ELECTRON MICROSCOPIC OBSERVATIONS ON THE ACCUMULATION OF DIVALENT CATIONS IN INTRAMITOCHONDRIAL GRANULES

LEE D. PEACHEY, Ph.D.

From the Department of Zoology, Columbia University, New York

ABSTRACT

Electron microscopic evidence is presented, from mitochondria in whole cells of toad urinary bladder and from isolated rat kidney mitochondria, indicating that the divalent cations calcium, strontium, and barium are accumulated in granules localized in the mitochondrial matrix. This accumulation occurs under conditions in which divalent ions are present in the medium bathing either whole cells or isolated mitochondria. The evidence indicates that the divalent ions are deposited on, or in a pre-existing granule, possibly in exchange for other ions. It suggests a possible role of the intramitochondrial granules in the regulation of the internal ionic environment of the mitochondrion. Certain biochemical and physiological implications of this phenomenon are discussed.

INTRODUCTION

Calcium and magnesium have long been considered important in the maintenance of normal cell structure and function. Obviously, an important part of understanding the role of these divalent cations in cellular processes is knowledge of their intracellular distribution. Studies of metal distribution in isolated cell fractions have generally implicated mitochondria as one site of divalent ion accumulation. Mitochondria, when isolated, are known to contain relatively large amounts of calcium and magnesium, and to be able to accumulate these ions, strontium, and manganese *in vitro* (Cleland, 1953; Slater and Cleland, 1953; Bartley and Amoore, 1958; De Luca and Engstrom, 1961; Mraz, 1962; Brierley *et al.*, 1962; Vasington and Murphy, 1962; Chappell *et al.*, 1962; Lehninger *et al.*, 1963; Saris, 1963). Manganese administered intravenously to rats has been found to reach a high level in the mitochondrial fraction of liver (Maynard and Cotzias, 1955), and the unusually high calcium content of livers of rats poisoned with carbon tetrachloride has been shown by Thiers *et al.* (1960) to be due to a twentyfold increase in mitochondrial calcium.

Histochemical studies have also suggested a localization of calcium in mitochondria. Ash techniques (Scott, 1932; Carey and Zeit, 1939) have shown that calcium is localized in certain striated muscles in A and Z bands: this is the same as the distribution of mitochondria in these muscles (Bullard, 1912). Both ash and staining techniques were used by Reynolds (1960) to localize calcium, in liver cells of carbon tetrachloride–poisoned rats, to dense, granular masses that are, presumably, mitochondria.

Thus it is clear that mitochondria have the ability to accumulate divalent cations under a variety of circumstances. Questions naturally arise as to the functional significance of this mitochondrial potentiality, and to the form in which the metals are retained. Brierly *et al.* (1962) consider that magnesium could not concentrate in isolated beef heart mitochondria, to the extent observed, without the formation of an insoluble precipitate within the mitochondria. Lehninger

et al. (1963) reported that the ratio of calcium to inorganic phosphate taken up by isolated rat liver mitochondria is similar to that of hydroxyapatite and that the solubility product of hydroxyapatite is far exceeded under conditions of maximum calcium uptake, implying that hydroxyapatite precipitates within the mitochondria. Those workers have observed dense masses as large as 500 to 1000 A in electron micrographs of their preparations, thus supporting the idea of metal retention in the form of a precipitate.

An initial observation of dense intramitochondrial granules in electron micrographs of intact cells incubated in the presence of strontium and barium has led to an electron microscopic study of the relative densities of these granules after incubation of whole cells and isolated mitocondria in the presence of various concentrations of magnesium, calcium, strontium, and barium. The results of this study suggest that the granules normally observed within the mitochondrial matrix have the ability to accumulate divalent cations in relatively large amounts. A preliminary report of this work has already appeared (Peachey, 1962).

METHODS

Whole Cells

The dense intramitochondrial granules were first found in epithelial cells of urinary bladders removed from toads, *Bufo marinus*, and incubated in a physiological system for measuring permeability (Bentley, 1958). In this system, the serosal surface of the bladder is bathed in Ringer's solution and the mucosal surface in contact with a diluted (20 per cent) solution of the same.[1] The Ringer's solution was composed of: NaCl, 110 mM; KCl, 2.0 mM; NaH_2PO_4, 0.2 mM; Na_2HPO_4, 1.0 mM; and $NaHCO_3$, 2.0 mM (pH 7.50 $\pm$ 0.05); usually also added were the acetates of magnesium, calcium, strontium, or barium, to a final concentration of 2.0 to 20 mM. The incubation lasted for 5 to 7 hours, during which both the inside and outside solutions were replaced with fresh solutions every 22 minutes. In some cases, antidiuretic hormone was present in the serosal solution during the latter part of the incubation. After the incubation, the bladders were fixed for 20 minutes in cold (0–4°C) 1 per cent osmium tetroxide in Ringer's solution containing 2 mM calcium chloride, dehydrated, and embedded in methacrylate.

Isolated Mitochondria

Mitochondria were isolated from rat kidneys by a method similar to that of De Luca *et al.* (1960). Kidneys were removed from ether-anesthetized animals, chilled in 0.44 M sucrose buffered to pH 7.4 with Michaelis's M/7 acetate-veronal buffer, and homogenized in 9 times their weight of buffere 0.44 M sucrose using a Teflon pestle in a Potter-Elvehjem homogenizer. In some experiments, 5×10^{-3} M ethylenediaminetetraacetic acid (EDTA) was present in the homogenizing medium. Six ml of this homogenate were layered over 5 ml of 0.52 M sucrose and centrifuged at 750 g[2] for 12 minutes to sediment nuclei and cell debris. The supernatant was recentrifuged at 3100 g for 12 minutes to sediment the mitochondria. When EDTA had been present in the homogenate, it was washed out at this point by twice resuspending the mitochondria in 0.44 M buffered sucrose and centrifuging at 3100 g for 12 minutes. These washed mitochondria, or the ones sedimented without EDTA, were resuspended in 2 to 3 ml of 0.44 M buffered sucrose.

The incubation mixture contained:

0.5 ml mitochondrial suspension prepared as above.
6 μmoles ATP (di-sodium).
45 μmoles sodium succinate.
10 μmoles magnesium acetate.
2.7 ml 0.44 M acetate-veronal-buffered sucrose.

This medium was derived from the one used by DeLuca and Engstrom (1961), but certain modifications were made. First, the components were omitted that those authors reported to be non-essential for calcium uptake. These included KCl, which Weiss (1955) has shown to accumulate in mitochondria of absorbing intestinal cells and which might be expected to interfere with the identification of divalent cations. Second, the phosphate buffer of the DeLuca and Engstrom system was replaced by the acetate-veronal buffer to avoid the possibile formation of precipitates of calcium phosphates either inside the mitochondria or in the medium. Various amounts of divalent cations (as acetates) were added to this mixture, which was incubated in air at 30°C for 20 to 30 minutes and quickly chilled to 0°C.

After chilling, the mixture was centrifuged in a high centrifugal field (10,000 g) for 12 minutes to assure sedimentation of even swollen mitochondria and fixed by resuspending in 2 per cent OsO_4 in 0.44 M sucrose. The fixed mitochondria were dehydrated

[1] This system was used in a series of experiments to be reported elsewhere (Peachey and Rasmussen, in preparation). Inclusion of the details is considered not important for the results presented here.

[2] All centrifugal forces refer to the bottom of the tube. A Sorvall type SS-4 centrifuge with a type HB-4 swinging bucket rotor was used in a cold room at about 4°C.

in a series of ethanol/water mixtures, infiltrated with methacrylate or Epon, and embedded in gelatin capsules in an oven at 60°C. The mitochondria were resuspended in each solution and then centrifuged, in a table model clinical centrifuge at speeds sufficient to sediment all visible material, before the solution was poured off and replaced by the next in the series. For samples embedded in Epon, it was necessary to centrifuge the capsules containing the mitochondria in the final embedding mixture to assure the formation of a pellet before the mixture became too viscous. This was done by spinning the capsules in centrifuge tubes partially filled with sand.

Electron Microscopy

Sections were cut on a Cambridge-Huxley microtome with glass knives, mounted on carbon substrates, and examined in an RCA EMU-3F fitted with a 25 μ objective aperture in the lower focal plane of the objective lens.

In most cases, the sections were not stained in order to avoid obscuring changes in density due to the accumulation of the divalent ions. Where staining was used, details are given in the figure legend.

RESULTS

As will be reported elsewhere (Peachey and Rasmussen, in preparation), incubation of toad urinary bladders in solutions in which calcium has been replaced by magnesium, barium, or, to a lesser extent, strontium, reduces cellular adhesion so that the integrity of the epithelium is disrupted. Many cells are thus loosened from the *lamina propria* and are found free in the bladder lumen. Nevertheless, cytological detail is usually well preserved in these detached cells and this loosening effect is not considered to be relevant to the present discussion.

The fine structure of the epithelial cells of the toad's urinary bladder has already been described (Peachey and Rasmussen, 1961).

Whole Cells

Strontium and Barium

Both the epithelial and smooth muscle cells of urinary bladders incubated with 2 mM strontium or barium acetate in the Ringer's solution had unusually dense granules in their mitochondria (Figs. 1 to 5). Mitochondria in these preparations otherwise had a sufficiently normal fine structure to be easily identifiable. The dense granules were found in the mitochondrial matrix, between the *cristae*, the same location of the "usual" intramitochondrial granules (see Fig. 4). In many of the granules the dense material formed a shell around a less dense core (Figs. 2 to 5). At high magnification (Fig. 5) the granules were seen to consist of finer granules about 40 A in diameter.

