EFFECT OF ACTIVE ACCUMULATION OF CALCIUM AND PHOSPHATE IONS ON THE STRUCTURE OF RAT LIVER MITOCHONDRIA

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ABSTRACT

Rat liver mitochondria allowed to accumulate maximal amounts of Ca^{++} and $HPO_4^{=}$ ions from the suspending medium *in vitro* during respiration have a considerably higher specific gravity than normal mitochondria and may be easily separated from the latter by isopycnic centrifugation in density gradients of sucrose or cesium chloride. When the mitochondria are allowed to accumulate less than maximal amounts of Ca^{++} and $HPO_4^{=}$ from the medium, they have intermediate specific gravities which are roughly proportional to their content of calcium phosphate. Maximally "loaded" mitochondria are relatively homogeneous with respect to specific gravity. Correlated biochemical and electron microscopic studies show that Ca^{++}-loaded mitochondria contain numerous dense granules, of which some 85 per cent are over 500 A in diameter. These granules are electron-opaque not only following fixation and staining with heavy metal reagents, but also following fixation with formaldehyde, demonstrating that the characteristic granules in Ca^{++}-loaded mitochondria have intrinsic electron-opacity. The dense granules are almost always located within the inner compartment of the mitochondria and not in the space between the inner and outer membranes. They are frequently located at or near the cristae and they often show electron-transparent "cores." Such granules appear to be made up of clusters of smaller dense particles, but preliminary x-ray diffraction analysis and electron diffraction studies have revealed no evidence of crystallinity in the deposits. The electron-opaque granules decrease in number when the Ca^{++}-loaded mitochondria are incubated with 2,4-dinitrophenol; simultaneously there is discharge of Ca^{++} and phosphate from the mitochondria into the medium.

INTRODUCTION

The "active" or respiration-linked accumulation of Ca^{++} from the suspending medium by isolated mitochondria, first demonstrated by Vasington and Murphy (1, 2), has been studied in some detail in a number of recent investigations (1–11). The accumulation of Ca^{++} during respiration has been found to be quite massive, attaining values as high as 3.0 μmoles Ca^{++} per mg mitochondrial protein (2–6). It is accompanied by uptake of inorganic phosphate from the medium; the molar ratio Ca:P of the accumulated salt is about 1.67, or approximately that of hydroxyapatite (4, 5). The amounts of Ca^{++} and P_i accumulated bear a simple relationship to electron transport and oxidative phosphorylation; Rossi and Lehninger have shown that, for the passage of a pair of electrons

through each of the three phosphorylation sites of the respiratory chain, one molecule of phosphate and 1.67 molecules of Ca^{++} are accumulated from the medium (4, 5). They have also shown that the oxidative accumulation of Ca^{++} and phosphate is a process that replaces the oxidative phosphorylation of ADP (5). The ability to accumulate Ca^{++} and phosphate in large amounts has been found in mitochondria of rat liver, kidney, and brain (5), as well as in beef heart (6), and may therefore be characteristic of mitochondria in most cell types. Finally, it has been found that isolated mitochondria accumulate Mg^{++} (12–14) in a respiration-linked process. The accumulation of Mn^{++} (7), Ba^{++} (7, 11) and Sr^{++} (7, 11) has also been briefly reported.

This paper describes some aspects of the structure and properties of isolated rat liver mitochondria as they are influenced by the respiration-linked accumulation of Ca^{++} and phosphate. This information has been obtained by correlating biochemical studies of ion accumulation with biophysical methods such as isopycnic centrifugation, electron microscopy, x-ray, and electron diffraction analysis.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of Carworth Farms albino rats (Wistar strain) by the method of Schneider (15). Protein was determined by a biuret method (16). Incubation of the mitochondria in Ca^{++}-containing medium was carried out as previously described (2, 5). After incubation, the system was quickly chilled at 0°, centrifuged 4 minutes at 20,000 *g*, and the mitochondrial pellet washed once with cold 0.25 M sucrose.

ISOPYCNIC CENTRIFUGATION IN A DENSITY GRADIENT: The sucrose gradients were prepared by layering 0.50 ml volumes of sucrose solutions of decreasing concentrations in 5 ml lusteroid tubes of the SW 39L swinging-bucket rotor of the Spinco Model L ultracentrifuge. The concentration sequence was 3.00, 2.50, 2.20, 1.75, 1.60, 1.40, 1.00, 0.80, and 0.25 M. Density gradients were also prepared by layering 55, 50, 45, 40, 35, 30, and 22 per cent solutions, w/v, of cesium chloride in the order listed. The mitochondrial suspension was layered on the density gradient and the tubes centrifuged at 165,000 *g*. Centrifugation speeds reflect values calculated to the bottom of the tubes. After the centrifugation, the position of the sedimented particles in the tube was recorded. The zones were then removed through the bottom of the tube by mounting it in a device which perforated the tube with a needle about 1 mm above the bottom. The contents of the tubes were then run out as small droplets at a controlled rate. The small volume of each droplet (*ca.* 0.01 ml) permitted the collection of 0.05 ml fractions by drop counting. The fractions were analyzed for Ca^{45} and for protein as described below.

DISCHARGE OF Ca^{++} AND P_i FROM LOADED MITOCHONDRIA: The mitochondria, previously incubated in the usual medium for maximum respiration-linked accumulation of labeled Ca^{++} and phosphate, were sedimented, washed once with cold 0.25 M sucrose, resuspended in 0.25 M sucrose and then incubated at 30° for different times in various media, as shown in the figures. Aliquots of the system were removed at different times, quickly chilled at 0°, and centrifuged 4 minutes at 20,000 *g*. Ca^{45} and P_i discharge was measured either by following appearance of the radioactivity in the suspending medium or by plating and counting of a heated formic acid extract of the sedimented mitochondria.

ELECTRON MICROSCOPY: For correlative electron microscope studies of ion accumulation, mitochondria were centrifuged from the suspending media after appropriate incubation and the pellets fixed for 1 hour at 0°C with 1 per cent OsO_4 in Veronal-acetate buffer (pH 7.4) or with 10 per cent formaldehyde. For controls, mitochondria were suspended in the complete incubation medium and fixed at zero time or incubated in the complete medium minus ATP or Ca^{++}, as indicated. The fixed mitochondrial pellets were then dehydrated by rapid passage through a cold (−10°C) ethanol series and embedded in Epon 812 according to the procedure of Luft (17). Thin sections were cut with glass knives on the Porter-Blum or LKB microtome and collected, unsupported, on grids. Most sections were stained with lead by Method B of Karnovsky (18). Unstained, formaldehyde-fixed sections were also examined to determine whether the deposited calcium phosphate contributed significant electron opacity to the large, dense granules observed in Ca^{++}-loaded mitochondria exposed to osmium tetroxide and lead.

To follow by electron microscopy the discharging of accumulated ions from loaded mitochondria by DNP, aliquots of mitochondria were removed at specified time intervals and were fixed with OsO_4, dehydrated, and embedded as described above.

