THE ORGANIC-INORGANIC RELATIONSHIP IN CALCIFIED MITOCHONDRIA

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ABSTRACT

Experimentally induced calcification within mitochondria has been studied electron microscopically. Cells investigated comprise hepatic cells damaged by CCl_4 intoxication, myocardial cells damaged by prolonged dihydrotachysterol (DHT) administration, and cells from skeletal muscle (gastrocnemius) damaged by DHT sensibilization and local injury. Cells from a human bowel carcinoma were studied too. Two types of intramitochondrial inorganic inclusion have been found. The first consists of clusters of apatite-like, needle-shaped crystals (crystalline aggregates), the second of clusters of very fine granules (granular aggregates). The former have been found mainly in mitochondria in apparently normal myocardial and muscular cells, the latter in mitochondria of degenerated hepatic, neoplastic, and myocardial cells. Crystalline aggregates are closely related to the membranes of cristae at first, but they later spread to occupy the whole mitochondrial matrix. Granular aggregates are initially found in the mitochondrial matrix near, but perhaps not touching, cristae; by growing they come into close contact with cristal membranes. Both types of aggregate show intrinsic electron opacity, which disappears after formic acid decalcification. Only the crystalline aggregates give an electron diffraction pattern of crystallinity. Uranium and lead staining of decalcified sections shows that both types of aggregate are intimately connected with an organic substrate. The substrate of crystalline aggregates consists of very thin, elongated structures shaped like the inorganic crystals. The substrate of granular aggregates consists of amorphous material gathered in clusters, with the same roundish shape and intercristal position as the inorganic granules. Both types of substrate are stained by phosphotungstic acid at low pH and by silver nitrate-methenamine after periodic acid oxidation. These results show that the organic content of the substrates includes glycoproteins; they have been confirmed by the periodic acid-Schiff (PAS) method under the optical microscope. These findings have been discussed in relation to the recent discovery of organic Ca^{2+}-binding sites in mitochondria and to the general problems of soft tissue calcification.

INTRODUCTION

It has repeatedly been shown that if isolated mitochondria, whole cells, and excised tissues are incubated in Ca^{2+}-containing solutions, widespread intramitochondrial accumulation of inorganic material takes place (27, 33, 39, 44, 50, 51, 56, 61, 102, 113, 121, 139, 145). The same kind of accumulation has been reported in vivo in a wide variety of physiological (7, 31, 59, 69, 91–94) and pathological (16, 17, 24, 25, 35–38, 40, 41, 45, 47, 53, 54, 62, 65, 76, 77, 81, 83, 100, 115, 128, 143, 150) conditions, especially those where levels of intracellular calcium are abnormally high.

The number of situations already known to involve intramitochondrial accumulation of calcium;

and the list can be expected to grow as our knowledge increases, testifies to the urgency of the need to understand this process in depth.

There is fair scope for the use of the electron microscope in research which aims to do this, because calcium accumulates in mitochondria in electron-dense aggregates. Many ultrastructural studies concur in describing these aggregates as roundish clusters of very fine, high electron-dense granules (16, 17, 27, 31, 36, 40, 47, 50, 53, 56, 61, 65, 69, 76, 91–93, 102, 115, 121, 139, 144). In some cases clusters of needle-like crystals have also been described (24, 25, 37, 41, 54, 62, 75, 77, 83, 100, 150).

Recent research has shown that the relationship between organic and inorganic substance in calcified cartilage and bone is very close, so close that the inorganic material seems to model its shape to that of the organic structures (4–6, 18, 19, 22, 23, 129, 134). This suggests that the shape of the inorganic aggregates largely depends on an intramitochondrial reaction between inorganic material and specific organic substrate. This view has recently been strengthened by the discovery of organic "low-" and "high-affinity" Ca^{2+}-binding sites in mitochondria (78, 79, 114).

There is therefore every reason to suppose that advances in knowledge of how calcium binding and uptake occurs will come from research on the relationship between inorganic aggregates and mitochondrial substructures and, even more probably, between inorganic material and the organic structures of the mitochondrial matrix. This is why we have given first priority to an electron microscope investigation of various types of tissues. We have concentrated on four topics: (*a*) the ultrastructure of the inorganic aggregates; (*b*) their relationship with the mitochondrial structures, especially cristae and membranes; (*c*) their relationship, if any, with the organic material of the mitochondrial matrix; (*d*) the histochemical properties of this organic substance.

MATERIALS AND METHODS

Sprague-Dawley rats weighing 120–150 g and fed with a standard diet were injected with a single intraperitoneal dose of analytical grade carbon tetrachloride (0.25 ml in an equal volume of mineral oil per 100 g body weight). The animals were killed by decapitation after 20 h. Their liver was immediately removed, cut into small fragments, and further processed for fixation as specified below.

It is known that this method induces widespread calcification of the mitochondria in hepatic cells (35, 115).

Another group of rats was sensitized with 1 mg dihydrotachysterol (DHT) in 0.5 ml corn oil administered through a stomach tube. After 24 h, the animals were subjected to mild trauma (soft but prolonged massage) on the right gastrocnemius. They were sacrificed 48 h after trauma. The gastrocnemius was immediately removed and cut into small fragments for fixation.

This method induces widespread muscle calcification (125) and accumulation of inorganic substance in mitochondria (24).

Another group of rats was treated for 2 wk with six parenteral injections of DHT (0.25 mg), and sacrificed 30 days after the first DHT administration. Specimens of myocardium were prepared for fixation.

This method induces high and persistent hypercalcemia and widespread calcification, chiefly in the heart, blood vessels, lung, kidney, and intestinal tract (64, 74, 97). Calcification of the heart leads to the build up of large quantities of inorganic material within mitochondria (25).

Additional material came from a carcinoma of the bowel of a woman aged 69. Previous electron microscope investigation had shown that the mitochondria of the neoplastic cells in this carcinoma contained inorganic material (45).

The specimens were fixed in three different ways: (*a*) 1% OsO_4 in 0.01 M phosphate buffer, pH 7.4, for 1 h; (*b*) 4% formaldehyde mixed with equal volume of 2.5% glutaraldehyde in 0.01 M phosphate buffer for 2 h (glutaraldehyde was sometimes omitted); (*c*) 4% formaldehyde for 2 h followed by 1 h postfixation with 1% OsO_4, both buffered as above. For controlling the effect of fixation on the inorganic material eventually accumulated in mitochondria, specimens of myocardium were left for 15 days in 4% formaldehyde and then postfixed in 1% OsO_4 for 1 h.

After fixation, some specimens were decalcified for 2 h in a 2% solution of formic acid (pH about 2), and others were left in the buffer for 2 h. Both groups were washed with renewed buffer, and then embedded either in Araldite alone or in Epon-Araldite mixture (96).

Ultrathin sections from undecalcified specimens were examined under the electron microscope: (*a*) without treatment of any kind, to examine the fine structure of the untreated mineral; (*b*) after "staining" with uranyl acetate and lead citrate, to study the relationship between mineral substance and mitochondrial substructures; (*c*) after decalcification by 30 min flotation on a 2% solution of formic acid, to observe the structure of mitochondria after removal of the inorganic material (it would have been easier to obtain decalcified sections from specimens treated with formic acid before embedding; the more difficult

and time-consuming process of decalcification by flotation of undecalcified sections was preferred because, as explained below, organic structures are preserved better in this case); (*d*) after decalcification as in (*c*) and poststaining with uranium and lead, to study the structure of the organic matrix in previously calcified areas; (*e*) after decalcification as in (*c*) and poststaining with 1% phosphotungstic acid (PTA) in 0.1 N hydrochloric acid (87, 88), to determine whether glycoproteins were present in calcified areas; (*f*) after decalcification as in (*c*) and treatment with 1% HIO_4 for 10 min, followed by staining with silver nitrate-methenamine (84, 85), again to see whether glycoproteins were present in mitochondria. The specificity of this reaction was checked under the optical microscope by comparing two sets of seriated sections, both treated with 1% HIO_4 ; the first was stained with silver nitrate-methenamine in the same way as the ultrathin sections and the second with the periodic acid-Schiff reagent (PAS). Other semithin sections were stained either with alizarin red S or by the von Kossa method, to determine whether and where calcified areas were present.

Selected area electron diffraction of calcified mitochondria was performed: (*a*) in untreated sections, to examine the degree of crystallinity of the intramitochondrial inorganic substance and its constitution; and (*b*) in decalcified sections, to make sure that the crystalline inorganic substance had been completely removed during decalcification. Selected area electron diffraction was carried out at 80 kV and at a magnification of × 22,000 in a Siemens Elmiskop 1A electron microscope equipped with a field-limiting diaphragm of 50 μm. Calibration for determination of crystal spacings (d_{hkl} values) was obtained by selected area electron diffraction of magnesium oxide crystals.

Sections from osmium-fixed specimens were treated with 2% H_2O_2 for 20 min. This removes the reduced osmium linked to the organic structures (95), so making comparison between osmium- and aldehyde-fixed structures possible.

To avoid acid-metal interaction and contamination of the ultrathin sections on the grids, both decalcification and staining were carried out on free-floating sections (85, 86).

Sections from specimens decalcified with formic acid before embedding were examined after staining with uranyl acetate and lead citrate.

RESULTS

Untreated Sections

Two types of inclusion were found within mitochondria, the first consisting of roundish aggregates of very small granules (Figs. 1, 2, 4, 5, 8), and the second of clusters of filament- or needle-like structures resembling thin crystals (Figs. 3, 6–8). These two types of inclusion will from now on be called *granular aggregates* and *crystalline aggregates*, respectively, and their smaller component units *granules* and *crystalline structures*.

Granular aggregates were found mainly in the mitochondria of hepatic cells in CCl_4-treated rats and in those of human neoplastic cells; very few crystalline aggregates were present in either case. Granular aggregates were found in myocardial mitochondria too, but not in the mitochondria of skeletal muscle.

Each granular aggregate appeared as a cluster of very electron-dense granules, often enclosing a less electron-dense area in which only a few granules could be found (Figs. 1, 2, 5). Other aggregates consisted of a very electron-dense center surrounded by a clear halo, itself surrounded by a very electron-dense border (Fig. 2). In some cases mitochondria were almost completely full of randomly distributed granules which entirely masked the mitochondrial structures.

Granular aggregates were recognizable in sections from aldehyde-fixed specimens, showing that the granules have an intrinsic electron opacity (Fig. 4). A slight difference was observed, however, between granules in aldehyde-fixed and those in osmium-fixed specimens. The former were more sharply outlined and therefore more clearly resolvable under the electron microscope. Treatment of ultrathin sections from osmium-fixed specimens with H_2O_2 increased the resolution of granules without altering their morphology (Fig. 5). Osmium-treated, H_2O_2-oxidized granules and aldehyde-treated ones had practically the same structure.

Electron diffraction of even the greatest granular aggregates gave no evidence of crystallinity. Only two broad diffraction rings were visible, both due to the embedding resin (Fig. 10).

Crystalline aggregates have mostly been found in the mitochondria of muscular and myocardial cells. Very few were present in hepatic cells in CCl_4-treated rats. Hardly any were found in neoplastic cells.

Each aggregate was made up of filament- or needle-like structures, sometimes branched and frequently curved (Figs. 6, 7). They were about 20 Å thick; their length could not be measured accurately because of their irregular orientation in the section.