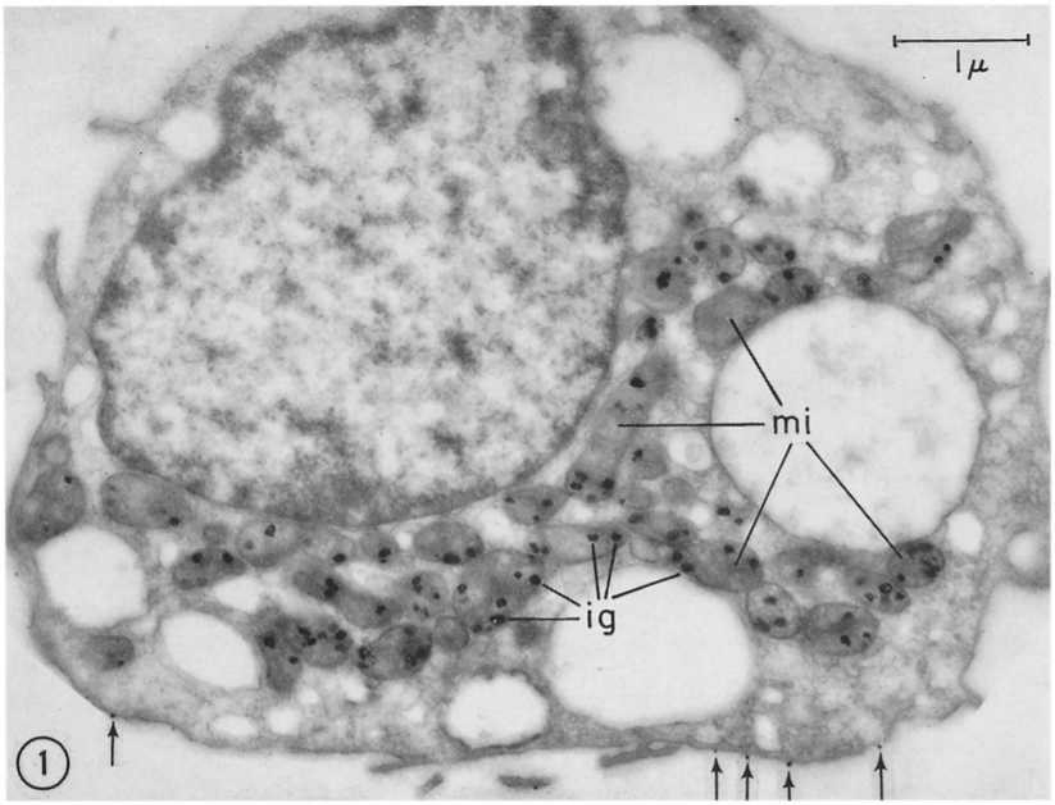
The dense intramitochondrial granules in cells incubated with strontium or barium were usually between 200 and 800 A in diameter. They tended to be larger and more numerous in epithelial cells following treatment with antidiuretic hormone, but the details of hormone response in in the absence of calcium and in the presence of these heavier divalent cations are not sufficiently clear at the present time to permit a discussion of the hormone effect. It is clear, however, that the effect of hormone on the intramitochondrial granules is a quantitative one and that the hormone is not required for the appearance of dense granules.

A rough estimate of the number of granules per mitochondrion can be made from the electron micrographs, such as those shown in Figs. 1 to 3. Knowing the section thickness, and approximating the mitochondria as cylinders 1 μ long and 0.3 μ in diameter, one obtains an estimate of 15 granules per mitochondrion.

Calcium and Magnesium

An attempt was made to extend these observations to the lighter, and thus less visible, elements, calcium and magnesium. Cells of bladders incubated in 2 mM calcium acetate (Fig. 6) had intramitochondrial granules whose density was judged to be not different from that of similar granules in cells of bladders fixed without incubation. However, when the calcium level was raised to 20 mM and the bladders were incubated, both the granules of the "usual" density and many considerably denser granules were found (Fig. 7). Again, some of the dense granules had less dense cores. These granules were 150 to 300 A in diameter and were more often found in bladders incubated with antidiuretic hormone than without hormone.

Incubation of bladders in Ringer's solutions containing 2 to 20 mM magnesium did not increase the size or density of the intramitochondrial granules (Fig. 8) above that seen in cells of bladders incubated in calcium-free solutions without added divalent cations (Fig. 9).


1 μ
mi
ig
1

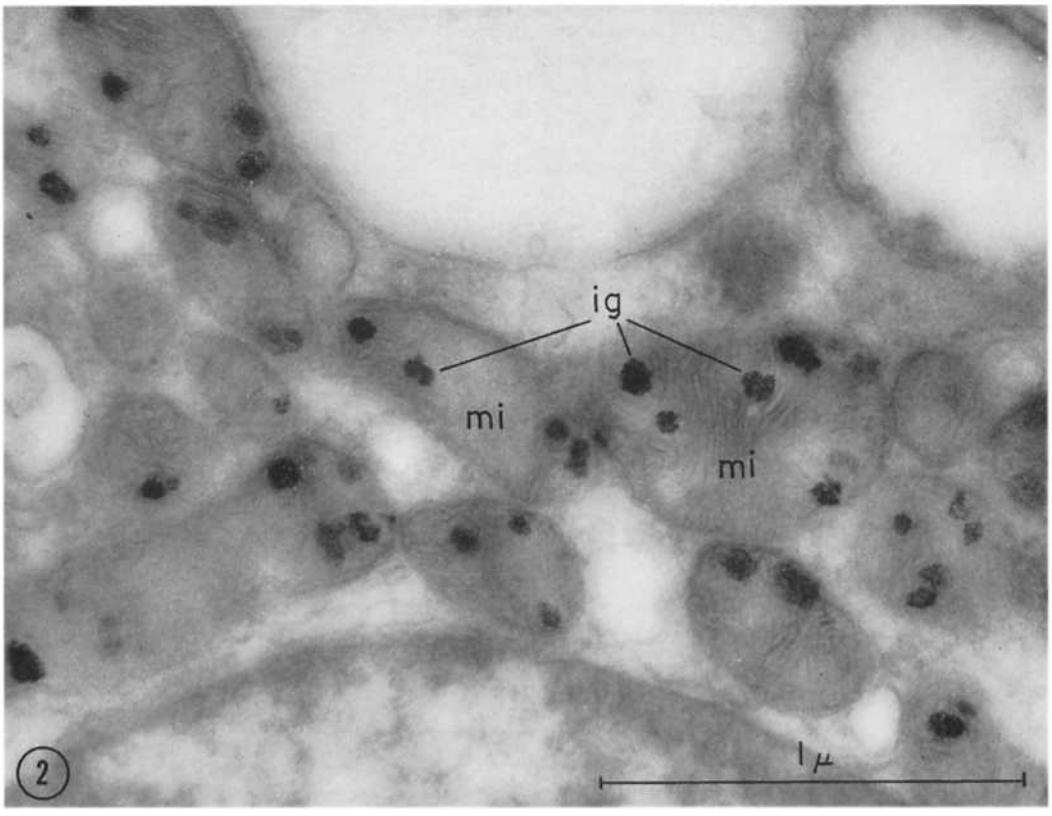

ig
mi
mi
1 μ
2

FIGURE 1 Electron micrograph of a cell found free in the lumen of a toad urinary bladder incubated for 6 hours in a calcium-free Ringer's solution containing 2 mM barium acetate. A maximal dose of lysine vasopressin was present in the medium for the last 20 minutes of incubation. This cell, of a type containing many mitochondria, is found in the epithelium of the amphibian urinary bladder (Peachey and Rasmussen, 1961). Note the many dense granules (*ig*) and their localization only in the mitochondria (*mi*). The arrows indicate small, dense granules adhering to the surface of the cell. These probably are a precipitate of barium phosphate formed in the incubating medium. 800 A thick section, not stained but covered with a layer of collodion. × 17,000.

FIGURE 2 Higher magnification of the field shown in Fig. 1. A finely granular substructure can be seen in some of the dense granules (*ig*) within the mitochondria (*mi*). × 57,000.