Quantitative as well as qualitative differences between experimental and control mitochondria were recorded; standard statistical methods were applied in evaluating the significance of the values obtained (19). In studies of the so called dense granules of the mitochondria, the average number of mitochondria containing one or more electron-opaque granules, the average number of granules per granule-containing mitochondrion and the mean size of granules found in mitochondria from the various systems were determined. The diameters of individual electron-

opaque granules were measured on micrographs magnified at least 20,000 times using the method described by Andersson-Cedergren (20); since the granules rarely have perfectly circular profiles, the largest dimension was considered an estimate of granule size. Dense granules of normal rat liver mitochondria rarely approach 500 A in diameter. However Ca^{++}-loaded mitochondria contain not only such "small" granules, but also much larger granules. Therefore, the number of granules in the size ranges of 1 to 500 A and >500 A were recorded separately. Measurements and counts were made on a minimum of 100 mitochondria for each preparation studied; systematic electron microscopic examination was made on mitochondria from seven different experiments.

X-RAY DIFFRACTION ANALYSIS: Specimens of dry, calcium phosphate–loaded rat liver mitochondria were prepared for x-ray diffraction analysis as follows. Some 50 batches of rat liver mitochondria (1 mg protein per ml) were incubated simultaneously with shaking for 15 minutes at 23° in a 10 ml medium containing 10 mM succinate, 4.0 mM ATP, 4.0 mM phosphate, 10 mM Tris-HCl buffer (pH 7.4), 3.0 mM $CaCl_2$, and 10 mM $MgCl_2$. The Ca^{++}-loaded mitochondria were harvested by centrifugation and washed once with cold 0.25 M sucrose. In preparation I, the mitochondria were immediately frozen and dried, then subjected to analysis. Preparation II was frozen-dried and then extracted with chloroform-ethanol to remove lipids, essentially by the procedure of Folch *et al.* (21). The content of Ca^{++} and phosphate in each specimen was analyzed before and after x-ray analysis.

RESULTS

ISOPYCNIC CENTRIFUGATION OF MITOCHONDRIA LOADED WITH CALCIUM PHOSPHATE: The large amounts of calcium and phosphate accumulated by respiring rat liver mitochondria would be expected to increase the specific gravity of the mitochrondia significantly because of the relatively high specific gravity of the deposited salt.

Fully loaded mitochondria may contain some 2.7 μmoles Ca^{++} and about 1.6 μmoles phosphate per mg mitochondrial protein (2, 4, 5), or about 0.27 mg hydroxyapatite ($[Ca_3(PO_4)_2]_3 \cdot Ca(OH)_2$) per mg protein (4, 5). Liver mitochondria also contain about 0.6 mg total lipid per mg protein; therefore the calcium phosphate present in fully loaded mitochondria may make up some 14 per cent of their dry weight. Since the specific gravity of calcium phosphate is about 3.14, it would be expected that calcium phosphate-loaded mitochondria have a higher specific gravity and might be separated from normal mitochondria by equilibrium centrifugation in a properly chosen density gradient.

The data in Fig. 1 show the results of an isopycnic centrifugation of "loaded" and normal rat liver mitochondria carried out in a gradient of sucrose (specific gravity range 1.02 to 1.37) employing the swinging-bucket rotor SW 39L in the Spinco Model L ultracentrifuge. The mitochondria were first incubated in a medium yielding maximum respiration-linked accumulation of Ca^{++} and phosphate; the Ca^{++} was labeled with Ca^{45}. Layers of the gradient were recovered and analyzed for Ca^{45}, phosphate, and protein as indicated above, and the position of the optically opaque zones in the density gradient noted. Comparable experiments were carried out on mitochondria incubated in a similar medium from which Ca^{++} had been omitted.

The findings summarized in Fig. 1 show that mitochondria incubated with Ca^{++} and P_i sedimented into three separate layers of different specific gravities. The major layer, which separated as a sharp buff-colored band having a specific gravity of about 1.37, contained 79 per cent of the total mitochondrial protein, 98 per cent of the total bound Ca^{++}, and about 98 per cent of the total bound phosphate. A second sharp band of significantly lower specific gravity than the major band (about 1.32), contained only 6 per cent of the total protein, about 0.3 per cent of the total bound Ca^{45}, and about 0.4 per cent of the total phosphate. The mitochondria in the intermediate band evidently have not accumulated significant amounts of calcium phosphate and cannot be regarded as partially-loaded mitochondria. The third band, also sharp, appeared to be identical with the control mitochondria in specific gravity (*i.e.* 1.20); it contained about 20 per cent of the total protein, about 0.3 per cent of the total bound Ca^{++}, and about 0.7 per cent of the bound phosphate.

The almost complete absence of bound Ca^{++} and phosphate in the second and third bands makes it apparent that a rather large fraction of the preparation does not deposit calcium phosphate to any great extent. One explanation might be that one or more of the bands consists not of mitochondria but of other subcellular particles such as lysosomes. It seemed unlikely, in view of the relative infrequency with which such contami-

nating particles are observed in thin sections of control preparations, that they could account for 20 per cent of the total protein of the preparation. On the other hand, it could be that some mitochondria fail to accumulate ions either because they represent a subpopulation in the original preparation (see Discussion) or because they are damaged during treatment. In separate experiments the individual bands separated in sucrose or cesium chloride gradients (see below) were collected, fixed, embedded, and observed as thin sections in the electron microscope. Although the distortion and loss of structural integrity observed, chondria are damaged when incubated in loading media containing high concentrations of Ca^{++} (see Fig. 3).

In some experiments the intermediate band (specific gravity 1.31) was absent but the loaded and unloaded mitochondria always sedimented into sharp, narrow bands with the specific gravities given above. Although no quantitative densitometric or optical measurements were made to assess the spreading of the calcium phosphate, loaded mitochondria consistently showed the same specific gravities and thus occurred in a relatively homogeneous state.

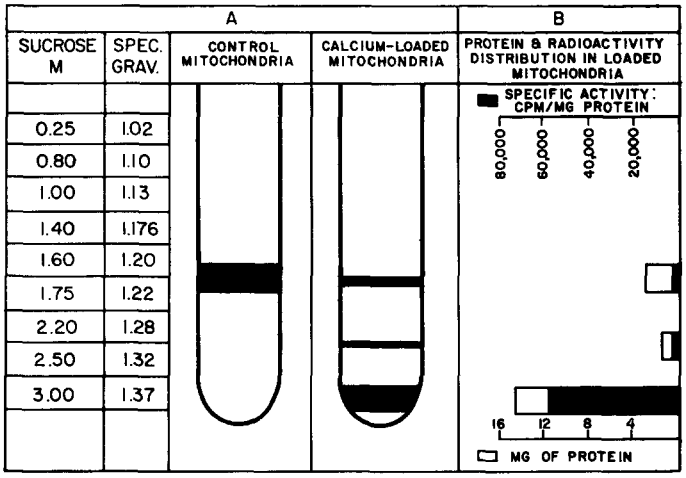

FIGURE 1 Isopycnic centrifugation of Ca^{++}-loaded mitochondria in a sucrose density gradient. Rat liver mitochondria (15 mg protein) were incubated for 20 minutes at 25° in 15.0 ml of a medium containing 3.0 mM ATP, 10 mM sodium succinate, 4.0 mM P_i, 10 mM $MgCl_2$, 10 mM Tris-HCl buffer pH 7.0, and 4.0 mM $CaCl_2$ labeled with Ca^{45}. The mitochondria were harvested by centrifugation and resuspended in 3.0 ml 0.25 M sucrose. One ml of this suspension was layered on the sucrose gradient (see text) and centrifuged at 115,000 *g* for 180 minutes. The location of the mitochondria was noted as shown above; the gradient was analyzed for Ca^{45}, P_i, and total protein.