These structures were either isolated or grouped in clusters of varying size. When isolated, in

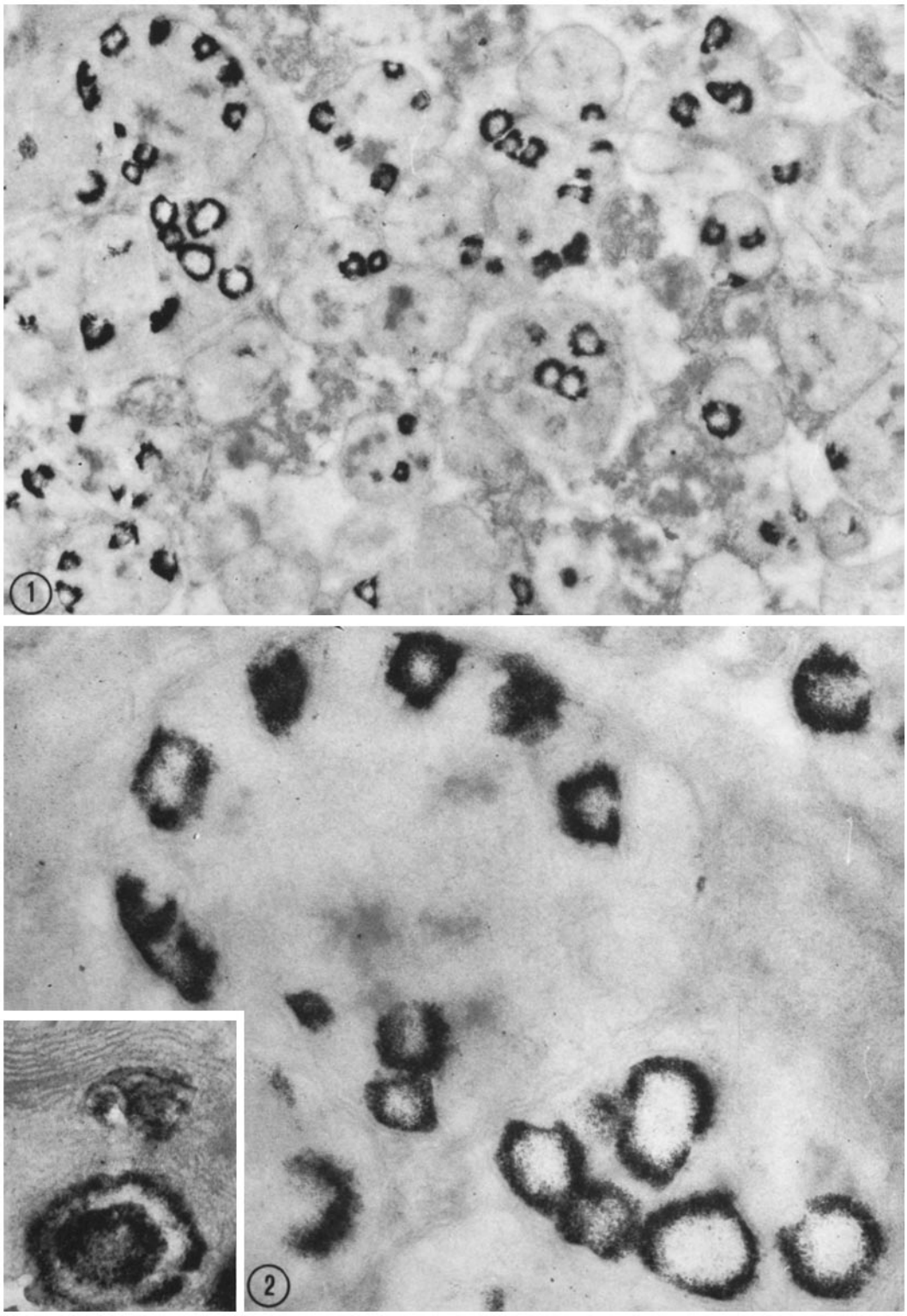
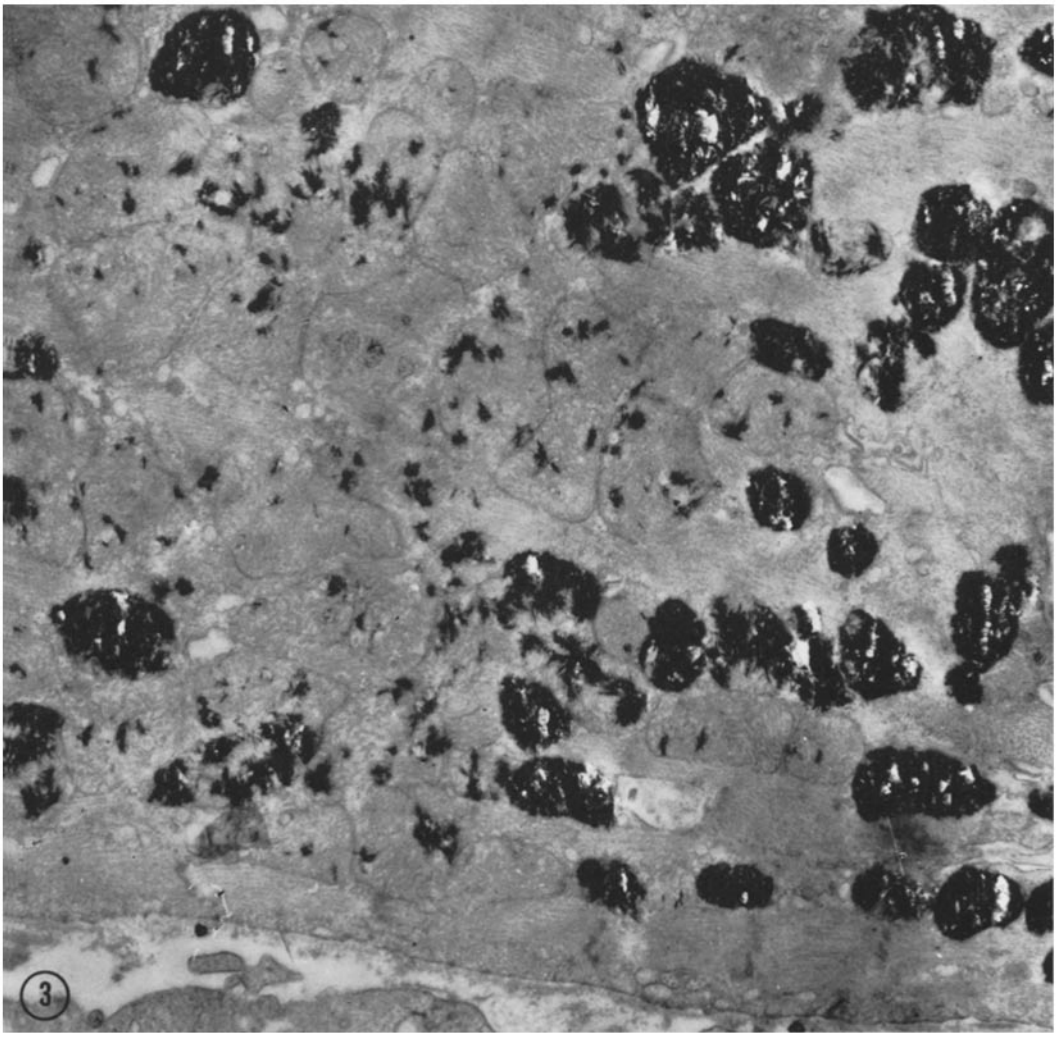
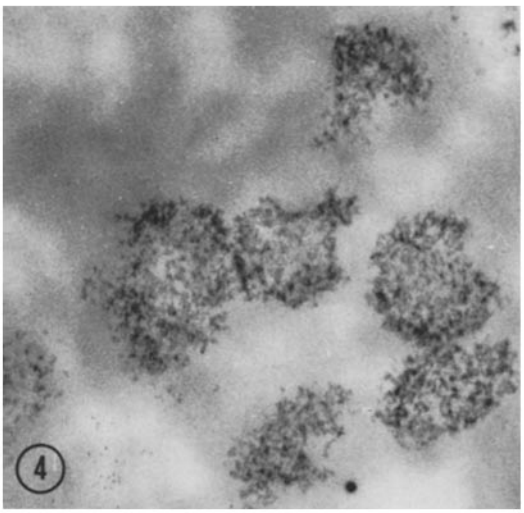


FIGURE 1 Mitochondria of liver cell of CCl_4-treated rat. Osmium fixation. The mitochondria contain roundish electron-dense structures often having a clear center (granular aggregates). Unstained section. × 21,000.

FIGURE 2 Detail of Fig. 1. × 66,000. The *inset* shows an intramitochondrial electron-dense structure having a cockade-like shape. Unstained section. × 75,000.





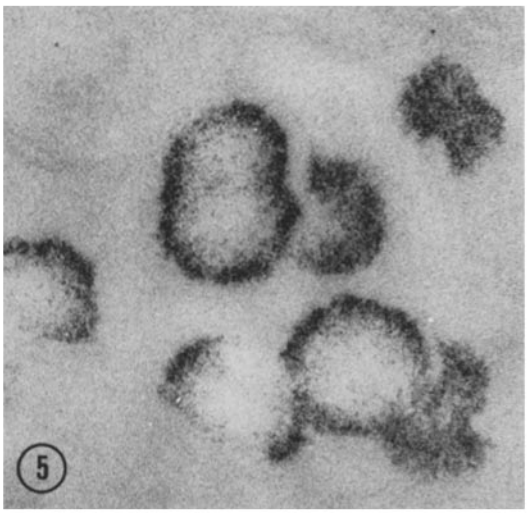


FIGURE 3 Myocardial cell with diffuse intramitochondrial calcification. Osmium fixation. Mitochondria contain both granular and crystalline aggregates which sometimes completely mask the mitochondrial structures. Unstained. × 17,000.

FIGURE 4 Granular aggregates in hepatic mitochondria of CCl_4-treated rats. Aldehyde fixation. The inorganic substance consists of very small granules having intrinsic electron density. Unstained section. × 80,000.

FIGURE 5 Intramitochondrial inorganic aggregates of the same type as that of the aggregates shown in Fig. 4. Osmium fixation; section oxidized with H_2O_2 and unstained. The inorganic substance has a finely granular structure. × 80,000.