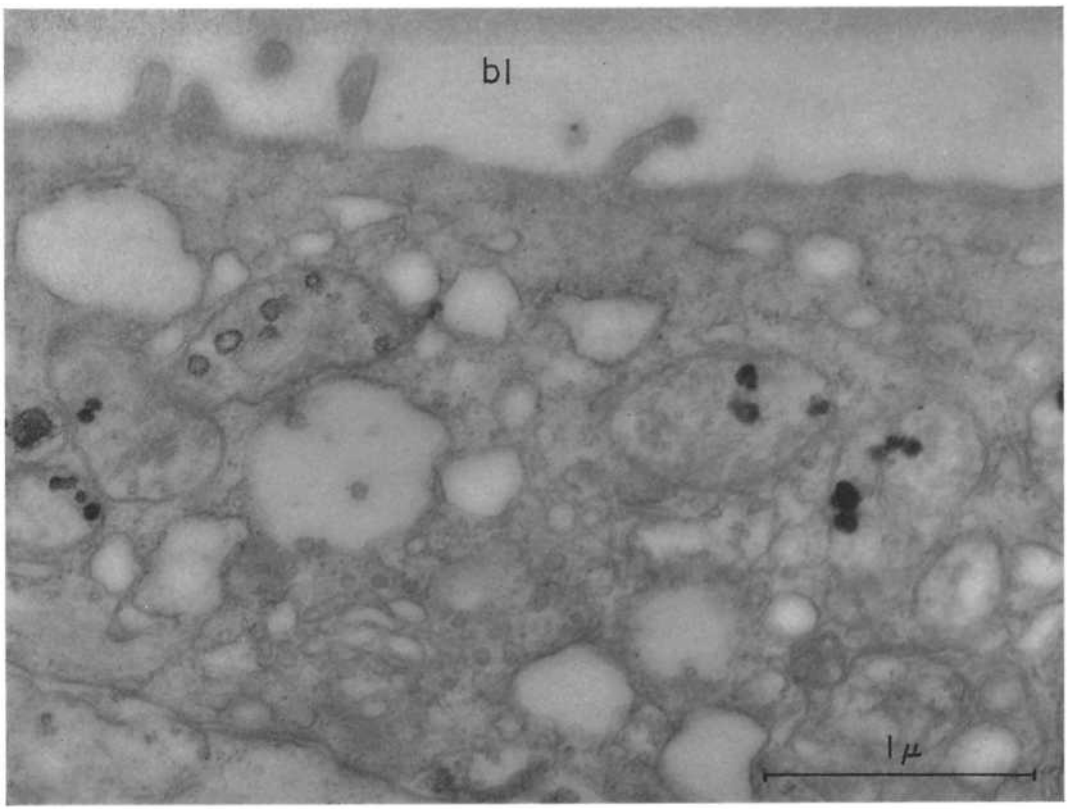


FIGURE 3 An attached epithelial cell from a urinary bladder incubated for 6 hours in calcium-free Ringer's solution containing 3 mM strontium acetate, with a maximal dose of oxytocin present during part of the last 2 hours of the incubation. The free surface of the cell, facing the bladder lumen (*bl*), is at the top of the figure. The intramitochondrial granules display a variety of sizes and densities. The smallest granule is 300 A in diameter; the unusually large one at the left measures 1200 A. Some of the granules are very dense, while some are less dense and hollow. 700 to 800 A thick section stained for 6 minutes on 80 per cent saturated lead subacetate and covered with collodion. × 36,000.

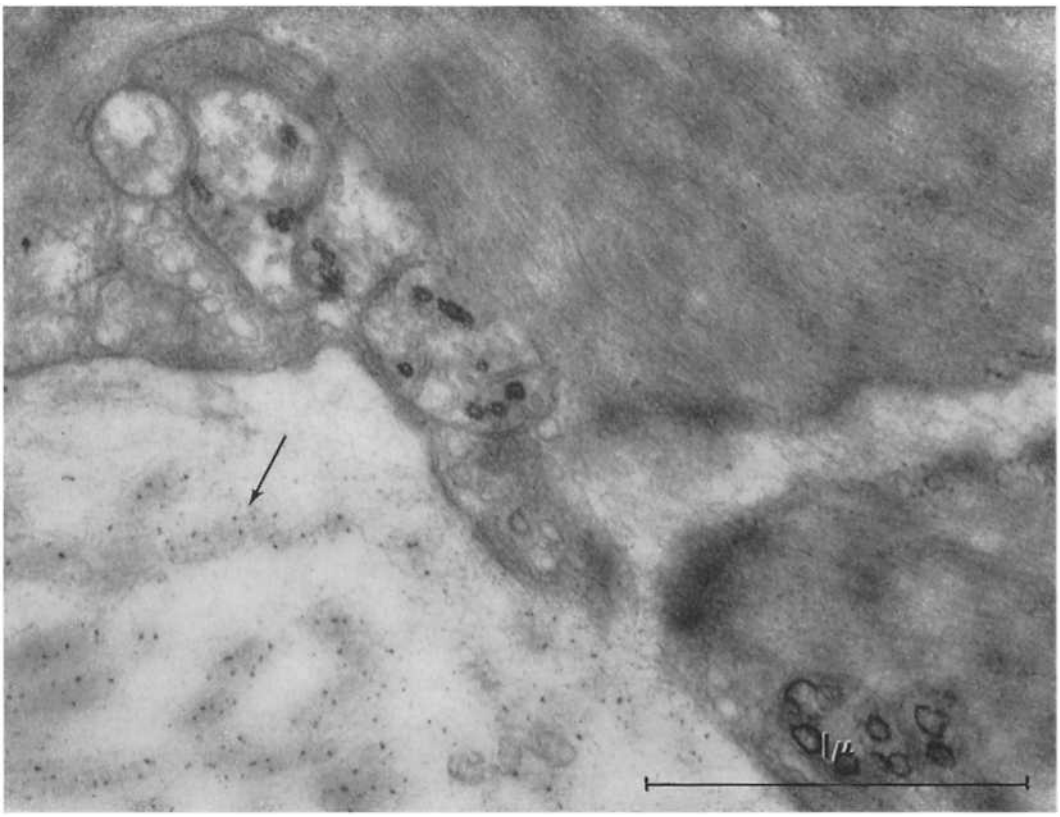
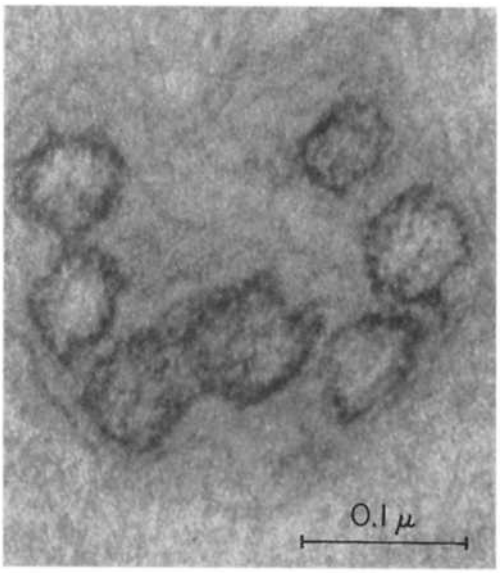


FIGURE 4 Smooth muscle cell from a urinary bladder incubated for 6 hours in calcium-free Ringer's solution containing 2 mM barium acetate, showing dense intramitochondrial granules, most of which appear hollow. Some dense precipitate (arrow), probably of barium phosphate, is seen in the connective tissue. 600 A thick section stained for 6 minutes on saturated lead subacetate and covered with collodion. × 36,000.



Isolated Mitochondria

Electron microscopic examination of mitochondria isolated from rat kidney in various ways indicated that the mitochondria were better preserved structurally when the isolation medium was buffered to pH 7.5 with 0.05 M sodium acetate-veronal buffer than when no buffer was used. Therefore, buffer was routinely used in all later isolations. The inclusion of EDTA in the isolation medium, reported by Slater and Cleland (1953) to improve the preservation of sarcosome structure in rat heart muscle, had no apparent effect on the structure of kidney mitochondria isolated in buffered media.

FIGURE 5 Higher magnification of granules from preparation shown in Fig. 4. The fine granules making up the larger deposits measure about 40 A across. × 210,000.

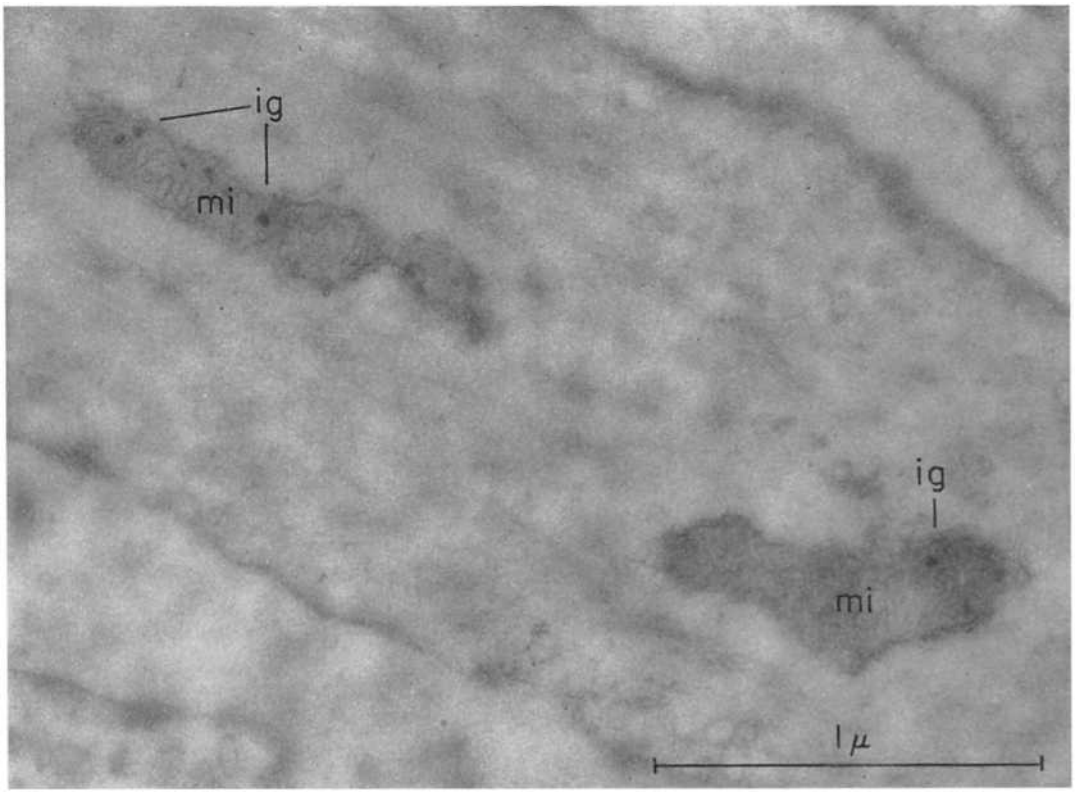


FIGURE 6 Epithelial cell from a urinary bladder incubated for 7 hours in ordinary Ringer's solution containing 2 mM calcium acetate. Only low density granules (*ig*) are seen in the mitochondria (*mi*). 600 A thick section, not stained but covered with collodion. × 51,000.

