MICROINCINERATION, ELECTRON MICROSCOPY, AND ELECTRON DIFFRACTION OF CALCIUM PHOSPHATE-LOADED MITOCHONDRIA

RICHARD S. THOMAS and JOHN W. GREENAWALT

From the Western Regional Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Albany, California 94710, and the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

Isolated rat liver mitochondria were incubated in vitro under conditions supporting the massive accumulation of calcium and phosphate. Samples were embedded, thin sectioned, and examined in the electron microscope. The intramitochondrial distribution of insoluble or structure-bound mineral substances was studied by electron microscopy coupled with recently developed techniques of high resolution microincineration. As shown previously, the ion-loaded mitochondria acquire large, internal granules which have inherent electron opacity indicative of high mineral content. Study of ash patterns in preselected areas of sections directly confirmed the high mineral content of the granules, and the appearance of the residues was consistent with the copresence in the granules of some organic material. Other mitochondrial structures were almost devoid of mineral. Thin sections of unincubated control mitochondria also were incinerated. They were found to contain appreciable amounts of intrinsic mineral, seemingly associated with membranes. The normal, dense matrix granules commonly seen in unaltered mitochondria could be seen in intact sections of these control preparations, but after burning no definite correspondence of any ash to the granules could be demonstrated. The normal granules perhaps do not contain mineral. Heating experiments on ash patterns of all the preparations demonstrated the thermal stability and crystallizability of the ash. The crystallized ash of the in vitro-produced dense granules was tentatively shown by electron diffraction to be β-tricalcium phosphate (whitlockite). This, together with evidence from the literature, suggests that the original, noncrystalline mineral may be a colloidal, subcrystalline precursor of calcium-deficient hydroxyapatite. Experiments were performed on synthetic calcium phosphates for comparison. Other possible applications of the microincineration techniques are briefly discussed.

INTRODUCTION

Isolated mitochondria incubated in vitro in a suitable ionic medium can utilize either ATP or an oxidizable substrate to actively accumulate large amounts of calcium and inorganic phosphate (Pullman and Schatz, 1967; Lehninger, 1966). Concomitantly with the massive accumulation of ions, large opaque granules are formed inside the mitochondria, often in association with the inner membranes (Greenawalt et al., 1964; Brierley and Slautterback, 1964).

It has seemed likely, a priori, that the granules must consist largely of some form of precipitated calcium phosphate, since (*a*) the calculated internal concentration of calcium and phosphate ions

in the mitochondria after maximal uptake greatly exceeds the solubility product for calcium and phosphate, and (*b*) the electron-opacity of the granules does not depend upon heavy metal staining but is intrinsic. Mitochondria heavily loaded with the granules accumulate calcium and phosphate with an average molar ratio of about 1.67:1 under the conditions initially described by Vasington and Murphy (1962). This has suggested that the mineral of granules might be hydroxyapatite (Rossi and Lehninger, 1963 *a*, *b*). However, no evidence of crystallinity was detected in the granules *in situ* (Greenawalt et al., 1964) and so the exact nature of the mineral has not been determinable by diffraction analysis.

In the present investigation, we have examined the nature of the granules by applying recently developed techniques of high resolution microincineration (Thomas, 1964, 1968) to the granule-loaded mitochondria. These procedures, carried out on thin sections on electron microscope grids, burn away all organic material in the specimen but leave *in situ* the inorganic constituents. The liberated mineral residues can be visually compared with the original, intact structures, and can also be subjected to further chemical or physical treatments. In this way it has been possible to (*a*) confirm directly the high mineral content of the granules in contrast to that of other structures in the mitochondrial sections; and (*b*) induce the mineral residues of the granules to crystallize and tentatively identify the crystals by electron diffraction and comparison with the properties of known minerals.

Microincineration experiments were also performed on control mitochondria not loaded in vitro with calcium and phosphate. These studies revealed residual deposits of intrinsic, bound mineral, possibly associated with the membranes of the organelles. They also provided new information on the chemical nature of the dense, matrix granules commonly seen in normal, unmodified mitochondria and regarded by some investigators as sites for physiological deposition of minerals in vivo (Weiss, 1955; Peachey, 1964; Bruni and Porter, 1965).

Preliminary accounts of the present work were reported earlier (Thomas and Greenawalt, 1964; Greenawalt and Carafoli, 1966). The latter report describes application of the microincineration technique to strontium phosphate–loaded mitochondria but also gives further details of results with calcium phosphate–loaded organelles. Since these reports appeared, calcium phosphate granules produced in vitro have been isolated from mitochondria and subjected to chemical and physical analysis in bulk (Weinbach and von Brand, 1965, 1967). This latter study is in essential agreement with our findings on the granules. This is reassuring for both investigations since the possible sources of artifact in the two approaches are rather different.

MATERIALS AND METHODS

Mitochondrial Isolation and Ion Accumulation

Mitochondria were isolated from the livers of Carworth Farms (New City, N. Y.) albino rats (Wistar strain) in 0.25 M sucrose by the method of Schneider (1957) and were then washed three times with 0.25 M sucrose.

The washed mitochondria were incubated in the medium described by Vasington and Murphy (1962) for the substrate-supported accumulation of Ca^{++} and P_i. The complete reaction mixture contained 80 mM NaCl; 4 mM sodium phosphate, pH 7.0; 10 mM Tris maleate (or Tris-chloride), pH 7.0; 10 mM succinate; 10 mM $MgCl_2$; 4 mM $CaCl_2$ (labeled with ^{45}Ca); 3 mM ATP; and 3–5 mg of mitochondrial protein. The total volume was 3.0 ml. Incubation was for 20 min at 30°. Controls consisted of preparations incubated in the complete medium minus Ca^{++}, in the complete medium minus ATP, or in the complete medium but removed from incubation at zero time. The incubation was terminated by quickly chilling the media to 0°C and centrifuging the mitochondria at 20,000 *g* for 4 min. The pellet was washed once with 0.25 M sucrose.

The accumulation of Ca^{++} was determined by measuring the amount of isotope which disappeared from the media by plating and counting aliquots following sedimentation of the mitochondria. In the experiments described here, over 90% of the added Ca^{++} was accumulated. Phosphate uptake was not measured but it has been shown that the ratio of Ca^{++}/P_i taken up under these conditions ranges from about 1.52 to 1.88 with an average value of about 1.67 (Rossi and Lehninger, 1963 *a*, *b*).

Preparation of Specimens for Electron Microscopy and Microincineration

Samples were taken from the pellets of control and "loaded" mitochondria and fixed with 10% aqueous formaldehyde or with 1% OsO_4 in Veronal-acetate buffer, pH 7.4, for 1 hr at 0°C. Samples were taken from the top, middle, and bottom regions of each

pellet; no major differences were observed in the mitochondria from these different regions. Mitochondria were dehydrated by rapid passage through a cold (−10°C) ethanol series and embedded in Epon 812 according to the procedure of Luft (1961).

All of the embedded preparations, both osmium-fixed and formalin-fixed, were routinely thin-sectioned and examined in the electron microscope to confirm that their morphology was similar to that previously reported (Greenawalt et al., 1964). However, for the microincineration study reported here, only one of the controls, the unincubated "zero time" preparation, was utilized, and most experiments were confined to formaldehyde-fixed material so possible artifacts could be avoided in the ash patterns which might arise from the introduction of osmium. In experiments on the "normal" matrix granules of control mitochondria, it was necessary to use osmium-fixed material, since the granules are not seen in formaldehyde-fixed organelles (see below in Results).

Embedded preparations for microincineration experiments were thin-sectioned at about 50–90 mμ, or thick-sectioned at 250–500 mμ, with a diamond knife and a Porter Blum MT-1 microtome. Thin sections were necessary to obtain good preservation of structural detail in the ash patterns but thick sections were advantageous for electron-diffraction experiments since they yielded a greater amount of ash per unit area which could contribute to a diffraction pattern (Thomas, 1968). To avoid the leaching of dense granules from the mitochondria which can occur on the surface of slightly acidic distilled water, we collected the sections from the knife edge on a dilute volatile buffer, 0.001 M ammonium bicarbonate, pH 7.5 (Thomas, 1968; Boothroyd, 1964). The sections were picked up on 200-mesh stainless steel grids bearing a thin collodion membrane on which had been deposited a film of silicon monoxide about 200 A thick (Thomas, 1968). The SiO film provided a heat- and oxidation-resistant supporting surface for the sections. The collodion on the backside imparted additional mechanical stability to the film during manipulation of the grids and was subsequently burned away during incineration. It was not necessary to remove the embedding medium from the sections; this also burned away easily during incineration.

It was directly demonstrated by viewing in the electron microscope that the total procedures used for preparation of mitochondria for microincineration were adequate for the retention of the mineral in the in vitro-acquired dense granules. However, the procedures almost certainly did not retain all of the original mineral content of the organelles, and in general, only inherently insoluble minerals or minerals bound to insoluble structures were maintained in place. It was necessary to keep this in mind when interpreting ash patterns of the mitochondrial specimens.

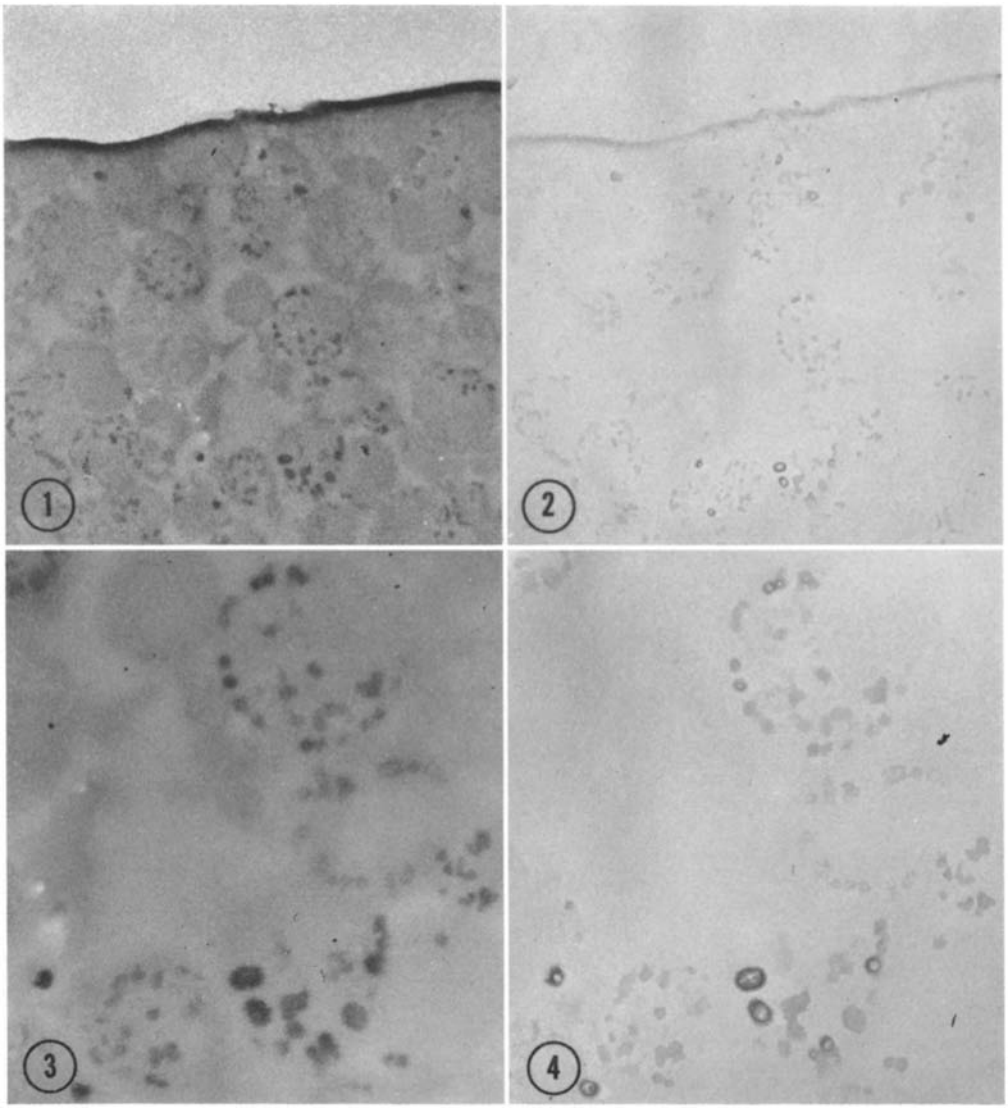
Samples of synthetic calcium phosphates were also prepared for electron microscopy for comparison with the mitochondrial specimens. The mineral powder was mixed with 1.5% (w/v) collodion in amyl acetate to form a thick slurry, and a drop of this mixture was allowed to spread on a water surface, forming a film enclosing the dispersed crystals (Bradley, 1961). Stainless steel grids (200 mesh) bearing silicon monoxide–collodion membranes were placed membrane side down on the upper surface of the crystal-containing film, and the grids with the film were picked up in the usual way and allowed to dry. Alternatively, the mineral powder was mixed with methyl-butyl methacrylate (inhibitor-free) and centrifuged into a pellet, and the pellet was polymerized overnight under an ultraviolet lamp. Thin sections (50–100 mμ) were cut with a diamond knife onto ammonium bicarbonate buffer and picked up on grids filmed with silicon monoxide–collodion or carbon-collodion. The methacrylate was removed from the sections by brief immersion of the grids in benzene.