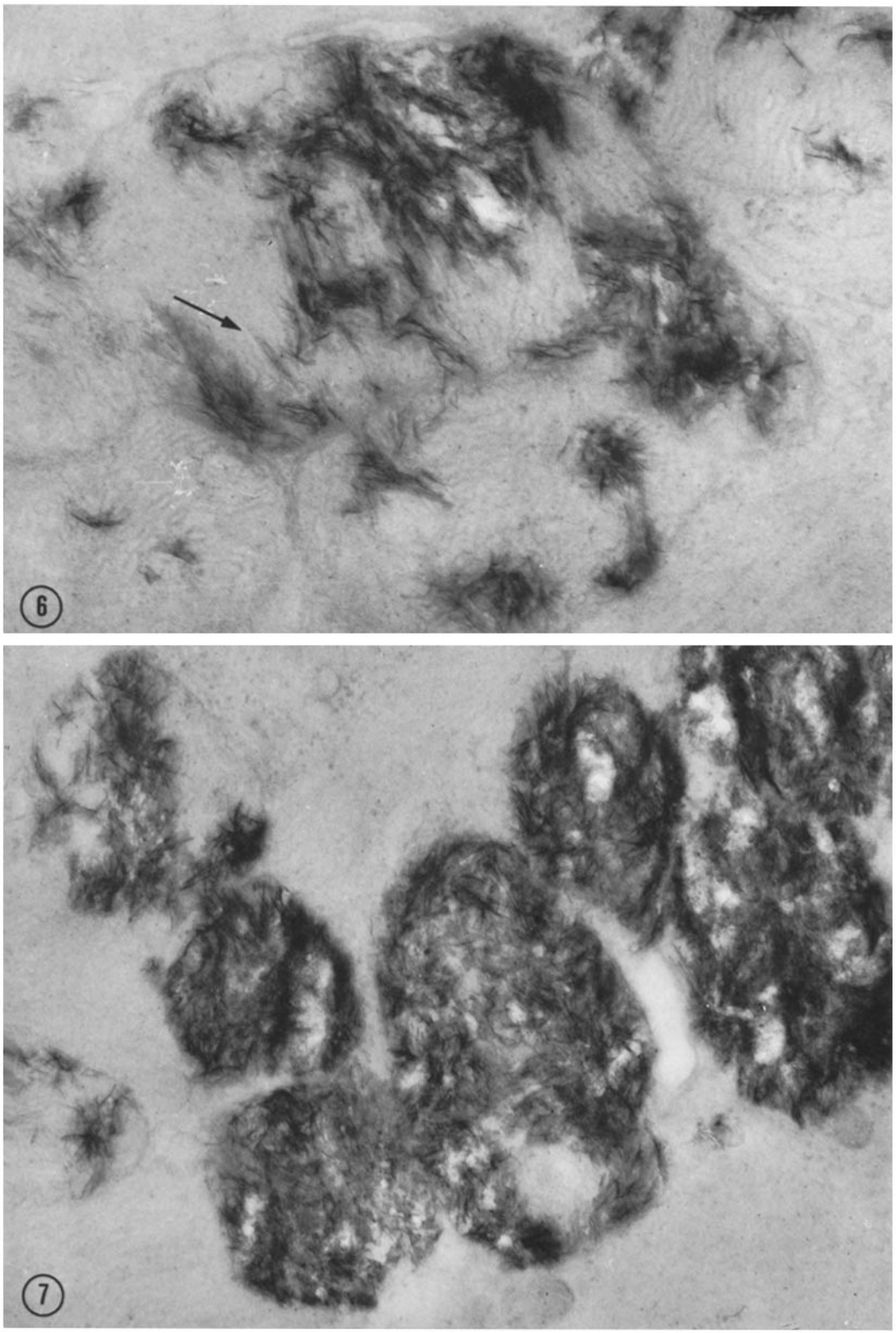
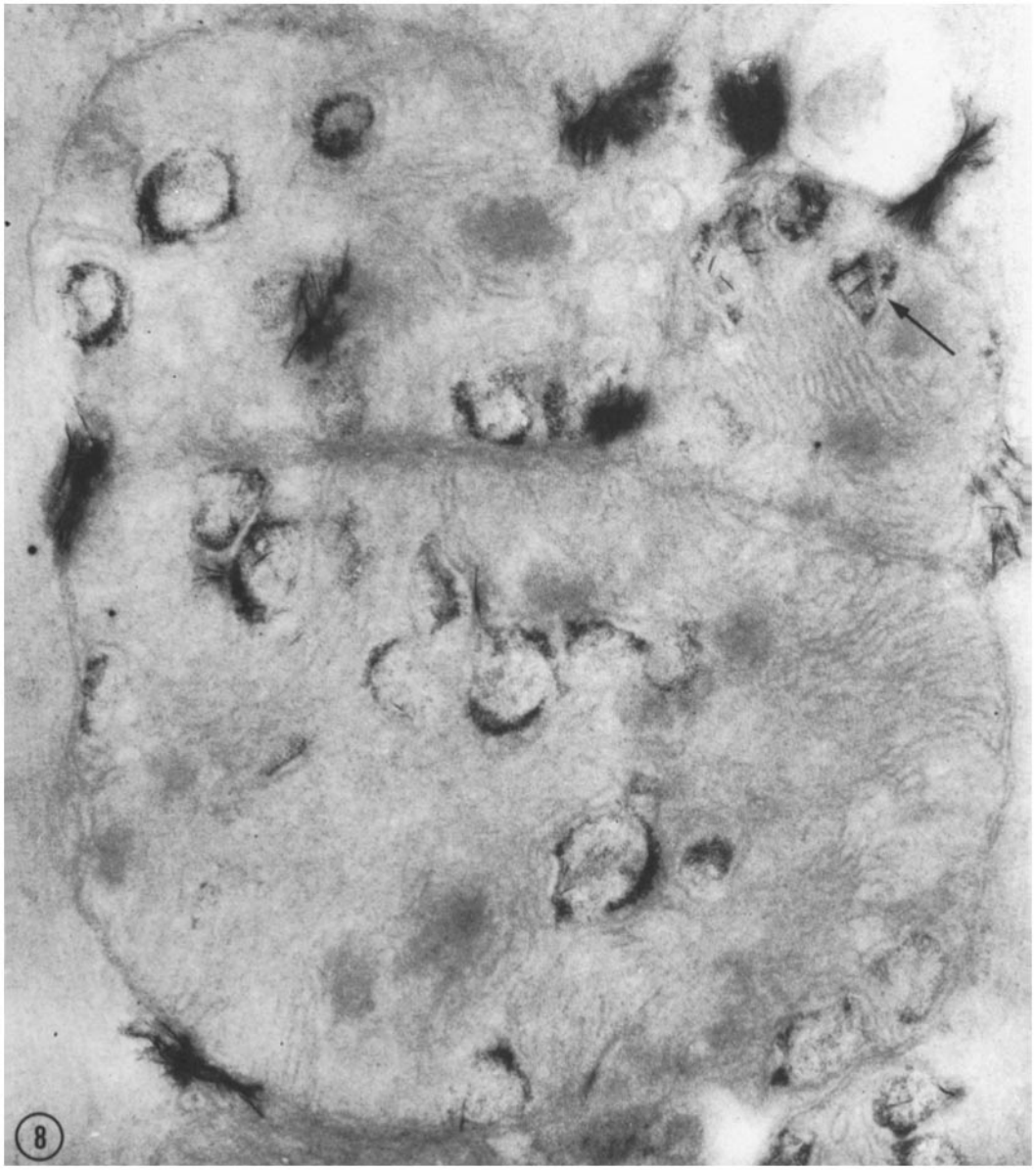
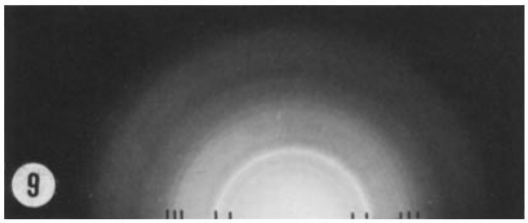


FIGURE 6 Calcified mitochondria of myocardial cell. Osmium fixation. The inorganic substance appears as elongated, needle-like crystalline structures, some of which (arrow) seem to be related to mitochondrial cristae. Unstained section. × 84,000.

FIGURE 7 Group of deeply calcified mitochondria in myocardial cell. They appear as roundish aggregates of crystalline structures and are recognizable as calcified mitochondria only from their roundish shape. Partially calcified mitochondria on the left. Unstained section. × 66,000.





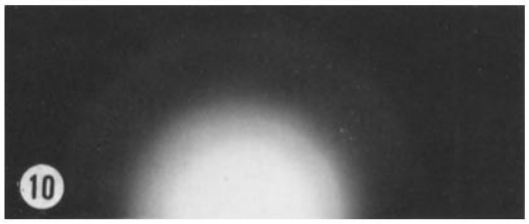


FIGURE 8 Early stage of intramitochondrial calcification. Osmium fixation. The mitochondria contain roundish aggregates of granular inorganic material and a few clusters of needle-shaped crystalline structures. Arrow points to a cluster which exceptionally contains both granular and crystalline structures. Unstained section. × 90,000.

FIGURE 9 Electron diffractogram obtained from a group of calcified mitochondria containing clusters of needle-like structures. Although the diffraction rings are weak (five of them are shown by dashes), an unmistakable pattern of crystallinity is recognizable.

FIGURE 10 Electron diffractogram obtained from a group of calcified mitochondria containing granular aggregates. No evidence of crystallinity is recognizable.

sections from osmium-fixed specimens, they often appeared closely related to the membranes of cristae and were sometimes arranged in doublets (Fig. 6). When grouped in clusters, they were gathered in irregular aggregates. The smallest clusters comprised only a few structures (Figs. 3, 6, 8), the largest many (Figs. 3, 6, 7). The largest clusters contained so many structures that they sometimes completely filled mitochondria, which could only be recognized by their roundish shape (Figs. 3, 7).

It was these mitochondria almost completely full of crystalline structures which appeared von Kossa and alizarin red S positive under the optical microscope.

The single crystalline structures had the same general appearance in sections from both osmium- and aldehyde-fixed specimens. But in the latter and in H_2O_2-treated sections from osmium-fixed specimens they were more sharply outlined.

Selected area electron diffraction of crystalline aggregates gave results which varied with the size of aggregates. The smallest gave no evidence of crystallinity. But when the crystalline aggregates completely or almost completely filled the mitochondria, especially when many of these mitochondria appeared within the field-limiting diaphragm, evidence of crystallinity was very clear (Fig. 9). Measurements of crystal spacings then gave the following d_{hkl} mean values: 3.25, 2.80, 1.96, 1.86, and 1.74. It must be stressed that though the diffraction ring with d value of 2.80 was always very prominent and clearly visible, the other diffraction rings were often weak and indistinct, so that their d values were extremely hard to measure and are certainly not absolutely reliable. In general, the fewer the crystalline aggregates within the field-limiting diaphragm, the weaker the photographic contrast of the diffraction rings.

No exact relationship could be found between granular and crystalline aggregates. They were occasionally found together in the same mitochondrion; in this case some crystalline structures were found within the granular aggregates (Fig. 8). No morphological changes were induced by prolonged fixation both in granular and crystalline aggregates.

Sections Treated with Uranium and Lead

Staining with uranium and lead does not greatly change the morphology of intramitochondrial aggregates, if it is not continued for too long.

Granular aggregates were mostly found in cells at a fairly advanced stage of degeneration, whose mitochondria appeared damaged to some extent too. In general, these were swollen and sometimes contained an unusually low number of cristae (Figs. 11, 12). Interestingly, granular aggregates were never found in the central zone of swollen mitochondria, and always remained closely related with cristae (Fig. 12).

The largest granular aggregates appeared either as roundish electron-dense structures (Fig. 13) or as electron-dense rings enclosing a less electron-dense area, itself containing dispersed granular material (Fig. 14). In both cases, these aggregates were in close contact with two adjacent cristae which were bent around them. Two cristae were usually curled around a granular aggregate, forming an incomplete ring around it (Fig. 13).

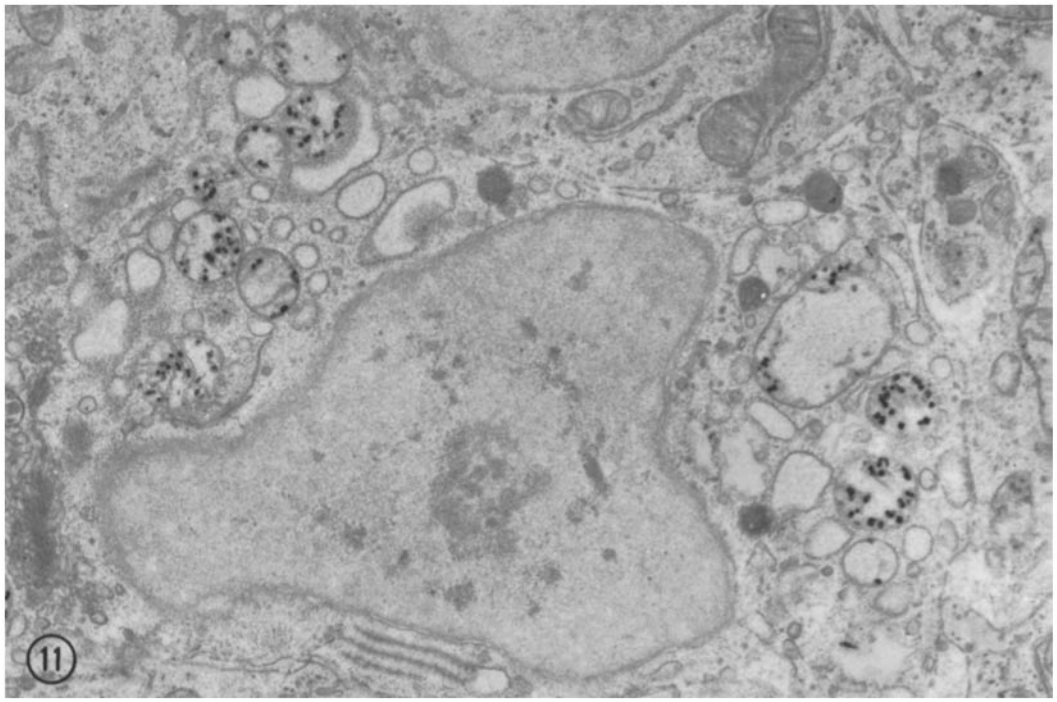
The largest aggregates of granular material were found in the mitochondria of degenerated myocardial cells in DHT-treated rats. They were between about 500 and 5,000 Å in diameter and were sometimes so large that they almost completely masked a mitochondrion (Fig. 15).

