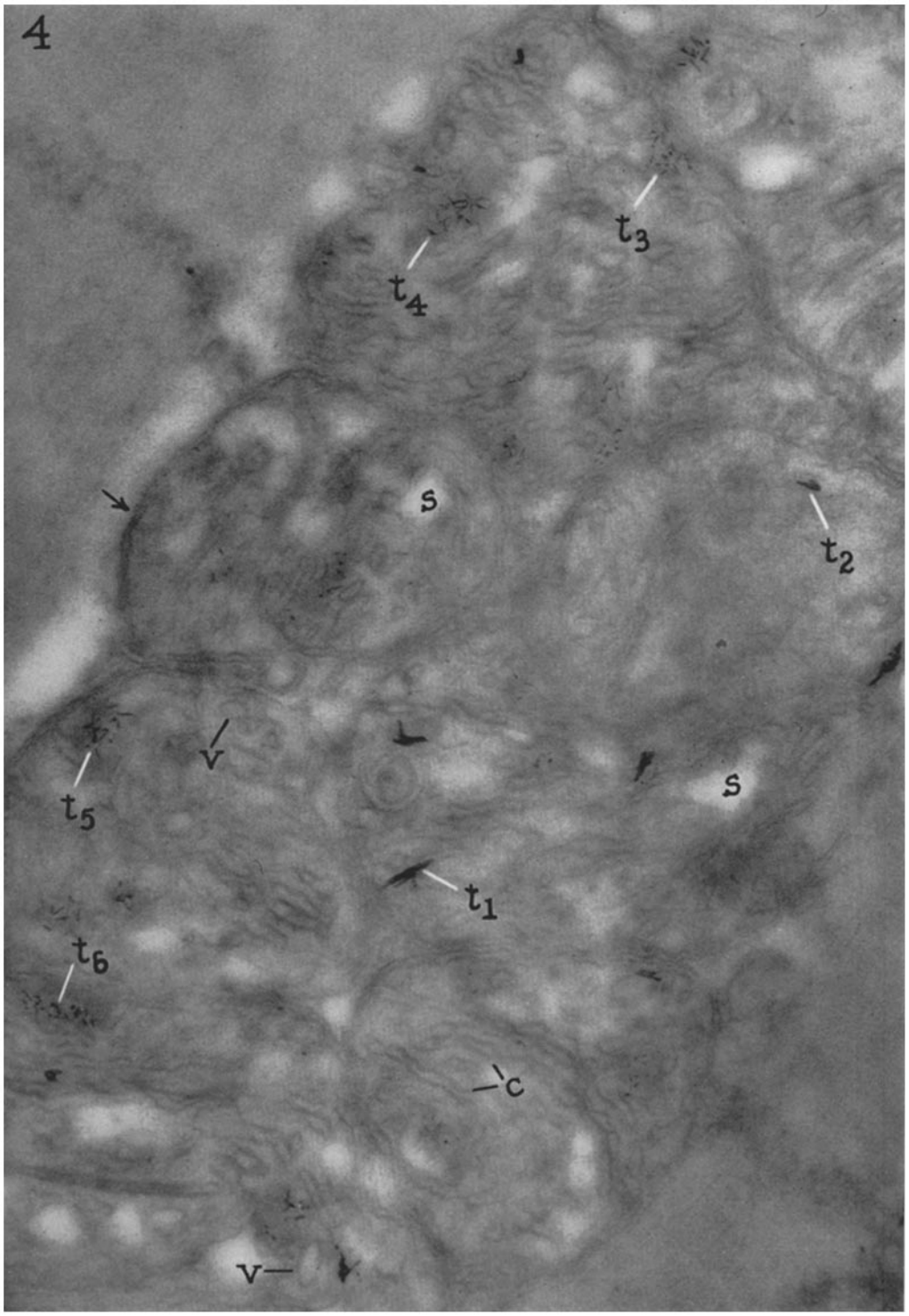
PLATE 182

FIG. 4. Cluster of mitochondria in a heart muscle fiber. Specimen incubated for 2 hours at 37°C. in a medium containing tellurite, succinate, phosphate buffer (pH 7.6), and sucrose; fixed thereafter for 15 minutes in 1 per cent OsO_4 in acetate veronal buffer, pH 7.4, with sucrose added to a total osmolar concentration of 0.44 M.

The clustered mitochondria occupy a broad diagonal band in the center of the field; myofibrils appear in the upper left and lower right corners.