Microincineration Techniques

The techniques for high resolution microincineration are presented in detail elsewhere (Thomas, 1968), and are only briefly described here. The organic material of the mounted thin sections was burned away in one of two ways: (*a*) by heating the grids in air to high temperature either in a muffle furnace or on a special heating stage in a vacuum evaporator (Thomas, 1964); (*b*) by exposing the grids to a gaseous plasma of electrically excited oxygen in a modified Tracerlab model LTA 500 low temperature asher (Tracerlab Div. of Laboratory for Electronics, Inc., Richmond, Calif.) (Gleit, 1963). The latter technique permits complete combustion of the specimen at a temperature below 100°C and minimizes or eliminates the melting and/or volatilization of minerals and mineral-organic complexes which can occur at high temperatures.

For high temperature ashing the grids were heated to either 500° or 600°C for 30 min. Previous experience has shown that this is more than adequate to completely combust the specimens. The higher temperature was used when it was desired to view the same area of the specimen in the electron microscope both before and after burning. A thin film of contamination from the electron beam persisted after burning at the lower temperature even though the organic material of the section under the film was obviously burned away completely.

The low temperature asher, model LTA 500 (single sample version) was modified for continuous rather than stepwise adjustment of the power input



Figs. 1–4, thin-sectioned pellet of formaldehyde-fixed, calcium phosphate–loaded mitochondria before and after incineration for 30 min at 600°C.

FIGURE 1 Before incineration. The dense granules are easily seen and the mitochondrial profiles which contain them are also visible, but with very low contrast. The edge of the section is seen at the top. ×12,000.

FIGURE 2 Same field as Fig. 1, but after incineration Except for a line (presumably contamination) tracing the edge of the section, nothing remains but residues of the dense granules. Lightly shadowed. ×12,000.

FIGURE 3 Before incineration. Area selected from Fig. 1, enlarged to show granules in more detail. ×29,000.

FIGURE 4 Same field as Fig. 3, but after incineration. The residues correspond exactly to the dense granules and only to the granules. The larger residues contain a bubble, however, indicating melting and some decomposition of the granules. Lightly shadowed. ×29,000.

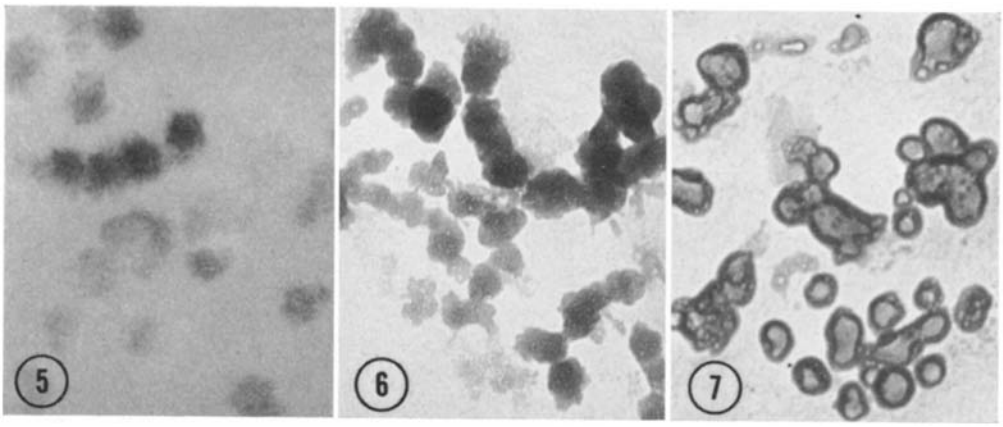
and also for monitoring of the input (by reading the plate current of the final stage amplifier.) Specimens were usually incinerated for 30 min at 30 ma. (Highest power input reads 300 ma.) Again, previous experience has shown this to be more than adequate for total combustion of the thin electron microscope specimens. Complete combustion could be confirmed, however, by heating the low temperature-ashed specimen to 600°C and noting no change (see below in Results). The low temperature asher did not completely burn away electron-beam contamination films from previewed specimens, and so its use in connection with before-and-after viewing experiments was not satisfactory.

Specimens were usually shadowed with uranium immediately after incineration, unless they were destined for electron-diffraction examination. The shadowing angle was nominally $\tan^{-1} \frac{1}{2}$, but undulations in the support film introduced some uncertainty in the local shadowing angles. Shadowing applied before exposure of the ash to the moist air of the laboratory served to monitor the possibility of hygroscopy in the ash (Thomas, 1968). None of the ash patterns in the present experiments showed any evidence of hygroscopy.

Electron Microscopy, Electron Diffraction, and X-ray Diffraction

Electron microscopic observations were made with a Hitachi HU-10 with a 35 μ objective aperture at 50 kv. Micrographs were taken at magnifications ranging from 2700 to 11,000. Higher magnifications were usually not warranted since very high resolution was limited by the relatively thick support films. Special procedures for mapping specimen grids for before-and-after-incineration viewing experiments are described elsewhere (Thomas, 1968). Dark-field observations were made by decentering the objective aperture.

Selected area electron diffraction observations (Bigelow, 1960) were made at 50 kv, with a magnification of 11,000 and a rectangular (or square) field-limiting aperture which usually included about 20 μ^2. The mitochondria tended to occur in clusters in the section and the 20 μ^2 area was usually about optimum to include a single cluster but exclude relatively empty support film which contributed unduly to background scattering. The ratio of Bragg diffraction/background scattering was in all cases



Figs. 5–7, effect of incineration temperature on fine structure of dense granule residues. Sections of formaldehyde-fixed, loaded mitochondria. ×63,000.

FIGURE 5 Untreated thin section (similar to that of Figs. 1 and 3). The native granules show a somewhat diffuse electron density; they apparently are aggregates of very fine mineral grains, 60 A or less in diameter, embedded in some sort of organic matrix.

FIGURE 6 Section, somewhat thicker than in Fig. 5, after incineration for 30 min at 500°C. The mineral residues of the granules show a fine porosity, which seems to correspond to the diffuse density of the native granules.

FIGURE 7 Section same thickness as for Fig. 6, after incineration for 30 min at 600°C. The granule residues are bubble-like. Apparently the granule mass fuses at this higher incineration temperature and bubbles are formed as the organic component vaporizes.

low, but varying the exposure of the plates caused the contrast between the two components to be maximized at different radii of the powder pattern. Thus, although no one plate showed all of the diffraction arcs to best advantage, a fairly complete assemblage of arcs could be observed from a collection of plates. The powder patterns were calibrated for determination of d_{hkl} values by reference to the diffraction from silicon monoxide membranes bearing vacuum-evaporated gold. Measurements of diffraction rings and arcs and visual estimations of intensities were made on the original electron microscope plates. For publication, plates were printed through appropriate contrast-cancellation masks (out-of-focus positives in register with the negative plates) for suppression of background and enhancement of the visibility of the crystalline diffraction spots (Gonzales, 1962).

X-ray diffraction powder photographs of calcium phosphate samples were obtained with a cylindrical camera of a radius 7.184 cm with copper K_α radiation, $\lambda = 1.5418$ A. Measurements of line spacings and visual estimates of intensities were made on the films.

RESULTS

Microincineration of Loaded Mitochondria

HIGH TEMPERATURE INCINERATION–EXAMINATION OF THE SAME SECTION BEFORE AND AFTER ASHING: Figs. 1 and 3 show a thin section of the formaldehyde-fixed, calcium phosphate–loaded mitochondria, before incineration. In the absence of heavy metal staining, little of the membrane structure of the organelles can be seen, but the granules stand out clearly owing to their intrinsic density. After incineration for 30 min at 600°C (Figs. 2 and 4) an ash residue remains precisely at the site of each granule but all other substance, of both the mitochondria and the embedding, is essentially missing. The association of ash with the granules directly confirms the high mineral content of the latter, and the absence of everything else in the burned section produces what is evidently a rather clean preparation of this particular ash.

DECOMPOSITION OF THE GRANULES–THE EFFECT OF TEMPERATURE: Although the mineral of the granules is retained in place, many of the granules, particularly the larger ones, appear somewhat altered by the 600°C treatment; they have a "bubble-like" aspect. Fig. 7, which shows a thicker section containing more large granules, demonstrates this strikingly. This evidence of some decomposition is not surprising. As reported previously (Greenawalt et al., 1964) and as shown in Fig. 5, high resolution examination of the granules before incineration indicates that they are not solid masses of mineral but rather a complex of very fine dense particles, 60 A or less in diameter, which seem to be held together in some sort of low density matrix, presumably organic in nature. The presence of the organic matrix has been confirmed in isolated granules (Weinbach and von Brand, 1965, 1967). Apparently the mineral-organic mass fuses at the high temperature, and bubbles are formed as the organic material vaporizes.