Like the granular aggregates, the crystalline aggregates too had a variable relationship with the mitochondrial structures, depending on their size.

The smallest of them were not true aggregates, because as few as one or two crystalline structures were sometimes found in a whole mitochondrion (Fig. 16). In this case, although they obscured the underlying structures, they often appeared to lie over one or both membranes of the cristae (Fig. 16), leaving the intracristal space free. The cristae did not seem to be damaged and their arrangement was not distorted.

The medium-size crystalline aggregates showed no clear relationship with the cristae. They usually consisted of clusters of filament- or needle-like structures which were apparently oriented at random within mitochondria (Fig. 16). It was, however, difficult to determine whether they were related to cristae, because they lay over areas of high electron density which completely obscured the underlying structures. This masking effect was even stronger in mitochondria completely full of crystalline aggregates. The crystalline structures were in this case very irregularly oriented and placed over areas of high electron density which, if considered as a whole, had the roundish outline of a mitochondrion.

Crystalline aggregates were mostly found in apparently normal mitochondria in apparently normal cells.



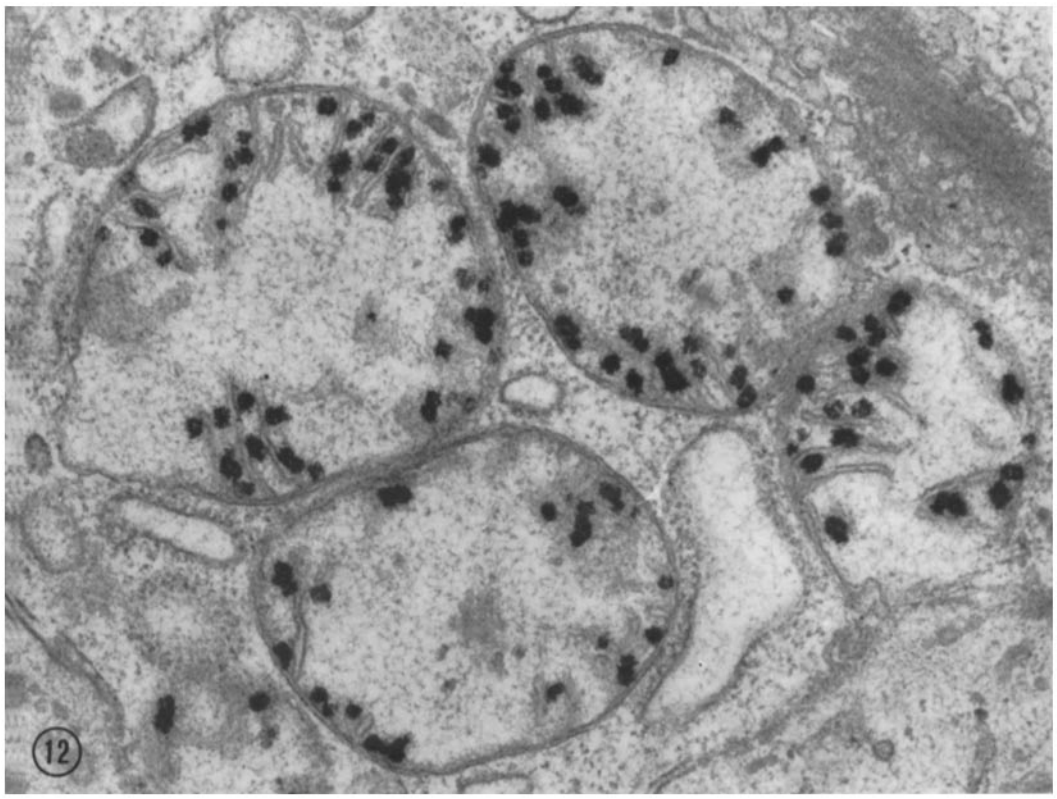


FIGURE 11 Neoplastic cell of bowel carcinoma. Many mitochondria contain inorganic aggregates. Uranyl acetate and lead citrate. × 12,000.

FIGURE 12 Mitochondria of neoplastic cell with many granular aggregates. These are roundish structures usually placed between two adjacent cristae. Note that mitochondria are swollen and that no inorganic aggregates are present in the swollen area. Uranyl acetate and lead citrate. × 50,000.

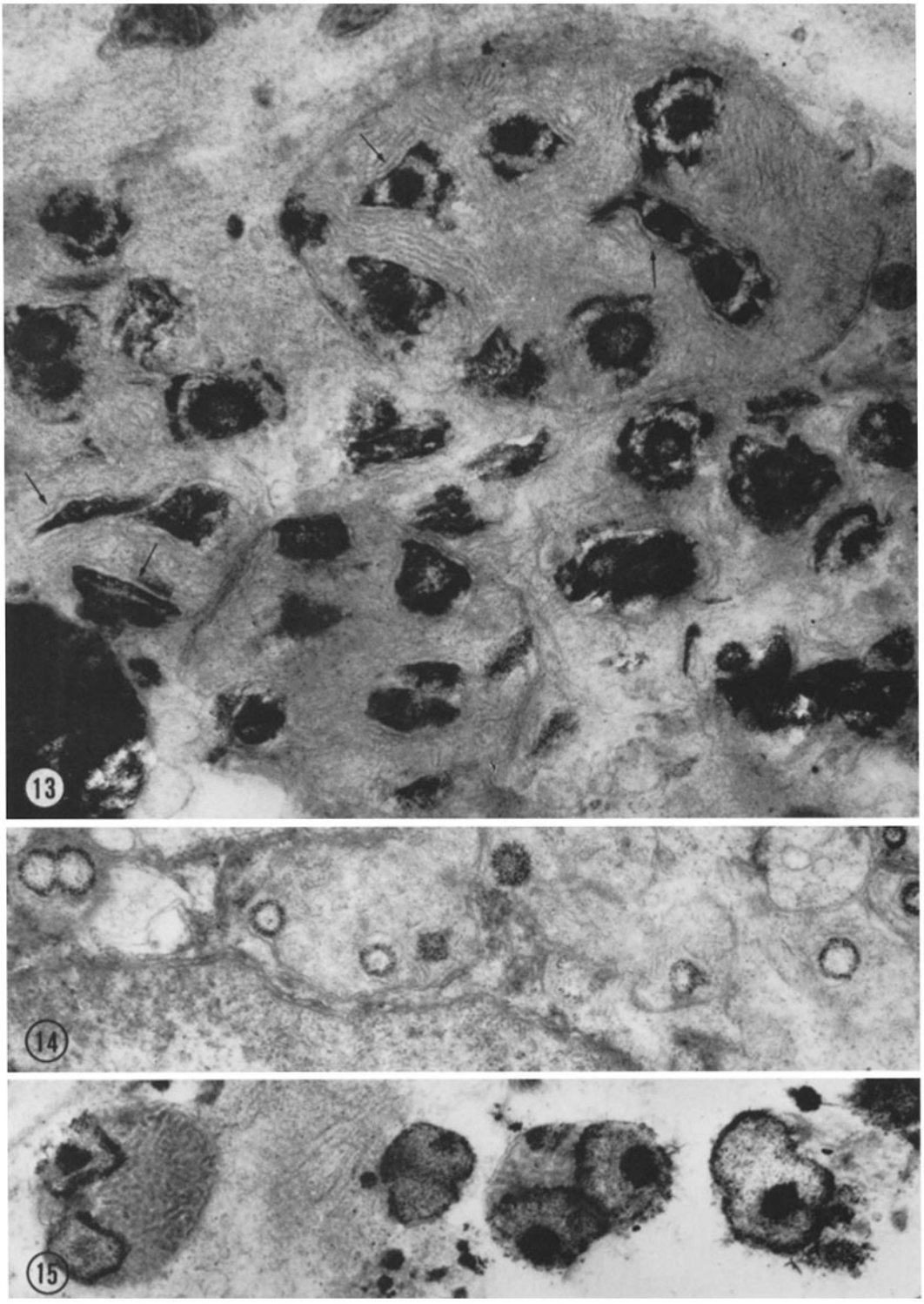
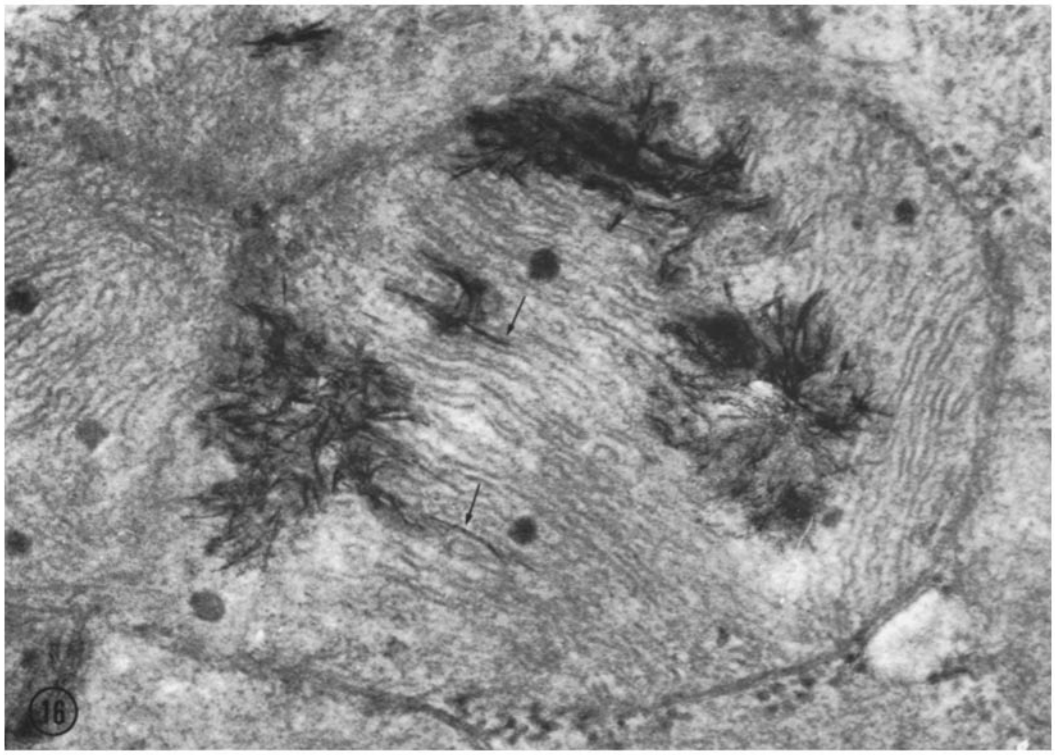


FIGURE 13 Myocardial mitochondria containing granular aggregates of inorganic substance. Some aggregates have a cockade-like shape; many of them are closely surrounded by mitochondrial cristae (arrows). Uranyl acetate and lead citrate. × 85,000.

FIGURE 14 Mitochondria of liver cells of CCl_4-treated rats, containing aggregates of granular inorganic material. Uranyl acetate and lead citrate. × 73,000.

