ELECTRON MICROSCOPE STUDIES ON THE ACTIVE ACCUMULATION OF Sr^{++} BY RAT-LIVER MITOCHONDRIA

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ABSTRACT

Electron-opaque granules are deposited in isolated rat-liver mitochondria concomitant with the energy-linked accumulation of Sr^{++} by these organelles. High temperature micro-incineration (600°C) of thin sections of mitochondria containing different amounts of Sr^{++} shows that a clear qualitative correlation exists between the number of inorganic residues remaining after incineration and the amount of Sr^{++} translocated into the mitochondria. By loading the mitochondria with consecutive pulses of small amounts of Sr^{++} ("multiple-pulse" loading), very early stages of granule formation can be detected; the first detectable deposits are seen closely associated with the cristae. The evidence presented supports the hypothesis that mineral deposition following or during the in vitro accumulation of ions by mitochondria occurs, at least initially, at sites on these membranes and not as nonspecific precipitates in the mitochondrial matrix. The large number of electron-opaque deposits (100 to 200) seen in single thin sections of individual mitochondria having accumulated intermediate levels of Sr^{++} clearly exceeds the number of normal dense granules in rat-liver mitochondria, indicating that the normal matrix granules per se do not constitute sites essential for deposition. At the highest levels of Sr^{++} uptake studied in the multiple-pulse loading experiments, needlelike deposits are seen, a result which suggests that the structural form ("crystallinity") of the mineral deposits may be determined by the rate of accumulation.

INTRODUCTION

Following the observation of Vasington and Murphy (1, 2), a number of investigators have shown that mitochondria isolated from various tissues accumulate massive amounts of divalent cations in vitro (3–6) in an energy-dependent process linked to electron transport (7–9). Rossi and Lehninger (10) have shown that the number of Ca^{++} ions accumulated during respiration-supported uptake bears a precise stoichiometric relationship to the number of energy-conserving sites traversed by electrons flowing down the respiratory chain. In the presence of excess Ca^{++}, no oxidative phosphorylation of ADP occurs; instead, Ca^{++} and P_i are stoichiometrically accumulated by the mitochondria, showing that respiratory energy can be diverted from the phosphorylative reactions to those supporting ion transport.

Electron microscope investigations have shown that the massive accumulation of Ca^{++} accompanied by uptake of P_i leads to the deposition within the mitochondria of electron-opaque

granules, which often reach huge dimensions (11, 12). Measurement of the Ca^{++}:P_i accumulation ratio after massive loading has shown it to be around 1.67, which is equal to that of hydroxyapatite ($[Ca_3(PO_4)_2]_3$, $Ca(OH)_2$); however, neither electron- nor x-ray diffraction analyses have revealed crystallinity in the dense intramitochondrial granules (11). After melting and cooling the deposits in thin sections, Thomas and Greenawalt (13), using microincineration techniques, obtained an electron-diffraction pattern similar to that of colloidal β-tricalcium phosphate ($Ca_3(PO_4)_2$). Also, Weinbach and von Brand (14) have reported that crystallization of granules isolated from Ca^{++} + P_i-loaded mitochondria can be induced by incineration at 600°C and then cooling; x-ray diffraction analyses showed the presence of hydroxyapatite and also whitlockite ($Ca_3(PO_4)_2$) as major constituents after the incineration treatment.

The conditions under which massive accumulation of ions occurs are obviously nonphysiological. For example, calcium at high concentrations is a potent uncoupler of oxidative phosphorylation, and massive loading with Ca^{++} and P_i causes irreversible structural and biochemical damage to mitochondria (10, 11). It is possible, however, that massive loading represents an exaggeration of the normal flux of ions across the mitochondrial membrane necessary for ionic homeostasis in the cytoplasm. The microincineration studies of Thomas and Greenawalt (13) suggest that minerals are indeed normal constituents of mitochondrial membranes and that localized deposits can be detected by this technique. Furthermore, the studies of Greenawalt et al. (11) and of Reynolds (15) indicate that a high proportion of the accumulated dense granules are associated with the mitochondrial membranes. On the other hand, Peachey (16) has suggested that the dense granules normally found in the matrix of mitochondria in situ serve as the actual nuclei for deposition of minerals. Vasington and Greenawalt (17, 18) have shown that water-washed mitochondria, which are devoid of these normal matrix granules, accumulate Ca^{++} and P_i and form electron-opaque deposits closely associated with the inner mitochondrial and cristal membranes. Massive ion accumulation by mitochondria is known to occur in vivo under specific conditions such as in CCl_4-poisoning (15, 19), in osteoclasts following bone fractures (20), and in cases of tumoral calcinosis (21).

These findings indicate the need to determine the nature of these deposits and to investigate the possibility that specific binding sites which could be of significance in understanding mitochondrial function play a role in granule formation. This paper presents evidence for the involvement of mitochondrial membranes in the deposition of strontium following the in vitro uptake of this ion; Carafoli et al. (30–32) and Peachey (16) have shown strontium to be readily transported and accumulated by isolated rat-liver mitochondria with no signs of biochemical damage and with mitochondrial structure preserved. In addition, strontium has a higher atomic number than calcium, a fact which suggests the suitability of this metal for use in studies attempting to visualize the initial binding sites of small amounts of transported ions.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of Carworth Farms albino rats (Sprague-Dawley strain) by the method of Schneider (22). Protein was determined by a biuret method (23).

Biochemical Methods

SINGLE-PULSE LOADING: In these experiments the medium supporting ion accumulation was placed in a polarographic cuvette so that the rate of oxygen consumption induced by the accumulation of a single addition ("pulse") of Sr^{++}, the amount of Sr^{++} transported, and the effect of its accumulation on mitochondrial structure could be determined on the same mitochondrial sample. The cuvette contained 10 mM Tris-HCl (pH 7.4), 80 mM KCl, 4 mM orthophosphate, 10 mM succinate (sodium salt), and 5 mg mitochondrial protein. The final volume was 2.0 ml; incubation was carried out at 26°C. Oxygen uptake was measured with the conventional Clark electrode described by Kielley and Bronk (24). One minute after the addition of mitochondria, 320 mμmoles of $SrCl_2 \times mg^{-1}$ protein (a total of 1.6 μmoles $SrCl_2$), labeled with $^{85}Sr^{++}$, was added to the reaction cuvette; the final concentration of Sr^{++} equalled 800 μM. During the incubation time (ca. 1 min), more than 95% of the added Sr^{++} was accumulated. At this level of loading, mitochondria are functionally intact as shown by the sharp return of the respiration curve to the resting rate after the Sr^{++}-induced activation (see Results, and Fig. 1).

At the end of the activated phase of respiration, the content of the vessel was rapidly poured into a precooled centrifuge tube and spun down in the cold at 20,000 × *g* for 4 min. The supernatant was collected and counted for the residual $^{85}Sr^{++}$ in a crystal

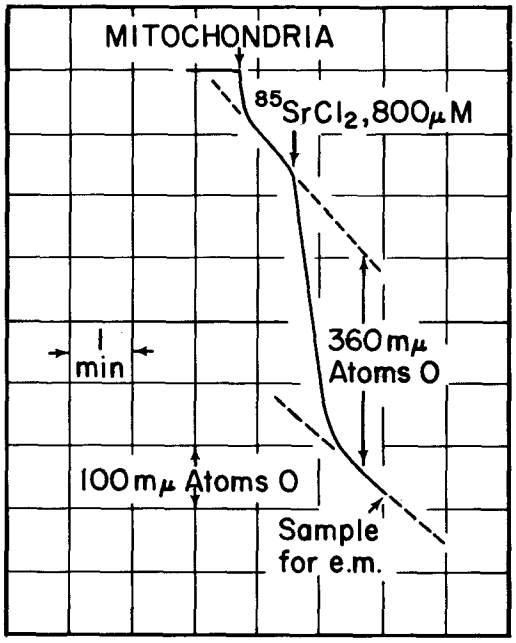


FIGURE 1 Polarographic tracing of respiration-linked accumulation of Sr^{++}. Additions of mitochondria and $^{85}SrCl_2$ and removal of samples for electron microscopy are indicated by arrows. Stimulation of respiratory rate by Sr^{++} and return to "resting" rate are shown. For details, see text.

scintillation detector against an appropriate $^{85}Sr^{++}$ standard. The pellets were used for electron microscopy.

MULTIPLE-PULSE LOADING: The reaction medium contained 10 mM Tris-HCl (pH 7.4), 80 mM NaCl or KCl, 4 mM orthophosphate, 10 mM succinate (sodium salt), 3 mM ATP (disodium salt), and 125 mg of mitochondrial protein. The final volume was 50 ml and incubations were carried out at 30°C. Mitochondria were added last, and 60 sec after the addition of the mitochondria the first of 7 consecutive pulses of $SrCl_2$ labeled with $^{85}Sr^{++}$ was added; the subsequent pulses were added at intervals of 60 sec, 60 sec, 120 sec, 120 sec, 4 min, and 5 min after each preceding addition. The last addition of $SrCl_2$ labeled with $^{85}Sr^{++}$ was 100 μmoles; all others were 20 μmoles.

The final concentrations of $SrCl_2$ following the addition of each pulse (after samples were removed for the measurement of ion uptake and for electron microscopy) were estimated to be 0.400, 0.816, 1.252, 1.707, 2.184, 2.683, and 5.313 mM as indicated in Fig. 4 *b*. It should be pointed out, however, that these values are not corrected for the Sr^{++} removed from solution by binding or precipitation within the mitochondria. Since deposition of Sr^{++}, probably as insoluble strontium phosphate, does occur at least at the higher levels of uptake, a more accurate description of the uptake conditions used in the multiple-pulse experiments may be expressed in terms of the μmoles of $SrCl_2$ added per pulse per mg mitochondrial protein. These data are given above.

Aliquots (2 ml) were withdrawn from the incubation medium just prior to the addition of each pulse of $^{85}SrCl_2$ and 10 min after the last addition; the mitochondria were incubated for a total of 26 min. The aliquots were transferred into prechilled centrifuge tubes and immediately spun down at 20,000 × *g* for 4 min at 0°C. The supernatants were collected and counted for the residual $^{85}Sr^{++}$ in a crystal scintillator as in the single-pulse experiments; pellets were fixed for electron microscopy as described below.

In the multiple-pulsing of mitochondria for microincineration studies, in which fixation with only 12.5% glutaraldehyde was used, the same loading procedure was followed, but only 3 pulses of Sr^{++} were added to the incubation medium. The levels of Sr^{++} accumulated and retained in this procedure are shown in Table II.

Effect of Fixation Procedures on Retention of Labeled Sr^{++}

The finding that water-washed mitochondria are greatly altered structurally when fixed with OsO_4 (25) and the report that radioactive Mg^{++} is lost from loaded heart mitochondria during preparation for electron microscopy (12) emphasized the need to determine the amount of Sr^{++} lost in the present experiments during fixation. Mitochondria were loaded with $^{85}Sr^{++}$, as described above under Biochemical Methods, and fixed in suspension with a number of different fixatives as indicated in the following section (see Results). The mitochondria were centrifuged, and aliquots of the supernatant fluids were removed and counted for radioactivity. These procedures were used also to determine whether or not radioactivity was removed from the mitochondria during dehydration and infiltration.

Electron Microscopy

SINGLE-PULSE LOADING OF MITOCHONDRIA: At the end of the incubation time (see Fig. 1), mitochondria were rapidly sedimented, as described under Biochemical Methods, and the pellets were fixed for electron microscopy. Occasionally, the washed mitochondria were fixed in suspension, in which case equal volumes of washed mitochondrial suspension and fixative were mixed so that the resulting 1:2 dilution gave the same final concentration as that used to fix the pellets. Comparison of the two methods showed no differences in the quality of structural preservation or in the amount of radioactivity dis-

charged from the loaded mitochondria. Three methods of fixation were used in these experiments: 1% OsO_4 in Veronal-acetate buffer, pH 7.4; 6.25% glutaraldehyde in 0.01 M phosphate buffer, pH 7.4; and double fixation with 6.25% glutaraldehyde in 0.01 M phosphate buffer, pH 7.4, followed by postfixation with 1% OsO_4 in 0.01 M phosphate buffer, pH 7.4. Two controls were run simultaneously with test samples: mitochondria incubated in uptake medium lacking inorganic phosphate, and mitochondria showing resting respiratory rates, i.e., prior to the addition of Sr^{++}. When fixed as suspensions, mitochondria were centrifuged to give a pellet. All samples were dehydrated as pellets by rapid passage through a cold (−10°C) ethanol series and then embedded in Epon 812 according to the procedure of Luft (26). Thin sections were cut with glass knives on the Porter Blum or LKB microtome and collected, unsupported, on grids. Sections were stained with lead monoxide by Method B of Karnovsky (27) or with lead citrate (28) or were observed unstained.