especially in the third band, discouraged a detailed structural study, a major portion of each band could be identified as mitochondria or membrane fragments derived from mitochondria. Thus, it seems that 20 to 26 per cent of the mitochondria in the test system failed to accumulate Ca^{++} and P_i; this finding is consistent with the electron microscope observations (see Fig. 4) that, in pellets of loaded mitochondria, an appreciable fraction of the mitochondria in any given section appears to contain no large dense granules. Results of our experiment designed to show intermediate levels of Ca^{++} and phosphate accumulation by mitochondria suggest that some mito-

While sucrose gradients have been successfully used in many types of equilibrium centrifugation applications, sucrose solutions are quite viscous and are also known to affect the structural state of mitochondria (22), to inhibit oxidative phosphorylation (22), and to penetrate mitochondria (23). For this reason it appeared desirable to carry out the isopycnic centrifugations in a medium containing a different solute. Accordingly, similar experiments on normal and calcium phosphate–loaded mitochondria were carried out in density gradients of cesium chloride. The results of a typical experiment are shown in Fig. 2. It was found that Ca^{++}-loaded and un-

loaded mitochondria were readily separated into sharp zones in the CsCl gradient also. The agreement on the specific gravities of the separate zones was rather good, as shown on comparing Figs. 1 and 2. In fact, the CsCl gradients yielded sharper zones and thus the more accurate values for the specific gravities when measurements were made immediately. On the other hand, it was found that the zones separated in the CsCl gradients were less stable than those separated in sucrose gradients. On standing, the Ca^{++} spread rather rapidly in the CsCl gradients, suggesting that it leaked readily in the presence of the high ionic strength of CsCl. Lehninger has observed

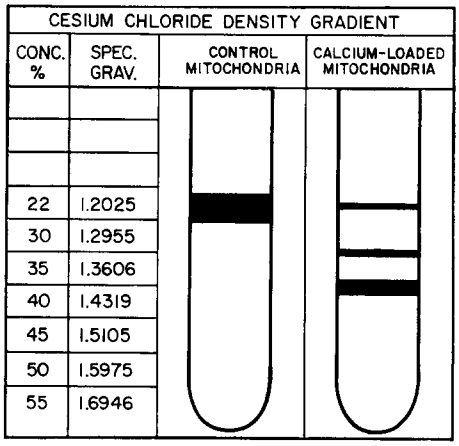

FIGURE 2 Isopycnic centrifugation of Ca^{++}-loaded mitochondria in a gradient of CsCl. The mitochondria were loaded as shown in Fig. 1, harvested, suspended in 0.15 M CsCl, and layered on the CsCl gradient. The centrifugation took place at 110,000 *g* for 180 minutes.

that Cs^{+} is an inhibitor of mitochondrial contraction by ATP (24); furthermore, Vasington and Murphy have found that Cs^{+} is somewhat inhibitory to active ion accumulation by rat liver mitochondria (2). Nevertheless, the excellent agreement in the observed specific gravities obtained after isopycnic centrifugation indicates that the methods give valid measurements of the specific gravities.

It must be pointed out, however, that the density gradient columns are highly hypertonic and cause considerable osmotic shrinkage and thus loss of water from both the normal and loaded mitochondria. This is evident from the relatively high specific gravity of the normal mitochondria in these gradients (*i.e.* ~1.20), in contrast to the substantially lower values which can be calculated from the known water, protein, and lipid content. Although osmotic equilibration may be assumed to be complete during the time scale of the isopycnic centrifugation (*cf.* reference 25), it is not feasible to make further quantitative deductions regarding the solids and water content of normal and loaded mitochondria by this method. On the other hand, the isopycnic centrifugation procedure makes possible clear empirical separation of normal and calcium phosphate–loaded mitochondria.

ALL-OR-NONE VERSUS GRADUAL LOADING OF MITOCHONDRIA: Since the isopycnic centrifugation experiments indicated that the calcium-loaded mitochondria separated in a sharp band having a very narrow range of specific gravity, without occurrence of Ca^{++}-loaded mitochondria having intermediate specific gravities, the question arose as to the manner in which individual mitochondria acquired deposits of calcium phosphate. The process could occur during respiration by gradual loading of all mitochondria, with gradually increasing specific gravity of all, to a limiting value, or by rapid loading of single mitochondria on an "all-or-none" basis, to account for the appearance of a single band of "heavy" mitochondria, with none of intermediate specific gravity. The question is not decisively resolved by the experiments shown in Figs. 1 and 2, since it is probable that the mitochondria in these experiments were already loaded to capacity when harvested.

Because maximal "loading" of mitochondria with Ca^{++} takes place in a relatively short time (10 to 15 minutes at 23°) compared with the time scale of the isopycnic centrifugation (up to 3 hours), it was not feasible to recover mitochondria at intermediate stages of loading following very short incubation periods. Therefore an experiment was designed in which the amount of calcium phosphate taken up by the mitochondria was adjusted to intermediate levels by limiting the amounts of Ca^{++} available in the test medium. The data in Fig. 3 show that mitochondria could be recovered in which intermediate degrees of loading existed, as observed by chemical analysis of the mitochondria harvested from the medium. Such chemical measurements indicate only a statistical load of calcium phosphate, and do not indicate the behavior of single mitochondria or of subpopulations. However, isopycnic centrifugation

of mitochondria harvested from these media (Fig. 3) showed that, at each intermediate level of loading produced by limiting the available Ca^{++} and phosphate in the medium, most of the mitochondria possessed an average specific gravity intermediate between the specific gravity of normal and that of maximally-loaded mitochondria; the increase in specific gravity of the mitochondria was approximately proportional to the amount of Ca^{++} and phosphate accumulated. At each intermediate stage of loading, the mitochondrial population showed significant spreading in the density gradient. At maximal loading, the amount of spreading was considerably less, but a separate band sedimenting at a specific gravity identical with that of unloaded, control mitochondria was observed as shown in the tube at the extreme right in Fig. 3. Sometimes a similar but less well defined band was seen in the tube containing 600 mμmoles Ca^{++}/mg protein. At lower concentrations of Ca^{++} this band was not seen. It seems reasonable to assume, since both Ca^{++} and phosphate are known to be potent swelling agents of isolated rat liver mitochondria, that during loading in the higher concentrations of Ca^{++} some of the mitochondria are damaged and thus unable to accumulate or to maintain the calcium phosphate deposits. These experiments demonstrate the feasibility of analyzing and separating mitochondria at different stages of loading with calcium phosphate by means of continuous and perhaps less steep gradients of sucrose and CsCl, in order to characterize in more detail the growth in specific gravity, size, and number of the dense granules of calcium phosphate during the accumulation process. As noted below, some mitochondria show numerous but relatively small granules, whereas others show less numerous but very large granules. It appears possible that more refined isopycnic centrifugation, combined with electron microscopy, may yield further information about the dynamics and structural aspects of the accretion of calcium phosphate deposits.

ELECTRON MICROSCOPY OF CALCIUM PHOSPHATE-LOADED MITOCHONDRIA: Electron microscopic examination of rat liver mitochondria loaded with Ca^{++} and phosphate provides evidence that (*a*) Ca^{++} and P_i are transported into the interior of isolated rat liver mitochondria, (*b*) these ions are retained within these organelles as extremely large, electron-opaque granules, and (*c*) these granules in many cases are closely associated with intramitochondrial membranes.