The two mitochondrial membranes can be seen in close apposition (*e.g.*, arrow) at the periphery of many mitochondrial profiles. The cristae (*c*), cut at various angles, have a central light layer of unequal thickness. Local distentions appear as intramitochondrial vacuoles (*v*). The matrix shows lighter irregular spots (*s*) probably due to unequal extraction. The vacuolization of the cristae and the unequal extraction of the matrix are both responsible for the spotted appearance of mitochondria in incubated specimens.

The micrograph illustrates the two forms taken by the deposits of reduced tellurite inside the mitochondria. Relatively large needles appear at t_1 and t_2 disposed in pairs, small sheaves, or heaps. Fine dense particles of round or rod-like shapes, can be seen at t_3, t_4, t_5, and t_6. They occur in clusters usually located in denser areas of the mitochondria. Magnification, 80,000.



(Barrnett and Palade: Activity of dehydrogenase systems)

PLATE 183

FIG. 5. Cluster of mitochondria in a heart muscle fiber. Specimen treated as the one in Fig. 4 and demonstrating, therefore, both endogenous and succinic dehydrogenase activity.

The elements of the sarcoplasmic reticulum (*sr*) at the periphery and within the cluster of mitochondria show extensive swelling. The mitochondria appear less damaged and show only a limited amount of vacuolization of their cristae and of spotty removal of the matrix.

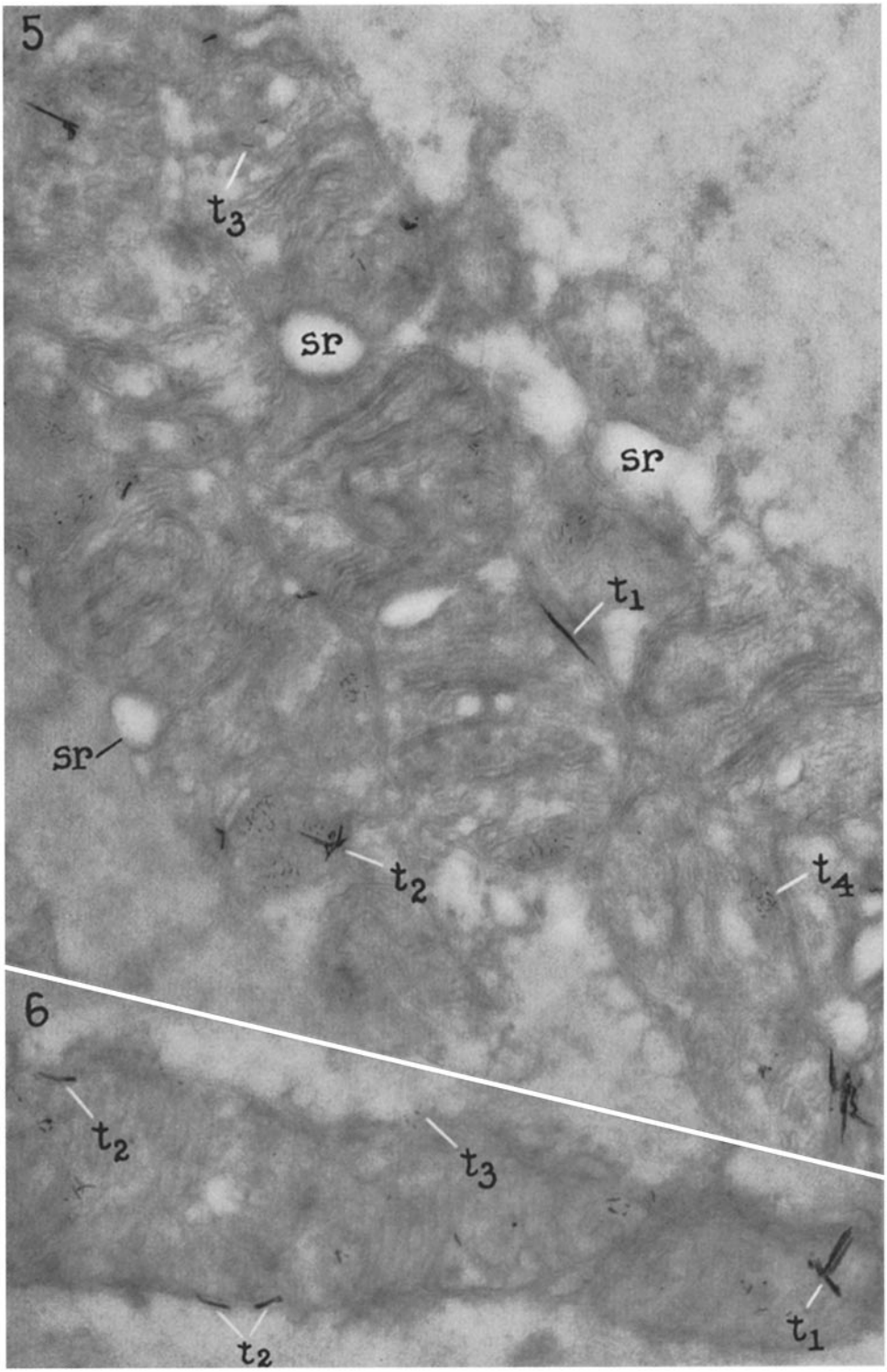
The two types of reduced tellurite are again clearly demonstrated. The relatively regular shape, the straightness, and the mode of aggregation of the "needles" (t_1, t_2) suggest that they are crystals of acicular or narrow, plate-like form.