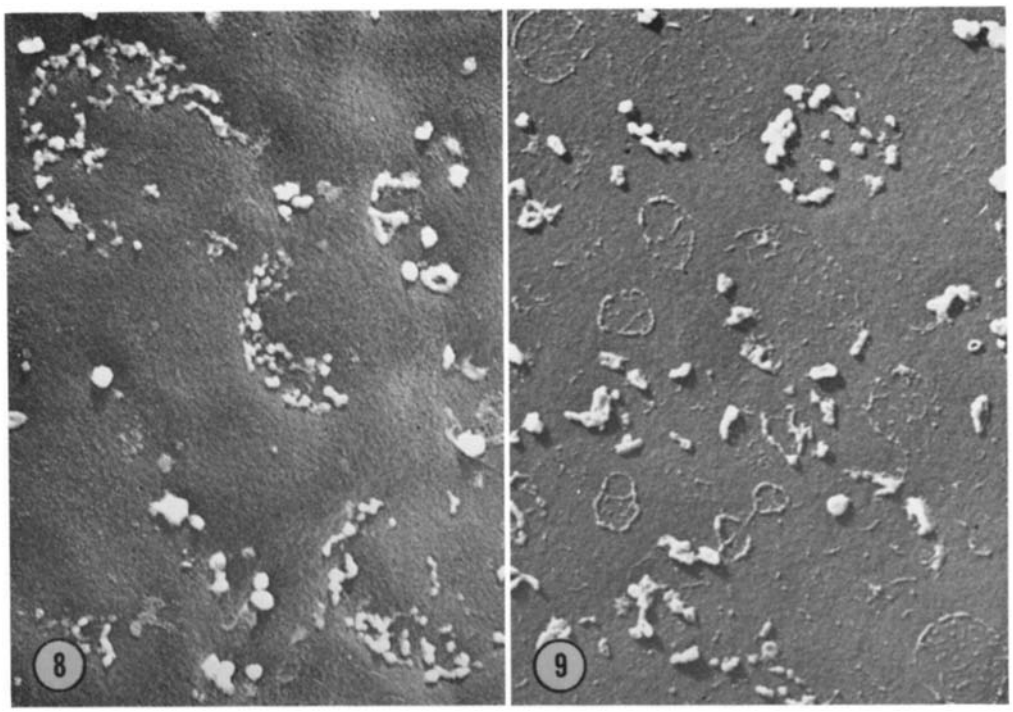
If microincineration is carried out at 500°C rather than 600°C this fusion of the granule mass does not occur, or is greatly minimized. Fig. 6 shows the result of 30 min treatment at this lower temperature. The mineral residues of the granules now have a flocculent, porous appearance which is not unlike that of the mineral in native granules. Apparently the organic matter is burned out at this lower temperature without greatly disturbing the inorganic structure.

Except for the improved preservation of granule fine structure, microincineration at 500°C gives results similar to 600°C burning. As shown in Fig. 8, nothing remains of the mitochondrial sections after 500°C but the residues of the granules.

LOW TEMPERATURE (LTA) INCINERATION–NONGRANULE ASH: Incineration of thin sections of the loaded mitochondria for 30 min at 30 ma in the low temperature asher results in porous granule residues which are quite similar in appearance to those of the 500°C treatment (Fig. 6). When one views the incinerated sections over-all, however, there is a difference. Fine strands or granules of ash other than the dense granule residues are seen scattered here and there. In favorable areas, as shown in Fig. 9, this ash is sufficiently well organized to form a just recognizable pattern. It evidently corresponds to mitochondrial cross-sections and must represent trace amounts of mineral, other than the dense granules, remaining in the organelles. This mineral is evidently volatilized during high temperature ashing (500°–600°C).

Microincineration of Control Mitochondria

HIGH TEMPERATURE ASHING OF FORMALIN-FIXED AND OSMIUM-FIXED MATERIAL—THE FATE OF NORMAL DENSE GRANULES:



Figs. 8 and 9, comparison of 500°C incineration and low-temperature ashing (LTA) on thin sections of formaldehyde-fixed, loaded mitochondria. Preparations shadowed, and micrographs printed with reverse contrast. ×27,000.

FIGURE 8 A section incinerated 30 min at 500°C. Only the mineral residues of the dense granules remain.

FIGURE 9 A section incinerated in the low temperature asher for 30 min at 30 ma. The granule residues appear similar to those from 500°C ashing. In addition, a slight ash remains from the rest of the mitochondrion and delineates its profile in favorable cases.

A typical thin section of formaldehyde-fixed unincubated control mitochondria is seen before ashing in Fig. 10. The organelles appear somewhat smaller and denser on the average than their incubated counterparts (see Figs. 1 and 3), which are known to swell during incubation. As would be expected, the control mitochondria contain none of the in vitro-produced dense granules, and in the absence of heavy metal staining, they display little evidence of internal structure. The "normal," dense, matrix granules seen in osmium-fixed mitochondria (see below in this section) are not observed. This agrees with previously reported descriptions of aldehyde-fixed mitochondria (Sabatini et al., 1963; André and Marinozzi, 1965). Whether the granules are not present or are simply invisible in this preparation is not known, but the former seems most likely (Ashworth et al., 1966).

After 30 min at 500° or 600°C, sections of the control mitochondria are volatilized completely and leave no ash residue. They thus behave similarly to the loaded mitochondria exclusive of their dense granules. Their complete volatilization is in agreement with results of classical microincineration studies (Kruszynski, 1966, pages 125 and 129).

So that some sure indication of the fate of the normal dense matrix granules during incineration could be gained, thin sections of control mitochondria fixed with osmium also were ashed. Before incineration (Fig. 11) the normal granules are clearly visible in this preparation, in contrast to formalin-fixed material. The same preparation, same field of view, after 30 min at 600°C is seen in

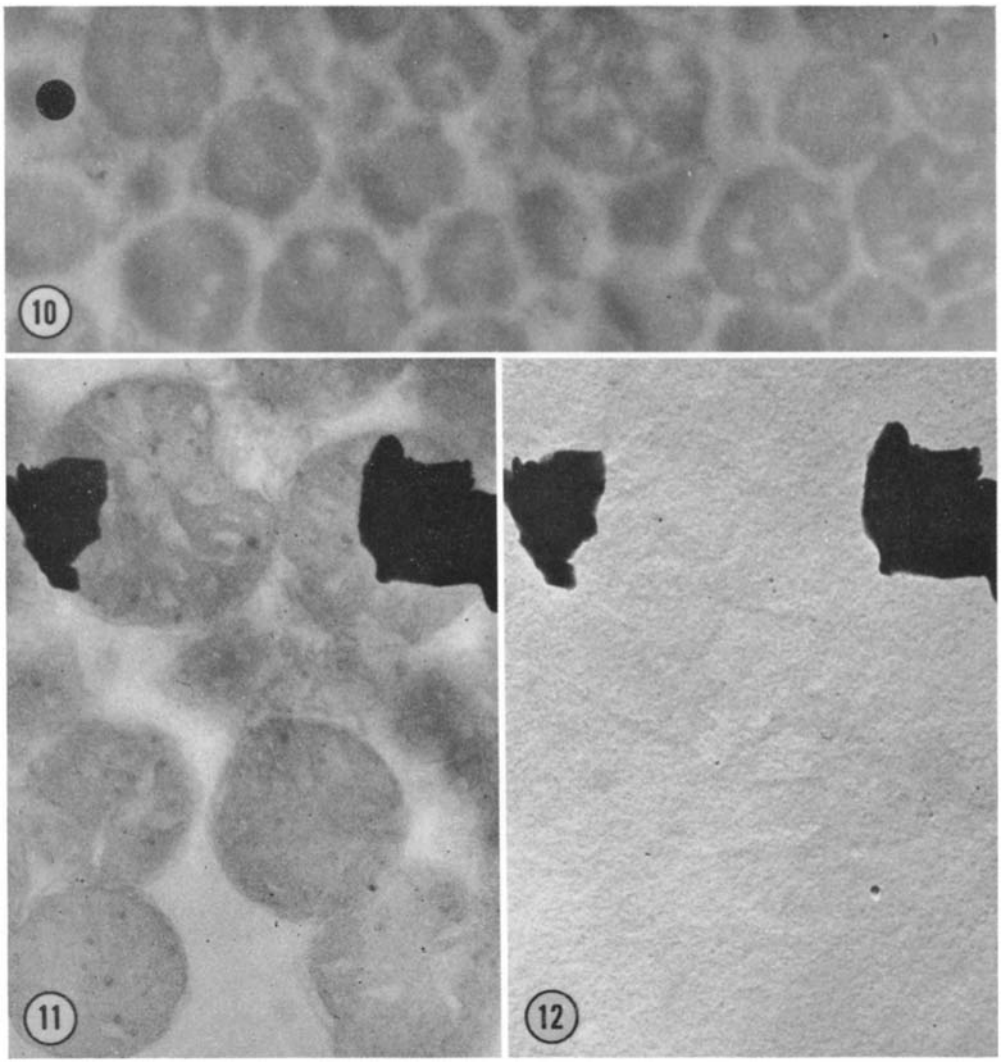


FIGURE 10 Thin-sectioned pellet of formaldehyde-fixed, control mitochondria. The organelles are somewhat denser and more distinct in appearance than similarly fixed loaded mitochondria. Intracrystate spaces can be recognized but still no definite membrane structure is visible. In vitro-acquired dense granules are absent, of course, and, similarly, there is no evidence of normal dense matrix granules. Section sprayed with 0.264 μ polystyrene latex for focusing aid. ×20,000.

Figs. 11 and 12, thin-sectioned pellet of osmium-fixed, control mitochondria before and after incineration for 30 min at 600°C. Diatom fragments (SiO_2) were sprayed on the backside of the grid to serve as permanent location markers. ×34,000.

FIGURE 11 Before incineration. Membranes are clearly seen in the mitochondrial cross-sections, as are also the normal dense granules. The latter, although distinct, are of considerably lower density than the in vitro-acquired granules of loaded mitochondria. In the lower right quadrant a granule is seen that is somewhat denser than the others and lies at the margin between two mitochondrial profiles rather than within the matrix of either organelle. It is probably a contaminant particle, either in or on the section.

FIGURE 12 Same field as Fig. 11, but after incineration and shadowing. Nothing remains of the normal dense granules or, in fact, of the mitochondria. The only granule left in the field is the suspected contaminant particle lying in the lower right quadrant.

Fig. 12. Just as with formaldehyde-fixed material after 600°C, nothing remains of the mitochondria. The osmium is evidently not retained anywhere and the normal dense granules leave no ash from osmium or anything else.

LOW TEMPERATURE (LTA) INCINERATION—MINERAL IN THE MEMBRANES (?): In contrast to preparations ashed at high temperature, an appreciable mineral residue does remain in LTA-incinerated sections of control mitochondria. Fig. 15 shows a thin section of formaldehyde-fixed control mitochondria after 20 min at 10 ma in the low temperature asher. These conditions were evidently sufficient for complete ashing since identical results were obtained from more strenuous treatments, e.g., 30 min at 30 ma. Cross-sections of individual mitochondria are easily recognized by their ash patterns. The patterns are somewhat sketchy, owing to fine scale migration of the mineral to form grains (Thomas, 1968), but nevertheless the deposition of the ash definitely suggests an origin from the membranes of the mitochondria.

This interpretation is strengthened by an examination of sections in various stages of the incineration process. Fig. 13 shows a typical initial preparation, an unincinerated section which, in this case, was shadowed. Some indication of internal membrane structure is given by slight surface detail, apparently induced by local differences in cutting properties during sectioning, but the section surface is nevertheless relatively smooth and continuous. Fig. 14 shows a thin section after incineration for 5 min at 10 ma. The embedding Epon and mitochondrial substance are partially etched away. The most resistant structures are seen emerging from the etched surface, and their organization indicates fairly convincingly a kinship both with the membranes in intact mitochondria and with the final ash pattern.

Osmium-fixed preparations also were LTA-incinerated. The ash patterns look essentially the same as from formaldehyde-fixed material, e.g., Fig. 15. The ash patterns contain no particles which are clearly identifiable as residues of the normal, dense matrix granules. It should also be stated, however, that the general grain size of the patterns is the same as the granules, and thus residues of the granules, even if present, might be indistinguishable from the general inorganic residues.