MULTIPLE-PULSE LOADING OF MITOCHONDRIA: Samples were removed at the end of each uptake interval, the mitochondria were centrifuged, and aliquots of the supernatant fluid were removed and counted for radioactivity as described above. The mitochondria were immediately fixed for electron microscopy. Experiments were designed to follow the deposition of ions and localization of dense granules as small additions of $^{85}Sr^{++}$ were added to the uptake medium and were transported into the mitochondria. The amount of loading ranged from very low levels ("micro" loading) to intermediate levels of uptake. The amount of radioactivity accumulated and that retained by the mitochondria after fixation differ significantly (see Effect of fixation on retention of labeled Sr^{++} in Results). However, preparations fixed with OsO_4 and with glutaraldehyde were studied in detail in order to (*a*) compare the results with those of previous studies on the massive loading of Ca^{++} in which OsO_4 was used (11), and (*b*) determine whether localization of dense granules in OsO_4-fixed mitochondria, from which 50% of the Sr^{++} was lost during fixation, differs significantly from that in mitochondria fixed with glutaraldehyde. In the experiment using OsO_4 fixation, samples were removed after each of 7 successive pulses of $^{85}Sr^{++}$; and with glutaraldehyde fixation, after each of 3 levels of $^{85}Sr^{++}$ was accumulated.

MICROINCINERATION STUDIES: The methods were essentially those reported previously by Thomas (29) and by Thomas and Greenawalt (13). A thin film of silicon monoxide was evaporated onto formvar-covered, stainless steel grids. Thin sections of glutaraldehyde-fixed mitochondria loaded with three different levels of $^{85}Sr^{++}$ were cut and then collected on the coated grids. Specimens were incinerated in a muffle furnace at 600°C for 15 min, cooled, and then shadowed with platinum-palladium at an angle of about 20°. Low temperature incineration with excited oxygen (29) was not carried out in this study.

RESULTS

Effect of Fixation on Retention of Labeled Sr^{++}

The results of the experiment testing for the loss of $^{85}Sr^{++}$ during fixation are shown in Table I. Large amounts of radioactivity are lost with all of the methods of fixation tested. When no additions of succinate or ATP are made to the standard buffers, label is best retained in the loaded mitochondria fixed with cold 12.5% glutaraldehyde for 10 min, in which case about 75% of the label accumulated is retained. Micrographs indicate that good structural preservation of the mitochondria is obtained by this relatively short fixation time. Longer fixation times increase the amount of label lost. Postfixation with OsO_4 also removed additional amounts of label. The addition of ATP or ATP + substrate to the 12.5% glutaraldehyde fixative does not improve the retention of label in the loaded mitochondria. The addition of substrate alone does appear to aid in the retention of label. After fixation with 12.5% glutaraldehyde, mitochondria loaded with Ca^{++} retain 95% of the label taken up (unpublished observations). Insignificant amounts of radioactivity are found in the dehydration and infiltration solutions regardless of the fixatives used. The loss of label encountered during preparation for electron microscopy, which can be more than 50% of the label accumulated, occurs almost entirely during fixation and not during the subsequent steps.

Single-Pulse Loading of Mitochondria

BIOCHEMISTRY: Carafoli (30) has demonstrated that Sr^{++} ions stimulate respiration in either the presence or absence of added inorganic phosphate (P_i), but that P_i is required in order that much more than 10% of the added Sr^{++} be accumulated. In the present experiments the stimulation of respiration by Sr^{++} is shown in the polarographic tracing (Fig. 1). Inorganic phosphate was present in the reaction medium, and over 95% of the added Sr^{++} or about 0.304 μmole of $Sr^{++} \times mg^{-1}$ mitochondrial protein was accumulated. This corresponds to about the second level of accumulation effected in the multiple-pulse loading experiments (see below). Arrows in

the tracing (Fig. 1) indicate the sequence of additions of mitochondria and Sr^{++} and of the removal of samples for electron microscopy and the measurement of residual radioactivity. The rate of oxygen uptake returns to the prestimulated rate concomitant with the disappearance of added Sr^{++} from the uptake medium. The Sr^{++} and P_i are probably deposited as insoluble strontium phosphate salts to form the visible granules (11, 30).

ELECTRON MICROSCOPY: Fig. 2 shows the typical appearance of all OsO_4-fixed, control mitochondria used in these studies. No electron-opaque granules other than those normally occurring in the matrix of mitochondria can be seen. Some distortion in morphology is observed due to the tight packing of mitochondria in the pellet but, in general, the structural organization is that of rat-liver mitochondria isolated in 0.25 M sucrose. The occurrence of irregular profiles of the outer mitochondrial membranes and cristae which appear distended in some mitochondria varies from sample to sample and mitochondrion to mitochondrion.

A mitochondrion loaded with a single pulse of 0.320 μmole of $^{85}Sr^{++}$ × mg^{-1} protein and fixed with OsO_4 is shown in Fig. 3 at intermediate magnification. Results (Fig. 4, below) suggest that about one-half of the Sr^{++} was retained in the mitochondria after fixation. Numerous, large electron-opaque deposits which appear to be composed of smaller particles are clearly seen. Again, the mitochondria are tightly packed causing some distortion to the outline of the outer mitochondrial membranes and possibly to the cristae. The matrix is relatively dense, a fact which suggests little swelling (increase in diameter) or dilution of the matrix. In fact, no increase in the diameters of $^{85}Sr^{++}$-loaded mitochondria is observed. This observation is in accord with the findings of Caplan and Carafoli (32), Carafoli et al. (31), and Peachey (16) who found that Sr^{++} inhibits mitochondrial swelling and stabilizes the structure and biochemical activities of mitochondria. This is in contrast to the gross swelling which is seen when mitochondria accumulate large amounts of Ca^{++} (11). In this regard, it should be noted that the electron-opaque granules after Sr^{++} uptake are generally smaller and less compact than those found in mitochondria massively loaded with Ca^{++}. In addition, the distribution and size of the deposits of Sr^{++} observed here in the single-pulse experiments (Fig. 3) differ markedly from those in mitochondria loaded with Sr^{++} under multiple-pulse conditions (Fig. 6) even though the same amount of Sr^{++} has been accumulated (about 0.320 μmole of Sr^{++} × mg^{-1} protein). This ob-

TABLE I

Effect of Various Fixatives on the Retention of $^{85}Sr^{++}$ in Loaded Mitochondria

Fixative	Time of fixation	Amount of $^{85}Sr^{++}$ lost
	min at 0° C	*% of total taken up*
1. 1% OsO_4 in Veronal-acetate buffer, pH 7.4	3	50
2. 10% formaldehyde, (± succinate and ATP),* pH 7.4	30	40
3. 6.25% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4	3	25–35
4. 12.5% glutaraldehyde in 0.1 M PO_4 buffer, pH 7.4	10	25
5. 12.5% glutaraldehyde (as in No. 4) + OsO_4 in 0.1 M PO_4 buffer, pH 7.4	10 + 3‡	63
6. 1% OsO_4 (as in No. 1) + sucrose, pH 7.4	10	60
7. 12.5% glutaraldehyde (as in No. 4) + succinate,* pH 7.4	10	13
8. 12.5% glutaraldehyde (as in No. 4) + ATP,* pH 7.4	10	26
9. 12.5% glutaraldehyde (as in No. 4) + succinate + ATP,* pH 7.4	10	28
10. 1% OsO_4, aqueous in uptake medium (see text)	No fixation§	—

Effect of various fixatives on retention of radioactivity after accumulation of $^{85}Sr^{++}$ in single-pulse experiments. Measurement of accumulation and retention of radioactivity were made as described in Materials and Methods.

* Succinate and ATP in concentrations used in uptake medium (see text).

‡ 10 min in glutaraldehyde followed by 3 min in OsO_4.

§ Addition of aqueous OsO_4 to uptake medium resulted in immediate discoloration of fixative.

servation may be of considerable significance in relation to the mechanism and site of action of granule formation (see Discussion).

Multiple-Pulse Loading of Mitochondria

BIOCHEMISTRY: As already indicated, about 50% of the label taken up by mitochondria during loading is lost during fixation with OsO_4. This is true at all levels of uptake tested even when $^{85}Sr^{++}$ is spontaneously released during incubation (Fig. 4 *a*). In this study the amount of label remaining after fixation ranged from 0.08 μmole of $Sr^{++} \times mg^{-1}$ protein to about 0.3 μmole of $Sr^{++} \times mg^{-1}$ protein, or from "micro" levels to intermediate levels of loading. The nearly constant proportion of label released due to OsO_4-fixation is interesting and could mean that about one-half of the $^{85}Sr^{++}$ is more tightly bound than the rest; it cannot be presumed, however, that some of the Sr^{++} is nonspecifically precipitated within the mitochondria and is therefore necessarily more easily liberated than bound ions. It does not seem likely, in view of the well preserved structure of Sr^{++}-loaded mitochondria, that the loss of 50% of the radioactivity is due to gross destruction, especially at the lower levels of accumulation.

Fig. 4 *a* shows that the amount of Sr^{++} accumulated increases rapidly until about 0.6 μmole of $Sr^{++} \times mg^{-1}$ protein has been accumulated. Under these experimental conditions this level of uptake has not occurred until 6 min after the addition of the first pulse. It should be noted that in 2 min about 0.320 μmole of $Sr^{++} \times mg^{-1}$ protein has been taken up and that about the same amount of Sr^{++} is accumulated by single-pulsing in about one-half this time. A comparison of Fig. 3 with Figs. 6 and 7 shows that the dense deposits which are detected under multiple-loading conditions are much smaller and fewer in number than when the same amount of Sr^{++} is accumulated as a single pulse. This suggests that, when Sr^{++} is taken up as relatively small pulses over longer time intervals, it is distributed more widely throughout the mitochondria. This is suggested also by the very large number of small deposits observed at higher levels of uptake.

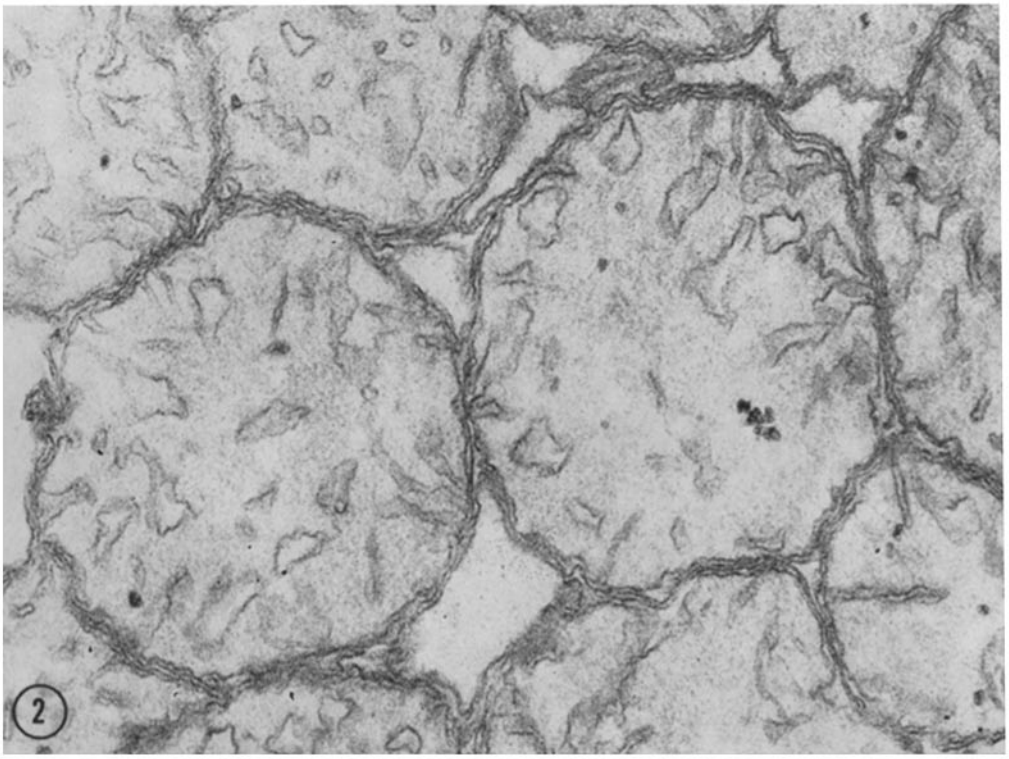
Fig. 4 *b* shows that more than 95% of the Sr^{++} added to the uptake medium during the first 3 pulses is accumulated. At the fourth level of uptake, however, the percentage of Sr^{++} accumulated decreases markedly and continues to drop even though the amount of $Sr^{++} \times mg^{-1}$ protein taken up increases and remains at a maximum (ca. 0.6 μmole of $Sr^{++} \times mg^{-1}$ protein) during the next two uptake intervals. This indicates that at the fourth and subsequent levels a significant fraction of the added Sr^{++} is not accumulated by the mitochondria. It is of interest that the needlelike deposits shown in Figs. 12 and 13 are observed only after this maximum level of uptake is achieved.