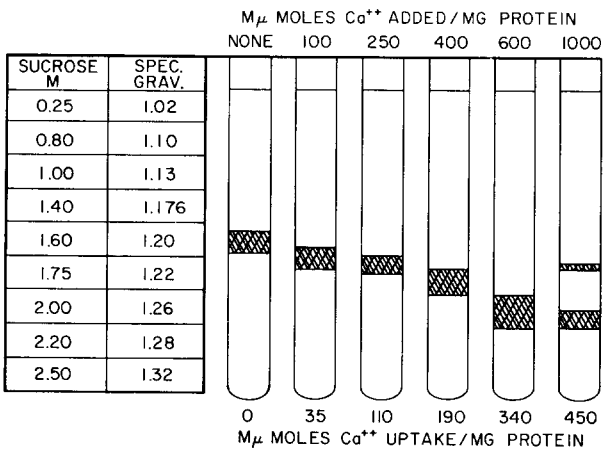

FIGURE 3 Increase in specific gravity of mitochondria with increasing Ca^{++} content (experiment carried out by Dr. Ernesto Carafoli). Rat liver mitochondria (15 mg protein) were added to 15.0 ml reaction medium containing 3.0 mM ATP, 10 mM succinate, 10 mM $MgCl_2$, 4.0 mM P_i, 10 mM Tris-HCl buffer pH 7.0, and the amounts of $Ca^{45}Cl_2$ shown above. Incubated 20 minutes at 25°. The mitochondria were harvested by centrifugation, and resuspended in 3 ml 25 M sucrose; 1 ml was layered on the sucrose gradient shown. The centrifugation was carried out at 115,000 *g* for 3 hours and the radioactivity of Ca^{++} taken up in the bands of mitochondria determined.

The active accumulation of massive amounts of Ca^{++} and P_i by isolated rat liver mitochondria is accompanied by the formation of large electron-opaque granules, which are sometimes massive, attaining a diameter of 3000 A, as shown in Fig. 4. In confirmation of the experiments on isopycnic centrifugation of mitochondria following respiration-linked loading, not all of the mitochondria in a given pellet recovered from the loading medium contain these granules. It may be significant that those mitochondria not containing such dense granules are generally several diameters larger than those mitochondria with granules and that a corresponding decrease in the density of their matrices can be seen. The large granules in those loaded mitochondria which show a wide separation between the inner and outer surrounding

membranes are located exclusively within that portion enclosed by the inner mitochondrial membrane; granules are not found in the space between the surrounding membranes, which appears structureless.

Maximal accumulation of Ca^{++} and P_i is dependent upon the presence of ATP, Mg^{++}, and

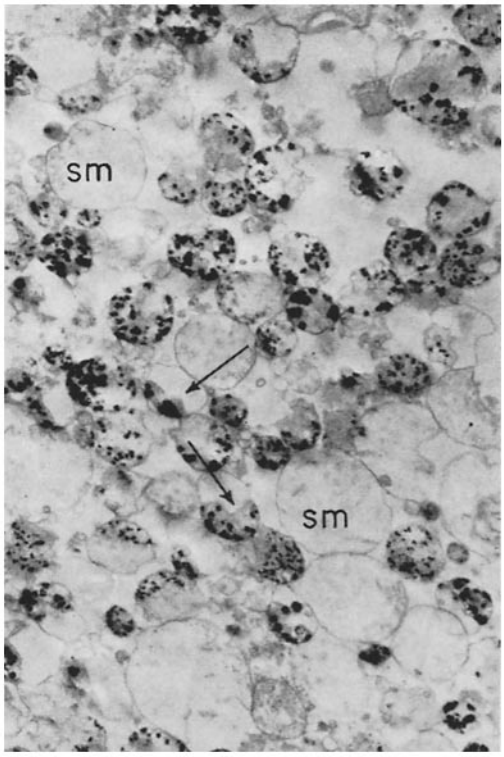

FIGURE 4 Isolated rat liver mitochondria fixed after the active accumulation of Ca^{++} and P_i. Extremely dense granules of variable sizes can be seen but are not present in all mitochondria. Mitochondria in which granules are not seen appear extremely swollen (*sm*). In some mitochondria the inner and outer surrounding membranes are widely separated but the granules are exclusively located in that portion containing the mitochondrial structures (arrows). Fixed with 1 per cent OsO_4 and stained with lead. × 8,000.

substrate (2, 3, 5). In Figs. 5 to 8, sections of mitochondria loaded with Ca^{++} and P_i by incubation in the complete medium for 20 minutes at 30°C (Fig. 5) are compared with those of mitochondria (*a*) removed from the loading medium prior to incubation, *i.e.* at zero time (Fig. 6), (*b*) incubated for 20 minutes at 30°C in the absence of Ca^{++} (Fig. 7), and (*c*) incubated in medium to which ATP was not added (Fig. 8). Fig. 5 shows that very large dense granules, which often appear to be composed of smaller particles, accumulate within mitochondria incubated with Ca^{++} and P_i in the presence of succinate, ATP, Mg^{++}, and phosphate; *i.e.*, conditions under which a large respiration-dependent accumulation of calcium phosphate occurs. Electron-opaque granules are also seen in mitochondria incubated in a medium containing no Ca^{++} (Fig. 7), but it is shown in Table I that they are much smaller on the average than those accumulating in the complete system and are much fewer in number; they are seen in only relatively few mitochondria. Furthermore, as can be seen by comparing Figs. 5 and 7, the distribution of the granules within mitochondria from these two systems is considerably different; granules seen in mitochondria from the system containing no Ca^{++} are typically arranged in clusters. It may be that these granules reflect the accumulation of Mg^{++} and P_i which are present in this control medium (see legend, Fig. 1); these ions are known to accumulate in isolated mitochondria under similar conditions of incubation (12). Such clusters are sometimes seen in isolated, unincubated mitochondria also. Mitochondria incubated in a medium containing Ca^{++}, but no ATP, which is necessary for Ca^{++} uptake (5), are distinctly different from the others (Fig. 8). They are larger in diameter, have less dense matrices than the zero time control mitochondria (Fig. 6), and the cristae are not well defined. However, the outer surrounding membranes appear continuous and extremely well preserved.

Fig. 9 illustrates the appearance of granules in unstained, formaldehyde-fixed sections of loaded mitochondria. Structural details, *i.e.* cristae and surrounding membranes, are not easily detected due to the low contrast, but the large dense granules are seen readily and the small particles composing each large granule are quite distinct. It is clear that the electron-opacity of the intramitochondrial granules of calcium phosphate-loaded mitochondria is intrinsic and is not merely the result of staining by or precipitation of either the osmium tetroxide or the lead used as fixative and stain. Presumably the electron-opaque granules of the formaldehyde-fixed mitochondria are localized deposits of calcium phosphate.