INCUBATION OF ISOLATED MITOCHONDRIA

Mitochondria isolated either without EDTA or with 0.005 M EDTA and then incubated without divalent cations (except magnesium) had, as do unincubated mitochondria, a variety of appearances ranging from normal (as in intact cells) to highly swollen (Fig. 10). A similar structural heterogeneity was observed by Amoore and Bartley (1958) in isolated rat liver mitochondria. An intermediate form, with slightly swollen *cristae* and a dense matrix, was most common. The very swollen forms seemed to have lost most of their matrix. However, many relatively normal appearing mitochondria were present, and the over-all appearance of these preparations in the electron microscope was not much different from that of freshly isolated, unincubated mitochondria. The intramitochondrial granules were only slightly denser than the mitochondrial membranes and were about 200 A in diameter.

Incubation in the medium containing calcium caused a drastic alteration of mitochondrial structure, resulting in swelling of the mitochondrial volume and, in some cases, rupture of the mitochondrial membranes and loss of matrix material. This effect occurred in both non-EDTA and EDTA (Fig. 11) preparations, although the effect was somewhat more pronounced in the preparations isolated in the presence of EDTA. The calcium-induced swelling agrees with earlier observations on isolated liver mitochondria (Hunter and Ford, 1955; Tapley, 1956) and heart muscle sarcosomes (Slater and Cleland, 1952). The intramitochondrial granules of these calcium-incubated preparations appeared to be somewhat larger and to show an increase in density, especially in the EDTA-isolated preparations, but one must be cautious in judging such small differences in density in electron microscope sections, where contrast can be influenced by a variety of factors.

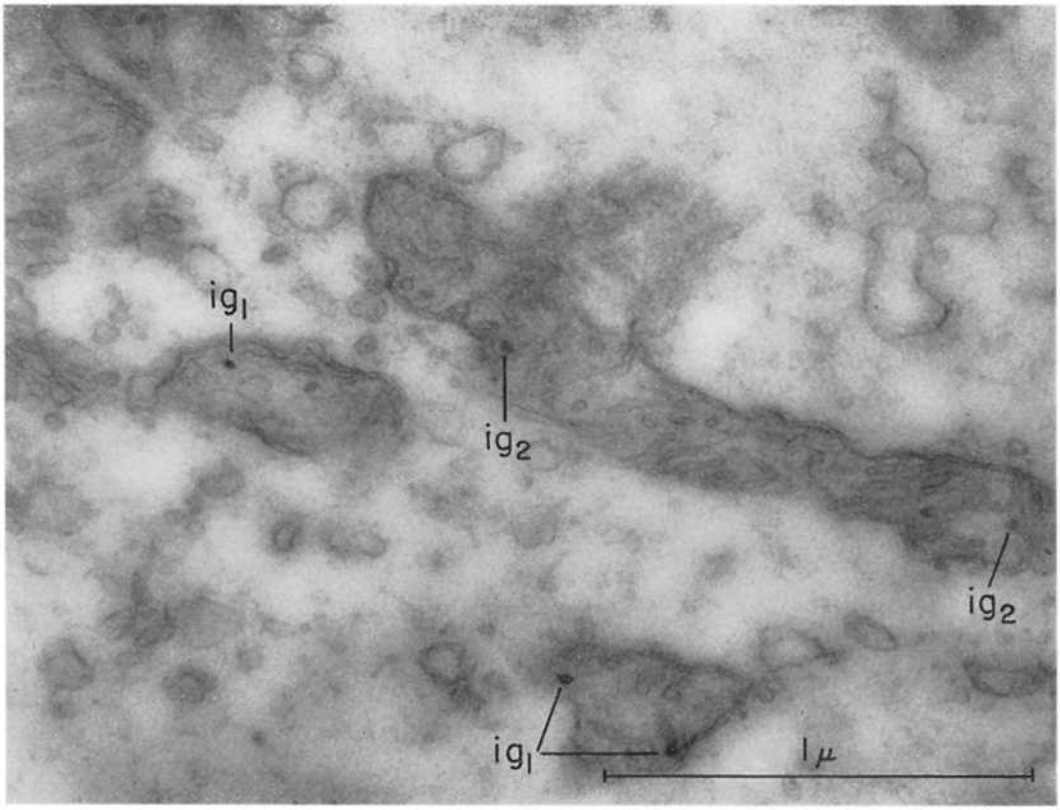


FIGURE 7 Epithelial cell from a urinary bladder incubated for 2 hours in a high-calcium Ringer's solution (20 mM calcium acetate). Beginning at 20 minutes and ending at 65 minutes after the start of the incubation, a maximal dose of lysine vasopressin was present in the serosal medium. In addition to granules of the usual density (ig_2), very dense granules (ig_1) are found in the mitochondria of these cells. 600 A thick section, not stained but covered with collodion. × 57,000.

Incubation with strontium (Fig. 12) and barium (Fig. 13) caused an obvious increase in the density of the intramitochondrial granules. The size of these granules is about double (400 A) that of the granules in the unincubated preparations. There was slight structural damage to mitochondria incubated in strontium, and the damage in barium, although greater than in strontium, was considerably less than in calcium.

DISCUSSION

The first question brought to mind by these results is whether the observed changes in density of the intramitochondrial granules can reasonably be attributed to an accumulation of divalent cations. Since the only difference between experiments in which granules of different density were observed was the divalent cation present in the incubation medium, the divalent cations must be considered at least the indirect cause, if not the direct cause, of the observed densities. Furthermore, the denser granules were observed when the heavier the divalent cations were present in the medium. This would be expected if it were the divalent cations themselves which accounted for the increased density, since electron-scattering power, and thus density in electron micrographs, increases with atomic number. Thus barium and strontium, with atomic numbers of 56 and 38, respectively, would be very apparent against a background of organic cellular and embedding material consisting largely of hydrogen, carbon, nitrogen, and oxygen, whose atomic numbers range from 1 to 8. Calcium, with an atomic number of 20, would also show up dark if sufficiently concentrated, while magnesium,

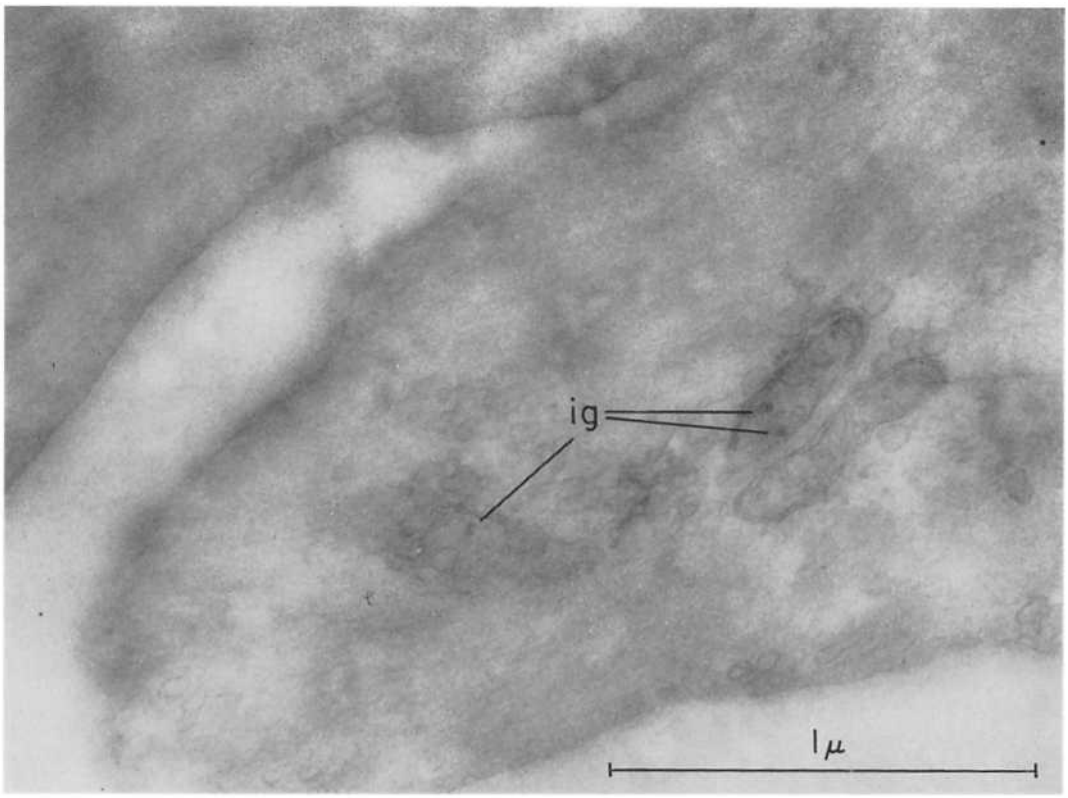


FIGURE 8 Smooth muscle cells from a urinary bladder incubated for 6 hours in a calcium-free Ringer's solution containing 2 mM magnesium acetate. Intramitochondrial granules (*ig*) in both smooth muscle and epithelial cells (not shown) are of low density. 800 A thick section, not stained but covered with collodion. × 57,000.

whose atomic number is 12, is probably not sufficiently dense to be distinguished from the background. The presence of osmium, with an atomic number of 76, might have been expected to interfere with the identification of these less heavy elements, but apparently their concentration in the granules is so high that they clearly stand out even in the presence of the osmium, which is probably more diffusely distributed.