ELECTRON MICROSCOPY: Samples taken at each point of the curve (Fig. 4 *a*) were fixed with OsO_4 and examined in the electron microscope. The appearance of control mitochondria taking up no Sr^{++}, the first point on the curve, has been described in Fig. 2. No significant difference can be seen between the control mitochondria removed at "zero" time (Fig. 2) and mitochondria accumulating Sr^{++} during the first uptake interval (Fig. 5), although the cristae in the latter figure do not appear distended. No electron-opaque granules other than the normal granules of the matrix can be seen.

Large electron-opaque granules are not present at the second level of uptake either (0.315 μmole

FIGURE 2 Control mitochondria. Fixed with 1% OsO_4 (see text, Table I) prior to stimulation of respiration by addition of Sr^{++} as shown in Fig. 1. Structural features are typical of all control mitochondria observed in this study. The distended appearance of cristae is not constant and varies from mitochondrion to mitochondrion within the same sample. Only dense granules normally present in mitochondrial matrix are seen. Stained with lead. × 65,000.

FIGURE 3 Single-pulse experiment: mitochondrion fixed with 1% OsO_4 after accumulation of Sr^{++}. Respiration has returned to resting rate. About 30 electron-opaque granules can be seen; some are deposited on or near cristae. The larger granules appear to be composed of smaller particles. Stained with lead. × 110,000.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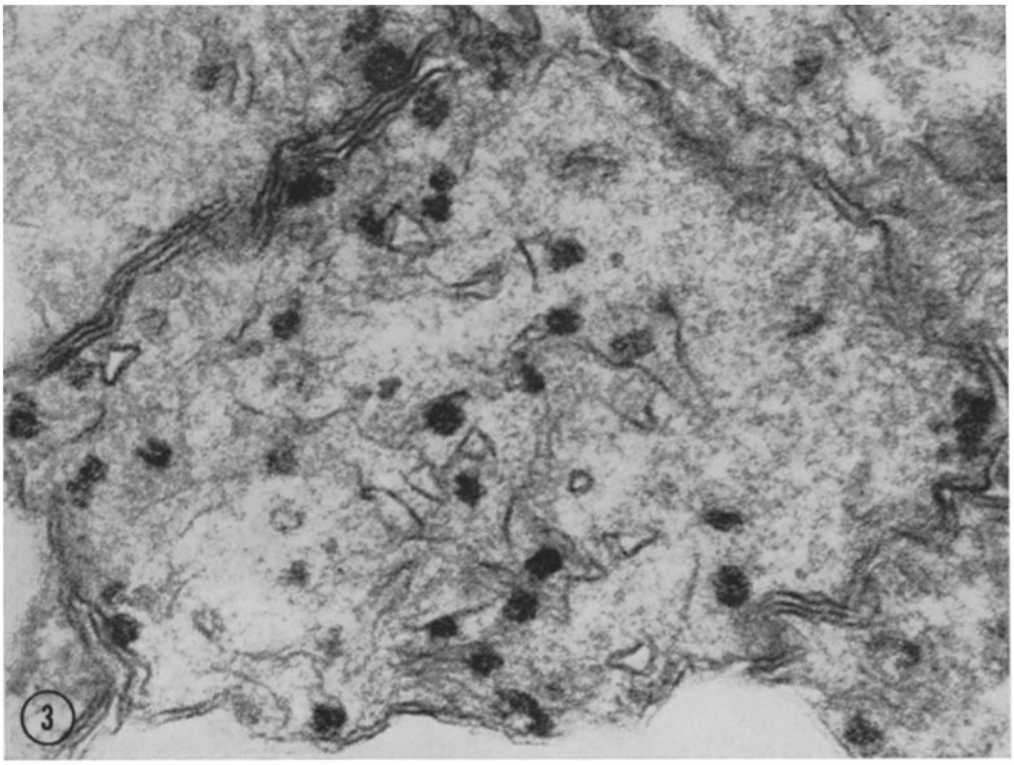
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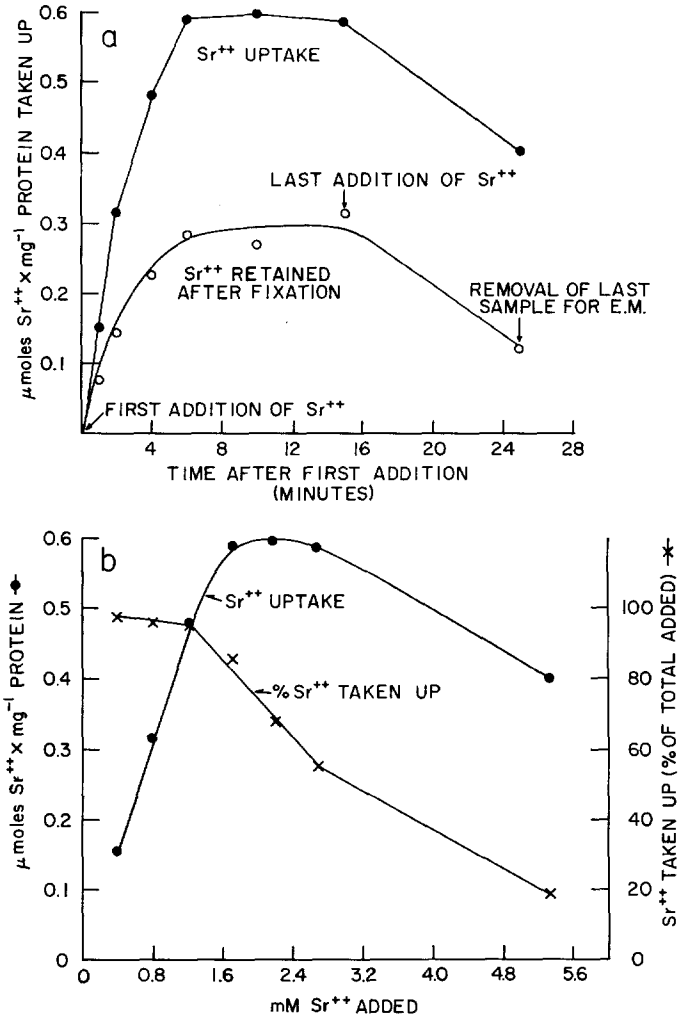
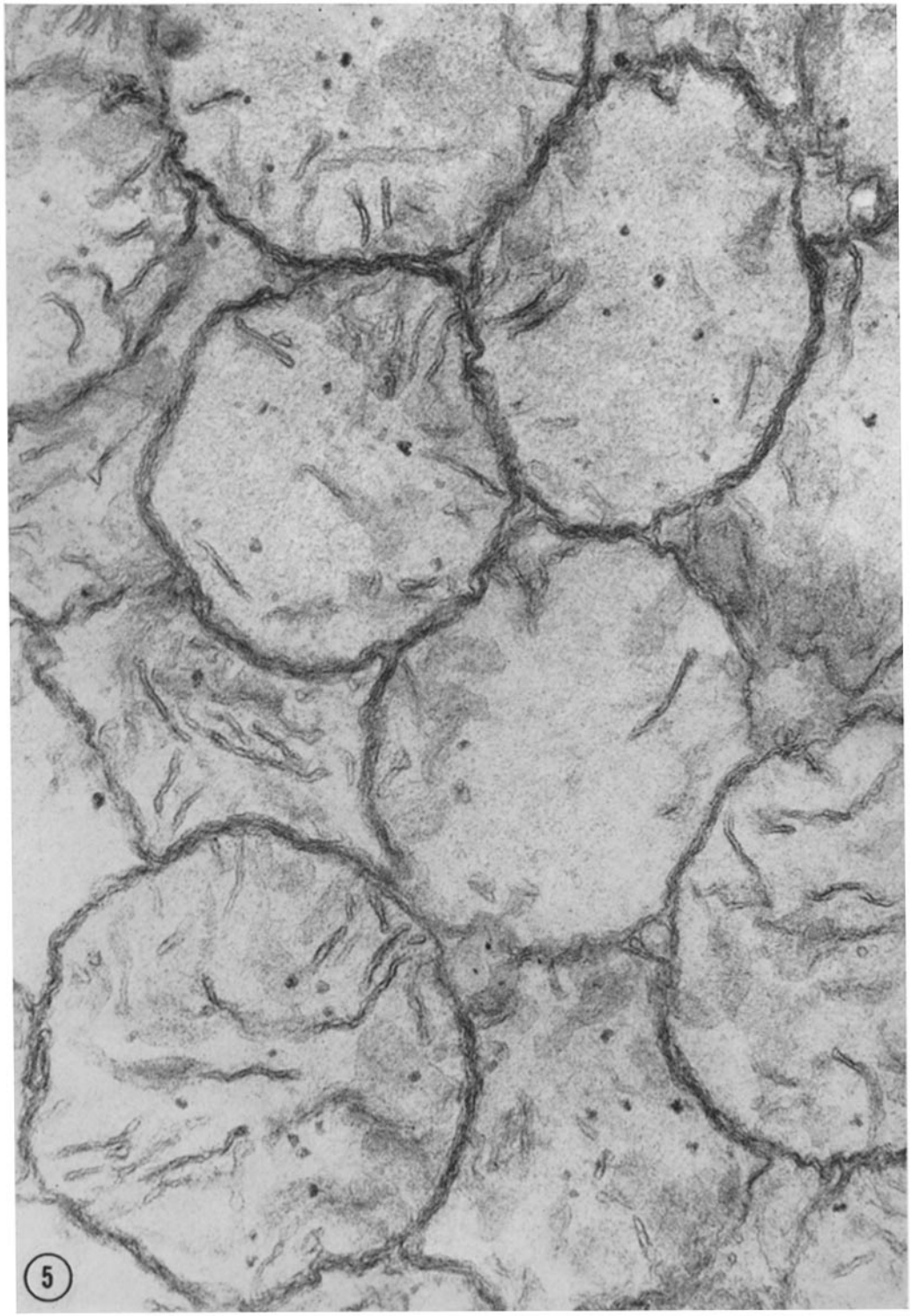


FIGURE 4 Accumulation of Sr^{++} during multiple-pulse loading and effect of OsO_4 on retention of $^{85}Sr^{++}$. For details, see Materials and Methods.

FIGURE 4 *a* The μmoles of Sr^{++} taken up and the amount of Sr^{++} retained per mg mitochondrial protein after fixation of the mitochondria with OsO_4 are given as a function of time.

FIGURE 4 *b* The amount of Sr^{++} accumulated per mg mitochondrial protein and the percentage of the added Sr^{++} accumulated are plotted as a function of Sr^{++} concentration (see Materials and Methods).

FIGURE 5 Multiple-pulse loading of mitochondria: level 1. Mitochondria fixed with 1% OsO_4 60 sec after addition of first pulse of $^{85}Sr^{++}$; about 0.15 μmole of $Sr^{++} \times mg^{-1}$ protein was taken up and 0.075 μmole of $Sr^{++} \times mg^{-1}$ protein was retained after fixation (see Fig. 4 *a*). Tight packing has caused some distortion of mitochondria, but structures in general are normal. No abnormal electron-opaque deposits can be seen. Stained with lead. × 65,000.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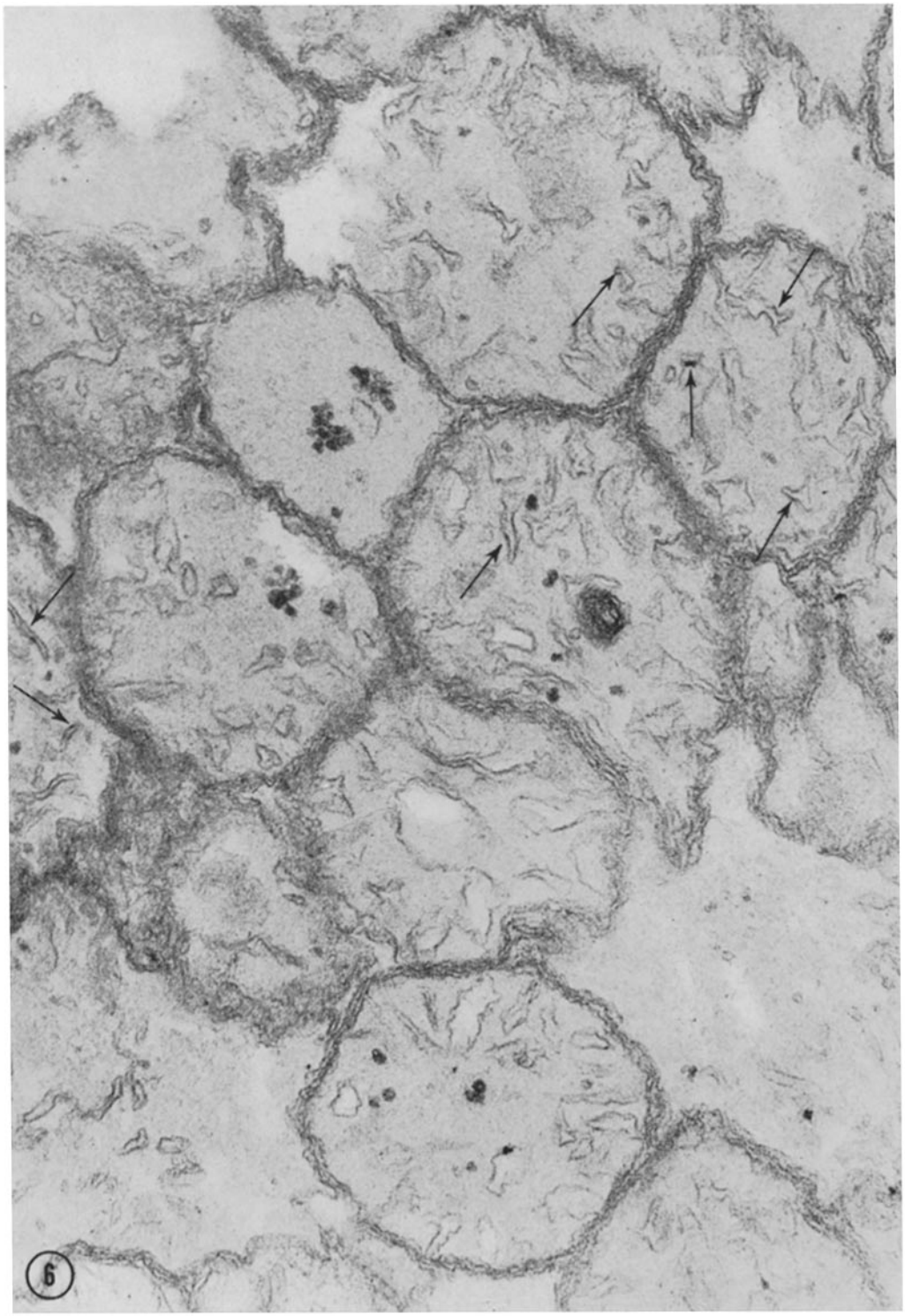