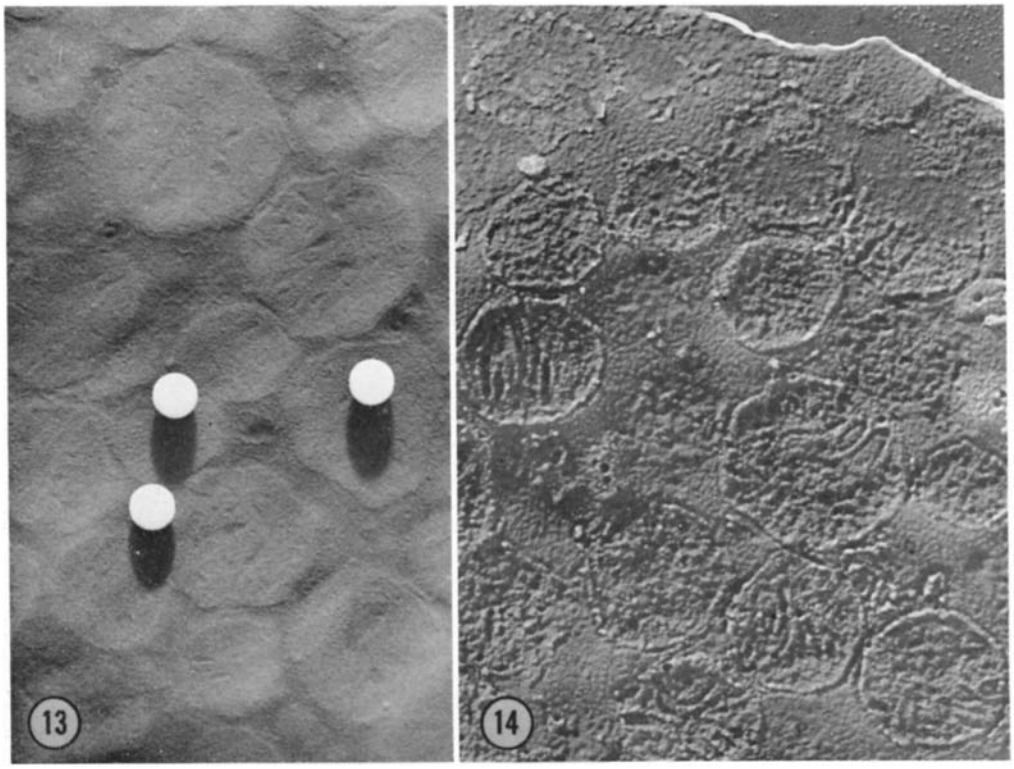
Heating Experiments on the Ash Patterns

HEATING OF THE LOW-TEMPERATURE (LTA) ASH PATTERN TO 600°C: For determination of the stability of the ash resulting from low temperature LTA incineration and also for verification of the absence of organic material, thin sections of both loaded and control mitochondria were incinerated in the low temperature asher for 30 min at 30 ma, and then the resultant residues were reincinerated in the furnace for 30 min at 600°C.

After this double treatment the specimens look no different than after low temperature ashing alone. The residues of loaded mitochondria are similar to those shown in Fig. 9 and those of the control mitochondria are as seen in Fig. 15. From this it seems reasonable to conclude that (*a*) the LTA-ashed specimens are, in fact, free of organic material; (*b*) the melting and bubble formation seen in dense granules initially incinerated at 600°C probably results from the presence of organic material, the mineral itself being stable; (*c*) the absence of nongranule mineral in both loaded and control mitochondria seen after initial incineration at high temperature probably results from a volatile mineral-organic complex, since the mineral alone is not volatile.

HEATING OF ASH PATTERNS TO HIGHER TEMPERATURES—CRYSTALLIZATION OF THE ASH AND ELECTRON DIFFRACTION: The ash patterns resulting from both 500° and 600°C burning as well as LTA incineration were routinely examined by dark-field electron microscopy and by electron diffraction for detection of crystallinity in the ash if such were present. Usually the ash gave no indication of crystallinity, and in the few instances when crystals were detected the presence of contamination in the specimen was strongly suspected. In the hope of inducing crystallinity, the ash patterns were heated for a few minutes to higher temperatures, ranging from 600° to 1000°C and then allowed to cool slowly for several minutes.

The results of these experiments are summarized in Table I, and the appearance of some of the ash patterns after heating is shown in Figs. 16–19. As may be seen, strong heating melts the ash of all the preparations and does induce it to crystallize. In most cases, however, the diffraction spots are relatively weak, and with one exception (group G) the number of individual spots in the diffraction pattern is insufficient to form recognizable arcs



Figs. 13–15, LTA incineration of thin sections of formaldehyde-fixed control mitochondria. Preparations shadowed, and micrographs printed with reverse contrast.

FIGURE 13 Intact, unincinerated section, sprayed with 0.264 μ polystyrene latex before shadowing. The mitochondrial profiles and some indications of internal membrane structure are seen by slight surface detail (apparently induced by local differences in cutting properties during sectioning), but the surface is relatively smooth. ×24,000.

FIGURE 14 A section after incomplete LTA incineration (5 min at 10 ma). The embedding plastic and mitochondrial substance have been partially, and differentially, etched away. The most resistant, emergent material has a configuration strongly suggesting origin from mitochondrial membranes. The edge of the section is seen at the upper right. ×24,000.

which can be used for identification. The spottiness is due to an insufficient number of individual crystals in the selected area field. A contributing factor here is the tendency of the ash to fuse to form a relatively few, relatively large crystals, rather than to produce a fine dispersion of small crystals. This tendency is pronounced with treatment at the higher temperatures, and thus the best diffraction patterns, at least in the case of the loaded mitochondria, are obtained at 700°C. The relative weakness of the spots is partly due to relatively high scattering from the necessarily thick support film, but may also result from a poor degree of crystallinity in most of the particles.

Figs. 18–20 show the bright- and dark-field images and diffraction pattern of a 600°C-incinerated section of loaded mitochondria, subsequently heated to 700°C (experiment group G, Table I). The ash pattern consists solely of residues of dense granules, which although somewhat melted, are still recognizable. As shown by dark-field, most, if not all, of the residues are detectably crystalline. Although somewhat weak and grainy, the diffraction pattern shows four fairly easily recognized arcs at relatively low angles, corresponding to crystal spacings (d_{hkl}) of 5.15, 3.18, 2.86, and 2.57 A. All the arcs recognized in this pattern, as well as those in three other patterns of

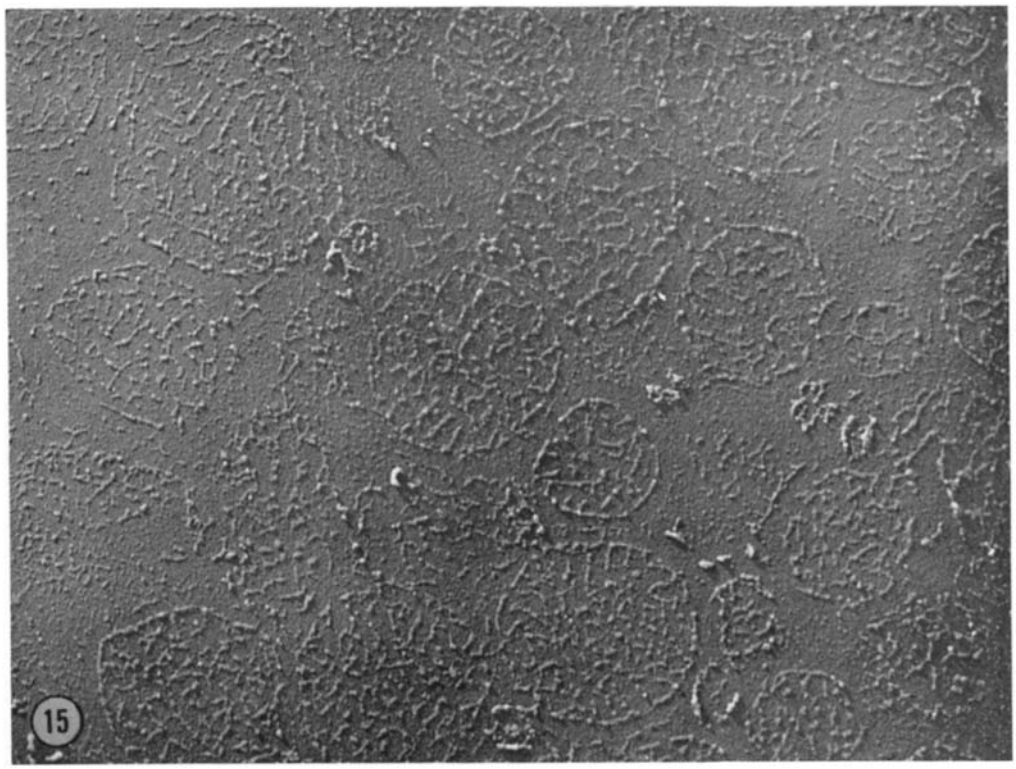


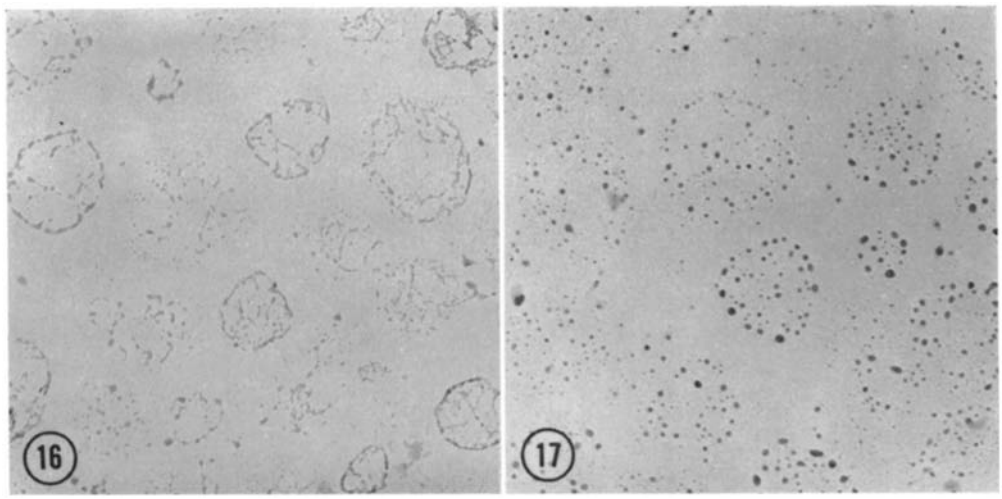
FIGURE 15 A section after complete LTA incineration (20 min at 10 ma). The mitochondrial cross-sections each leave a distinct ash pattern. The patterns are somewhat more sketchy than those of the incompletely incinerated preparations but, still, they suggest origin from the mitochondrial membranes. ×30,000.

similar preparations, are listed by d_{hkl} values in Table II. As may be seen, the crystal spacings and the roughly estimated relative intensities of the arcs agree fairly well with powder X-ray data for β-tricalcium phosphate otherwise known as whitlockite. Owing to the weak and spotty character of the patterns, the measurements and particularly the judgements of relative intensity were difficult and should be considered as subject to some error. Nevertheless, the identification is probably correct. There is no evidence for the presence of a major amount of any other crystalline substance, and in particular, the prominent, complex line of hydroxyapatite at 2.78 A is not seen. However, small amounts of crystalline impurities could escape detection.

The diffraction patterns obtained from the heated ash of loaded mitochondria incinerated by the LTA procedure (group E, Table I) were not of sufficient quality for measurement, but nevertheless the patterns appear similar to those of the high temperature ashed preparations (group G) recorded in Table II. It thus seems likely that residues of dense granules ashed by LTA are also thermally convertible to whitlockite.