The fine particulate deposits consist of rods and dots, unevenly distributed individually (t_3) or in clusters (t_4) throughout the mitochondria. They may represent smaller crystals or micelles of a different compound.

As in the case illustrated by Fig. 4, the crystals are exclusively located inside the mitochondria. Magnification, 55,000.

FIG. 6. Longitudinally sectioned mitochondrion in a heart muscle fiber. Specimen incubated for 2 hours in a medium containing tellurite, phosphate buffer (pH 7.6) and sucrose for the demonstration of endogenous dehydrogenase activity.

The micrograph illustrates the localization of reduced tellurite inside the mitochondria (t_1), on the limiting mitochondrial membrane (t_2), or in its immediate vicinity (t_3). Magnification, 65,000.



(Barrnett and Palade: Activity of dehydrogenase systems)

PLATE 184

FIG. 7. Rat myocardium frozen and thawed to eliminate endogenous dehydrogenase activity; incubated thereafter aerobically for 2 hours in a medium containing tellurite, succinate, activators, potassium cyanide, phosphate buffer (pH 7.6), and sucrose; fixed finally for 15 minutes in 1 per cent OsO_4 in acetate veronal buffer (pH 7.6) and sucrose. The preparation demonstrates succinic dehydrogenase activity.

The micrograph shows a relatively large area in an obliquely sectioned muscle fiber. The field is occupied by clusters of mitochondria (*m*) and by myofibrils (*mf*).

The mitochondria show spotty extraction of the matrix and a certain amount of vacuolization of their cristae. Otherwise their structure is relatively well preserved. The cavities of the sarcoplasmic reticulum (*sr*) appear distended.

Reduced tellurite appears in the form of short needles of high density inside the mitochrondria (t_1) or on their surfaces (t_3). Fine individual rods are also encountered (t_2), but clusters of fine particles are absent.