FIGURE 6 Multiple-pulse loading: level 2. Mitochondria have retained 0.14 μmole of $Sr^{++} \times mg^{-1}$ protein (uptake = 0.315 μmole of $Sr^{++} \times mg^{-1}$ protein). Close inspection shows small opaque deposits associated with cristae (arrows, cf. Fig. 3). Fixed with 1% OsO_4; stained with lead. × 65,000.

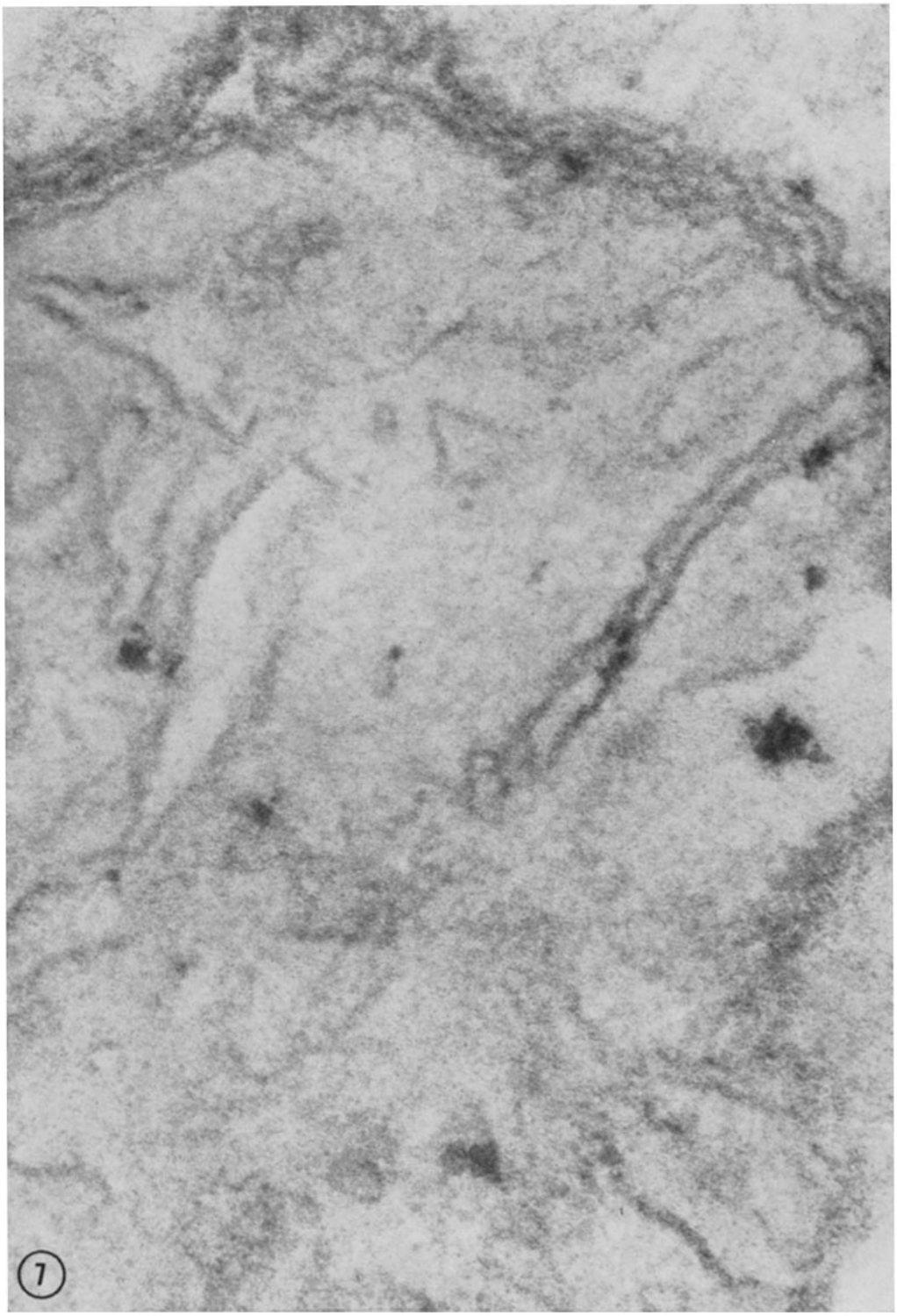


FIGURE 7 Multiple-pulse loading: same level of Sr^{++} as in Fig. 6. At this magnification, association of electron-opaque deposits with cristae is readily seen. Fixed with 1% OsO_4; stained with lead. × 260,000.

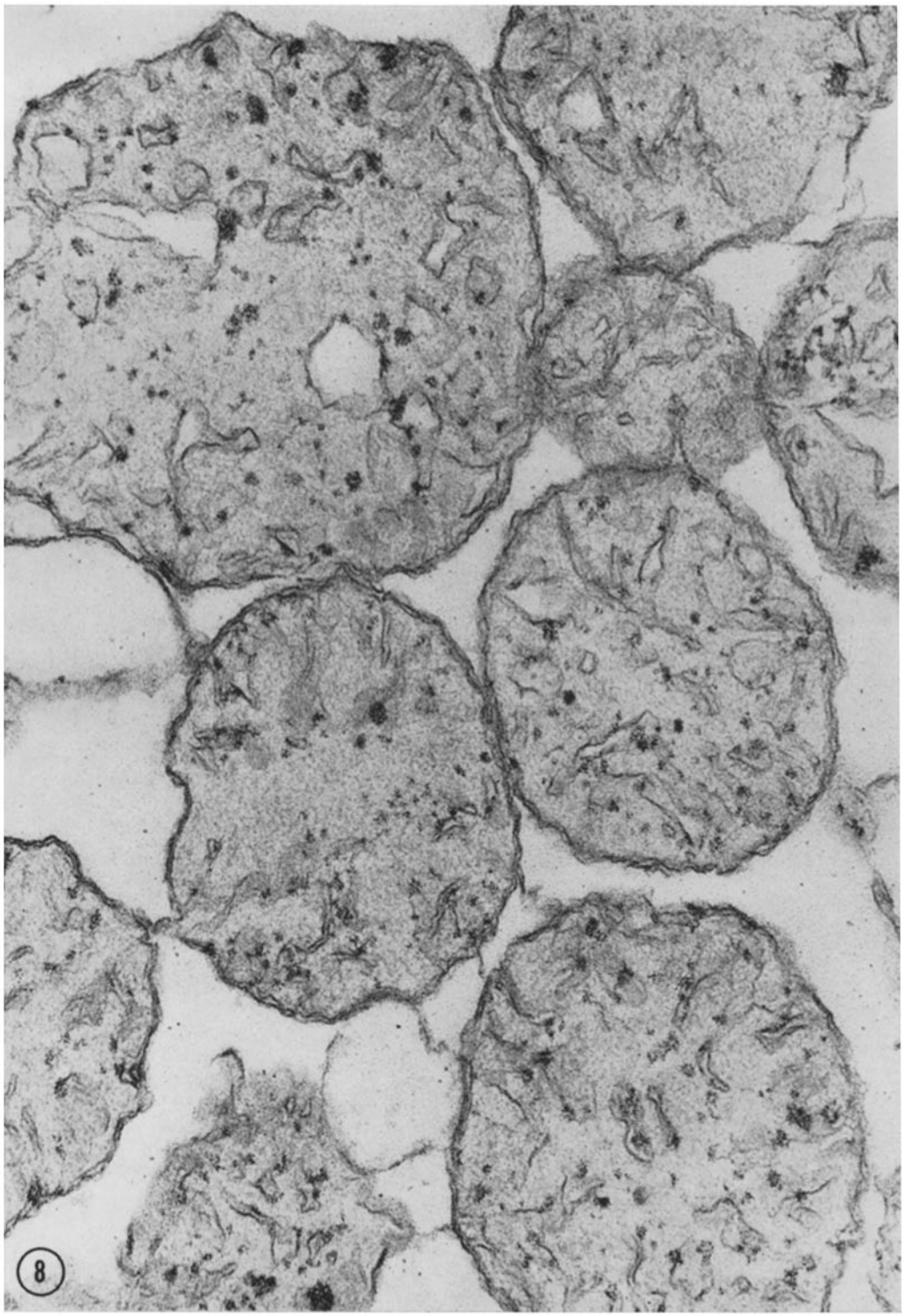


FIGURE 8 Multiple-pulse loading: 3rd level of Sr^{++} accumulation (0.475 μmole of $Sr^{++} \times mg^{-1}$ protein). Large numbers of electron-opaque deposits can be observed; 100 or more deposits are visible in a single section of some mitochondria. Many are associated with cristae. Fixed with OsO_4; stained with lead. × 65,000.

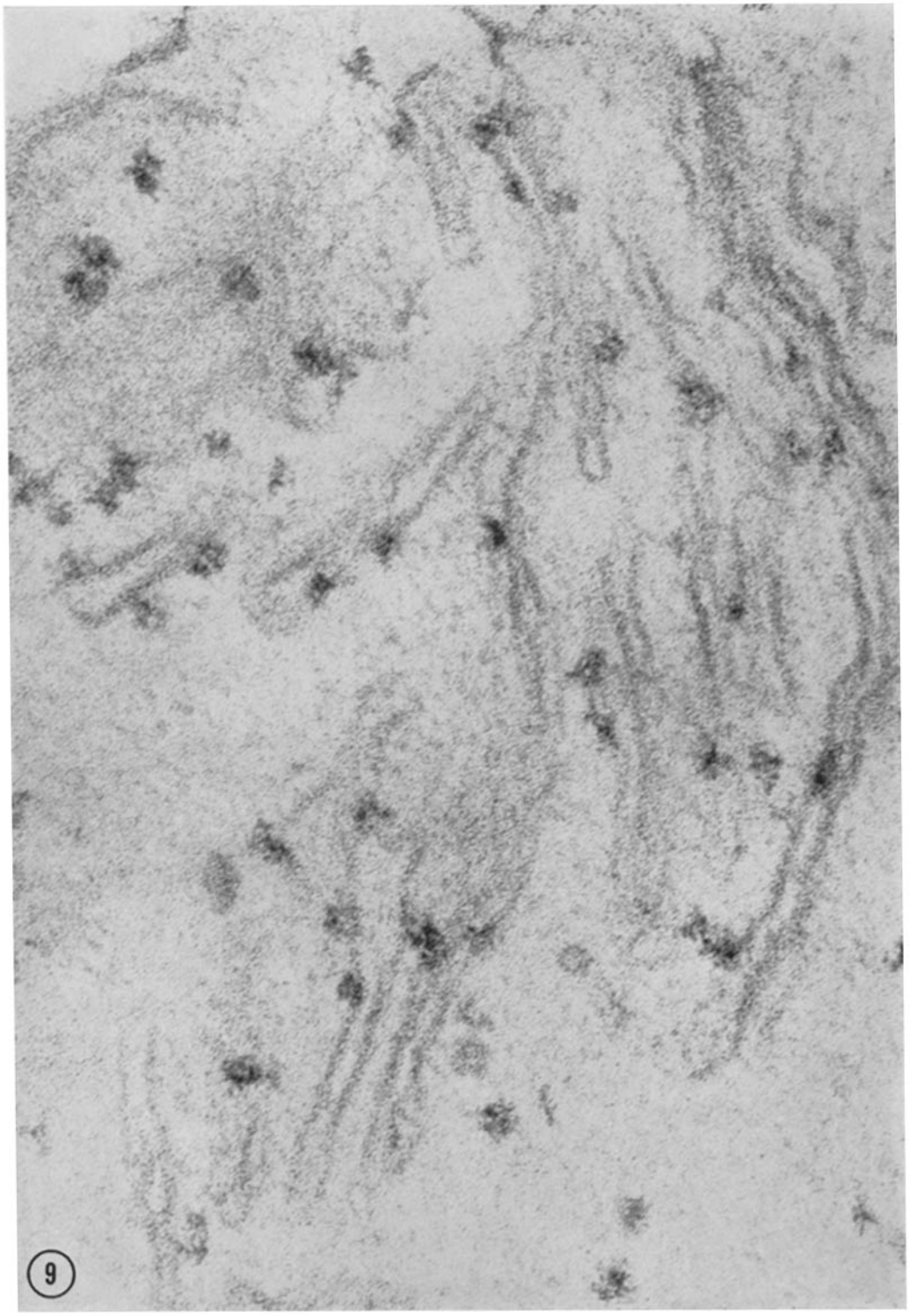


FIGURE 9 Multiple-pulse loading: as in Fig. 8. Distribution of granules proximal to cristae is shown. Fixed with 1% OsO_4; stained with lead. × 260,000.