Thus, there seems to be little reason to doubt that the observed increases in density of the intramitochondrial granules after incubation with barium, strontium, and calcium are due to accumulation of these elements. This conclusion agrees with the suggestion made by Poche (1959) that the intramitochondrial granules in the heart of the hibernating dormouse might be collections of cations.

The electron microscopic method for studying the localization of metals in mitochondria, while clearly inferior to chemical methods for quantitative work, has several distinct virtues. In the first place, one can be sure that the metal is in the mitochondrion and not on its surface or in the intermitochondrial spaces. Secondly, one can easily rule out the possibility that the metal is concentrated in a contaminant of the mitochondrial preparation. Furthermore, it is possible, using electron microscopy, to further localize the metal within the mitochondrion, in this case to intramitochondrial granules, without further disruption and fractionation of mitochondrial structures.

The hollow appearance of the granules was especially prominent in cells incubated in strontium or barium, in which the contrast was highest, but was observed also in cells incubated in high calcium concentrations. This findings sug-

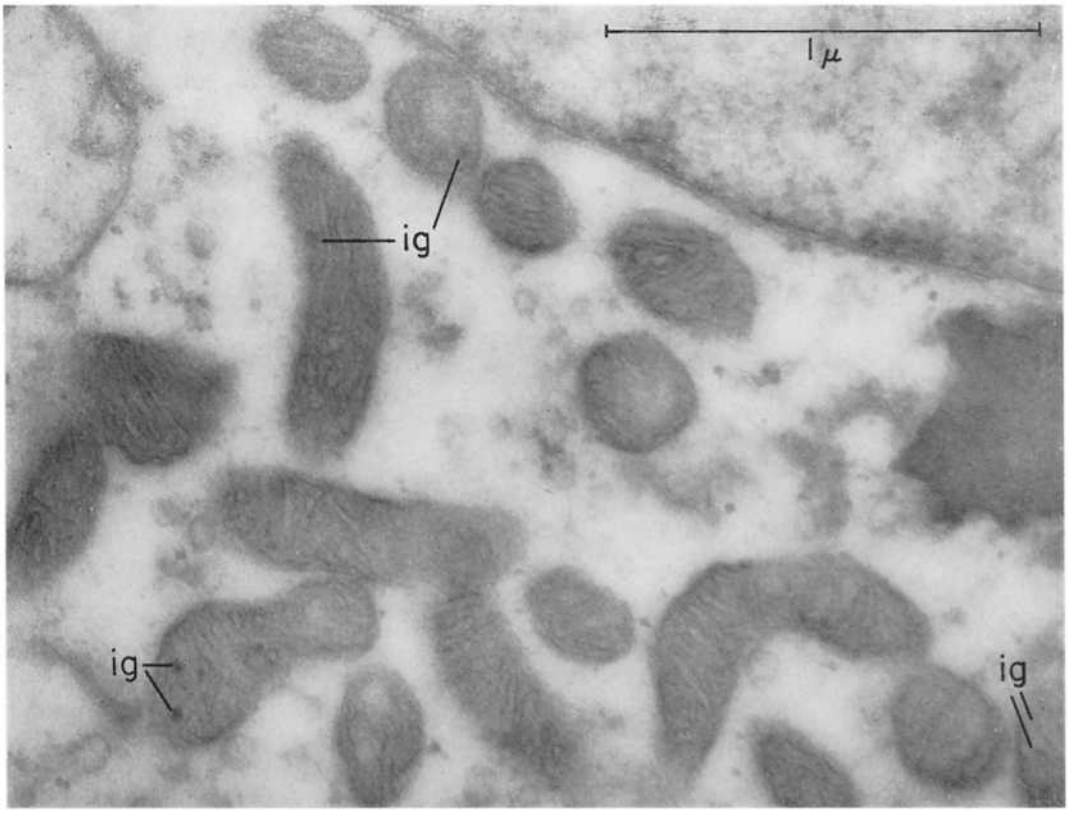


FIGURE 9 Free epithelial cell of the mitochondrion-rich type, obtained by centrifuging solutions collected from inside a urinary bladder incubated for 3 hours in calcium-free Ringer's solution. As in the previous figure, intramitochondrial granules (*ig*) are of low density. 800 A thick section, not stained but covered with collodion. × 57,000.

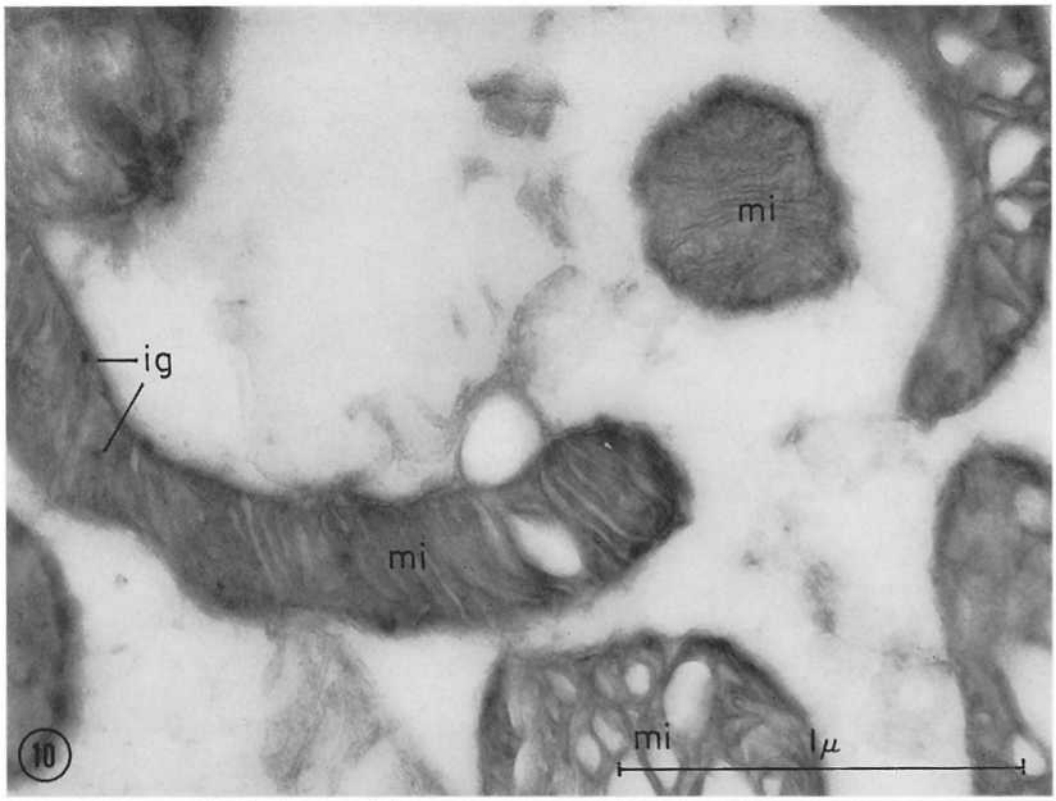
gests either that the incubation resulted in the formation of granules with surface-localized binding sites or that the denser cations are deposited selectively on the surfaces of pre-existing granules, possibly in exchange for other cations. The latter interpretation seems more likely to be the correct one, since, although before incubation intramitochondrial granules of normal density are present, in most cases after incubation, with heavy divalent cations, only dense granules are found. Therefore, one must conclude either that the pre-existing granules disappear during the incubation or, more likely, that they bind the heavy atoms. The increased size of the granules in either whole cells or isolated mitochondria incubated with strontium or barium indicates that the granules increase in size as more atoms are bound.