FIGURE 15 Myocardial mitochondria containing large inorganic granular aggregates having a cockade-like shape and almost completely obscuring the mitochondrial structures. Uranyl acetate and lead citrate. × 30,000.



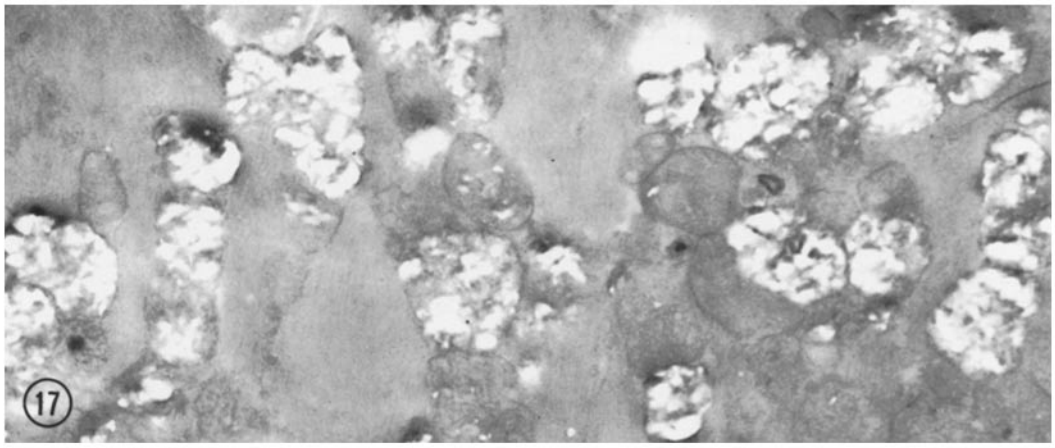


FIGURE 16 Myocardial mitochondrion containing crystalline aggregates of different size. The crystalline structures which form the largest aggregates seem not related to the cristae; they are either randomly or almost radially arranged. The crystalline structures which form the smallest aggregates are in close contact with the cristal membranes and seem to lie right over them (arrows). Note persistence of normal electron-dense granules in the mitochondrial matrix. Uranyl acetate and lead citrate. × 95,000.

FIGURE 17 Calcified mitochondria decalcified by flotation of the section on formic acid. The areas originally occupied by inorganic aggregates show a very low electron density and seem to be empty. Unstained section. × 18,000.

Section Decalcified by Flotation on Formic Acid

Granular and crystalline aggregates were no longer visible in mitochondria after flotation of ultrathin sections on formic acid solution. Mitochondria showed areas of very low electron density in areas where granular and crystalline aggregates had previously located (Fig. 17). These decalcified areas seemed to be empty and no ultrastructural details were visible in them.

Selected area electron diffraction of decalcified mitochondria gave no evidence of crystallinity.

Sections Decalcified by Flotation on Formic Acid and Stained with Uranium and Lead

The organic substance in calcified areas is unmasked by flotation on formic acid, which removes inorganic material. This substance can then be stained by uranium and lead and usefully examined under the electron microscope.

Mitochondria which had contained granular aggregates now showed aggregates of electron-dense material which were either granular or amorphous (Figs. 18, 19). It was sometimes found between cristae and sometimes between, and touching, the near sides of two adjacent cristae so being enclosed by a nearly complete ring (Figs. 18, 19). This material often included a less electron-dense central area containing fine granules (Fig. 18). In other cases, it had an electron-dense center surrounded by a clear halo, itself surrounded by electron-dense border (Fig. 19). The material revealed by uranium and lead staining therefore had almost the same shape and arrangement as untreated granular aggregates.

Mitochondria which contained crystalline aggregates now showed clusters of elongated filament-like or crystal-like structures (Fig. 20). These were about 20 Å thick and were either straight or, more often, bent and branched (Fig. 20). When isolated they appeared to be placed right over the membranes of the cristae. When there were more of them, and they were grouped in clusters, they had no clear relationship with the cristae; they seemed to be oriented at random, but to lie over areas of high electron density. Some of these filament-like structures, however, especially those at the edge of clusters, had the same orientation as adjacent cristae and were sometimes arranged in doublets. These filament-like structures were often so similar to the crystalline structures found in unstained sections (or in undecalcified sections stained with uranium and lead) that the two types of structures were hardly distinguishable.

Ultrastructural details in sections from specimens decalcified before embedding were not well preserved. Electron-transparent areas containing very fine granules and a few filaments were, however, recognizable in many mitochondria. These areas were often roundish and were surrounded by curved cristae (Fig. 21), i.e., they occupied the same position as granular aggregates in undecalcified mitochondria.

Sections Decalcified by Flotation on Formic Acid and Stained with PTA

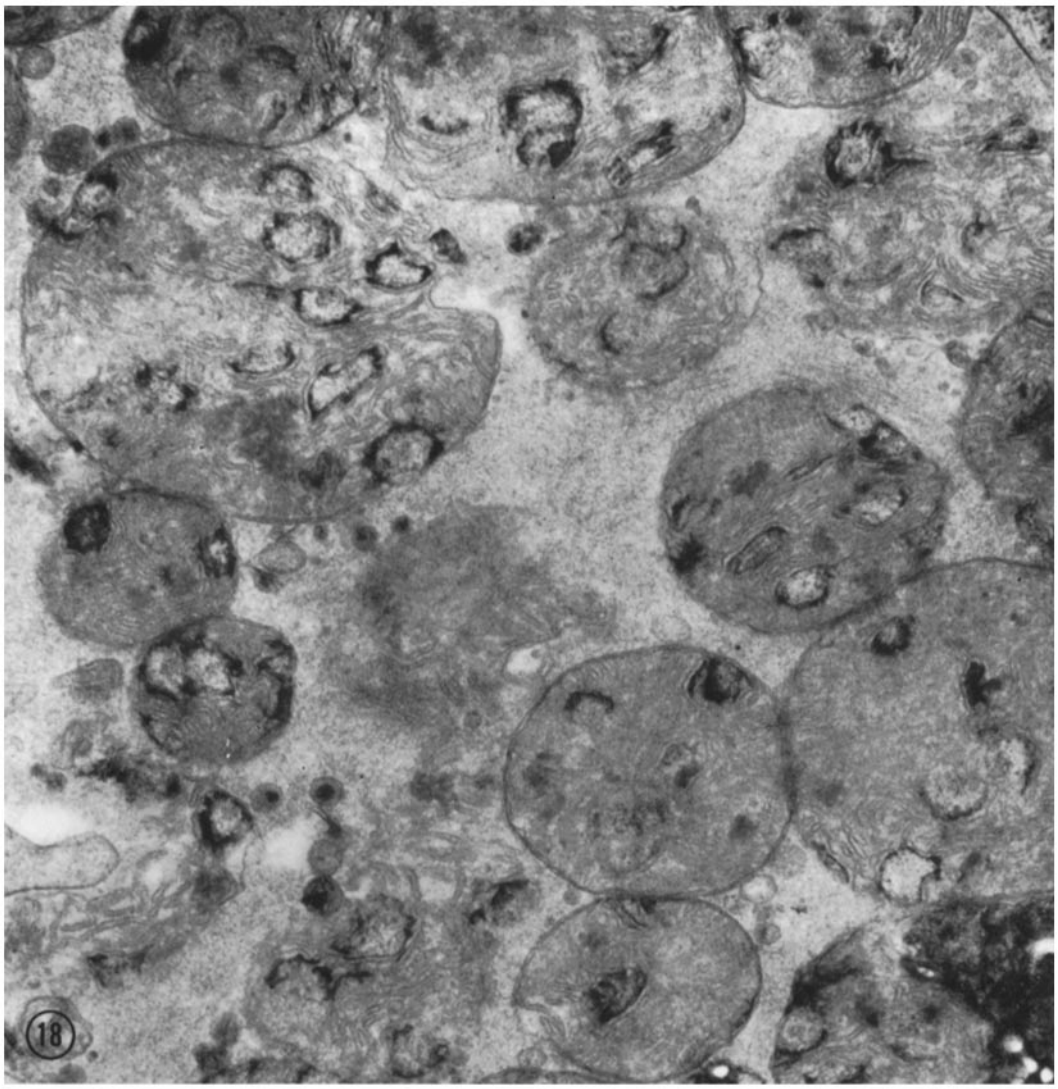
This technique gave almost the same results as those described in the preceding section. Mitochondria contained inclusions of very electron-dense material. This was either gathered in roundish aggregates (Figs. 22–24) or consisted of clusters of elongated, filament-like structures (Fig. 25). In sections from aldehyde-fixed specimens no other intramitochondrial structures were revealed by PTA (Fig. 24).

After decalcification and PTA treatment mitochondria which had contained granular aggregates showed roundish aggregates of this electron-dense material. When small, these appeared as electron-dense inclusions within mitochondria, near one or between two cristae. When larger, they appeared as rings of highly electron-dense material very near the membranes of curved cristae and enclosed by them. Aggregates like these were almost indistinguishable from those found in unstained mitochondria, mitochondria stained with uranium and lead, and mitochondria stained with uranium and lead after decalcification.

After decalcification and PTA treatment mitochondria which had contained crystalline aggregates showed clusters of filament-like structures like those described in the preceding section, except for their higher electron density and weaker outline (Fig. 25).

Section Decalcified by Flotation on Formic Acid, Oxidized with HIO_4, and Treated with Silver Nitrate-Methenamine

The residual material found in place of granular and crystalline aggregates in mitochondria after



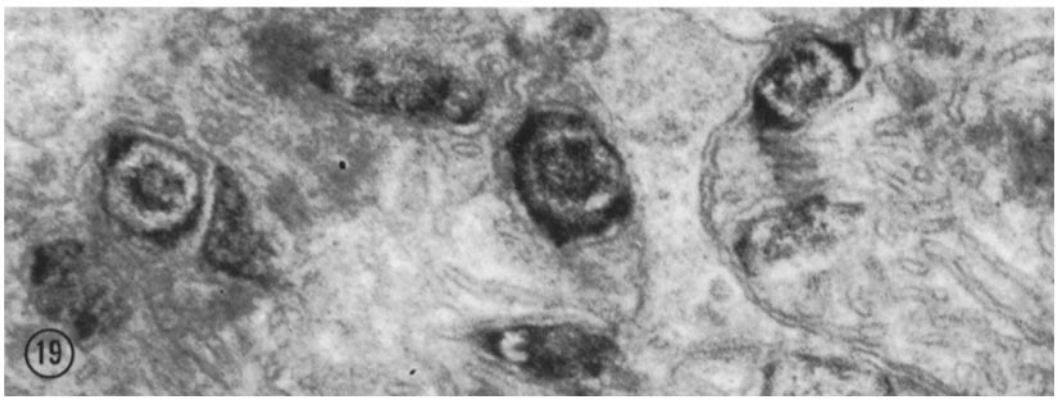
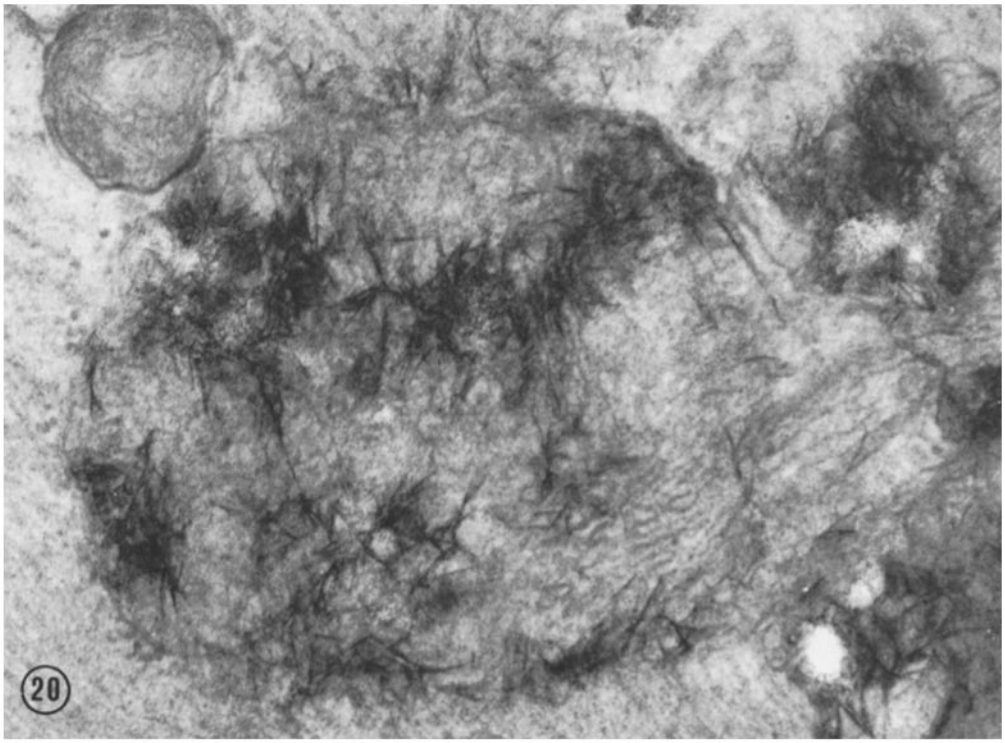


FIGURE 18 Section decalcified by flotation on formic acid and stained with uranyl acetate and lead citrate. The originally calcified mitochondria contain aggregates of electron-dense material showing the same shape, localization, and relationship with the cristae as the granular aggregates in undecalcified sections (compare with Figs. 1, 8, and 13). × 40,000.