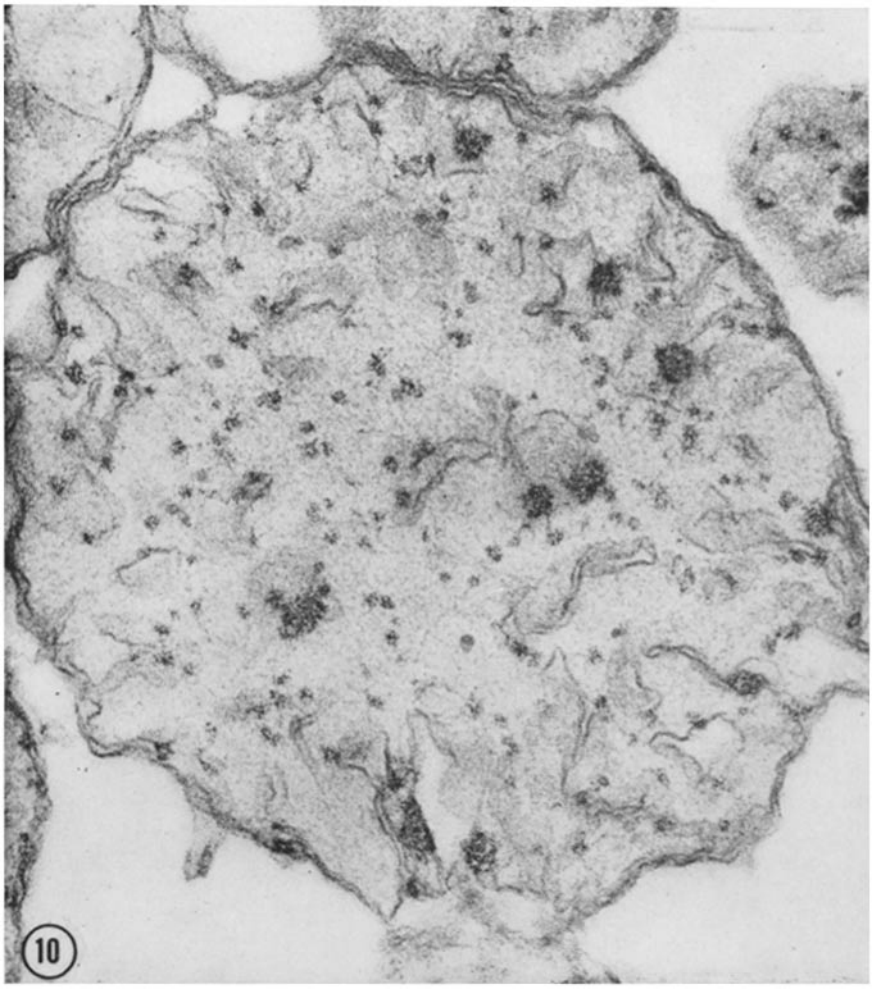


FIGURE 10 Multiple-pulse loading: level 3 (0.475 μmole of $Sr^{++} \times mg^{-1}$ mitochondrial protein). Wide range in size of granules is evident. Larger granules are about 700 A in diameter, and smaller ones about 150 to 200 A. Fixed with OsO_4; lead stained. × 80,000.

of $Sr^{++} \times mg^{-1}$ protein taken up and 0.14 μmole of $Sr^{++} \times mg^{-1}$ protein retained); however, definite although minute deposits are now seen in association with the cristae (see Fig. 6). The electron-opaque deposits associated with the cristae are prominent at higher magnifications (Fig. 7, arrows) but differ greatly in size, number, and distribution from the deposits observed when this same amount of Sr^{++} is taken up as a single pulse (Fig. 3).

At the level of 0.475 μmole of $Sr^{++} \times mg^{-1}$ protein a great increase in the number of electron-opaque deposits, many of which are obviously unassociated with the normal matrix granules, can be seen (Fig. 8). In a single section some mitochondria contain over 100 granules of varying sizes; at this level of uptake numerous granules are clearly associated with the cristae. The granules do not seem to be randomly deposited since large areas of the matrix do not have deposits and in some cases opaque granules seem to be arranged in a more or less linear array along the cristae (see Fig. 9). The granules vary greatly in size, but the majority are about 150 to 200 A in diameter and the largest have a diameter of about 700 A (Fig. 10). The largest accumulations of Sr^{++} noted in this study measure about $\frac{1}{4}$ to $\frac{1}{5}$ the size of the largest deposits seen in massive Ca^{++} uptake experiments (11).

As was seen in Fig. 4, no net accumulation of

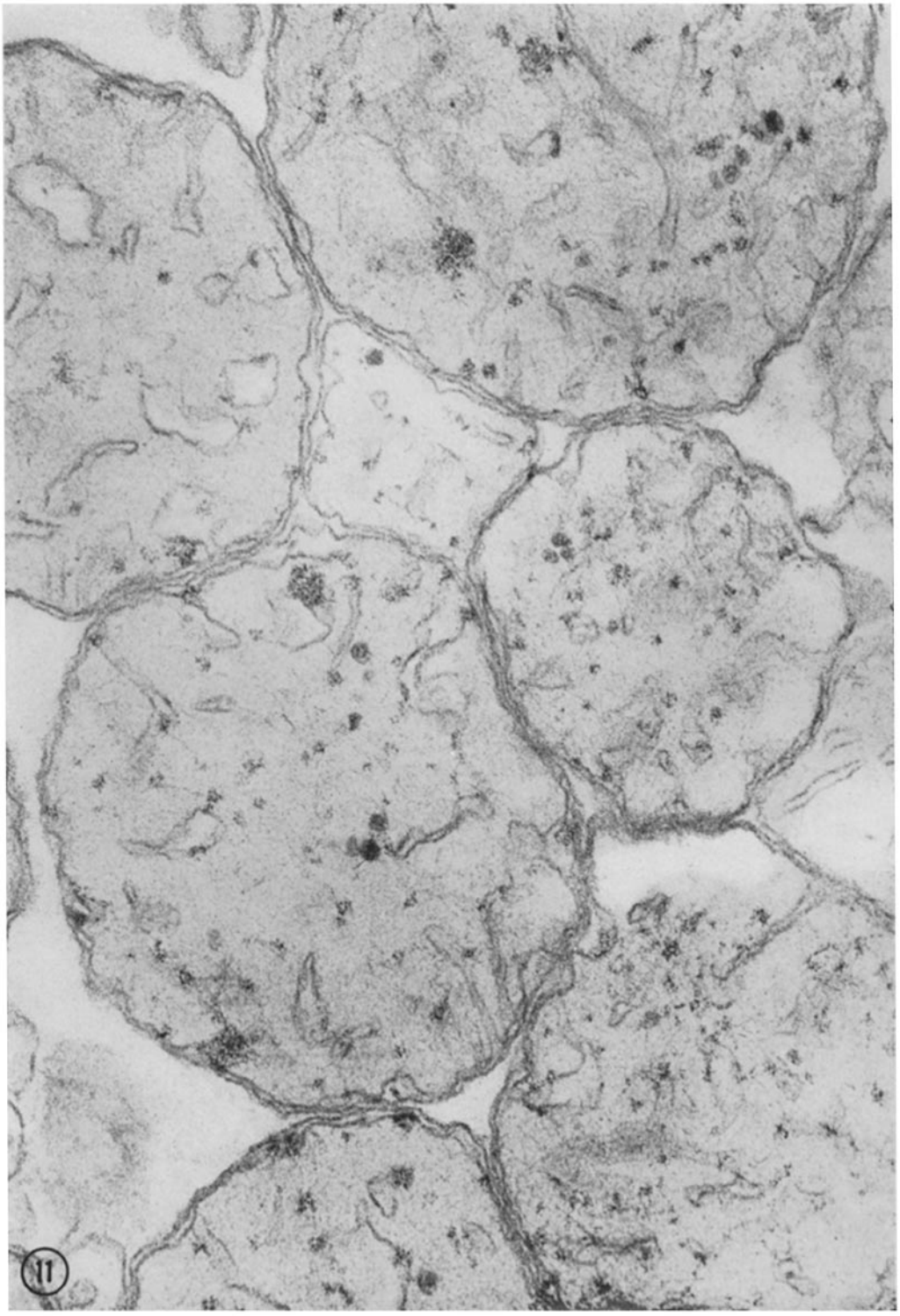


FIGURE 11 Multiple-pulse loading: level 4 (0.590 μmole of $Sr^{++} \times mg^{-1}$ protein). No further net uptake of Sr^{++} above this level occurred; this micrograph is representative of the last four points on the accumulation curve (see Fig. 4 *a* and *b*). The number of large granules appears to be maximal. Fixed with OsO_4; lead stained. × 97,500.

label occurs after the fourth addition of $^{85}Sr^{++}$ when about 0.6 μmole of $Sr^{++} \times mg^{-1}$ protein is accumulated. In fact, Sr^{++} was spontaneously lost from the mitochondria during the interval between the seventh and eighth samples. This is possibly due to the absence of Mg^{++} from the incubation medium; Carafoli et al. (31) have shown that Mg^{++} is not required for the rapid uptake of limited amounts of Sr^{++}, but that it greatly stimulates the slower accumulation of larger amounts of this ion. A few structurally damaged mitochondria are seen at higher levels of uptake, and this may also contribute to the poor retention of Sr^{++}. In this regard, it should be borne in mind that the mitochondria had been incubated for 26 min at the time of the removal of the last sample. Mitochondria taken from the fourth through the seventh levels of added strontium are qualitatively very similar in appearance; this might be expected since the amount of Sr^{++} accumulated becomes stationary at this point in the loading curve (Fig. 4). The tremendous numbers of granules seen in individual mitochondria at these levels make attempts to quantitate any differences in number impractical.