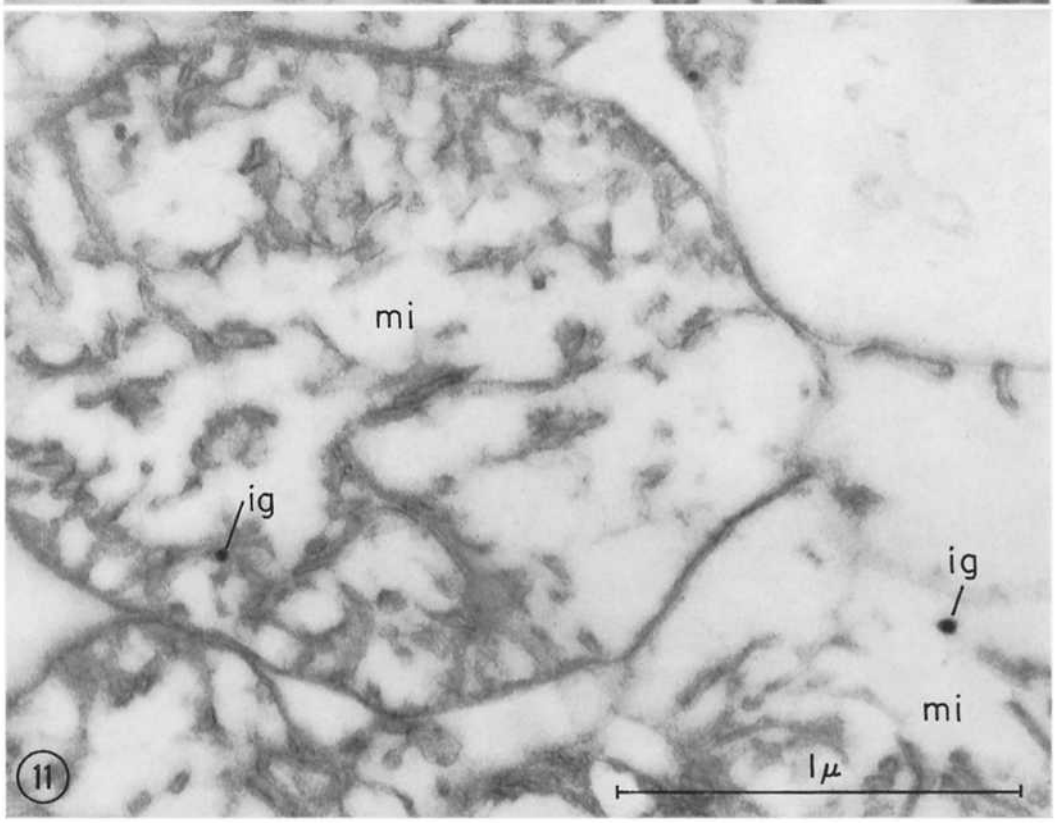
Of primary interest in these results is the accumulation of divalent cations in intramito-

FIGURES 10 to 13 Rat kidney mitochondria isolated in 0.44 M sucrose containing 5×10^{-3} M EDTA, washed twice without EDTA, and incubated in the standard medium with the addition of the following divalent ions:

Fig. 10, none.

Fig. 11, 2 mM calcium acetate.


mi
ig
mi
mi
1μ
10


mi
ig
ig
mi
1μ
11

chondrial granules of *intact cells*. This observation supports the notion that at least part of the chemically determined metal content of isolated mitochondria represents an *in situ* mitochondrial component.

As stated in the Introduction, it would be interesting to know the form in which calcium is retained in the mitochondria, a second question that should be considered. Slater and Cleland (1953) have argued that intramitochondrial calcium is probably in a bound form, rather than free in solution, and that the most likely group for binding calcium is phosphate. These workers point out that most of the phosphate present in mitochondria is in phospholipid, and that the amount of phosphate present (0.5 μg atoms/mg protein) is more than enough to bind the calcium (0.1 μmoles/mg protein) observed in heart muscle mitochondria in their experiments. In the experiments of Lehninger *et al.* (1963), in which the amount of calcium (up to 2.2 μmoles/mg protein) taken up by isolated rat liver mitochondria is many-fold greater than that present in freshly isolated mitochondria, it is suggested that the calcium is retained in the form of a precipitate of inorganic phosphate.

The present experiments on isolated rat kidney mitochondria were performed under conditions similar to those used by DeLuca and Engstrom (1961), except for a change in buffer systems and the omission of components of the incubation mixture which were shown to be non-essential for maximum uptake of calcium. The use of phosphate buffer and the addition of inorganic phosphate to the medium were avoided in the present experiments, in order to reduce the possibility of precipitation of phosphates of divalent cations in the medium or within the mitochondria. Thus, it seems reasonable to assume that the amount of calcium uptake in the experiments presented here did not exceed that observed by DeLuca and Engstrom and was not so great as that observed by Lehninger *et al.* (1963). Therefore, the calcium taken up in these mitochondria did not exceed the amount that could be bound by available organic phosphates in the mitochondria, and it is not necessary to assume that the dense granules observed are precipitates of inorganic phosphates. In fact, since no inorganic phosphate ions were added to the incubation medium, it seems unlikely that such precipitates could form. A more satisfactory explanation is that the divalent cations are bound to organic phosphate in the form of granules pre-existing within the mitochondria. These arguments would not, of course, apply to the experiments of Lehninger *et al.* (1963) in which inorganic phosphate was available and was taken up along with calcium in very large amounts.

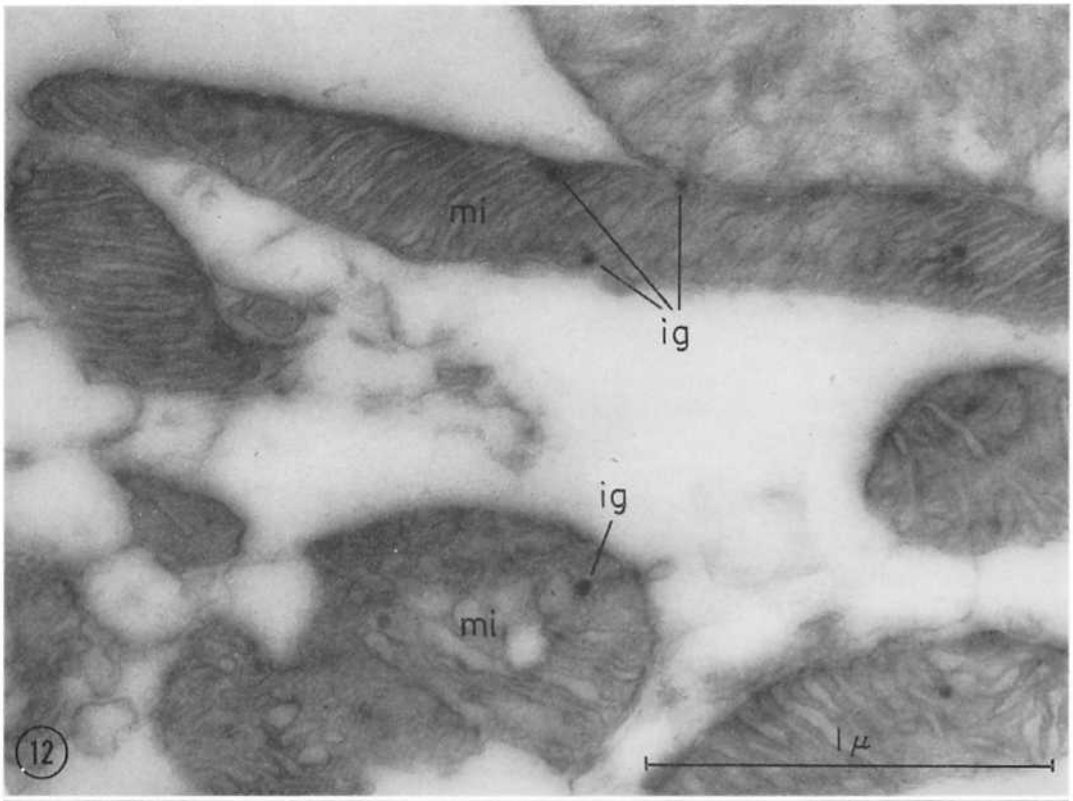
All of these considerations lead one to conclude that calcium may be retained within mitochondria in two possible forms: one organically bound and the other an inorganic precipitate. The former may be the usual form and the latter may occur only when unusually large amounts of calcium are accumulated.