FIGURE 19 Section decalcified by flotation on formic acid and stained with uranyl acetate and lead citrate. The originally calcified mitochondria contain aggregates of electron-dense material having the characteristic cockade-like shape which is typical of many granular inorganic aggregates (compare with FIG. 2, *inset*, and Fig. 13). Note the very close relationship between aggregates and cristal membranes. × 98,000.



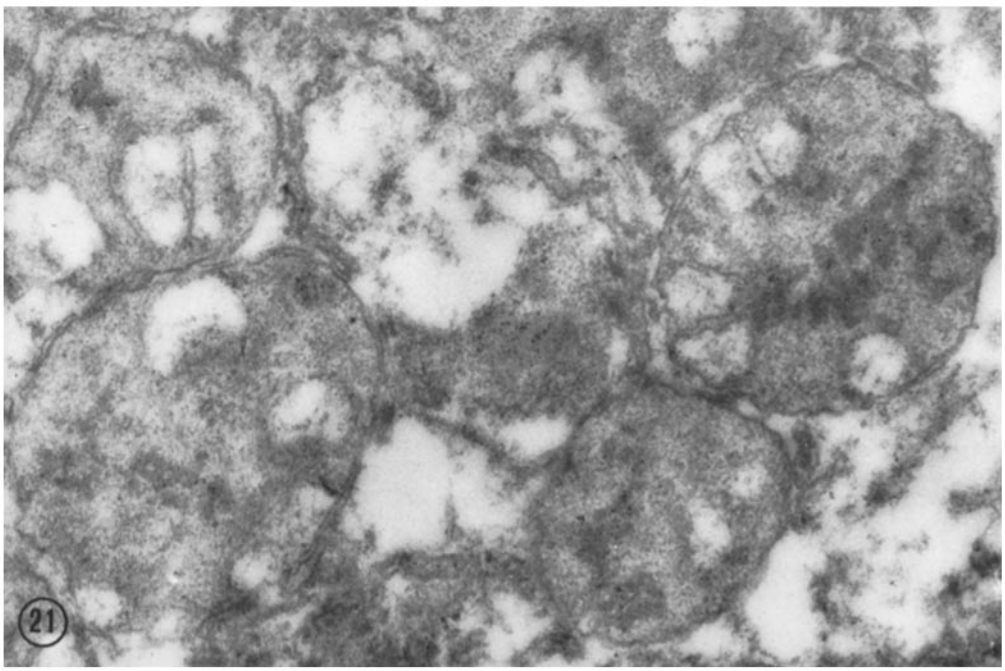
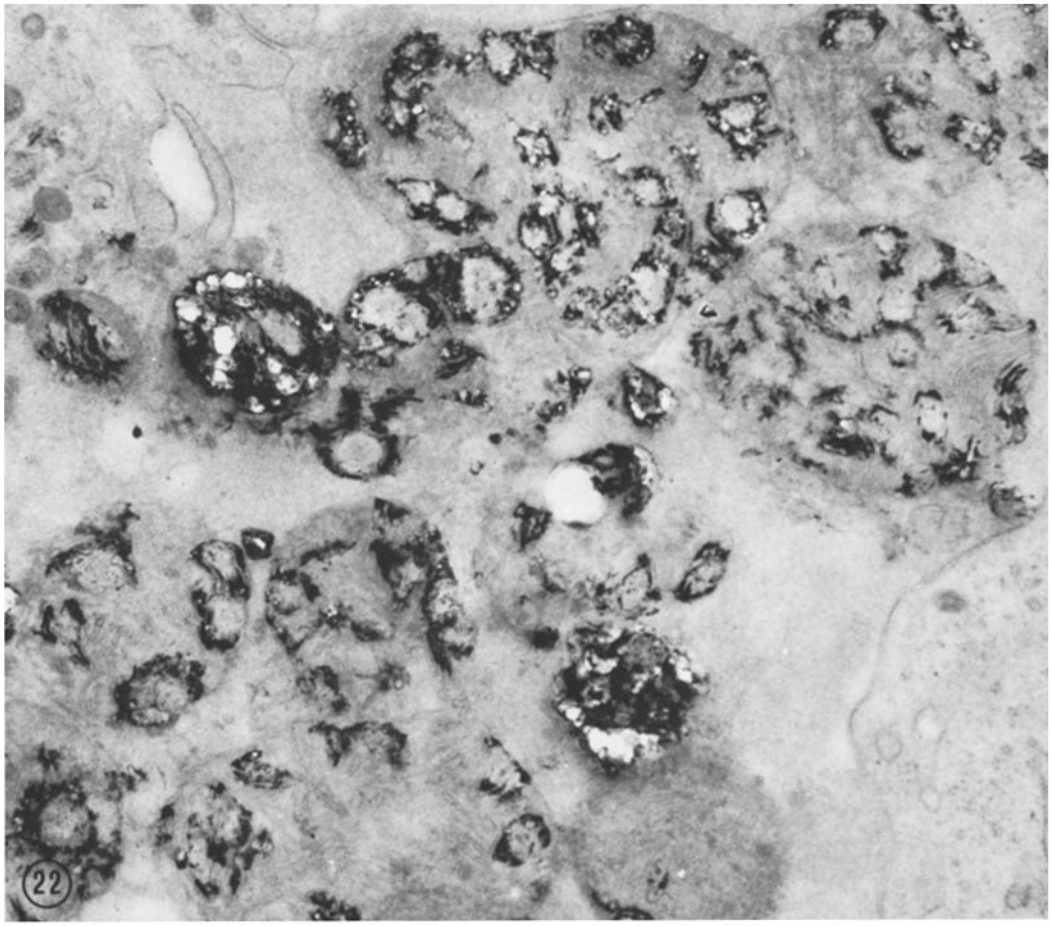
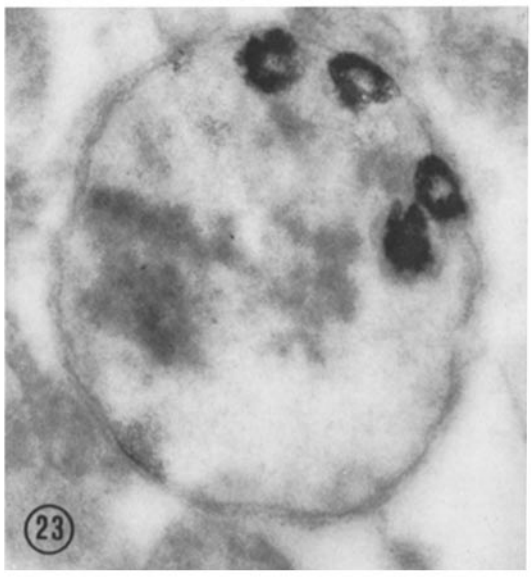


FIGURE 20 Myocardial mitochondrion, decalcified by flotation of the section on formic acid and stained with uranyl acetate and lead citrate. Elongated, needle-like structures (crystal ghosts) are visible. They have the same morphology and arrangement as crystalline structures in untreated sections (compare with Fig. 6) and in undecalcified sections stained with uranium and lead (compare with Fig. 16). × 90,000.

FIGURE 21 Liver of CCl_4-intoxicated rats. Section obtained from a specimen decalcified with formic acid before embedding. Roundish, electron-transparent areas surrounded by cristae and sometimes containing a granular material are evident. Uranyl acetate and lead citrate. × 60,000.





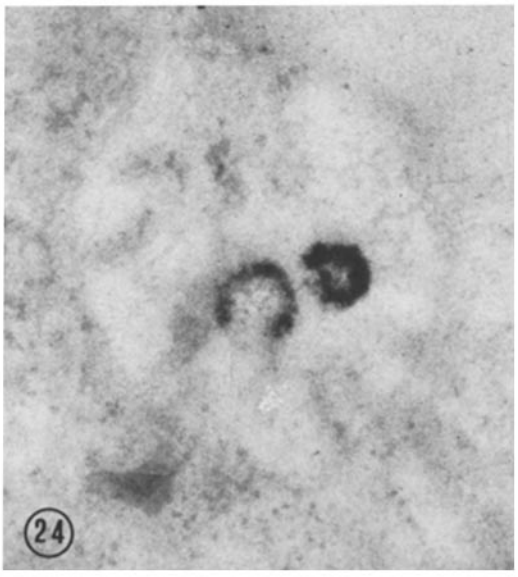


FIGURE 22 Section of myocardium decalcified by flotation on formic acid and stained with PTA at low pH. Roundish aggregates of very electron-dense material are present in the originally calcified mitochondria. These aggregates have the same shape, localization, and arrangement as the inorganic granular aggregates (compare with Figs. 1, 2, 8, 13, 18). × 32,000.

FIGURE 23 Mitochondrion of a neoplastic cell containing PTA-stained structures similar to those shown in Fig. 22. Osmium fixation; decalcification by flotation of the section on formic acid; staining with PTA at low pH. × 70,000.

FIGURE 24 Mitochondrion of a neoplastic cell containing PTA-stained aggregates similar to those shown in Figs. 22 and 23. Aldehyde fixation; decalcification by flotation of the section on formic acid; staining with PTA at low pH. Note that mitochondrial structures are not stained. × 60,000.

decalcification, oxidation, and staining by this method was less clearly defined than after staining with uranium and lead or with PTA. This was partly due to the granular background produced by aspecific silver precipitation. Even so, two types of aggregates were recognizable. Some mitochondria contained roundish aggregates of silver granules often surrounding a clear central zone (Fig. 26). The morphology of these silver aggregates was like that of the granular aggregates described in the preceding sections. Other mitochondria contained a lot of silver granules, which were either distributed at random or had a linear orientation and outlined the shape of the filament-like structures described in the preceding sections.

Semithin sections decalcified and stained by the PAS technique showed a lot of small red granules (Fig. 27), which were easily identifiable as calcified mitochondria under the electron microscope. By comparing such sections with those stained by the von Kossa method, it was found that only calcified mitochondria were PAS positive. Calcified mitochondria were also the only mitochondria stained by silver nitrate-methenamine after periodic acid oxidation (Fig. 28).