PREPARATIONS OF SYNTHETIC CALCIUM PHOSPHATE–X-RAY DIFFRACTION, ELECTRON MICROSCOPY, AND ELECTRON DIFFRACTION: For comparison with patterns from mitochondrial dense-granule ash, it seemed desirable to obtain diffraction powder patterns from samples of genuine whitlockite. Whitlockite is of relatively rare occurrence in nature (Frondel, 1943) but can be produced in the laboratory by high temperature dehydration of calcium-deficient synthetic hydroxyapatite (Hodge et al., 1938). The calcium/phosphorus molar ratio of the preparation must be about 1.5 to allow complete conversion to the anhydrous mineral (Hodge et al., 1938; Trautz, 1955), and the conversion requires a temperature of at least 600°–800°C (Kunin, 1958).

Multimilligram samples of a calcium-deficient



Figs. 16 and 17, heated ash patterns of LTA-incinerated thick sections of formaldehyde- fixed, control mitochondria. (Examples from groups B and C, Table I.) ×17,000.

FIGURE 16 Ash heated 2 min at 700°C and slowly cooled. The ash pattern still resembles more or less, that of an unheated preparation, but the individual strands of ash have begun to melt and from into droplets. Except for an occasional small granule or strand, the ash is not crystalline.

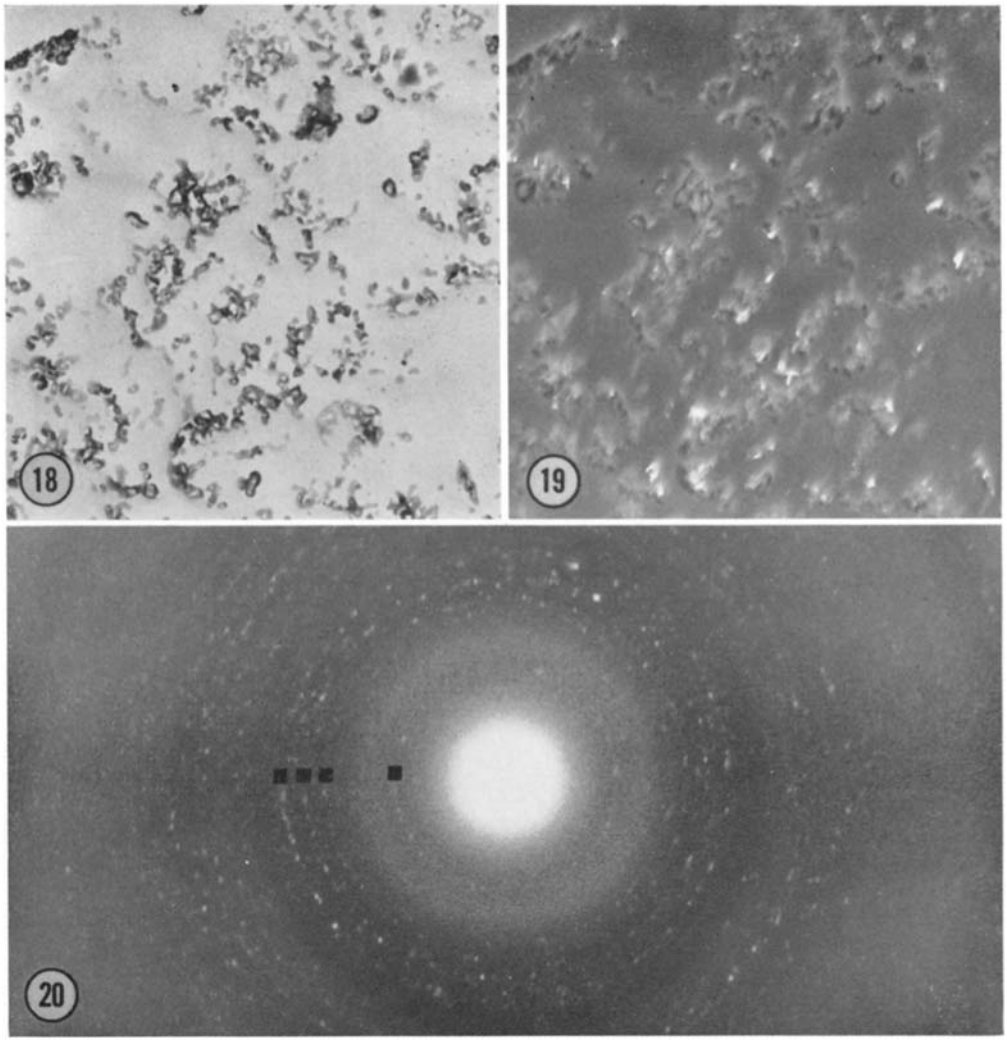
FIGURE 17 Adjacent area to that of Fig. 16, after grid was reheated for 2 min at 960°C and slowly cooled. The ash has now grossly melted and formed into distinct droplets. Many of the "droplets" are crystalline and show anomalous high opacity owing to diffraction contrast. (Compare the opacity of various droplets of similar diameter. The crystallinity of the more opaque droplets was confirmed by dark-field examination, not shown.)

hydroxyapatite (Baker Analyzed calcium phosphate, tribasic, powder, J. T. Baker Chemical Co., North Phillipsburg, N.J.) were heated for 1.5 hr to various temperatures in a platinum crucible in a muffle furnace, and then the X-ray powder pattern was determined. The calcium/phosphate molar ratio of the material was shown analytically to be 1.49 ± 0.015. Fig. 21 shows the powder pattern of the unheated material. It is a typical pattern of hydroxyapatite. Fig. 22 shows the pattern of the same material after heating to 1050°C. It is that of whitlockite (see Table II). A similar result was obtained on material heated to 825°C. If the sample was heated to only 720°, however, it did not dehydrate and the powder pattern remained that of hydroxyapatite.

For electron diffraction, various samples of the whitlockite, prepared as described above, were dispersed on electron microscope grids either by spreading in a collodion film or by embedding and sectioning. Unfortunately, all of these preparations were consistently unsuccessful; the crystals, even in thin section preparations, were much too large and unevenly distributed to be suitable for powder patterns. Evidently the microcrystals of hydroxyapatite had grossly fused during their recrystallization to whitlockite.

In the hope of overcoming this problem, samples of the original hydroxyapatite were dispersed by the collodion-spreading technique, mounted on silicon monoxide–filmed stainless steel grids, and heated to the same temperatures as before, with the aim of converting the hydroxyapatite to whitlockite *in situ* on the grid. The microcrystals were very well dispersed. Before heating, they showed the typical habit of hydroxyapatite (Fig. 23) and gave a typical and quite satisfactory powder pattern for hydroxyapatite (Fig. 24). After heating for 2.5 hr at 1050°C the crystals had a decidedly melted appearance (Fig. 25) but, curiously, their powder pattern, although grainy, remained that of hydroxyapatite (Fig. 26). Similar results were obtained at temperatures down to 700°C with heating times of a few minutes. Below 700°C, no apparent melting occurred, and the crystal morphology and diffraction pattern were completely identical with those of unheated specimens.

The *in situ* recrystallization experiments were



Figs. 18–20, ash of 600°C-incinerated thick section of formaldehyde-fixed, loaded mitochondria, subsequently heated 2 min at 700°C and slowly cooled. Inspection of the ash in the electron microscope before 700°C heating failed to detect any crystallinity.

FIGURE 18 Bright-field view of a selected area. The ashed granules look more melted than after their initial incineration, but they are still recognizable. Although they have no angular, obviously crystalline contours, they show marked variations in opacity owing to crystalline diffraction contrast. ×17,000.

FIGURE 19 Dark-field view of same area as Fig. 18. Many granules appear bright; this indicates that they are crystalline and contribute to that part of the diffraction pattern passed by the off-center aperture. Shifting the aperture to other parts of the diffraction pattern reveals that nearly all of the granules are crystalline. ×17,000.

FIGURE 20 Diffraction pattern from the selected area shown in Figs. 18 and 19. (The field-limiting aperture, $4 \times 5\ \mu$, included an area slightly greater than shown.) The diffraction spots form only a sketchy powder pattern; there are too few contributing crystals to completely fill out the rings. The four strongest inner rings, which are marked, correspond to crystal spacings of 5.15, 3.18, 2.86, and 2.57 A.

TABLE I

Effect of High Temperatures on Mitochondrial Ash Patterns

Experiment group	Initial ash pattern*	Treatment temperature	Appearance of ash	Crystallinity by dark-field exam	Quality of diffraction pattern
		°C			
A	Control mitochondria, LTA incinerated	600	unchanged	absent	—
B		700–800	melted	weak or absent	—
C		900–1000	grossly melted	present	a few weak spots, no arcs
D	Loaded mitochondria, LTA incinerated	600	unchanged	absent	—
E		700–800	melted	present	many weak spots but no clear arcs
F		900–1000	grossly melted	present	a few strong spots, no arcs
G	Loaded mitochondria, 500° or 600°C incinerated	700–800	slightly melted	present	many spots, mostly weak, *recognizable arcs*
H		900–1000	grossly melted	present	a few strong spots, no arcs

* All of the ash patterns were from formalin-fixed material. The sections before incineration were in most cases fairly thick (about ¼–½ μ), so as to contain a relatively large amount of mineral per unit area. None of the initial ash patterns were crystalline.

repeated with another sample of hydroxyapatite, a semicrystalline colloidal preparation (obtained from Dr. A. Posner) with a calcium/phosphate molar ratio of 1.51, and a crystallinity index of 30%. (The preparation represented an early stage in the in vitro formation of hydroxyapatite, and it was thought that this preparation might more closely mimic the successfully converted noncrystalline deposits in mitochondria. See Discussion.) Results were similar to those with the Baker preparation The colloidal particles fused in the temperature range, 700°–800°C, as before (See Figs. 27 and 28), but their amorphous-plus-hydroxyapatite powder pattern could not be converted to whitlockite even if the temperature was raised above 1000°C.

Why the conversion to whitlockite occurred on the macroscale but not in these microscale experiments remains a mystery. A possible explanation in connection with the mitochondrial results is discussed below (in Discussion).

It is noteworthy that in all of these preparations the temperature of apparent fusion of the crystals was far below their bulk melting point (1720°C for tricalcium phosphate, according to Bale et al., 1945) but in the same temperature range as for the mitochondrial dense granule ash.

Although the goal of these experiments, to obtain electron-diffraction powder patterns of genuine whitlockite, was not achieved, the experiments were valuable to illustrate possible pitfalls in applying macroscale data to events in the very small world of microincineration.