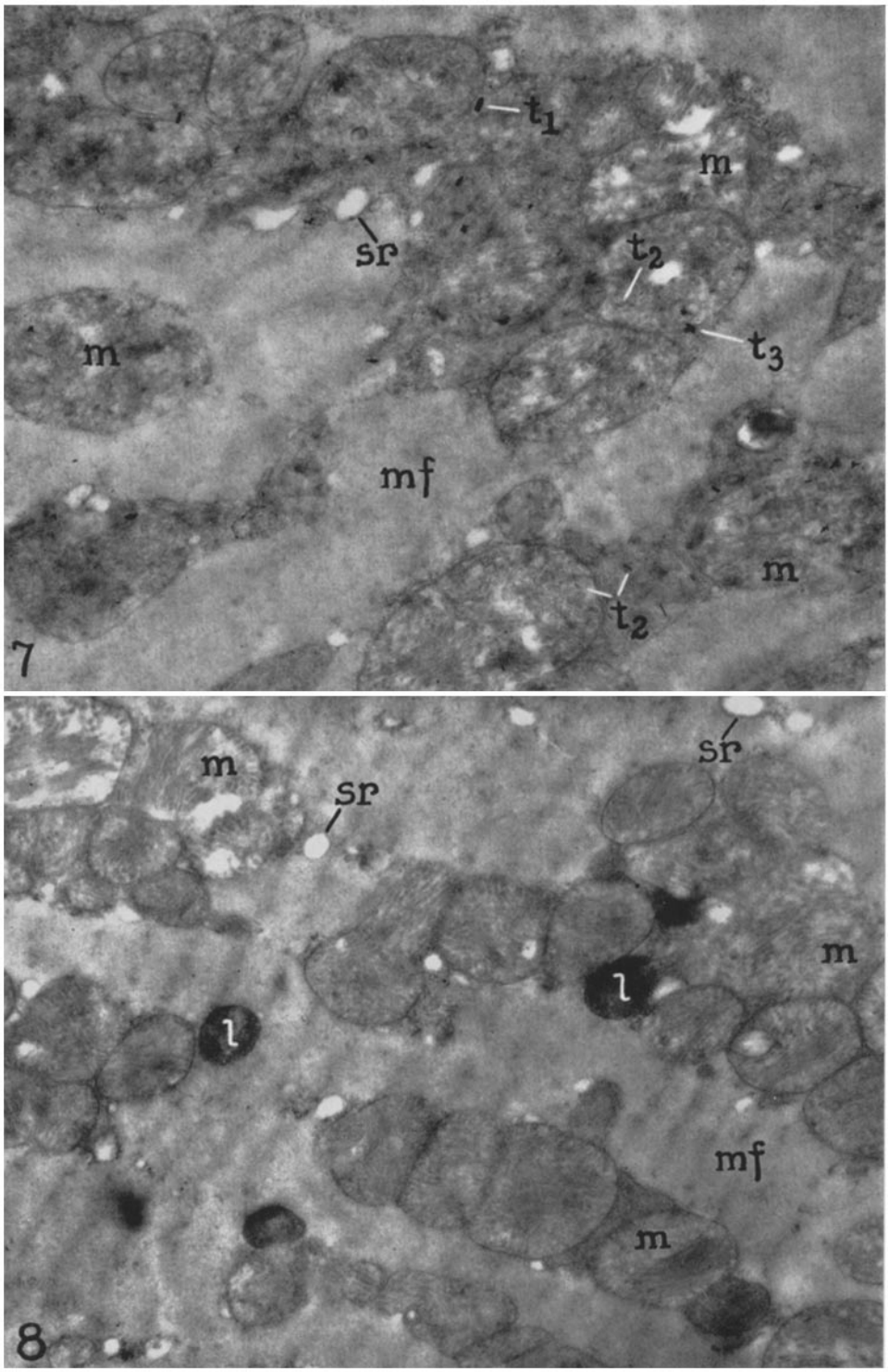
No reduced tellurite is found in the myofibrils. Magnification, 22,000.

FIG. 8. Rat myocardium treated as the specimen in Fig. 7 except that potassium tellurite was excluded from the incubating medium.

The micrograph shows a relatively large field in a muscle fiber containing rows and clusters of mitochondria (*m*), lipide inclusions (*l*), and contracted myofibrils (*mf*).

The damage produced by incubation (swelling of the sarcoplasmic reticulum, *sr*, spotty extraction of the mitochondrial matrix, and distention of the cristae) is comparable to that shown by the specimen in Fig. 7.

No deposits of material of high density are visible in the mitochondria or in the rest of the cytoplasm. The result indicates that tellurite is responsible for the formation of the dense needles and fine particulate deposits in the specimens shown in Figs. 1 to 7. Magnification, 20,000.