DISCUSSION

The present results show that mitochondria in hepatic cells in CCl_4-treated rats, those in myocardium and skeletal muscle in DHT-treated rats, and those in neoplastic cells in bowel carcinoma all contain two types of inclusion. The first consists of roundish aggregates of granular material (granular aggregates), the second of rather irregular clusters of filament-like or needle-like structures (crystalline aggregates).

Nature of Intramitochondrial Aggregates

Many findings show that both granular and crystalline aggregates consist of inorganic material.

First, both types of aggregates have intrinsic electron opacity. Second, the treatment of both types of aggregate with formic acid results in the complete removal of their granular and crystalline material, as discussed in detail below. Moreover, as far as the crystalline aggregates are concerned, their inorganic nature is proved by the fact that they are found in von Kossa-positive, alizarin red S-stained mitochondria and by the observation that they consist of structures which have the same morphology and give the same electron diffractograms as the inorganic crystals found in bone, cartilage, and dentine (8–10, 18, 19, 22, 23, 29, 30, 43, 46, 67, 117, 122, 132, 137) and in many calcified soft tissues (13, 16, 37, 48, 55, 68, 90, 98, 99, 116).

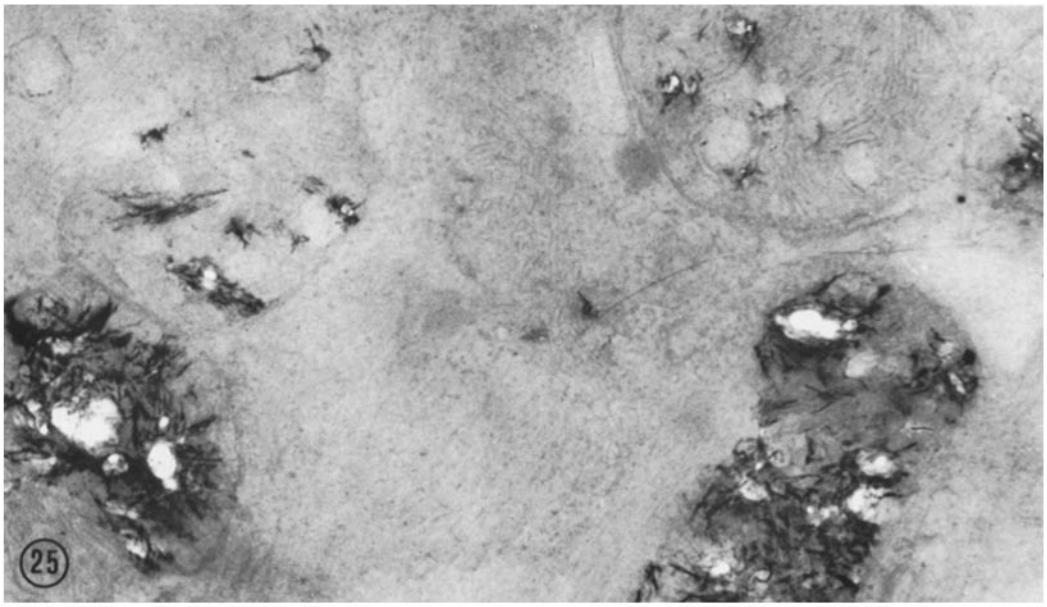
The granular aggregates, too, are to be considered inorganic. This conclusion depends not only on their intrinsic electron density and their solubilization by decalcifying solutions, but also on the consideration that they have the same ultrastructure as the inorganic inclusions found in in vitro calcium phosphate-loaded mitochondria (27, 61, 80, 102, 135, 139, 144, 148).

FIGURE 25 Section of skeletal muscle decalcified by flotation on formic acid and stained with PTA at low pH. Note elongated, needle-like structures having the same shape and arrangement as crystalline structures in untreated sections (compare with Fig. 6), in undecalcified sections stained with uranium and lead (compare with Fig. 16), and in sections decalcified by flotation on formic acid and stained with uranium and lead (compare with Fig. 20). × 50,000.

FIGURE 26 Section of myocardium decalcified by flotation on formic acid, oxidized with HIO_4, and treated with silver nitrate-methenamine. Roundish aggregates of silver granules are visible in many originally calcified mitochondria. They have the same localization as that of the granular aggregates. × 26,000.

FIGURE 27 Section of calcified myocardium as seen under the optical microscope after PAS staining. Only the calcified structures are PAS positive. They are two myocardial cells (*mc*), a small blood vessel (*bv*), and the mitochondria (*m*) of another myocardial cell. Section decalcified by flotation on formic acid and stained with PAS without counterstaining. Compare with Fig. 28. × 1,300.

FIGURE 28 Section of calcified myocardium pertaining to the same series of sections as that shown in Fig. 27. It has been decalcified by flotation on formic acid, oxidized with HIO_4, and treated with silver nitrate-methenamine. Compare with Fig. 27. Only the originally calcified and PAS-positive structures are stained. × 1,300.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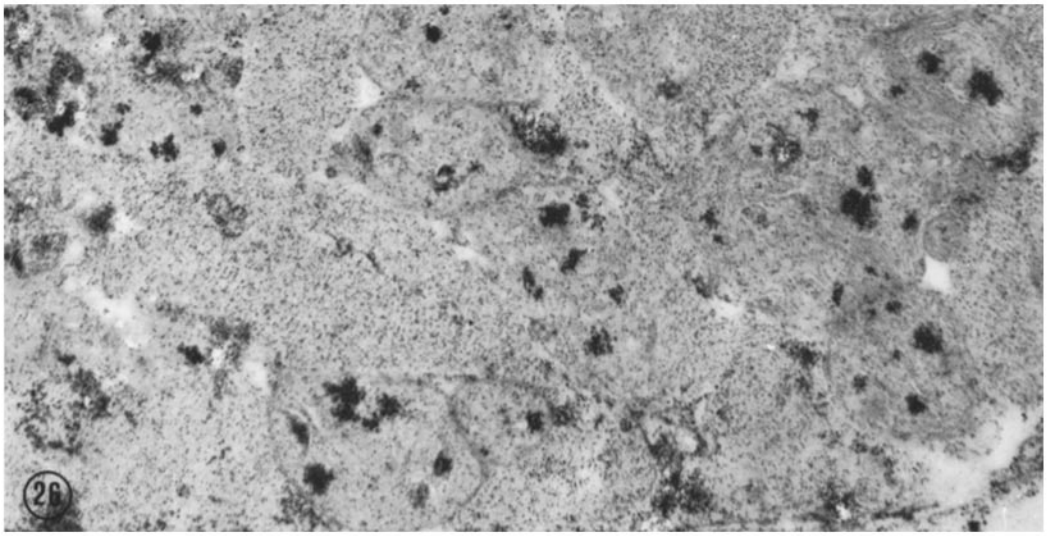
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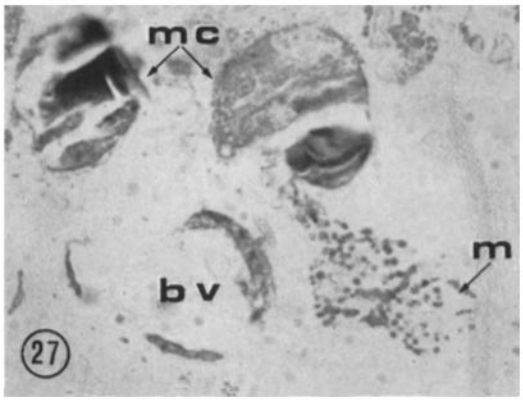
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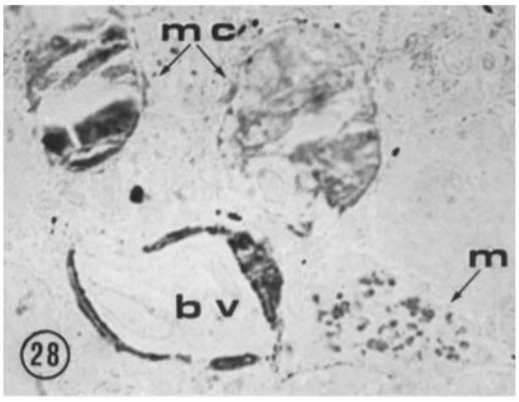
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Relationship between Intramitochondrial Inorganic Aggregates and the State of the Cells

The two types of intramitochondrial inorganic aggregates—crystalline and granular—differed in frequency in the various tissues examined. Crystalline aggregates were mostly found in the myocardium and skeletal muscle of DHT-treated rats; and granular aggregates mostly in the hepatic cells of CCl_4-intoxicated rats and in neoplastic cells. Moreover, crystalline aggregates were mostly found in normal mitochondria in normal cells, and granular aggregates in swollen mitochondria in degenerated cells. Granular and crystalline aggregates were only occasionally found in mitochondria in the same cell, or in the same mitochondrion.

None of the many investigations on mitochondrial calcification have been primarily devoted to exploring the relationship between type of inorganic inclusion and state of cells. Information on this topic in the literature is therefore scanty and imprecise.

Crystalline aggregates are less common than granular ones in calcified mitochondria. They have been reported both in normal and damaged cells and in preserved and degenerated mitochondria (37, 41, 54, 62, 77, 81, 100, 150). Granular aggregates have invariably been found in in vitro Ca^{2+}-loaded mitochondria (27, 61, 102, 128, 139, 145). Moreover, they have been found in mitochondria of normal cells, especially after fixation in solutions containing high concentrations of Ca^{2+} (31, 69, 91–93), in macrophagic cells engaged in resorption of normal and pathologically calcified tissues (24, 40, 59), and in damaged mitochondria of cells at various stages of degeneration and necrosis (25, 40, 47, 65, 76, 115, 119, 128).

These results seem to show that there is no relationship between the state of cells and mitochondria and the type of intramitochondrial inorganic aggregates. But it must be pointed out that the early stages of mitochondrial calcification have not been thoroughly studied, while it is precisely at this point that analysis of the relationship between type of inorganic aggregate and state of cells can be expected to yield decisive results. It has been suggested that mitochondria could protect the cells from damage caused by excessive build up of intracellular calcium (34, 140). Effectively, both myocardial and skeletal muscular cells have a normal structure as long as only a few of their mitochondria contain crystalline aggregates, but show signs of more or less advanced degeneration when most mitochondria are calcified (25).

The true relationship between type of aggregate and state of cells is still an unsettled question which calls for further investigation, above all on the early stages of mitochondrial calcification.

Relationship between Granular and Crystalline Aggregates

It is difficult to determine whether these two types of inorganic inclusion are closely related forms of the same basic material or whether they are distinct, unrelated forms of the same material or, even, of different materials.

Thomas and Greenawalt (139) have suggested that granular inorganic material could be a subcrystalline precursor of a calcium-deficient hydroxyapatite. Although it is not yet known whether this material can be further crystallized, it could be suggested that it might be the first stage of the intramitochondrial accumulation of inorganic substance (phase of nucleation) followed by further Ca^{2+} uptake and by the formation of crystalline structures (phase of crystal growth), a process similar to that proposed for bone calcification (105). Some findings seem to support the possibility that granular aggregates can be converted into crystalline aggregates simply on the basis of increased intramitochondrial Ca^{2+} concentration (60, 120). However, if this possibility is accepted, two findings in the present investigation would remain unexplained. First, the granular aggregates can spread throughout mitochondria until all the mitochondrial structures are masked by them. It is difficult to explain why such a situation, which should lead to local supersaturation of Ca^{2+} and P_i, does not invariably induce the formation of crystal structures. In practice, granular and crystalline structures are only occasionally found in the same aggregate. Second, crystalline structures, sometimes only one or two of them, have been found in mitochondria which are otherwise completely uncalcified and without granular aggregates, thus clearly showing that they can be formed directly, without any granular intermediate.