DISCUSSION

The Nature of the In Vitro-Produced Dense Granules

ORGANIC CONSTITUENT: The dense granules, when viewed with good resolution, are seen as aggregates of much finer dense grains. If the granules contain organic material serving as a matrix to hold the grains together this observation might be expected (Greenawalt et al., 1964). That this may be the case is supported by analytical results on isolated granules, showing a weight loss in incin-

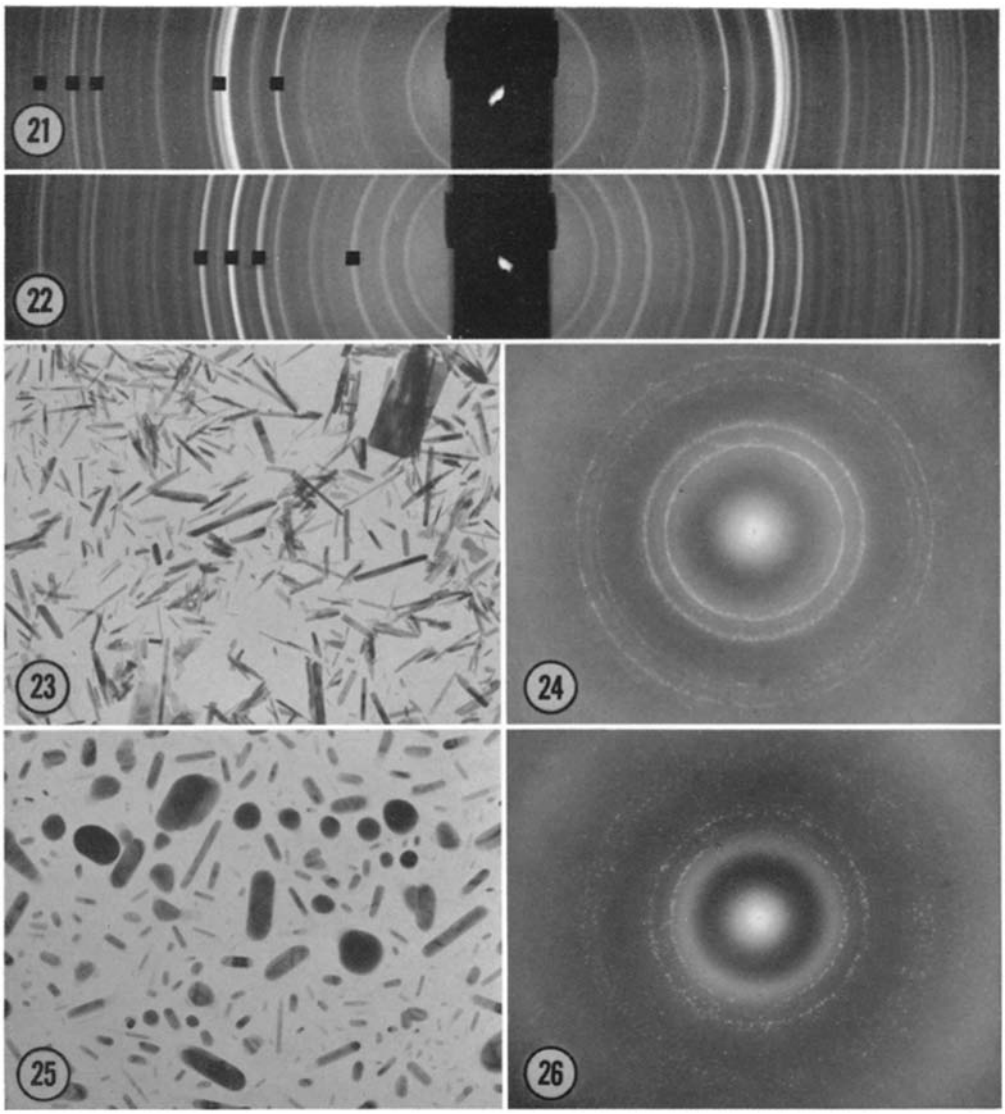
TABLE II

Crystal Spacings (d_{hkl}), and Relative Intensities from Electron-Diffraction Patterns of Dense Granule Ash Heated to 700° or 800°C*

Comparison with X-ray data from β-tricalcium phosphate (whitlockite).

Dense granule ash; electron diffraction						Whitlockite; X-ray diffraction					
Plate 8-28-64-19A (Fig. 20)		Plate 1-4-66-4A		Plate 1-5-66-8A		Plate 1-5-66-9A		Frondel (1943)		Plate C-102A (Fig. 22)	
d_{hkl}	Relative intensity	d_{hkl}	Relative intensity	d_{hkl}	Relative intensity	d_{hkl}	Relative intensity	d_{hkl}	Relative intensity	d_{hkl}	Relative intensity
A		*A*		*A*		*A*		*A*		*A*	
—	—	—	—	—	—	—	—	8.03	1	8.0	1
—	—	—	—	—	—	6.36	1	6.55	3	6.50	3
5.15	5	5.22	3	—	—	5.22	5	5.24	2	5.20	3
—	—	—	—	—	—	—	—	—	—	4.40	1
—	—	3.95	1	4.03	1	4.03	3	4.07	2	4.05	1
3.48	3	3.48	3	3.43	3	3.48	5	3.45	4	3.45	3
—	—	—	—	—	—	—	—	3.35	1	—	—
3.18	5	3.18	3	3.22	5	3.18	5	3.21	5	3.20	4
3.00	1	3.00	1	—	—	—	—	3.02	1	3.00	1
2.86	5	2.87	5	2.87	5	2.87	5	2.88	5	2.90	5
2.74	1	2.75	1	2.75	3	—	—	2.75	2	2.75	1
—	—	—	—	—	—	—	—	2.68	1	—	—
2.57	5	2.59	5	2.59	5	2.56	5	2.60	5	2.60	4
—	—	2.53	1	2.53	1	—	—	2.52	1	2.50	1
—	—	—	—	—	—	—	—	2.41	1	2.40	1
—	—	—	—	2.27	1	—	—	2.25	1	2.26	1
2.14	1	—	—	2.18	3	2.18	3	2.19	1	2.20	1
—	—	—	—	—	—	—	—	2.16	1	2.16	1
—	—	—	—	—	—	—	—	2.07	1	2.08	1
—	—	—	—	2.05	1	2.04	1	2.04	1	2.04	1
—	—	—	—	—	—	—	—	2.00	1	2.00	1
1.89	3	—	—	1.93	3	—	—	1.93	3	1.94	3
1.84	1	—	—	1.88	3	1.88	3	1.88	3	1.89	2
—	—	—	—	1.80	1	—	—	1.82	1	1.83	1
—	—	—	—	—	—	—	—	—	—	1.81	1
—	—	—	—	—	—	—	—	1.77	2	1.77	1
1.72	3	1.73	1	1.73	3	1.72	3	1.72	4	1.73	3
—	—	—	—	—	—	—	—	1.70	1	—	—
—	—	—	—	—	—	—	—	1.67	1	1.68	1
—	—	—	—	1.61	1	—	—	1.63	1	1.63	1
—	—	—	—	—	—	—	—	1.60	1	1.60	1
—	—	—	—	1.54	3	1.54	1	1.55	3	1.55	2
—	—	—	—	—	—	—	—	—	—	1.51	1
—	—	—	—	—	—	—	—	1.46	1	1.47	1
—	—	—	—	—	—	—	—	1.43	1	—	—
—	—	—	—	—	—	—	—	1.40	1	1.41	1
—	—	—	—	—	—	—	—	1.38	1	1.39	1

* Relative intensities are visual estimates on a scale of 1 to 5, 5 being the most intense. It was impossible to make five distinctions on the weak and discontinuous arcs of the electron diffraction patterns, so intensities are there given as 1, 3, or 5. Both the intensity and the number of spots in the electron-diffraction arcs were considered in assigning the intensity value. The relative intensities taken from the literature (Frondel, 1943) were converted by us to a scale of 1 to 5.



Figs. 21–26, effect of high temperature on a microcrystalline calcium-deficient hydroxyapatite (Baker Analyzed calcium phosphate, tribasic, powder, calcium phosphate molar ratio 1.49 ± 0.015).

FIGURE 21 X-ray powder pattern of unheated preparation. It is a typical pattern for hydroxyapatite. The strongest arcs which can also be easily recognized in electron-diffraction patterns (Fig. 24) are marked; they correspond to crystal spacings of 3.44, 2.78 (average of 3 spacings: 2.84, 2.79, and 2.72), 1.94, 1.84, and 1.72A.

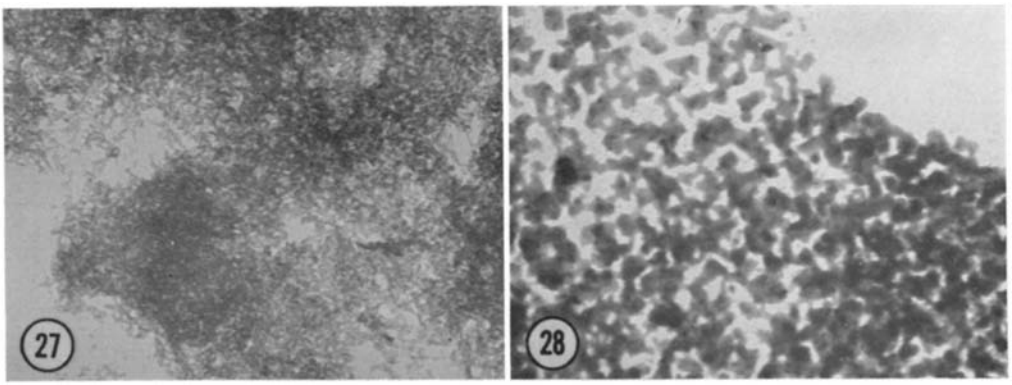
FIGURE 22 X-ray powder pattern of preparation after heating 1.5 hr at 1050°C. It is a typical pattern of whitlockite (see Table II). The strongest arcs which correspond to the four most easily recognized arcs in electron-diffraction patterns of crystallized dense granule ash (Fig. 20) are marked in the figure.

FIGURE 23 Electron micrograph of an unheated preparation dispersed on a silicon monoxide–collodion support film. Nonuniform opacity of some of the crystals is due to diffraction contrast. ×16,000.

FIGURE 24 Electron diffraction pattern of a 4 × 5 μ selected area including the field shown in Fig. 23. The five easily recognized rings correspond to the marked arcs in the X-ray powder pattern, Fig. 21.

FIGURE 25 Dispersed preparation similar to that of Fig. 23, but after heating for 2.5 hr at 1050°C. All of the particles have rounded contours, as though they were molten, but they are all quite crystalline. Dark striations across many of the crystals are due to diffraction contrast. ×16,000.

FIGURE 26 Electron diffraction pattern of a 4 × 5 μ selected area including the field shown in Fig. 25. The arcs of the pattern have become quite grainy owing to the increased size and decreased number of crystals contributing to them, but nevertheless the pattern is still recognizable as that of hydroxyapatite.



Figs. 27 and 28, electron micrographs showing the effect of high temperature on a dispersed preparation of semicrystalline, colloidal calcium phosphate. (The preparation was obtained from Dr. A. Posner. It was shown to have a calcium/phosphate molar ratio of 1.51, and it gave an X-ray powder pattern of hydroxyapatite. Detailed analysis of the pattern indicated that the preparation was about 30% crystalline.) ×39,000.

FIGURE 27 Preparation after heating for a few minutes at 600°C. The 600°C treatment did not alter the specimen, as could be shown by comparison with micrographs of the same grid before heating. The material consists of masses of tiny asymmetric particles with minimum dimension of about 60 A. (The preparation both before and after heating gave an excellent, continuous ring, electron-diffraction powder pattern of hydroxyapatite.)

FIGURE 28 Preparation, on same grid as for Fig. 27, after reheating for a few minutes at 800°C. The material now consists of a fused reticulum of highly irregular particles with rounded contours. The average minimum dimension of the particles is about 400 A. Occasional dense spots on the particles are apparently due to diffraction contrast. (The preparation still gave a continuous ring, electron-diffraction pattern of hydroxyapatite, but it was slightly grainy by comparison to that from the 600°C-heated material.)

erated granules of 16–60% depending on the preparation (Weinbach and von Brand, 1967). The present microincineration results are also consistent with this picture. The granules after initial rapid burning at 600°C are seen to have melted and formed bubbles during the escape of some volatilized component. The vaporized material could be partly a mineral constituent such as carbonate (carbonate content of isolated granules is 7–10%) or bound water. but probably most vaporized material is organic substance.

Granules which have been divested of their organic component at lower temperature, either by heating at 500°C or by low temperature plasma ashing, retain their inorganic fine structure in the ash with little or no evidence of melting and no bubbles. This structured ash shows no evidence of decomposition when it is subsequently heated to 600°C. The ash appears stable until nearly 700°C is reached, at which temperature it begins to melt without bubbles. Carbonate and bound water are generally retained after low temperature ashing (Gluskoter, 1965) but they are lost from the granules at 600°C (Weinbach and von Brand, 1967). Evidently their departure does not much affect the fine structure of the inorganic component of the granules.