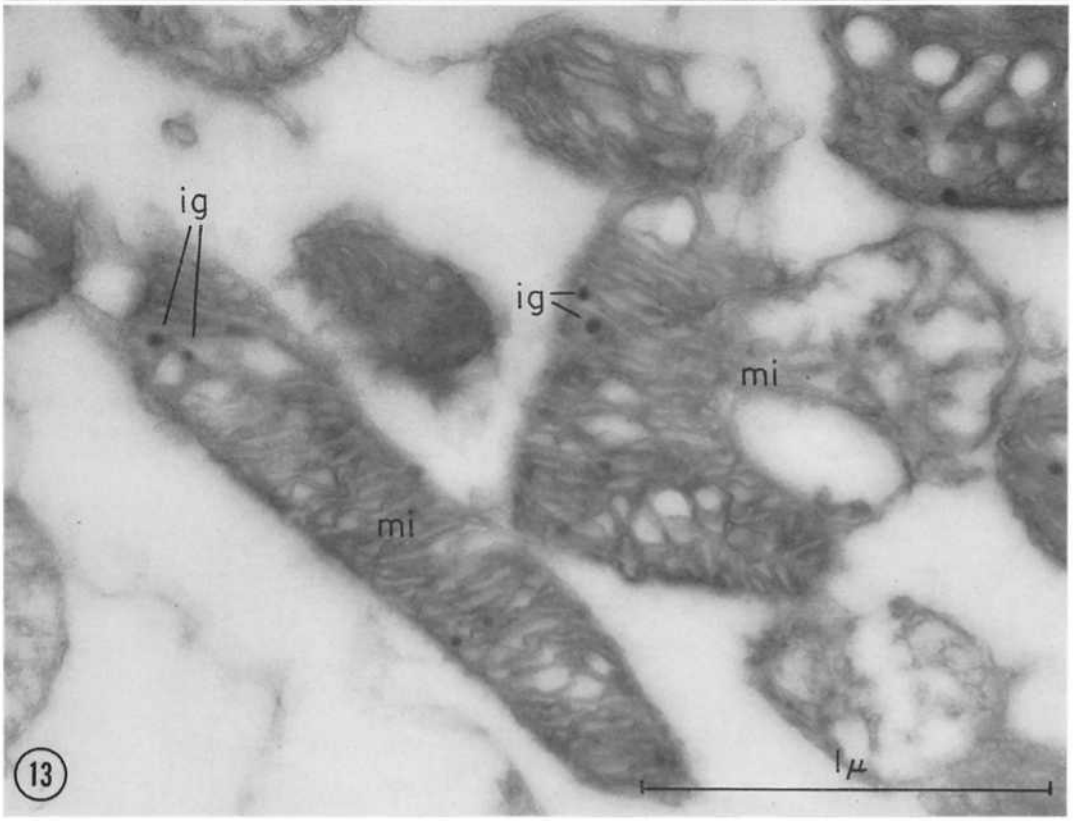
A third question to be answered concerns possible functions, in the cell's physiological activities of the ion-binding abilities of intramitochondrial granules. Mitochondria are being strongly implicated in mechanisms of calcium transport across cells (Engstrom and DeLuca, 1962; DeLuca *et al.*, 1962; Rasmussen and DeLuca, 1963). These workers have observed that vitamin D, which is apparently required for transport of calcium across the intestinal wall, and parathyroid hormone accelerate the release of accumulated calcium by isolated mitochondria. An intramitochondrial binding mechanism, such as that demonstrated here, might be part of an over-all mechanism by which mitochondria contribute to transcellular movements of ions. It is interesting to note, in this respect, that Roullier (1960), searching for a pattern in the variability of numbers of intramitochondrial granules in different tissues, observed that granules are especially prominent in tissues that transport large quantities of water or ions.

Fig. 12, 2 mM strontium acetate.

Fig. 13, 2 mM barium acetate.

For description, see text p. 101. *ig*, intramitochondrial granule. *mi*, mitochondrion. Sections 600 to 800 A thick, not stained. × 54,000.


mi
ig
ig
mi
12
1 μ


ig
ig
mi
mi
13
1 μ

The presence of binding sites for divalent cations also provides for a buffering of the concentration of these elements in solution within the mitochondrion. The sensitivity of mitochondrial oxidative enzyme systems to divalent cations and the demonstrated ability of intramitochondrial granules to bind these ions suggests a possible role of the granules in the regulation of mitochondrial metabolism.

Finally, the possibility that other binding sites for divalent ions exist, which are not shown by this method, must be considered. For example, one would expect divalent ions to bind to phospholipids in membranes, but, possibly because these binding sites are relatively diffuse, or because osmium is present in the fixed membranes, the membrane-bound elements do not show up in an ordinary preparation. Hopefully, the use of fixatives that do not contain heavy metals may allow visualization of these less concentrated deposits, especially when the denser elements, strontium or barium, can successfully be substituted for calcium.

This study was supported by grants from the National Science Foundation, Nos. G-13133 and GB-542.

Received for publication, March 26, 1963.

BIBLIOGRAPHY

AMOORE, J. E., and BARTLEY, W., 1958, *Biochem. J.*, **69,** 223.

BARTLEY, W., and AMOORE, J. E., 1958, *Biochem. J.*, **69,** 348.

BENTLEY, P. J., 1958, *J. Endocrinol.*, **17,** 201.

BRIERLEY, G. P., BACHMANN, E., and GREEN, D. E., 1962, *Proc. Nat. Acad. Sc.*, **48,** 1928.

BULLARD, H. H., 1912, *Am. J. Anat.*, **14,** 1.

CAREY, E. J., and ZEIT, W., 1939, *Proc. Soc. Exp. Biol. and Med.*, **41,** 31.

CHAPPELL, J. B., GREVILLE, G. D., and BICKNELL, K. E., 1962, *Biochem. J.*, **84,** 61p.

CLELAND, K. W., 1953, unpublished, quoted in Slater, 1957.

DELUCA, H. F., and ENGSTROM, G. W., 1961, *Proc. Nat. Acad. Sc.*, **47,** 1744.

DELUCA, H. F., ENGSTROM, G. W., and RASMUSSEN, H., 1962, *Proc. Nat. Acad. Sc.*, **48,** 1604.

DELUCA, H. F., REISER, S., STEENBOCK, H., and KAESBERG, P., 1960, *Biochim. et Biophysica Acta*, **40,** 526.

ENGSTROM, G. W., and DELUCA, H. F., 1962, *J. Biol. Chem.*, **237,** PC974.

HUNTER, F. E., and FORD, L., 1955, *J. Biol. Chem.*, **216,** 357.

LEHNINGER, A. L., ROSSI, C. S., and GREENAWALT, J. W., 1963, *Biophys. and Biochem. Research Comm.*, **10,** 444.

MAYNARD, L. S., and COTZIAS, G. C., 1955, *J. Biol. Chem.*, **214,** 489.

MRAZ, F. R., 1962, *Proc. Soc. Exp. Biol. and Med.*, **111,** 429.

PEACHEY, L. D., 1962, Proceedings Fifth International Congress for Electron Microscopy, (S. S. Breese, Jr., editor), New York, Academic Press, Inc., **2,** OO–3.

PEACHEY, L. D., and RASMUSSEN, H., 1961, *J. Biophysic. and Biochem. Cytol.*, **10,** 529.

POCHE, R., 1959, *Z. Zellforsch.*, **50,** 332.

RASSMUSSEN, H., and DELUCA, H., 1964, *Ergebn. Physiol.*, in press.

REYNOLDS, E. S., 1960, *J. Histochem. and Cytochem.*, **8,** 331.

ROUILLIER, C., 1960, *Internat. Rev. Cytol.*, (G. H. Bourne and J. F. Danielli, editors), **9,** 227.

SARIS, N., 1963, *Acta. Chem. Scand.*, **17,** 882.

SCOTT, G. H., 1932, *Proc. Soc. Exp. Biol. and Med.*, **29,** 349.

SLATER, E. C., and CLELAND, K. W., 1952, *Nature*, (London), **170,** 118.

SLATER, E. C., and CLELAND, K. W., 1953, *Biochem. J.*, **55,** 566.

SLATER, E. C., 1957, *Symp. Soc. Exp. Biol. and Med.*, **10,** 110.

TAPLEY, D. F., 1956, *J. Biol. Chem.*, **222,** 325.

THIERS, R. E., REYNOLDS, E. S., and VALLEE, B. L., 1960, *J. Biol. Chem.*, **235,** 2130.

VASINGTON, F. D., and MURPHY, J. V., 1962, *J. Biol. Chem.*, **237,** 2670.

WEISS, J., 1955, *J. Exp. Med.*, **102,** 783.

After completing this work, the author found that Porter and Hogeboom many years ago had observed dense granules in mitochondria of unfixed cells, but had not published their observations. Their evidence supports the conclusion reached in this paper that the densities observed were not the result of osmium deposition. Dr. Keith R. Porter has kindly supplied the following addendum, briefly describing their results, and the illustrations in Figs. 14 to 16. (See following pages.)

ADDENDUM

At the request of Lee Peachey I am including here a brief note on some observations made by George Hogeboom and me about 17 years ago. During the course of some studies on the morphological features of cultured cells following various procedures of fixation, we observed that the mitochondria showed small dense granules. These became especially prominent when the calcium ion concentration was increased. In experiments made subsequently on cells cultured from explants of heart tissue from day-old rats, the appearance of these granules was examined following variations in (*a*) concentration of ion, (*b*) temperature of incubation over a 20-minute period (38°C, 4°C), and (*c*) species of divalent cation (Ca^{++}, Sr^{++}, and Ba^{++}). In each instance the cells were dried from the saline solution without fixation. The findings demonstrated (*a*) that the size but not the number of the granules increased (up to 200 mμ in diameter) with increased concentration of ion, (*b*) that granule development in the presence of excess calcium (100 mM) was greatly inhibited at 4°C– to the extent that they never developed to a diameter greater than 50 mμ, and (*c*) that divalent ions besides calcium were equally capable of leading to prominent granule development at 38°C.

These observations, which were at the time (November, 1946) original, were, however, not interpretable and were put aside for future investigation. I very much appreciate this opportunity to bring them to the attention of students of mineral metabolism, and I am sure that my collaborator of those days would feel the same.