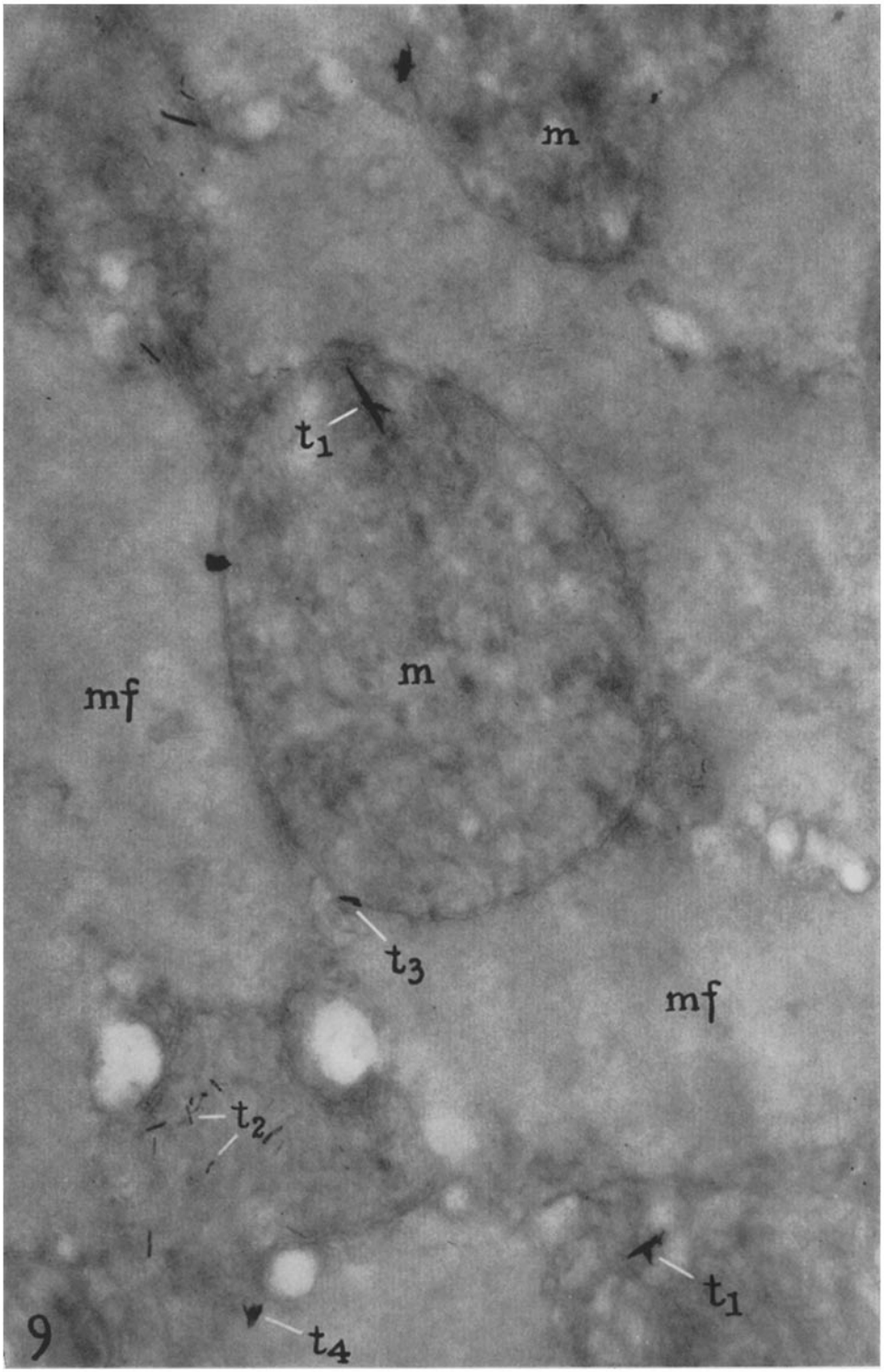
(Barrnett and Palade: Activity of dehydrogenase systems)

PLATE 185

FIG. 9. Rat myocardium treated as the specimen in Fig. 7. The preparation demonstrates therefore succinic dehydrogenase activity.

The micrograph shows at a higher magnification mitochondria (*m*), and transversely sectioned myofibrils (*mf*) in a heart muscle fiber.

The mitochondria contain sheaves of relatively large crystals (t_1) and smaller individual rods (t_2) of high density (smaller crystals?). Some of these rods show holes or discontinuities in their otherwise homogenous substance. Other crystals occur individually or in sheaves on the limiting membrane of mitochondria (t_3) or in its close proximity (t_4). Magnification, 65,000.



(Barrnett and Palade: Activity of dehydrogenase systems)

PLATE 186

FIG. 10. Rat myocardium treated as the specimens in Figs. 3 and 6 to demonstrate endogenous dehydrogenase activity.