It is noteworthy that the melting range of the intact granules, 500°–600°C, is appreciably lower than that of their mineral residues, 600°–700°C. This melting point lowering suggests an intimate association of organic and inorganic constituents in the granules.

INORGANIC CONSTITUENT: The tentative finding in the present study of a β-tricalcium phosphate (whitlockite) electron-diffraction pattern for the 700°C-heated ash of the dense granules agrees quite well with X-ray diffraction results obtained by Weinbach and von Brand (1967) on bulk preparations of isolated granules subjected to prolonged (18 hr) incineration at 600°C. In this latter study, four different preparations of isolated granules yielded X-ray powder patterns variously of hydroxyapatite alone, hydroxyapatite plus whitlockite, or whitlockite alone. However, the two preparations which were isolated under chemically mild

conditions, without the use of strong alkali, yielded patterns of only whitlockite. These latter preparations may have been impure in that they contained a large proportion of organic material, but this would only increase their similarity to our preparations of unisolated granules *in situ*. Any mineral associated with the organic impurity probably had little effect on the mineral analyses and X-ray diffraction results after 600°C-incineration since our results show that only the mineral of the dense granules remains after this high temperature processing.

A seeming anomaly in the identification of the ashed-granule mineral is the low melting temperature, 600–700°C, of the ash seen under the electron microscope both by Weinbach and von Brand (1967) and us. The bulk melting point of tricalcium phosphate is 1720°C (Bale et al., 1945). In defense of the identification, electron microscopic preparations of verified calcium phosphates showed similar behavior. Actually, the melting seen in microscale had a slight but detectable correlate on the macroscale; high temperature–treated calcium phosphate powders displayed a small change in texture which was evidently due to slight scintering. The apparent melting of electron microscopic specimens of "heat-stable" minerals is discussed elsewhere (Thomas, 1968).

The finding, in both Weinbach and von Brand's preparation and ours, of whitlockite in the crystallized ash, and also the low crystallization temperature, suggests that the original, noncrystalline, colloidal mineral of the dense granules may be a subcrystalline precursor of a *calcium-deficient* hydroxyapatite rather than the stoichiometric hydroxyapatite which had been previously suggested (Rossi and Lehninger, 1963 *a*, *b*). The calcium/phosphate molar ratio of the mineral would theoretically be about 1.50 rather than the 1.67 for stoichiometric hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. As has long been known, hydroxyapatite with the stoichiometric ratio, 1.67, maintains its stable, hydrated structure at temperatures up to 1450°C (Bale et al., 1945), but calcium-deficient hydroxyapatites dehydrate and recrystallize at 600°–800°C (Hodge et al., 1938; Kunin, 1958). If the calcium/phosphate molar ratio is between 1.67 and 1.50, a powder pattern showing both hydroxyapatite and whitlockite (in relative amount proportional to ratio) is obtained after heating. If the ratio lies between 1.50 and 1.33 (the minimum ratio for hydroxyapatites) then a pattern of varying amounts of whitlockite and pyrophosphate (β-$Ca_2P_2O_7$) is obtained. If, however, the ratio is quite close to 1.50, only whitlockite is found in the powder pattern after heating (Hodge et al., 1938; Trautz, 1955). These dehydration results, although obtained on preparations which were initially well crystallized, are probably applicable to subcrystalline or noncrystalline preparations as well. The important factor seems to be simply the calcium/phosphate molar ratio of the preparation (Trautz et al., 1954).

So that the interpretation would be reliable for a noncrystalline preparation, it would be necessary that the entire preparation crystallize; otherwise the deduced calcium/phosphate ratio might apply to only a biased fraction of the material. Whether Weinbach and von Brand's mitochondrial dense granule preparation and ours crystallized entirely is not known.

In the special case of our microincineration experiments, confidence in the interpretation requires an explanation of why electron microscopic specimens of calcium-deficient, synthetic hydroxyapatite *did not* dehydrate to whitlockite, even though the dense granule ash seemingly did. Slight moisture uptake from the atmosphere in these specimens with relatively enormous surface area, and a role of minor mineral components in the dense granule ash might provide an answer here. Even at 1050°C, calcium phosphates can react with water vapor (Van Wazer, 1958). Such a reaction might have prevented the dehydration of the pure, synthetic hydroxyapatite. In the case of the dense granule ash, 2–4% magnesium (shown to be present by Weinbach and von Brand, 1967) may have counteracted the effect of moisture uptake and facilitated the dehydration to whitlockite. Unlike pure calcium whitlockite, calcium-magnesium whitlockite can be formed in the presence of water (Trautz et al., 1954).

The actual molar ratio, calcium (plus magnesium)/phosphate, shown by Weinbach and von Brand for their dense granule preparations crystallized to whitlockite is 1.51 in one case, in excellent agreement with theory, but in the second case it is 1.31. Assuming this is not analytical error, it would represent anomalous behavior in crystallization. The molar calcium/phosphate ratio for our own preparation is unfortunately unknown. In other experiments done in the same way as ours (Rossi and Lehninger, 1963 *a*, *b*) the molar ratios for uptake of calcium and phosphorus ranged from about 1.52–1.88 with an average of 1.67. These values pertain to whole mitochondria, however,

and so whether they accurately reflect the ratios in the dense granules, alone, is not certain.

Quite aside from the crystallographic and analytical results but considering that the original mineral of the dense granules is colloidal calcium phosphate, it would be very reasonable to find that this mineral has a calcium/phosphate molar ratio of about 1.5. As shown by Watson and Robinson (1953) and more recently by Eanes et al. (1965), hydroxyapatite prepared in the test tube passes through a sequence of stages during its formation. First, a colloidal, noncrystalline (or subcrystalline) precipitate appears. This converts to minute, poorly crystalline particles or thin sheets, which, however, continue to grow and mature and finally form fully developed microcrystals; these give the sharp powder pattern of hydroxyapatite. The calcium/phosphate molar ratio of the initial, subcrystalline deposits is found to be about 1.5 (actually, 1.46 or 1.52 depending, on the method of preparation). Only as the preparation becomes detectably crystalline and the crystals mature does the molar ratio rise to the final figure, 1.67, the stoichiometric value for $Ca_{10}(PO_4)_6(OH)_2$ (Eanes et al.,1965).

Recently, Termine and Posner (1966) have produced evidence and have cited earlier observations by others to show that also in biological systems, such as bone, the formation of hydroxyapatite proceeds via initial deposition of a noncrystalline (or subcrystalline) colloid. The colloidal, noncrystalline mineral in the mitochondrial dense granules studied here might similarly represent this first stage of hydroxyapatite formation and one might expect the material to have a calcium/phosphate molar ratio of about 1.5.

The success of the present experiments in tentatively identifying the chemical nature of mineral deposits *in situ* in thin sections of isolated mitochondria suggests that the microincineration–electron-diffraction technique might be usefully applied to similar noncrystalline, mitochondrial mineral deposits (Gonzales and Karnovsky, 1961; Caulfield and Schrag, 1964; Heggtveit et al., 1964; Reynolds, 1965; Cameron et al., 1967) and to other mineral bodies seen in thin-sectioned whole cells and tissues. In such whole cell material it is usually impractical to attempt isolation of the mineral deposits, and thus an *in situ* technique offers a unique possibility for identifying the mineral. Some of the problems of the microincineration–electron-diffraction technique are discussed by Thomas (1968).

Ash Patterns from Control Mitochondria

"MEMBRANE" ASH: In contrast to the calcium phosphate–loaded mitochondria, which showed only traces of mineral other than from the dense granules, unincubated control mitochondria showed an appreciable amount of intrinsic mineral in low temperature ash patterns. The difference between these two preparations in this regard can not be explained with certainty. It could be, however, that the loaded mitochondria lose intrinsic mineral during incubation at the same time that they are accumulating vast amounts of calcium and phosphate in the granules. This could result from deterioration in the ultrastructure of the mitochondria. In addition, loss of ultrastructural integrity during incubation could predispose the organelles to loss of minerals from specific sites during subsequent fixation and embedding. It is known that, under the incubation conditions used here, oxidative phosphorylation in these mitochondria is uncoupled and appreciable deterioration of structural intactness occurs.

The low temperature ash patterns of the control mitochondria suggest that the origin of the ash is from the membranes of the organelle. This suggestion is strengthened by examination of partially ashed preparations, but unfortunately the poor definition in both types of preparations prevents a completely certain interpretation of this point. The membranous structure of the mitochondrion undoubtedly influences in some way the deposition of the ash to produce the patterns seen, but whether the ash actually derives from native mineral in the membranes or from mineral originally at adjacent sites cannot be answered at this time. It seems likely that soluble, unbound minerals localized within the matrix would be largely depleted during fixation and dehydration. Membrane-bound minerals, on the other hand, could be at least partially retained even though the formaldehyde-fixed membranes might well be stripped of most of their lipids during acetone dehydration (Ashworth et al., 1966). Rather uniquely, the ultrastructural appearance and macromolecular configuration of mitochondrial membranes appears to depend little, if at all, upon the presence of the lipid components (Fleischer et al., 1967).

It is noteworthy that ash patterns of osmium-fixed mitochondria looked very similar to those of formaldehyde-fixed preparations. Presumably, membrane lipid was largely retained with osmium (Ashworth et al., 1966). What effect osmium might

have on retention of cations bound to phospholipid is, of course, an unanswered question. The partially reduced osmium, itself, might well be lost by reoxidation to osmium tetroxide and volatilization during the low temperature ashing (Gleit, 1967).

Further work is in progress to determine the origin of the membrane ash patterns, and their configurations in other specimens, such as thin-sectioned whole cells. Better retention of minerals in the specimens presented for microincineration may improve not only the significance, but also the definition of the ash patterns (Thomas, 1968).

The chemical nature of the membrane ash remains unknown, but it is noteworthy that after initial formation by low temperature ashing, it proves to be stable at 600°C and it is not hygroscopic. This suggests that it is either a metallic oxide or a metallic phosphate. Carbonates, chlorides, sulfates, and probably other common salts could be retained by low temperature ashing (Gluskoter, 1965) but these anions would most likely be volatilized at 600°C. Loss of the mitochondrial mineral by initial high temperature ashing at 600°C is probably due to organic material formed into volatile complexes with the mineral. Alternatively, the initial state of the mineral itself, before low temperature oxidation, might be volatile at high temperature (Gleit, 1967). The apparent melting or scintering of the ash seen at 700°C is not inconsistent with a metallic phosphate (see previous section of Discussion) or even with a metallic oxide (see references in Thomas, 1968). The ash could, of course, be a mixture of several compounds, and this is actually suggested by its relatively great reluctance to crystallize at high temperature.

NORMAL DENSE GRANULES: The small, dense, matrix granules commonly seen in normal mitochondria within cells were not found in isolated mitochondria following active uptake of calcium and phosphate (Greenawalt et al., 1964) perhaps because of the swollen condition of the incubated mitochondria (Wlodawer et al., 1966). The small granules were found in osmium-fixed, unincubated control preparations, however, and this provided an opportunity to compare their behavior during ashing with that of the in vitro-produced granules. This comparison is of some interest since it has been suggested that the function of the normal granules in vivo is to sequester osmotically active, metallic cations (Weiss, 1955; Peachey, 1964) in a manner analogous to that of the larger, in vitro granules.