KEITH R. PORTER

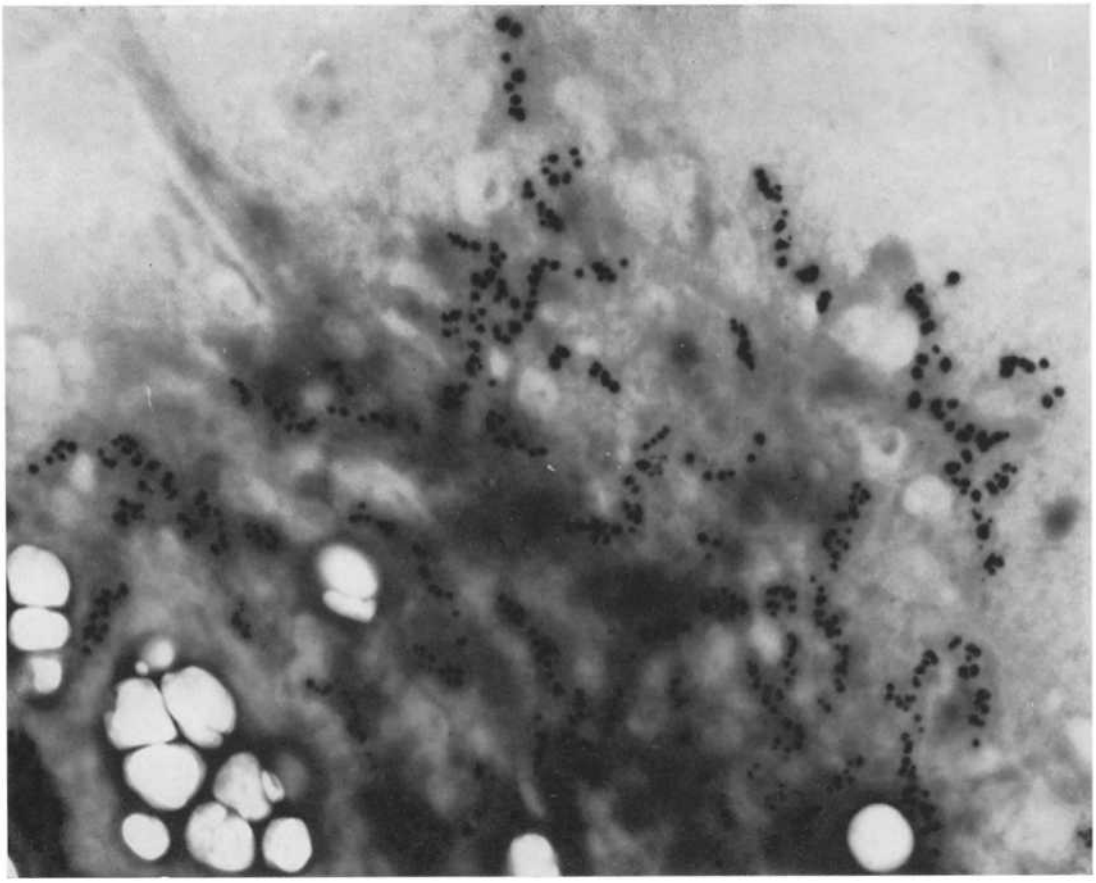
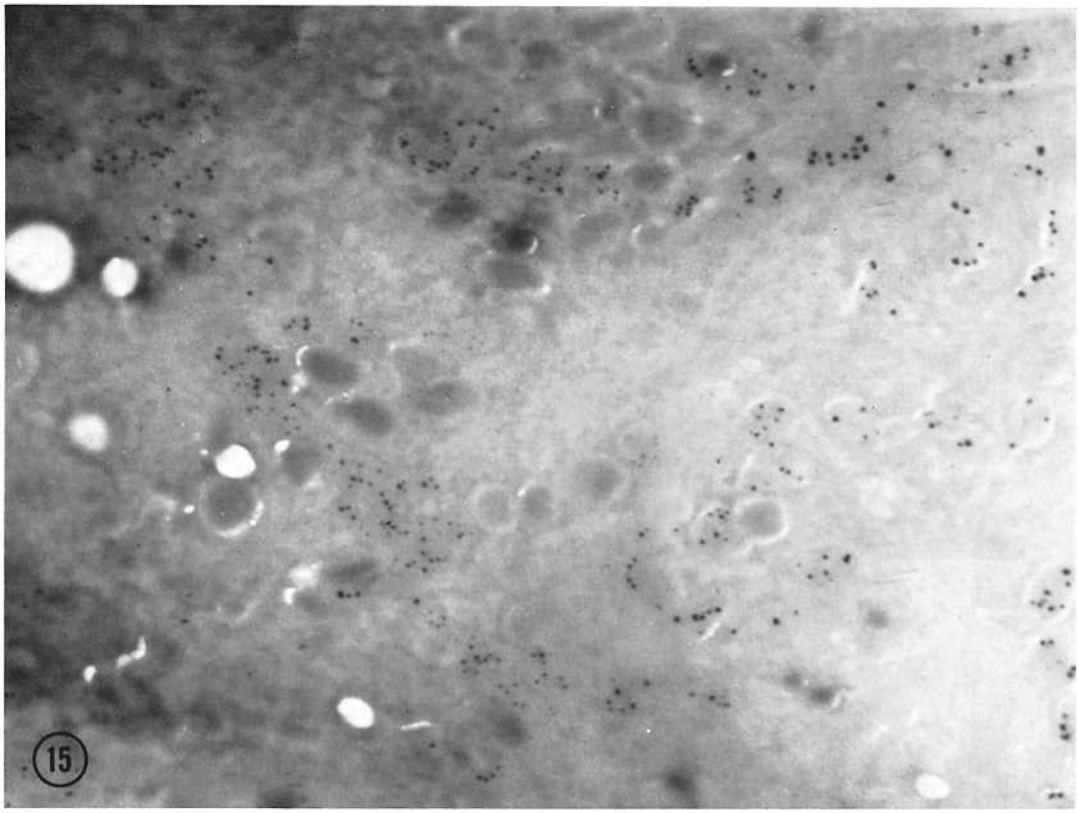
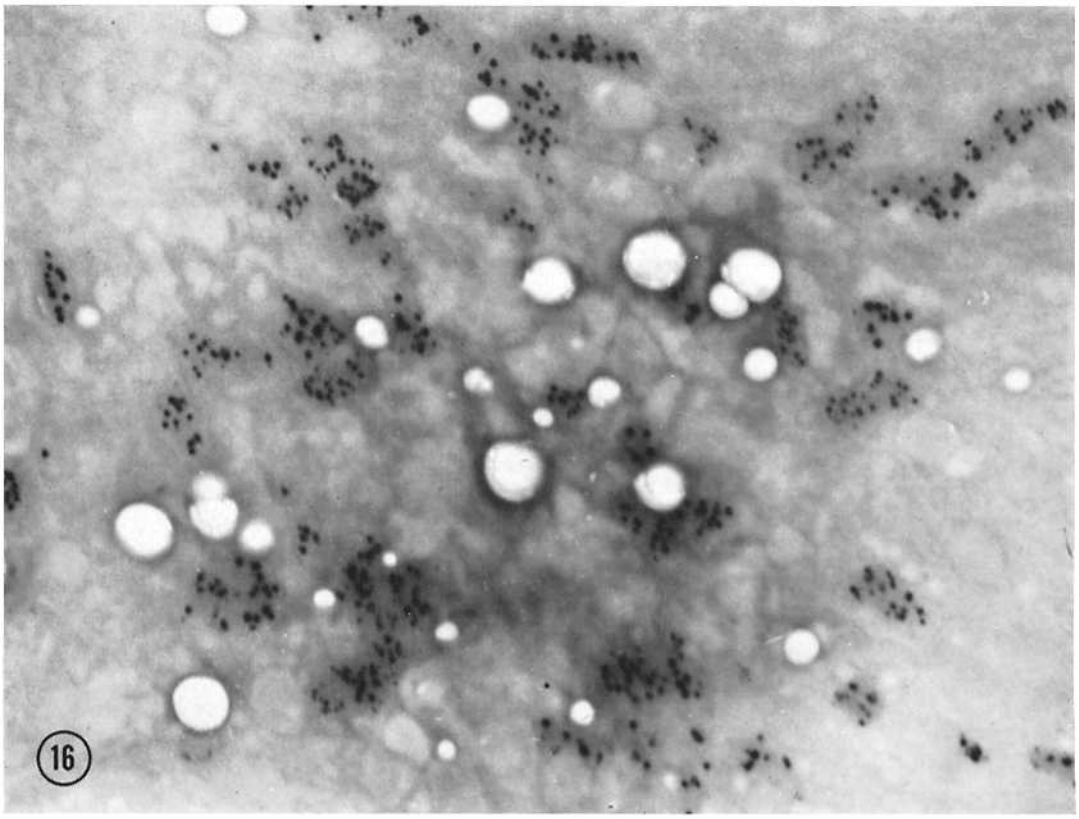


FIGURE 14 Cell incubated with 100 mM $CaCl_2$ for 20 minutes at 38°C. According to Hogeboom's notes, "Outlines of mitochondria are not distinctly visible, but the granules are very dense and numerous." It was further noted that the "outlines of mitochondria disappear in practically all cells which have been incubated at 38°C." About × 10,000.

FIGURE 15 Cell incubated with 10 mM $CaCl_2$ for 20 minutes at 38°C. The granules developed at this lower concentration are much smaller than those seen in the previous micrograph. About × 10,000.

FIGURE 16 Cell incubated with 100 mM $BaCl_2$ for 20 minutes at 4°C. Hogeboom, referring to this preparation, noted that the "outlines of mitochondria are about as distinct as ever obtained." The intramitochondrial granules in this preparation are very dense and moderately large. About × 10,000.


15


16