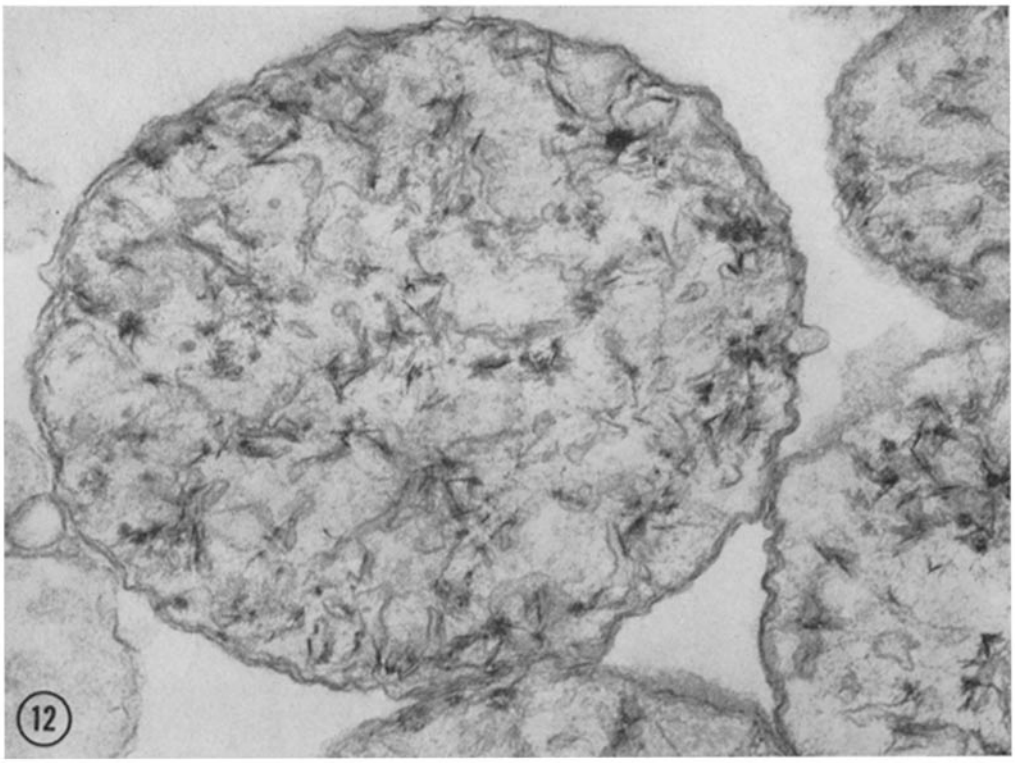
Samples from these last four points on the curve are discussed largely as a single sample. In these mitochondria, as in mitochondria from sample 4, many granules are observed which range widely in size but are still much smaller than those seen in mitochondria massively loaded with Ca^{++} (cf. reference 11). Many of these deposits also show clear association with the cristae (see Fig. 11). A most remarkable difference in the appearance of some of the electron-opaque deposits in mitochondria taken from these later points on the curve vs. those from lower levels of accumulation is the needlelike deposits observed. These are seen n many mitochondria at all levels above 0.48 μmole of $Sr^{++} \times mg^{-1}$ protein taken up, and also at the last point on the uptake curve in which spontaneous discharge has occurred. The needlelike deposits are shown at intermediate magnification in Fig. 12. Not all of the electron-opaque granules at these levels have this crystal-like appearance, and some mitochondria containing granules have none of these peculiar deposits. Such long, thin needles also are frequently associated with cristae as shown at higher magnification in Fig. 13, but their shape makes it difficult to localize them precisely. Fig. 14 shows that these needlelike deposits are present in many mitochondria taken from the last sample from which spontaneous discharge of Sr^{++} has occurred.

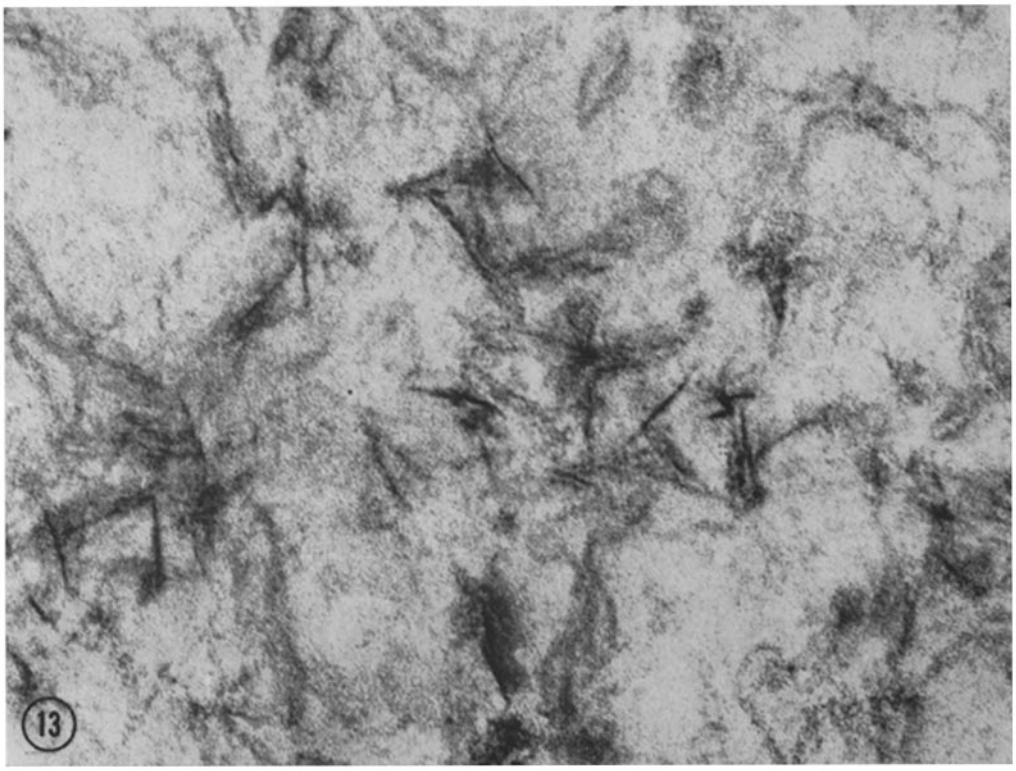
These crystalline-appearing deposits occur in mitochondria which have undergone multiple-pulse loading at intermediate levels of Sr^{++} uptake but have not been seen in mitochondria loaded by a single pulse nor in mitochondria accumulating smaller amounts of Sr^{++} (<0.6 μmole of $Sr^{++} \times mg^{-1}$ protein). This suggests that these needles occur as a result of the gradual deposition of strontium salts after a certain minimum concentration of ions has been transported into the mitochondria.

It seemed possible that displacement by OsO_4 or the deposition of osmium or lead during fixation or staining might have created these unusual deposits. Fig. 15 clearly shows, however, that long electron-opaque deposits are present in glutaraldehyde-fixed mitochondria unexposed to OsO_4 or to lead. It is of interest that in these mitochondria the elongated deposits appear more fibrous than needlelike. This is shown at higher magnification in Fig. 16. The mitochondrial preparation shown in this figure accumulated 0.745 μmole of $Sr^{++} \times mg^{-1}$ protein and retained 75% of it after glutaraldehyde-fixation (level No. 3, Table II). Fixation

FIGURE 12 Multiple-pulse loading of mitochondria. Electron-opaque, needlelike deposits are present in mitochondria from each of the last four points on the uptake curve (see Fig. 4 *a* and *b*). The shape of these deposits makes it difficult to assess the degree of association with the cristae but many appear proximal to the cristae. Fixed with OsO_4; stained with lead. × 65,000.

FIGURE 13 Multiple-pulse loading: same as in Fig. 12, but at higher magnification. Deposits are clearly needlelike and measure from about 200 to 900 A long. Fixed with OsO_4; lead stained. × 195,000.





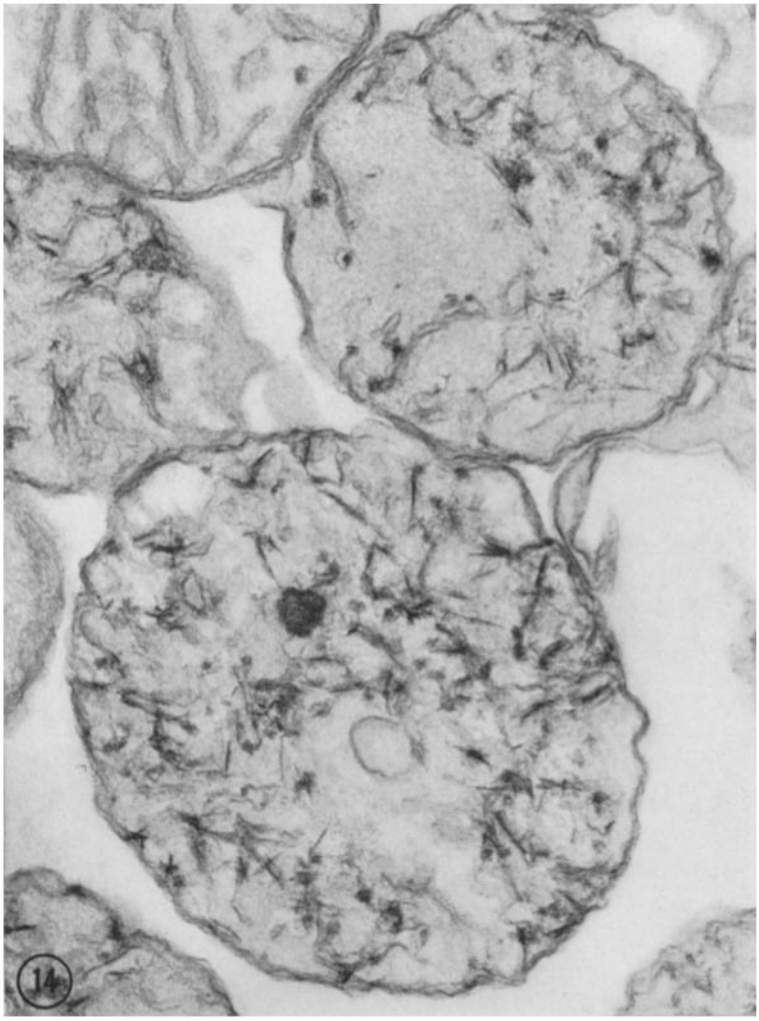
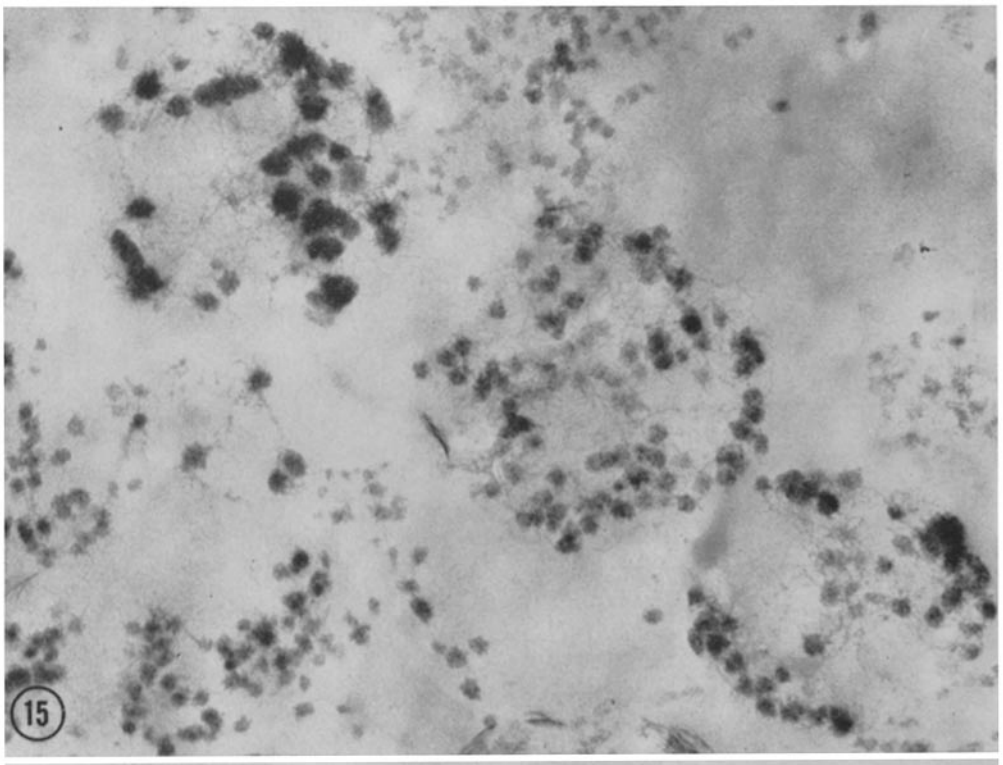


FIGURE 14 Multiple-pulse loading of mitochondria. Sample from eighth point on the curve. Needlelike deposits are present despite the spontaneous discharge of Sr^{++} from this sample. Fixed with OsO_4; stained with lead. × 65,000.

with 12.5% glutaraldehyde extracts about ½ the amount of Sr^{++} removed by OsO_4 fixation (Tables I and II), but the contrast of the mitochondria is inherently low. These long, fibrous deposits in glutaraldehyde-fixed preparations probably correspond to the needles seen in OsO_4-fixed samples and indicate that their presence is not dependent on the deposition of either osmium or lead.

FIGURE 15 Multiple-pulse loading of mitochondria: microincineration studies. Control mitochondria. Large and small deposits are present; much fibrous or threadlike electron-opaque material is visible, probably corresponding to needlelike deposits in OsO_4-fixed, lead-stained mitochondria (Figs. 12 to 14). Fixed with 12.5% glutaraldehyde; no poststain. × 65,000.

FIGURE 16 Same as Fig. 15 but at higher magnification. Granules and fibrous "whiskers" are shown within a single mitochondrion. Fixed with 12.5% glutaraldehyde; no staining. × 130,000.