The high temperature microincineration experiments showed unequivocally that the normal granules are completely volatilized at 600°C, a finding which is in sharp contrast to that for in vitro-formed dense granules. This perhaps indicates that the normal granules contain no mineral substances. On the other hand, the membrane mineral, demonstrated by low temperature ashing, is also completely volatilized from the same preparations during the high temperature ashing; the normal granules thus might contain mineral which was similarly volatilized. Low temperature ash patterns of the preparations do not display any particles clearly recognizable as residues of the normal granules, but the latter are so small that their residues might be overlooked among the ubiquitous small grains of the ash patterns. In conclusion, the present experiments do not rule out the possibility that the normal granules contain some mineral, but they do show that the granules lack the stability against high temperature incineration displayed by the in vitro-formed calcium phosphate granules. The normal granules behave during ashing like the membranes or other mitochondrial substances.

The failure to obtain any clear evidence of mineral in the normal granules is perhaps not surprising. The granules are seen only in osmium-fixed or aldehyde-osmium–fixed preparations and not at all in material fixed by aldehyde alone. Without additional heavy metal staining, the osmium-stained granules do not have an electron opacity which even approaches that of small in vitro-produced calcium phosphate granules. Ashworth et al. (1966) have shown that the aldehyde-fixed normal granules cannot be demonstrated even by osmium fixation and staining if this follows dehydration with nonpolar solvents and subsequent rehydration. This behavior is similar to that of membranes and other lipid-containing structures. Those workers conclude that the granules are lipid; the lipid is evidently not fixed by aldehyde but is extracted during dehydration of aldehyde-fixed specimens. André and Marinozzi (1965) find that the granules retained by osmium fixation disappear when the reduced osmium is removed by hydrogen-peroxide oxidation. They also conclude that the granules have no intrinsic density and are probably lipid bodies. Evidence that the normal granules are lipid bodies does not,

of course, prove that they do not serve as sites for native mineral deposition in vivo. However, failure to find any of this native mineral still attached in the specimens presented for examinations does tend to downgrade the special status of the granules with regard to mineral content and place them in the same category as other lipid-containing bodies in the cell which can be stained with heavy metals.

The present experience in trying to determine the nature of the normal dense granules in osmium-fixed mitochondria suggests that microincineration may be generally useful in discriminating between heavily OsO_4-fixed structures and mineralized structures seen in osmium-fixed thin sections. It may prove generally so, as here, that osmium is completely volatilized from high temperature ash patterns, and even from low temperature ashed specimens (Gleit, 1967; Thomas, 1968). Thus electron-opaque structures leaving an ash could be unambiguously categorized as mineral. Osmium removal by microincineration would offer at least one distinct advantage over other methods such as hydrogen-peroxide treatment (André and Marinozzi, 1965). The microincineration reagent is gaseous, rather than aqueous, and thus the possibility of leaching out native mineral along with the osmium is eliminated.

Other possible applications of the microincineration technique are discussed in Thomas (1968).

The authors thank Drs. C. Rossi and F. D. Vasington (formerly of Johns Hopkins University School of Medicine) for some of the mitochondrial preparations used in these experiments. Model preparations of colloidal calcium phosphate were gratefully received from Dr. A. Posner (Cornell University Medical College). The authors are indebted to Mr. A. Potter and Miss R. Young (Western Regional Research Laboratory) for chemical and X-ray analyses of calcium phosphates.

Reference to a company or product name does not imply approval or recommendation of the product by the United States Department of Agriculture to the exclusion of others that may be suitable.

This investigation was partially supported by research grant No. GM-12125 from the National Institutes of Health, United States Public Health Service.

Received for publication 18 March 1968, and in revised form 10 June 1968.

REFERENCES

André, J., and V. Marinozzi. 1965. Presence dans les mitochondries, de particules ressemblant aux ribosomes. *J. Microscopie.* **4**:615.

Ashworth, C. T., J. S. Leonard, E. H. Eigenbrodt, and F. J. Wrightsman. 1966. Hepatic intracellular osmiophilic droplets. Effect of lipid solvents during tissue preparation. *J. Cell Biol.* **31**:301.

Bale, W. F., J. F. Bonner, H. C. Hodge, H. Adler, A. R. Wreath, and R. Bell. 1945. Optical and X-ray diffraction studies of certain calcium phosphates. *Ind. Eng. Chem. (Anal. Edition).* **17**:491.

Bigelow, W. C. 1960. Electron diffraction. *In* Physical Methods in Chemical Analysis. W. G. Berl, editor. Academic Press Inc., New York. 2nd edition. 1:620–660.

Boothroyd, B. 1964. The problem of demineralization in thin sections of fully calcified bone. *J. Cell Biol.* **20**:165.

Bradley, D. E. 1961. Techniques for mounting, dispersing and disintegrating specimens. *In* Techniques for Electron Microscopy. D. Kay, editor. Blackwell Scientific Publications, Oxford. 63–81.

Brierley, G. P., and D. B. Slautterback. 1964. Studies on ion transport IV. An electron microscope study of the accumulation of Ca^{2+} and inorganic phosphate by heart mitochondria. *Biochim. Biophys. Acta.* **82**:183.

Bruni, C., and K. R. Porter. 1965. The fine structure of the parenchymal cell of the normal rat liver. *Am. J. Pathol.* **46**:691.

Cameron, D. A., H. A. Paschall, and R. A. Robinson. 1967. Changes in the fine structure of bone cells after the administration of parathyroid extract. *J. Cell Biol.* **33**:1.

Caulfield, J. B., and P. E. Schrag. 1964. Electron microscopic study of renal calcification. *Am. J. Pathol.* **44**:365.

Eanes, E. D., I. H. Gillessin, and A. S. Posner. 1965. Intermediate states in the precipitation of hydroxyapatite. *Nature.* **208**:365.

Fleischer, S., B. Fleischer, and W. Stoeckenius. 1967. Fine structure of lipid-depleted mitochondria. *J. Cell Biol.* **32**:193.

Frondel, C. 1943. Minerology of the calcium phosphates in insular phosphate rock. *Am. Mineralogist.* **28**:215.

Gleit, C. E. 1963. Electronic apparatus for ashing biologic specimens. *Am. J. Med. Electron.* **2**:112.

Gleit, C. E. 1967. The reaction of oxygen with carbonaceous compounds in the electrodeless ring discharge. *Preprints Div. of Fuel Chem.* **11**:211.

Gluskoter, H. J. 1965. Electronic low-temperature ashing of bituminous coal. *Fuel.* **44**:285.

Gonzales, F. 1962. A masking technique for contrast

control in electron micrographs. *J. Cell Biol.* **15:** 146.

GONZALES, F., and M. J. KARNOVSKY. 1961. Electron microscopy of osteoclasts in healing fractures of rat bone. *J. Biophys. Biochem. Cytol.* **9:**299.

GREENAWALT, J. W., and E. CARAFOLI. 1966. Electron microscope studies on the active accumulation of Sr^{++} by rat-liver mitochondria. *J. Cell Biol.* **29:** 37.

GREENAWALT, J. W., C. S. ROSSI, and A. L. LEHNINGER. 1964. Effect of active accumulation of calcium and phosphate ions on the structure of rat liver mitochondria. *J. Cell Biol.* **23:**21.

HEGGTVEIT, H. A., L. HERMAN, and R. K. MISHRA. 1964. Cardiac necrosis and calcification in experimental magnesium deficiency. *Am. J. Pathol.* **45:** 757.

HODGE, H. C., M. L. LEFEVRE, and W. F. BALE. 1938. Chemical and X-ray diffraction studies of calcium phosphates. *Ind. Eng. Chem. (Anal. Edition).* **10:**156.

KRUSZYNSKI, J. 1966. The microincineration technique and its results. *In* Handbuch der Histochemie. W. Graumann and K. Neumann, editors. Gustav Fischer Verlag, Stuttgart. 1 (Pt. 2):96–187.

KUNIN, R. 1958. *Reported by* Neuman, W. F., and M. W. Neuman, 1958. The Chemical Dynamics of Bone Mineral. University of Chicago Press, Chicago, 43–44.

LEHNINGER, A. L. 1966. Dynamics and mechanisms of active ion transport across the mitochondrial membrane. *Ann. N. Y. Acad. Sci.* **137:**700.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:**409.

PEACHEY, L. D. 1964. Electron microscopic observations on the accumulation of divalent cations in intramitochondrial granules. *J. Cell Biol.* **20:**95.

PULLMAN, M. E., and G. SCHATZ. 1967. Mitochondrial oxidations and energy coupling. *Ann. Rev. Biochem.* **36**(Pt. 2):539.

REYNOLDS, E. S. 1965. Liver Parenchymal cell injury. III. The nature of calcium-associated electron-opaque masses in rat liver mitochondria following poisoning with carbon tetrachloride. *J. Cell Biol.* **25:**53.

ROSSI, C. S., and A. L. LEHNINGER. 1963. Stoichiometric relationships between mitochondrial ion accumulation and oxidative phosphorylation. *Biochem. Biophys. Res. Commun.* **11:**441.

ROSSI, C. S., and A. L. LEHNINGER. 1963. Stoichiometric relationships between accumulation of ions by mitochondria and the energy-coupling sites in the respiratory chain. *Biochem. Z.* **338:**698.

SABATINI, D. D., K. G. BENSCH, and R. J. BARNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17:** 19.

SCHNEIDER, W. C. 1957. *In* Manometric Techniques. W. W. Umbreit, R. Burris, and J. E. Stauffer, editors. Burgess Publishing Co., Minneapolis. 188–201.

TERMINE, J. D., and A. S. POSNER. 1966. Infrared analysis of rat bone: age dependency of amorphous and crystalline mineral fractions. *Science.* **153:**1523.

THOMAS, R. S. 1964. Ultrastructural localization of mineral matter in bacterial spores by microincineration. *J. Cell Biol.* **23:**113.

THOMAS, R. S. 1968. Microincineration techniques for electron-microscopic localization of biological minerals. *In* Advances in Optical and Electron Microscopy. R. Barer and V. E. Cosslett, editors. Academic Press Inc., New York. **3:** In press.

THOMAS, R. S., and J. W. GREENAWALT. 1964. Microincineration of calcium phosphate-loaded mitochondria. *J. Appl. Phys.* **35:**3083.

TRAUTZ, O. R. 1955. X-ray diffraction of biological and synthetic apatites. *Ann. N. Y. Acad. Sci.* **60:**696.

TRAUTZ, O. R., E. FESSENDEN, and M. G. NEWTON. 1954. Magnesium whitlockite in ashed dental tissue—an identification by X-ray diffraction. *J. Dental Res.* **33:**687.

VAN WAZER, J. R. 1958. Phosphorus and Its Compounds. Interscience Publishers Inc., New York. 1:525–527.

VASINGTON, F. D., and J. V. MURPHY. 1962. Ca^{++} uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. *J. Biol. Chem.* **237:**2670.

WATSON, M. L., and R. A. ROBINSON. 1953. Collagen-crystal relationships in bone. II. Electron microscope study of basic calcium phosphate crystals. *Am. J. Anat.* **93:**25.

WEINBACH, E. C., and T. VON BRAND. 1965. The isolation and composition of dense granules from Ca^{++}-loaded mitochondria. *Biochem. Biophys. Research Commun.* **19:**133.

WEINBACH, E. C., and T. VON BRAND. 1967. Formation, isolation and composition of dense granules from mitochondria. *Biochim. Biophys. Acta.* **148:** 256.

WEISS, J. M. 1955. Mitochondrial changes induced by potassium and sodium in the duodenal absorptive cell as studied with the electron microscope. *J. Exptl. Med.* **102:**783.

WLODAWER, P., D. F. PARSONS, G. R. WILLIAMS, and L. WOJTCZAK. 1966. Morphological changes in isolated rat-liver mitochondria during swelling and contraction. *Biochim. Biophys. Acta.* **128:**34.