It may also be noted that the small dense granules seen by many observers in freshly iso-

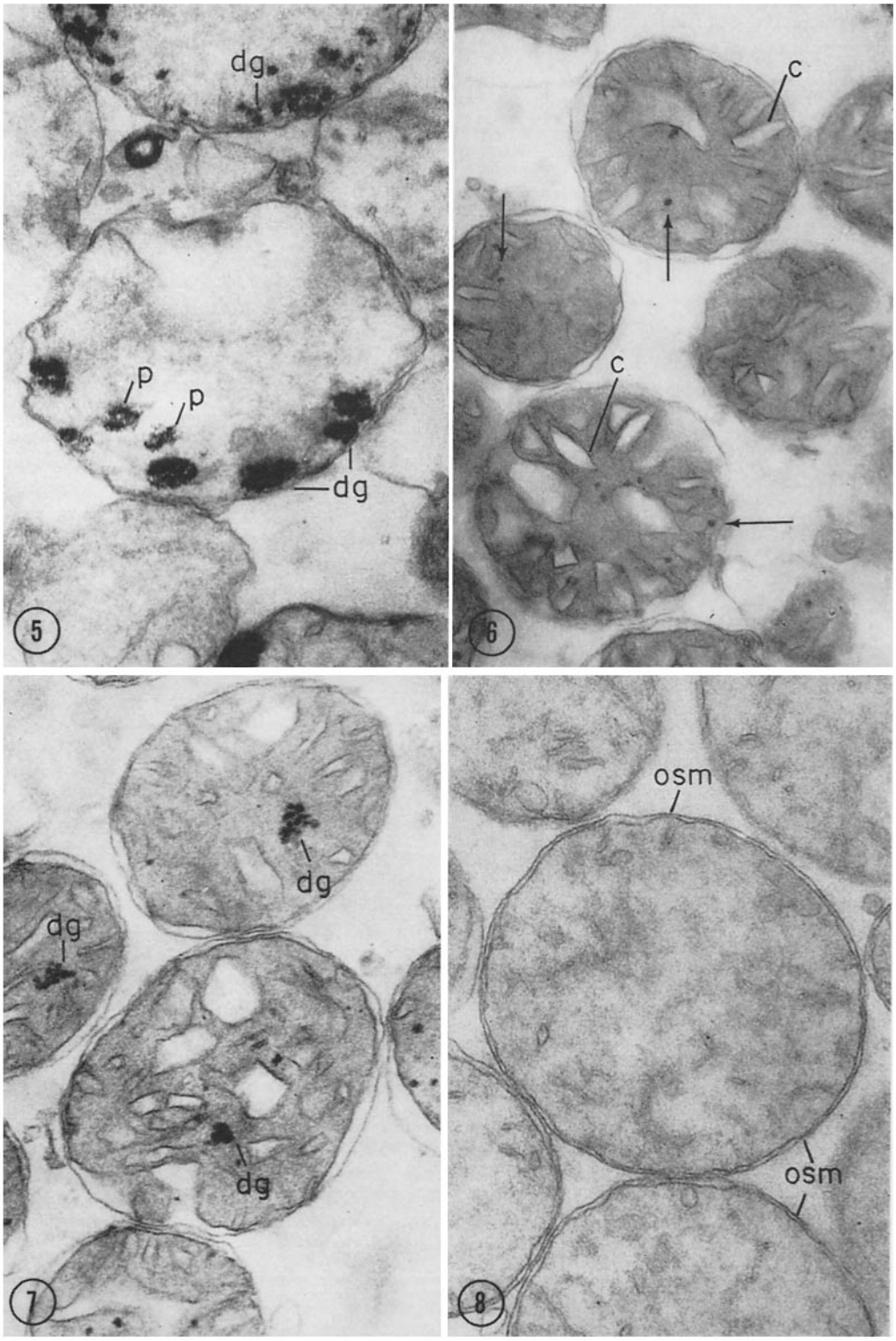
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TABLE I

Comparison of Diameters of Electron-Opaque Intramitochondrial Granules in Normal Mitochondria and After "Loading" with Ca^{++} and P_i

Condition of mitochondria	Mean diameter of granules*			Granules 500 A or larger
	<500	≧500	All	
	A	*A*	*A*	*Per cent*
1. Fresh, unincubated	355 ± 30	0	355 ± 30	0
2. Loaded with calcium phosphate	441 ± 72	1294 ± 143	1149 ± 21	83
3. Incubated without Ca^{++}	416 ± 33	667 ± 206	431 ± 36	6
4. Incubated without ATP	No granules present			—

* Mean diameters were determined for granules < 500 A, granules ≧ 500 A, and all granules present whether < 500 A or ≧ 500 A in diameter. The ± sign represents the standard error of the mean.

lated mitochondria or in mitochondria examined *in situ* (*cf.* Fig. 6), which may be termed normal granules, are not observed in mitochondria incubated in the presence of Ca^{++} or in the absence of ATP. The significance of this fact is considered in more detail below.

The size of the dense granules seen in loaded mitochondria varies widely, but only about 17 per cent of these electron-opaque granules are in the size range typical of the normal granules seen in freshly isolated mitochondria. In fresh mitochondria and in those incubated in the absence of Ca^{++}, almost all of the granules observed are <500 A in diameter. The mean diameter of granules in mitochondria after accumulation of Ca^{++} and P_i is over 1000 A, and in some it measured more than 3000 A (see Table I).

The qualitative variation of the size of granules in loaded mitochondria is apparent in Figs. 10 and 11, which show the extremes of size, from diameters of less than 500 A to as large as 3000 A. Granules in mitochondria incubated in media with no Ca^{++} are somewhat larger than normal dense granules seen in the zero-time control but do not approach the tremendous size of the granules accumulated in the presence of Ca^{++}. It is noteworthy that no granules, not even the small normal granules usually present in intact, unincu-

Figs. 5 to 8 compare mitochondria incubated in complete with control media. Fixed with OsO_4 and stained with lead. × 40,000.

FIGURE 5 Mitochondria from complete system loaded with Ca^{++} and P_i. The mitochondria are slightly swollen and contain dense granules (*dg*) which are composed of smaller particles (*p*).

FIGURE 6 Control. Mitochondria suspended in the complete system but fixed before incubation at zero time. Mitochondria are of normal size but cristae (*c*) are distended and sharply separate the dense matrix from the intracristal spaces. Normal dense granules can be seen within the matrix (arrows).

FIGURE 7 Control. Mitochondria incubated in medium complete except for added Ca^{++}; some increase in size can be seen. General appearance is similar to that of the control taken at zero time (Fig. 6). The electron-opaque granules (*dg*) shown here are seen in relatively few mitochondria and differ in size, distribution, and general appearance from those in mitochondria from the system containing Ca^{++} (Fig. 5).

FIGURE 8 Control. Mitochondria incubated in medium minus added ATP. The two surrounding membranes (*osm*) are well preserved and intact although the mitochondria are significantly larger than the zero time control; the matrix is less dense than normal and the cristae ill defined. No electron-opaque granules are present.

bated mitochondria, were detected in any sections of mitochondria incubated in the absence of ATP. However, a detailed study of numerous serial sections would be necessary to establish this observation firmly. This was not done.