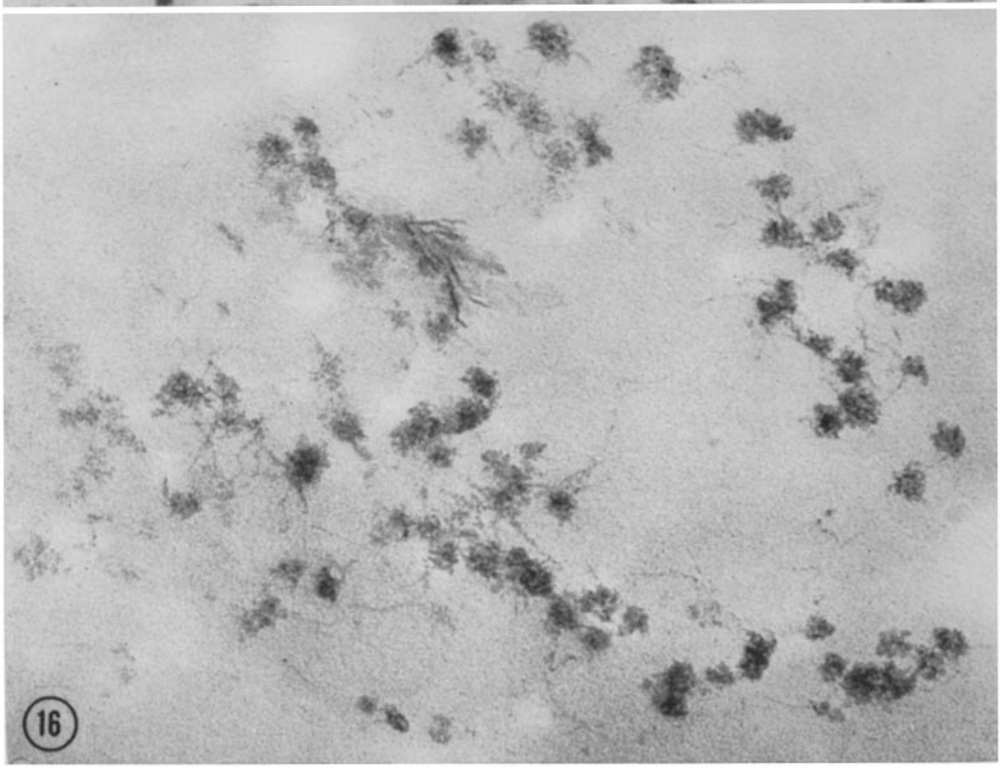


TABLE II

Multiple-Pulse Loading of Mitochondria: Microincineration Studies. Retention of $^{85}Sr^{++}$ after Glutaraldehyde Fixation

	A	B	C
	Amount of Sr^{++} accumulated	Amount of Sr^{++} retained after fixation with glutaraldehyde	Amount of Sr^{++} retained
	μmole of Sr^{++} × g^{-1} protein	*μmole of Sr^{++} × mg^{-1} protein*	*B/A × 100%*
Level 1	0.298	0.205	68.6
Level 2	0.596	0.428	72.3
Level 3	0.745	0.552	75.4

Multiple-pulse loading of mitochondria: microincineration studies showing levels of Sr^{++} accumulation and retention of radioactivity after fixation with 12.5% glutaraldehyde. The amount of radioactivity accumulated and retained was measured as described in Materials and Methods. Fixation as described in Table I (No. 4).

Microincineration Studies

Incineration of thin sections of Sr^{++}-loaded mitochondria fixed with 12.5% glutaraldehyde indicates that the electron-opaque deposits consist primarily of inorganic material, probably salts of strontium and phosphate (31). About 70 to 75% of the $^{85}Sr^{++}$ incorporated during uptake is retained in the mitochondria fixed with 12.5% glutaraldehyde for microincineration studies (Table II). This is in good agreement with the data given in Table I, which compares the effects of various fixatives on retention of Sr^{++}. Neither fixation with OsO_4 nor staining with lead was used in these experiments.

The appearance of electron-opaque deposits in glutaraldehyde-fixed mitochondria prior to incineration at 600° has been illustrated in Figs. 15 and 16. The mitochondria have retained 0.552 μmole of Sr^{++} × mg^{-1} protein (Table II), which is 80% greater than the highest level retained in the multiple-pulse loading experiments using OsO_4 fixation (Fig. 4). This may account in part for the appearance of some of the electron-opaque deposits in the glutaraldehyde-fixed mitochondria which resemble "whiskers" of mineral deposits. In some sections of the mitochondria large transparent holes were seen, presumably where deposits of salts had been. Upon close inspection it can be seen also that many of the granules themselves have "hollow" centers. It is thought that these effects are not the result of extraction of Sr^{++} by the fixative but that the larger holes, at least, occur during cutting of the specimen and are due to the extreme hardness and relatively poor infiltration of the deposits which allow them to fall out of the sections. This problem is largely eliminated when thick sections are cut. In addition, this effect is not seen in OsO_4-fixed preparations. It is possible, however, that some extraction does occur as the thin sections float on the trough prior to being picked up on the grids.

Figs. 17 to 19 are micrographs of incinerated sections of Sr^{++}-loaded, glutaraldehyde-fixed mitochondria which have retained 0.205, 0.428, and 0.552 μmole of Sr^{++} × mg^{-1} protein, respectively. Small, spherical, or nearly spherical granules remain after incineration although the mitochondria themselves are no longer visible; this indicates the mineral nature of these remnants. Comparison of Figs. 17, 18, and 19 shows that the number and size of the deposits increase with increasing uptake of Sr^{++}. At the lower levels of accumulation (Figs. 17 and 18) the concentration of granules is so low that it is difficult to know where the mitochondria had been; at the third level (Fig. 19), however, outlines of individual mitochondria are fairly well defined by the high concentration of mineral deposits remaining. Great significance should not be placed in the difference in size and precise number of the residues, since it is thought that high temperature incineration causes considerable coalescence of mineral deposits (13).

DISCUSSION

Sr^{++} offers some advantages over Ca^{++} in the study of active ion transport and accumulation by isolated rat-liver mitochondria. Like Ca^{++}, this ion is readily accumulated in vitro by these mitochondria, but, unlike Ca^{++}, Sr^{++}, once accumulated, does not grossly damage mitochondrial structures. Carafoli (30) has shown that rat-liver mitochondria containing as much as 0.3 to 0.4 μmole of Sr^{++} × mg^{-1} protein exhibit normal P/O ratios and normal respiratory control. Sr^{++} has the additional advantage of having a greater atomic number than Ca^{++} and is therefore more electron-scattering. Hence, it would be expected that the accumulation and deposition of small amounts of Sr^{++} would be visualized more readily in the electron microscope than would the deposition of equivalent amounts of Ca^{++}. Thus, the

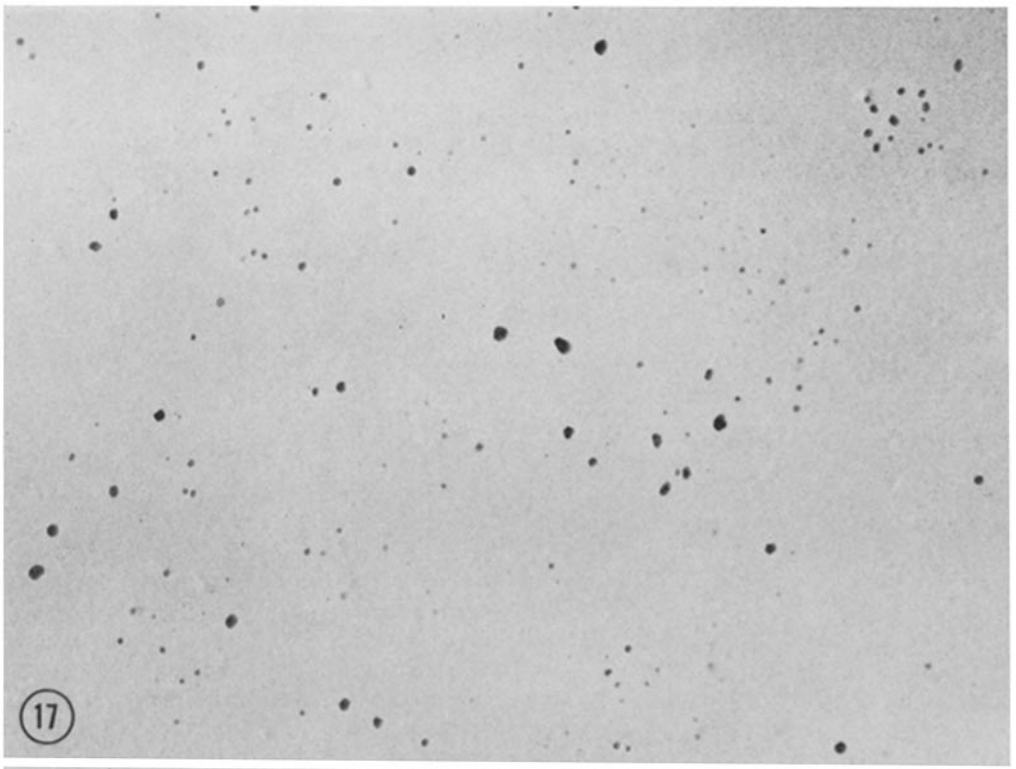
accumulation of Sr^{++} by isolated rat-liver mitochondria may prove a valuable model system for the study of ion uptake even though some differences in the mechanism of accumulation of different ions have been noted (31).

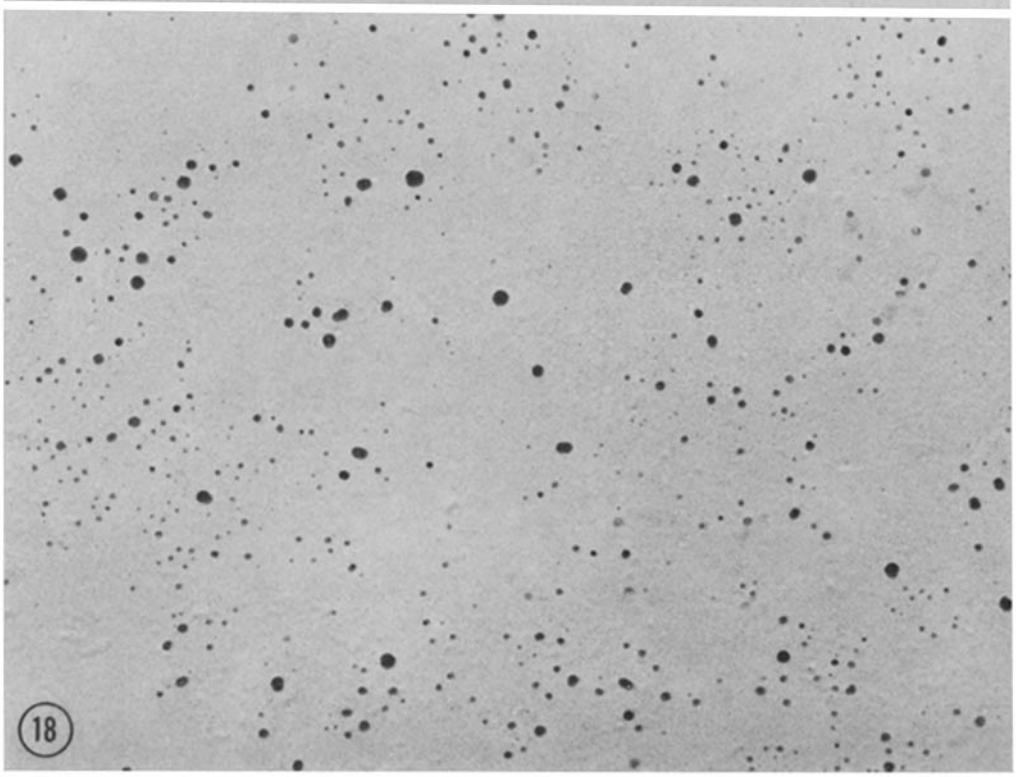
Recent studies have shown that the energy-dependent accumulation of large amounts of Ca^{++} by isolated mitochondria is accompanied by the formation of large electron-opaque granules within the mitochondria, indicating that the ions are actually translocated across the mitochondrial membranes (11, 12). The present report shows that the accumulation of Sr^{++} by isolated rat-liver mitochondria also results in the formation of electron-opaque deposits (Figs. 3, and 8 to 11) and that a clear, qualitative correlation exists between the amount of Sr^{++} accumulated and the amount of inorganic material (probably $Sr_3(PO_4)_2$) remaining after high temperature microincineration (Figs. 17 to 19).