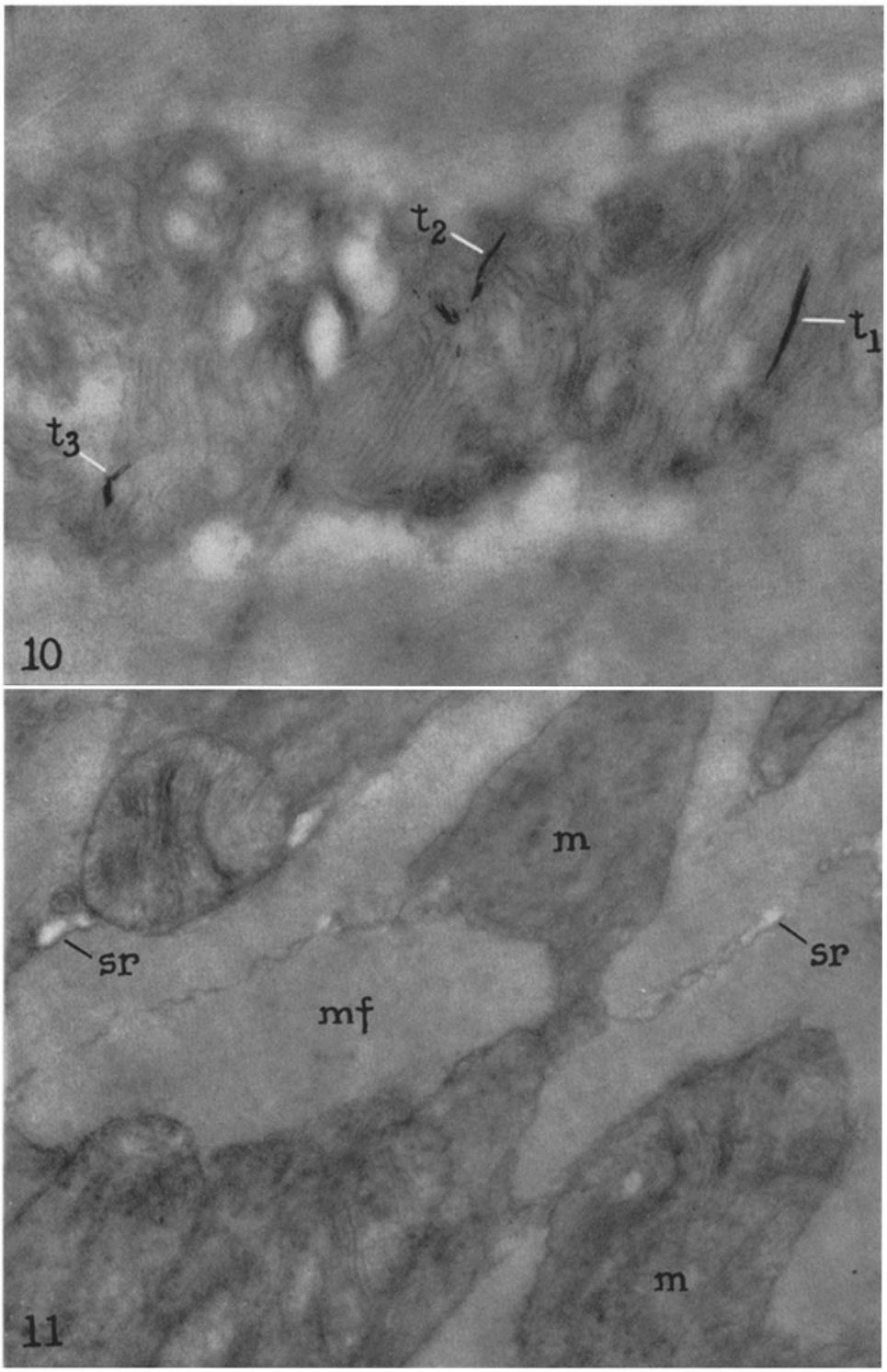
The central part of the field is occupied by an elongated mitochondrion whose peripheral membranes do not show clearly because of the obliqueness of the section. The numerous mitochondrial cristae, cut at a higher angle, appear distinctly. Note that the sheaf of relatively large crystals marked t_1 is disposed parallel to the cristae and in close proximity to their membranes. The relationship between the sheaf marked t_2 and adjacent cristae is less clear. The crystal marked t_3 shows a hole or discontinuity in its substance. Magnification, 75,000.

FIG. 11. Rat myocardium frozen, thawed, and then incubated for 2 hours at 37°C. in a mixture containing tellurite, phosphate buffer and sucrose, but no succinate.

The micrograph shows a field in a heart muscle fiber containing mitochondria (*m*), elements of the sarcoplasmic reticulum (*sr*), and myofibrils (*mf*).

Note that no deposits of dense material occur in the mitochondria or in the other cytoplasmic components.

Note also that with all the complicated history of the specimen the preservation of fine structural detail is relatively satisfactory. Magnification, 35,000.



(Barrnett and Palade: Activity of dehydrogenase systems)