In addition to their huge size, the electron-opaque granules formed by mitochondria actively

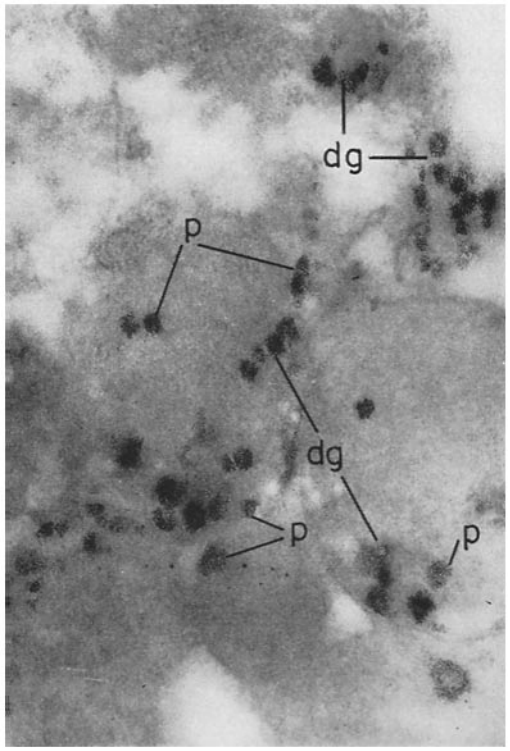


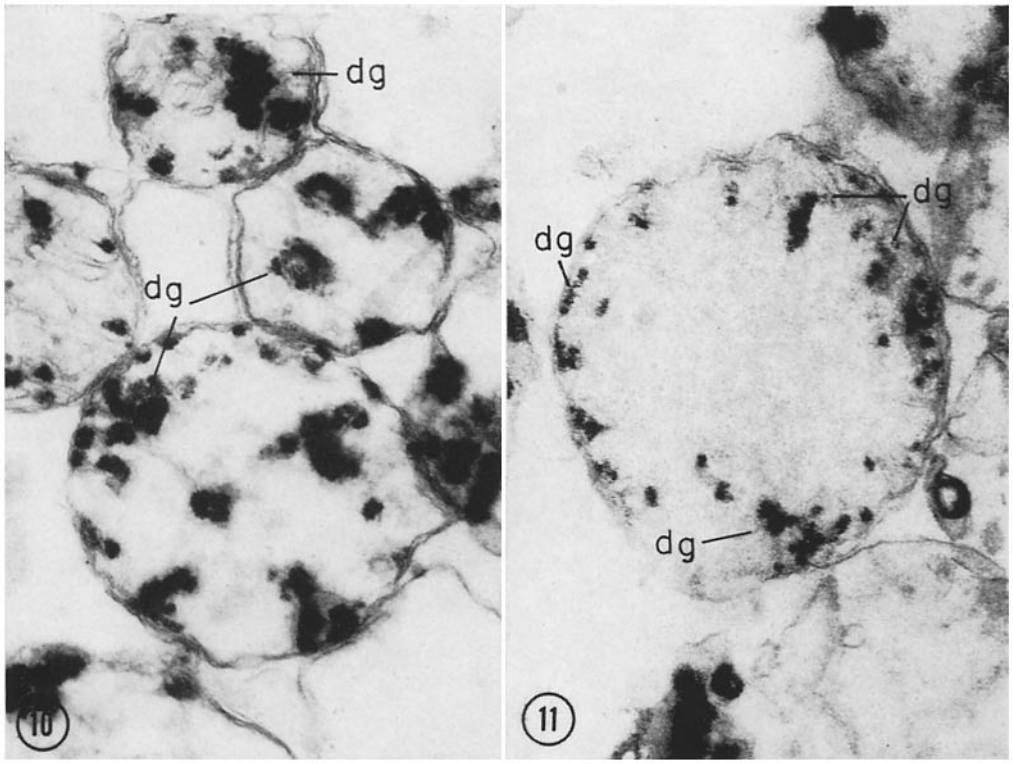
FIGURE 9 Mitochondria incubated in complete medium fixed with formaldehyde and unstained. Electron-opaque granules (*dg*) are readily discerned. Note small particles (*p*) comprising the dense granules. × 40,000.

accumulating Ca^{++} and P_i have two other remarkable morphological features. In many sections these granules are seen closely associated with intramitochondrial membranes; the intracristal spaces are clearly outlined as hyaline areas within or near the dense granular deposits. The proximity of granules to membranes are shown in Figs. 12 and 13. The high frequency with which this proximity occurs seems significant. The other striking morphological feature of many granules, shown in Fig. 13, is the central core around which small particles making up the large dense granules appear to have been deposited. Whether or not this type of profile is actually a morphological feature distinct from the association of these granules with cristae is not clear; the hollow appearance may be dependent on the cutting plane through the mitochondrion and may thus reflect the association of granules with membranes. The presence of "hollow" granules in close association with profiles of cristae suggests that this is not the case (Fig. 13); also, it is often observed that many granules in a single mitochondrion are likely to enclose a hollow central core. Granules with translucent centers and granules in close proximity to membranes are seen most frequently when they are about 500 to 1000 A in diameter; these morphological features are not observed in granules much larger or smaller.

The major qualitative observations concerning the structural nature of granules in loaded mitochondria are illustrated in Fig. 13; *i.e.*, (*a*) the large size, (*b*) the localization within the mitochondrial interior and absence from the space separating inner and outer membranes, (*c*) the frequent association with membranes, (*d*) the translucent central cores typical of many granules, and (*e*) the small particles of which the large electron-opaque granules are apparently composed.

PHYSICAL NATURE OF THE CALCIUM PHOSPHATE DEPOSITS: Preliminary examination of calcium phosphate–loaded rat liver mitochondria, prepared and isolated as described in this paper, by both electron diffraction and by x-ray diffraction methods revealed no evidence that the calcium phosphate was deposited in a predominantly crystalline form. In fact, no crystallinity was detected within the limits of detection of the methods employed.

Attempts to obtain selected area electron diffraction diagrams of calcium phosphate–loaded mitochondria were made with the Siemens-Halske Elmiskop I at 80 kv. utilizing the double condenser system, 80,000 × projector pole piece, and a 20 μ selecting aperture. Sections of formaldehyde- and osmium tetroxide–fixed loaded mitochondria embedded in Epon 812 were mounted on grids with no supporting films. No deflections other than those attributed to the epoxy embedding resin itself were obtained for either type of specimen examined; however, not all parameters governing the possible detection of crystallinity in these mitochondrial granules, such



Figs. 10 and 11 show variability of granule size. Over 80 per cent of the electron-opaque granules in mitochondria following Ca^{++} loading are > 500 A in diameter with a mean > 1000 A, although mitochondria occasionally contain smaller granules. See Table I. Fixed with OsO_4 and stained with lead. × 40,000.

FIGURE 10 Very large electron-opaque granules (*dg*) seen in loaded mitochondria.

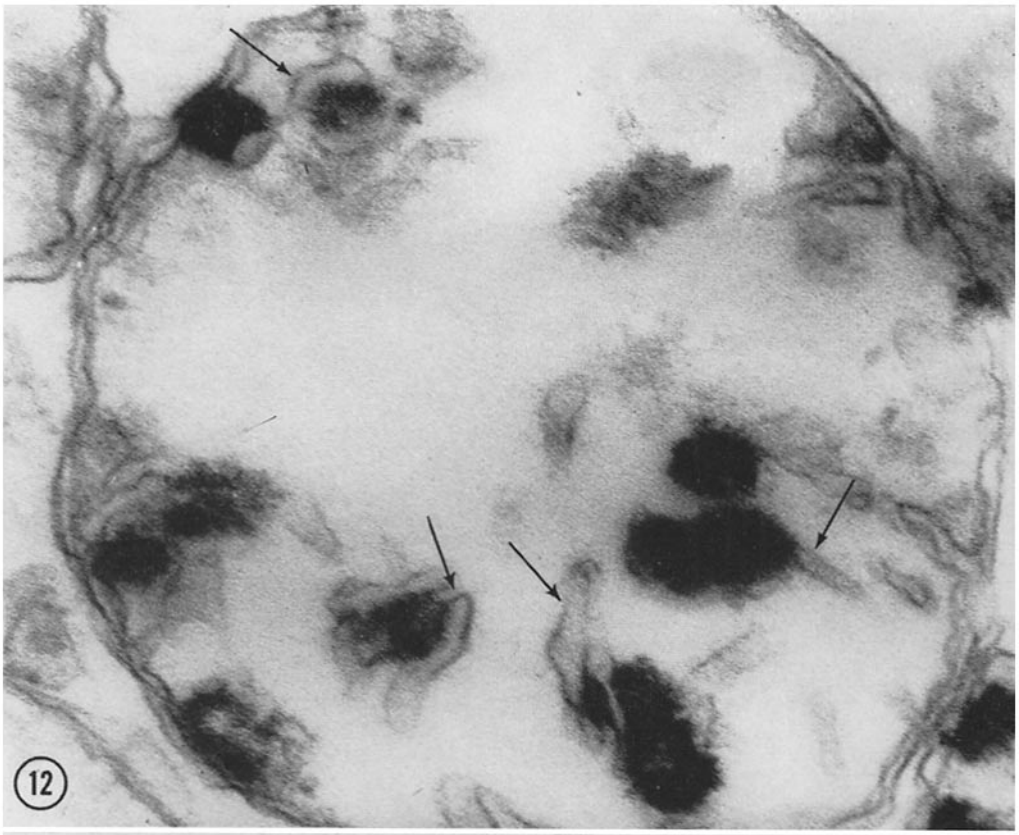
FIGURE 11 An example of Ca^{++}-loaded mitochondria containing small dense granules (*dg*). These granules typically are located around the periphery of the mitochondrion.