The intactness of strontium-loaded mitochondria contrasts markedly with the structural disorganization caused by the uptake of large amounts of Ca^{++} (cf. Figs. 2 and 11, and reference 11). Little swelling, i.e. increase in diameter, can be detected in mitochondria taking up Sr^{++} at the levels studied here; in fact, the diameters of mitochondria containing Sr^{++} are, in general, somewhat smaller than those of mitochondria isolated in 0.25 M sucrose. This may merely reflect the fact that 0.25 M sucrose is not optimal for structural preservation of rat-liver mitochondria (33), but it is also in agreement with the observation that Sr^{++} inhibits mitochondrial swelling (32). In the present study the cristae of control and loaded mitochondria frequently appeared distended (Figs. 2, 6, and 8), but this alteration is not a consistent feature of a given mitochondrial sample. In our preparations this aspect of mitochondrial structure varied from mitochondrion to mitochondrion in the same sample as well as in different samples (Fig. 8). The different profiles of cristae observed in our preparations may be a result of fixation with OsO_4, of the plane of section through individual mitochondria, or of actual differences in the respiratory state of mitochondria within the population (34). According to the polarographic tracing shown in Fig. 1, the mitochondria in both Figs. 2 and 3 are in a phase of "resting" respiration; obviously this measurement is statistical and cannot be applied directly to individual mitochondria. This variation in appearance of cristae does not appear to be related specifically to the level of Sr^{++} accumulated, since swollen cristae are seen in mitochondria freshly isolated in 0.25 M sucrose and in mitochondria containing small and large deposits of Sr^{++}.

The close and frequent association of calcium phosphate deposits with the cristae of mitochondria massively loaded with Ca^{++} noted in an earlier study (11) suggested that these ions might be bound, at least initially, to sites on the cristae. That study also showed that when mitochondria were loaded with increasing amounts of Ca^{++} the density of the mitochondria was correspondingly increased, a result which indicates that accumulation occurred, not as an "all or none" process, but by an increase in the load of Ca^{++} accumulated by the entire mitochondrial population. The massive accumulation of Ca^{++}, the huge dimensions of the granules, and the destruction of mitochondrial structure prevented an unequivocal demonstration that ion binding to membranes did, in fact, occur. This same circumstance is encountered to a lesser degree when mitochondria accumulate a large single-pulse of Sr^{++}. The deposits are large and the relationship between membranes and electron-opaque deposits is not always clear, although some granules are seen closely adjacent to cristae (see Fig. 3). As with the deposits found in Ca^{++}-loaded mitochondria, these large Sr^{++}-containing granules appear to be made up of smaller units. The accumulation of even reduced amounts of Sr^{++} as a single-pulse and the rapid formation of large granules could mask the initial binding of ions to membranes if, in fact, this occurred early in the accumulation process.

An attempt was made to "titrate" these binding sites by the procedure of multiple-pulse loading (see Methods). These experiments indicate that, at the earliest stages of Sr^{++} loading at which electron-opaque granules can be detected, granule formation does indeed occur in, on, or near the inner mitochondrial membranes (Figs. 6 and 7). The exact amount of Sr^{++} in a given mitochondrion cannot be estimated since differences within the population undoubtedly exist and loss of Sr^{++} occurs during OsO_4-fixation. However, about 0.315 μmole of $Sr^{++} \times mg^{-1}$ protein was accumulated, and after fixation about 0.14 μmole of $Sr \times mg^{-1}$ mitochondrial protein remained (Fig. 4 *a*). At lower levels of uptake (Fig. 5) no suggestion of deposition in association with the membranes or elsewhere can be detected; at the next higher level (0.475 μmole of $Sr^{++} \times mg^{-1}$ protein taken up) the number and size of granules





FIGURES 17 to 19 Multiple-pulse loading of mitochondria: microincineration studies. Sections of mitochondria, accumulating $^{85}Sr^{++}$ as 3 consecutive pulses. Incinerated at 600°C for 15 min. Details in text. Increasing accumulation and retention of Sr^{++} is accompaned by increasing number of inorganic deposits. Fixed with 12.5% glutaraldehyde; no stain. × 32,500.

FIGURE 17 Level 1: 0.205 μmole of $Sr^{++} \times mg^{-1}$ protein retained after fixation. Relatively few residues of inorganic deposits can be seen.

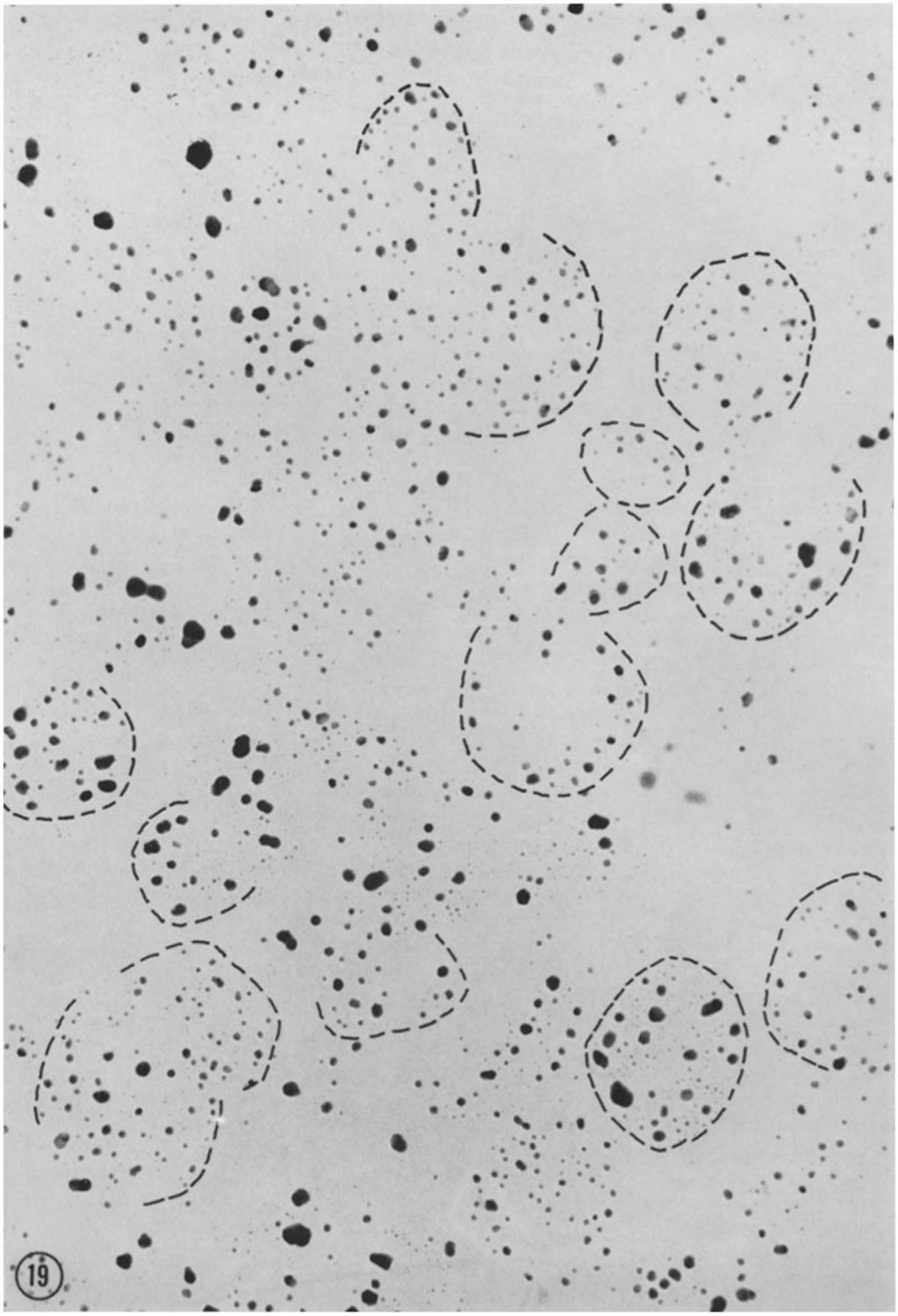


FIGURE 18 Level 2: 0.428 μmole of $Sr^{++} \times mg^{-1}$ protein retained. Comparison with Figs. 17 and 19 indicate intermediate numbers of residues remaining after incineration.

FIGURE 19 Level 3: 0.552 μmole of $Sr^{++} \times mg^{-1}$ mitochondrial protein retained. Many residues of varying sizes are seen. Increase in size may result in part from coalescence of smaller deposits. Outlines of mitochondria (indicated by dotted lines) can be visualized owing to the high concentration of deposits.

are increased so greatly that although a vast number of deposits are associated with membranes not all of the granules clearly show this association (Figs. 8 to 10). Therefore, electron-opaque deposits are first detectable in the electron microscope after OsO_4 fixation when mitochondria retain somewhere between 0.08 and 0.23 μmole of $Sr^{++} \times mg^{-1}$ protein; the preponderance of these granules is localized on or near the cristae.

The mechanism of granule formation has not been completely elucidated, but a possible sequence in the accumulation of Sr^{++} is suggested by the results of the multiple-pulse loading experiments reported here. It is proposed that under these conditions Sr^{++} is initially bound to sites uniformly distributed over the mitochondrial cristae, possibly by interaction with the phospholipids in these structures. Then, as additional Sr^{++} is translocated into the inner mitochondrial compartment, $Sr_3(PO_4)_2$ is formed at localized sites on the cristae. When the size and mineral concentration of the deposits are great enough, these can be detected as minute electron-opaque granules. Finally, as accumulation continues, the size and number of individual deposits are markedly increased (cf. Figs. 2, 6, and 8). Determining sequential events by electron microscopy is exceedingly difficult, but this proposal would account for the rapid uptake of "micro" amounts of Sr^{++} without visible deposits being formed and the exceedingly large number of small deposits observed as additional pulses of Sr^{++} are taken up. Granule formation could then occur anywhere on the cristae by accretion to these preformed nuclei of mineral, and deposition could be extended to exceedingly high levels as individual granules and more nuclei accumulated additional amounts of $Sr_3(PO_4)_2$. The localization of large numbers of granules in association with the cristae and the large areas of matrix which contain no deposits suggest that granules are not formed merely by nonspecific precipitation in the aqueous matrix as the solubility product of $Sr_3(PO_4)_2$ is exceeded. This reaction may play a role in the size and shape of the deposits, however, and could account for the differences in the distribution and size of granules in mitochondria loaded with Sr^{++} under multiple-pulse vs. single-pulse conditions. It is suggested that because of the rapid uptake of large amounts of Sr^{++} in the latter case, Sr^{++} is not evenly distributed in the mitochondria but deposited at relatively few sites with the formation of larger granules.

The present results do not support the contention that the normal matrix granules function in ion accumulation, although this possibility is not eliminated. It should be emphasized that inorganic phosphate is present in the uptake media used in the present studies. It is therefore possible that deposition is not identical to that reported by Peachey (16) who observed an increase in size and the density of staining of the normal matrix granules during ion accumulation in the absence of P_i. Evidence indicating that the normal "dense granules" per se are not essential for the formation of electron-opaque deposits accompanying active ion transport in isolated rat-liver mitochondria is summarized: (*a*) the normal dense granules are typically localized in the matrix and not associated with the cristae; (*b*) the number of Sr^{++}-containing deposits observed in the present study (as many as 200 per single thin section clearly exceeds the number of normal matrix granules); (*c*) water-washed mitochondria appear to lack normal matrix granules but accumulate large amounts of calcium and phosphate in the form of large electron-opaque deposits (17, 18); and (*d*) the normal matrix granules apparently are not extracted by OsO_4-fixation but, in fact, are increased in opacity by this fixative, whereas as much as 50% of accumulated $^{85}Sr^{++}$ is extracted by OsO_4-fixation. The present results provide evidence which implicates the cristae as sites of deposition, at least at certain stages of granule formation. At the earliest stages of deposition a clear association of granules with cristae is shown; large areas of the matrix of many mitochondria have no granules, a fact which indicates that random deposition does not occur. The interpretation presented here does not conflict with the view that nonspecific precipitation or aggregation may occur as well.

The presence of crystal like needles at the higher levels of multiple-pulse loading (Figs. 12 to 14) could be explained by the increase in size of the deposits by the accretion of salts around initial sites of binding at a rate sufficiently slow to permit needlelike growth to occur. The fact that needlelike deposits have been seen only in multiple-pulse loading suggests that the slow accumulation of small amounts of strontium phosphate at specific sites may be necessary before "crystallization" and needlelike formation can take place as additional amounts of Sr^{++} are accumulated. Whether or not these deposits are truly crystalline is not yet established; diffraction studies will be necessary to

determine this point. It seems likely that the needlelike deposits in OsO_4-fixed mitochondria and the fibrous material in glutaraldehyde-fixed samples represent different views of the same substances. The difference in appearance of these deposits in OsO_4-fixed vs. glutaraldehyde-fixed samples (Figs. 12 to 16) may be due in part to the difference in the amounts of Sr^{++} extracted by the two fixatives (Tables I and II) or to the enhancement of contrast due to the binding of osmium and/or lead. It is also possible that the fibrous materials seen in glutaraldehyde-fixed mitochondria are real structures partially masked in the OsO_4-fixed, lead-stained samples.

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