The ultrastructure of the inorganic aggregates does not seem to be changed by fixation, as shown

in sections from specimens fixed for 15 days. Nor does it seem to be changed by dehydration and embedding because, although exposed to identical chemical and physical conditions, the mitochondria of hepatic cells of CCl_4-intoxicated rats and those of the neoplastic cells almost exclusively contained granular aggregates and the mitochondria of the muscular cells of DHT-treated rats almost exclusively contained crystalline structures.

Relationship between Inorganic Aggregates and Mitochondrial Structures

Staining ultrathin sections with uranium and lead makes it possible to determine the exact relationship between inorganic aggregates and mitochondrial structures. In this connection, the present results confirm previous findings showing that both the granular (47, 53, 56, 61, 62, 115, 121, 144) and the crystalline (25, 60, 77, 83) aggregates have a very close relationship with the mitochondrial cristae.

The smallest granular aggregates are generally located in the space between two adjacent cristae, in contact with the membrane of one of them. It seems that when the granular aggregates grow by accretion of new inorganic granules, their volume gradually becomes greater than the space between the cristae so that these are pushed away and are forced to bend round the aggregates. The membranes of the cristae, however, remain visible and the inorganic substance does not lie over them.

The relationship between crystalline aggregates and cristae has often been close; so close that the underlying membrane has been completely masked, if only a few crystalline structures were present in a mitochondrion. If more of them were present, and they were grouped in large aggregates, they completely masked the mitochondrial structures, so that no relationship could be made out. In this case, however, the crystalline structures were so randomly oriented, and there were so many of them in each mitochondrion, that no relationship with the cristae was, presumably, feasible. The normal mitochondrial architecture is very probably completely disarranged by this stage, in any case.

Organic-Inorganic Relationship in Intramitochondrial Inclusions

The hypothesis that inorganic aggregates in mitochondria might be closely related to an organic substrate was first formulated by Peachey (102). He suggested that divalent cations could be deposited on, or in, preexisting organic granules (so-called "normal" electron-dense granules), possibly in exchange for other ions. These granules, however, are highly osmiophilic structures but have no intrinsic electron density (3, 89, 139). Moreover, microincineration does not give evidence that they contain inorganic substance (139); nor are they affected by treatment with 2,4-dinitrophenol, which releases about 80% of mitochondrial Ca^{2+} (101).

Leaving out this question, other findings show clearly that granular aggregates are closely related to an organic substratum. For instance, inorganic aggregates isolated from Ca^{2+}-loaded mitochondria contained substantial amounts of organic material (from 0.3 to 5% N, depending on the isolation procedure used) and some adenine nucleotides (148). The finding that intramitochondrial inorganic aggregates are stained by toluidine blue O prompted Reynolds (115) to suggest that the intramitochondrial aggregates may include an organic, Ca^{2+}-binding component. Similarly, from the fact that in microincineration experiments the melting point of intact inorganic granules (500°–600°C) is appreciably lower than that of their mineral residues (600°–700°C), Thomas and Greenawalt (139) deduced that the organic and inorganic constituents of intramitochondrial granular aggregates are closely associated.

The decalcification and staining of mitochondria in the present investigation has supplied direct evidence that both granular and crystalline aggregates consist of closely connected inorganic and organic material. As stated above, decalcification leaves apparently empty areas where either or both types of aggregates had been. But subsequent staining with uranium and lead reveals electron-dense structures of almost the same morphology, and occupying almost the same position, as the original aggregates. Amorphous structures are observed where granular aggregates had been and crystal- or filament-like structures where crystalline aggregates had been. Neither type of "stained" structures has intrinsic electron opacity. These structures can therefore be considered organic "ghosts" of the preexisting inorganic aggregates.

The same technique of decalcification plus staining gave similar results and led to the same conclusion when applied to calcifying cartilage

and bone (18, 19, 22, 23). The calcifying areas in these tissues contain needle-shaped crystals (1, 18, 19, 22, 23, 29, 117, 137) like those found in calcified mitochondria. If ultrathin sections of calcified cartilage and bone are decalcified by flotation on EDTA or acid solutions, the areas which originally contained crystals now appear to be empty (18, 19). If, however, decalcification is followed by staining with uranium and lead, organic crystal-like structures of the same morphology as untreated inorganic crystals are seen occupying the same positions (18, 19, 22, 23). These results have been confirmed using both similar and different techniques (4–6, 129, 134). Moreover, it has been shown histochemically that the organic crystal ghosts are proteic structures probably pertaining to glycosaminoglycan complexes (19, 129).

It might be objected that what appears to be organic substance may be undecalcified inorganic material left in partly decalcified mitochondria. This problem has been discussed in detail by Bonucci (22, 23) in relation to early cartilage and bone calcification. Without wishing to lay undue weight on the observation that ultrathin sections of calcified bone can be decalcified in a few minutes even by distilled water (26), so making it highly improbable that undecalcified areas could be left in sections floated for 20 min on a formic acid solution, it needs only be added here that the organic nature of the ghosts is clearly proved both by their lack of intrinsic electron opacity and by the fact that they can be digested by papain (19) and trypsin (6).

It might still be objected that the ghosts could consist of organic substance adsorbed on the inorganic material during fixation, dehydration, and embedding. Although such a phenomenon is a theoretical possibility, it seems unlikely here. As underlined by Bonucci (18) in relation to calcifying cartilage and by Rönnholm (118) in relation to the calcification of enamel, after decalcification and staining any organic material coating the surface of a needle-like crystal must appear as a hollow cylinder thicker than the crystal itself. Under the electron microscope, such a cylinder would appear as a ring in cross section and as a pair of slightly electron-dense structures separated by a clearer axial space in longitudinal section. These highly characteristic shapes have never been observed in calcifying cartilage, bone, or enamel, or calcified mitochondria containing crystalline aggregates. Moreover, the diameter of the ghosts found in these mitochondria was almost exactly the same as that of the untreated crystalline structures.

A useful hypothesis to account for these ghosts might be that a calcified organic material is partly or completely "embedded" in the inorganic substance, so acquiring a high degree of stiffness and hardness. It would then be protected against deformation, solubilization, and extraction during the preliminaries to electron microscopy. With such protection, the organic material might be easily demonstrable in calcified mitochondria, while, even if present, it might be undemonstrable in uncalcified mitochondria, where it would be lost during preparation of the specimens. Nor would it be surprising if, as actually happens, a decalcified area showed fewer ultrastructural details in sections from specimens decalcified before embedding than in sections decalcified by flotation after embedding. In the first case, the organic material unmasked by the removal of the inorganic substance would be directly exposed to the dangerous effects of dehydration and embedding and of decalcification itself, if continued for too long. In the second case, it would be initially protected by the inorganic substance. Once this has been removed, it would still be protected by the embedding resin so that most of it would remain intact in the sections. If such reasoning is accepted, there would be nothing startling in the discovery that an organic nidus or nucleating substrate to the inorganic substance had the same shape and morphology as the inorganic structures themselves.

Nature of the Organic Substratum of the Intramitochondrial Inclusions

Data on the nature of the organic substrata present in the intramitochondrial inorganic aggregates have been obtained by treating decalcified ultrathin sections with 1% PTA in 0.1 N hydrochloric acid and with silver nitrate-methenamine after periodic acid oxidation. Both these techniques deeply stain the organic framework of both granular and crystalline aggregates.

PTA staining at low pH, initially proposed by Pease (103) and by Marinozzi (87, 88) and later developed by Rambourg (109, 110, 112) for detecting complex carbohydrates in ultrathin sections, has been considered unspecific, because

PTA interacts with amino acids, proteins, acid glycosaminoglycans, and with any hydroxylic polymer (57, 107, 108, 123, 124). However, many investigations have shown that, in spite of this unspecificity, PTA staining at low pH is a very useful technique for revealing glycoproteins under the electron microscope and that there is a close parallel between the results of this technique and those of other staining methods considered specific for glycosaminoglycan complexes (12, 70, 104, 110–112, 149).

PTA at low pH deeply stains calcified areas within mitochondria, whether they contain granular or crystalline aggregates, and leaves cristae and the outer mitochondrial membrane unstained, unless sections from osmium-fixed specimens are used, when the membranes show the low electron density due to osmium fixation. This seems to be further evidence that proteins are not stained by this technique. But, most importantly, these results show that the organic substance in intramitochondrial calcified areas includes glycoproteins, a finding in complete agreement with previous results obtained with calcified cartilage and bone (15, 19, 22, 23), as also with those obtained in the present investigation by using the periodic acid-silver methenamine technique.

This staining technique is considered to be equivalent to the PAS method and can be used to visualize glycoproteins in both optical (73) and electron microscopy (84, 110, 111, 138). In the present investigation, the decalcified areas previously occupied by granular and/or crystalline aggregates were stained using this technique. By using serial sections and the optical microscope, it was also possible to show that the same calcified mitochondria were stained by both this technique and the PAS method.

Taken together, these results leave little doubt that the material found in intramitochondrial inorganic aggregates consists largely of glycoproteins, thus implicitly confirming that it is organic.

To deepen the analysis of the exact nature of this organic material, it is worth recalling that a glycoproteic material showing high-affinity Ca^{2+}-binding activity has been isolated from rat liver mitochondria after osmotic shock (42, 58, 79, 130, 131). These biochemical results, and those obtained in the present investigation, though not directly comparable, are in full agreement in showing that a glycoproteic material is largely responsible for calcium binding and uptake in mitochondria. The importance of this finding is not, perhaps, confined to mitochondria; it may involve the calcification process in general. In this context, it is certainly remarkable that glycoproteins are always present at sites of both normal (23, 28, 106, 141) and pathological (142) calcification, that a glycoprotein with Ca^{2+}-binding activity has been isolated from calcifying cartilage (66, 146), that early cartilage, bone, and dentin calcification occurs within roundish bodies (2, 14, 15, 18, 20–23, 49) containing glycoproteins (18, 20, 82), and that both in cartilage and bone the inorganic substance is intimately connected with a fibrillar organic substrate (4–6, 18, 19, 22, 23, 129, 134) which has the histochemical properties of glycosaminoglycans (19, 129).

In the early stages of intramitochondrial calcification, the crystalline structures appear to be closely related to the membrane of the cristae, unlike the granular aggregates, which, though found near the cristae, lie in the mitochondrial matrix. The exact interpretation to be given to this difference is not yet clear. But it would appear reasonable to infer that crystalline inorganic material might be related to the proteins and phospholipids of the cristal membranes. It would be extremely interesting to know if this is really so, especially when it is considered that the mitochondrial low-affinity Ca^{2+}-binding sites are believed to consist of nonspecific anionic groups of membrane proteins and lipids (32, 78), that both Ca^{2+}-binding factors recently isolated from mitochondria contain high percentage of phospholipids (58, 131), and that there is increasing evidence that a lipidic material is involved in the early stages of calcification in bone, cartilage, and soft tissues (52, 71, 72, 126, 127, 136, 147, 151, 152).

Evidence that a lipidic substance may be present in both organic substrates of intramitochondrial inorganic aggregates is given by the finding that both granules and crystalline structures are less clearly outlined in sections from osmium-fixed specimens than in those from aldahyde-fixed specimens. Osmium tetroxide is known to react with, and stain, phospholipids, but to leave protein molecules practically unstained (63, 133). It is therefore reasonable to suppose that the blurring of outlines of inorganic aggregates observed after osmium fixation is due to the staining of phospholipidic material in their organic substrates. Such phospholipidic material would have no intrinsic

electron opacity and would therefore not appear after aldehyde fixation.

Bearing in mind that it is now agreed that lipids are contained in the normal electron-dense mitochondrial granules (3, 11) which disappear just before, or during, the formation of inorganic aggregates (115), it seems likely that these lipids contribute to the formation of the organic substrate which permit deposition of inorganic material. At present, no reliable data on the formation of these substrates are obtainable with the electron microscope, and further research and new techniques are needed.

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