as thickness of section, exposure time and concentration of granules, have yet been fully investigated.

The preliminary x-ray diffraction measurements were carried out by Dr. Aaron Posner, Hospital for Special Surgery, Cornell University Medical College, New York, on lyophilized or solvent-extracted specimens of rat liver mitochondria loaded with calcium phosphate. They showed no evidence of crystallinity under conditions which could have revealed crystallinity of only a small percentage of the known calcium phosphate content.

It is interesting that the "calcareous corpuscles" of tapeworms appear amorphous *in situ* but upon isolation and treatment exhibit diffraction patterns indicating the crystallization of constituents within the granules (29, 30). It may be possible that under different conditions of loading or of harvesting and preparing mitochondria the deposits may appear crystalline. Further work is being carried out to examine such variables in detail. The possibility must be held open that under some circumstances the calcium phosphate in loaded mitochondria may crystallize as hydroxyapatite.

DISCHARGE OF INTRAMITOCHONDRIAL CALCIUM PHOSPHATE: Vasington and Murphy (2) first reported that Ca^{++} accumulated by kidney mitochondria may be discharged again into the medium if the incubation is continued for long periods or if 2,4-dinitrophenol is added to loaded mitochondria. In similar experiments, Brierley *et al.* (6, 11) have shown that accumulated Mg^{++}



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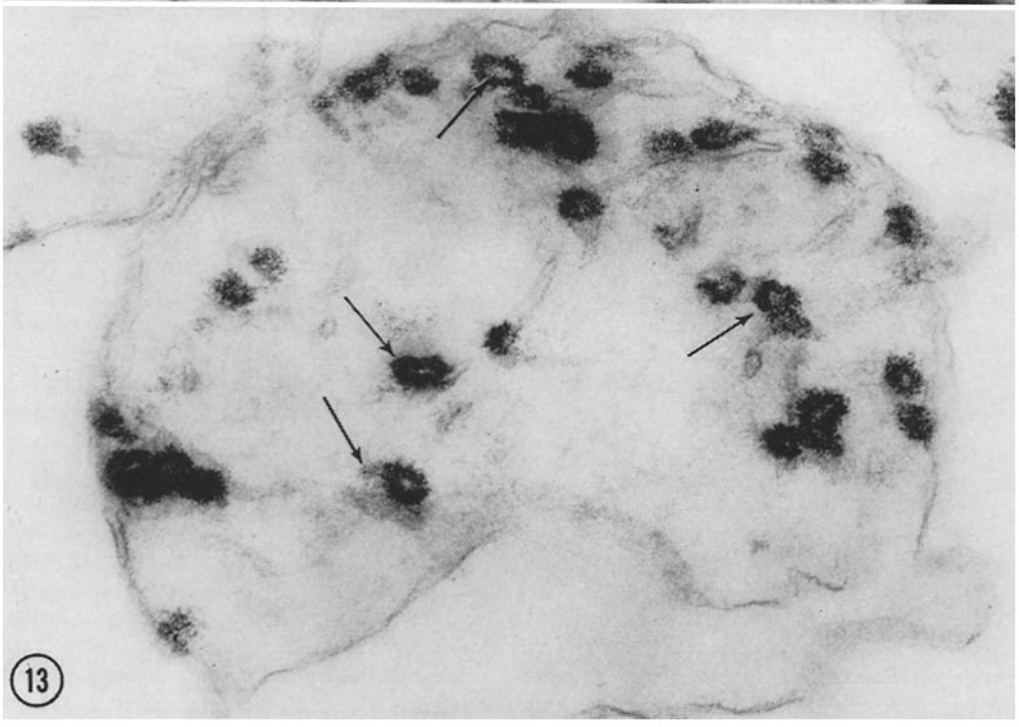

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or Ca^{++} may be discharged from heart mitochondria in the presence of dinitrophenol.

We have carried out quantitative experiments on the kinetics of discharging of accumulated calcium phosphate from fully loaded rat liver mitochondria. In these experiments the loaded mitochondria were harvested from the loading medium and then incubated in the presence and absence of 2,4-dinitrophenol in a fresh medium not containing Ca^{++}. A typical experiment (Fig. 14) shows that incubation at 30° of Ca^{++}-loaded mitochondria in a simple medium of NaCl and Tris buffer, pH 7.4, proceeds with only a relatively small loss of Ca^{++} into the medium, less than 30 per cent over a 90 minute period. However, when 0.1 mM 2,4-dinitrophenol was added, then loss of Ca^{++} was greatly stimulated, about 50 per cent being discharged at 10 minutes and nearly all in 60 to 80 minutes. These and the earlier findings cited above indicate that loss of Ca^{++} (and P_i) from loaded mitochondria may require breakdown of some high energy intermediate of oxidative phosphorylation or some other mitochondrial component whose integrity is dependent on oxidative phosphorylation. Similar results were obtained if loaded mitochondria were incubated in a medium identical with the loading medium, but with Ca^{++} omitted, as shown in Fig. 17. In this case, discharge in the presence of dinitrophenol was slower and less complete than in the case of the experiments shown in Fig. 14, presumably because the presence of ATP and substrate tended to preserve the intramitochondrial calcium phosphate.

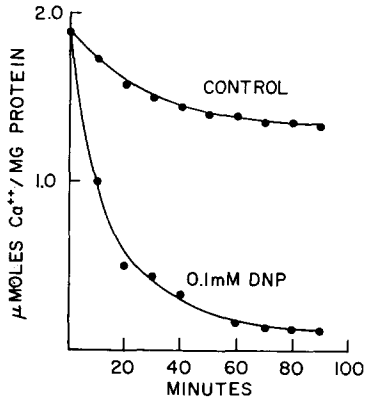
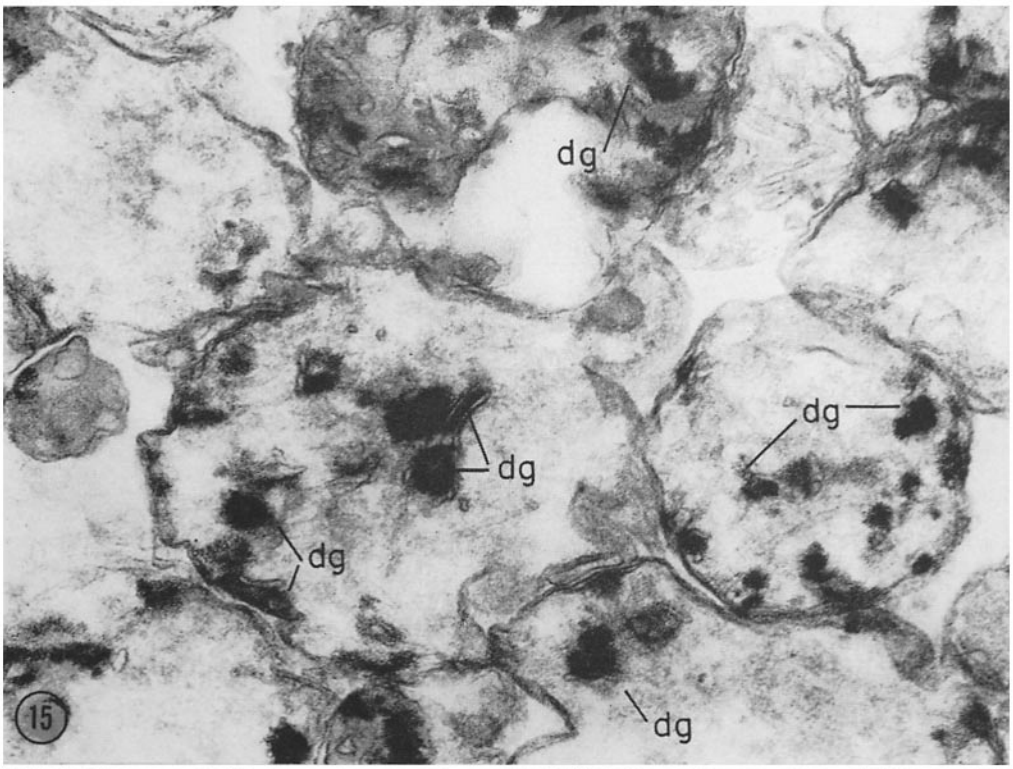


FIGURE 14 Discharge of Ca^{++} from loaded mitochondria by 2,4-dinitrophenol. Rat liver mitochondria (1 mg protein per ml), following incubation in a medium of 10 mM succinate, 4.0 mM ATP, 4.0 mM P_i, 3.0 mM $Ca^{45}Cl_2$, 8 mM $MgCl_2$ and 10 mM Tris-HCl buffer pH 7.0, for 20 minutes at 25°, were harvested by centrifugation and resuspended in 0.15 M KCl-0.01 M Tris-HCl buffer pH 7.0, with and without 0.1 mM 2,4-dinitrophenol. The mixture was incubated at 25° and the discharge at Ca^{++} measured as described in the text.

These findings presented the opportunity of correlating qualitative and quantitative electron microscopic observations on mitochondrial structure and granule morphology with chemical measurements of loss of Ca^{++} during the course of the discharging reaction. Results of such a correlated study of discharge of Ca^{++} show that the large dense intramitochondrial granules are formed during uptake of Ca^{++} and that they disappear when Ca^{++} is discharged. Fig. 15 shows the typical appearance of loaded mitochondria prior to discharge, whereas Fig. 16 shows such mitochondria after incubation with 2,4-dinitrophenol. It is seen that many of the dense granules have disappeared and those remaining are much less dense to the electron beam. Nevertheless, vestiges of the granules are still visible. The location of the granules adjacent to cristae after the discharge is also noteworthy. The combined biochemical and quantitative electron microscopic data in Fig. 17 show that the loss of Ca^{++} from the mitochondria owing to dinitrophenol-catalyzed discharge is closely correlated with (*a*) the decrease in numbers of mitochondria containing electron-opaque granules, and (*b*) the decrease in

FIGURE 12 Association of electron-opaque granules with intramitochondrial membranes (arrows). The proximity of granules to cristae is most frequently observed when granules are of intermediate size (500 to 1000 A). Fixed with OsO_4; stained with lead. × 120,000.

FIGURE 13 Mitochondrion containing dense granules encompassing a less dense, central core (arrows). All the major morphological features of the granules in calcium phosphate–loaded mitochondria can be seen. See text. Fixed with OsO_4; stained with lead × 80,000.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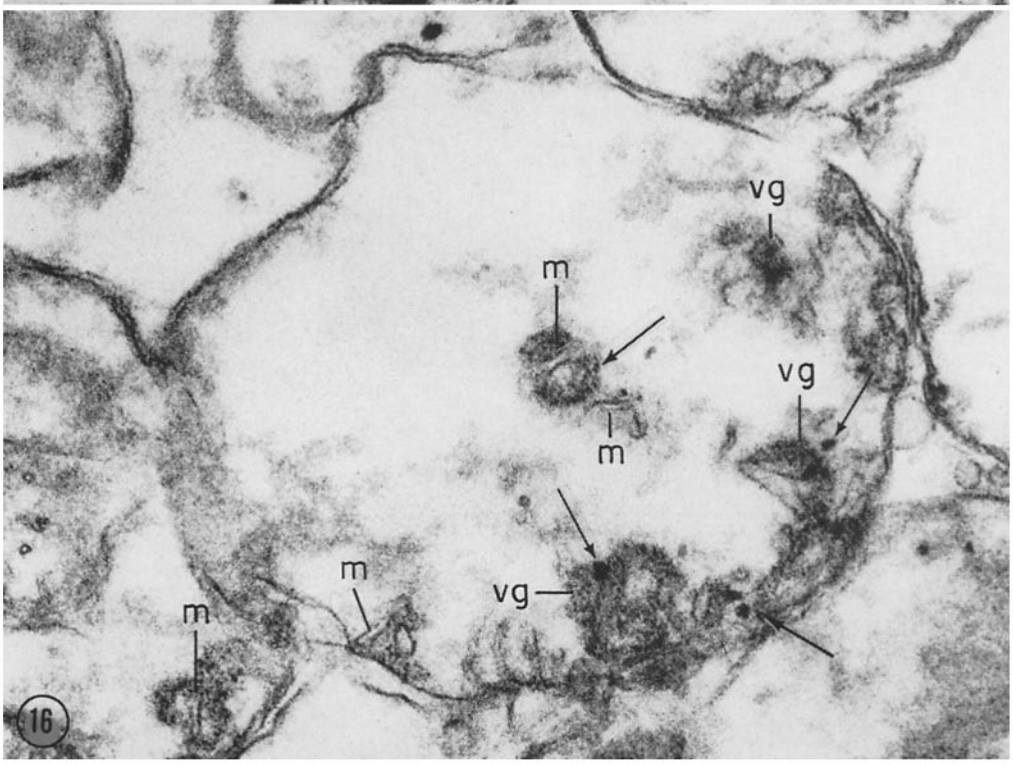
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the average number of dense granules per mitochondrial profile with time. These data thus clearly confirm and extend the observations on the appearance of dense granules during Ca^{++} uptake.

It may be mentioned that, even after discharge of 98 per cent or more of the Ca^{++}, vestiges of the granules often remain visible with an electron opacity which appears to be greater than could be accounted for by the remaining small amounts of Ca^{++}. It is possible that the specific Ca^{++}-binding sites in or near the cristae, in addition to the granules *per se*, accept osmium tetroxide or lead.

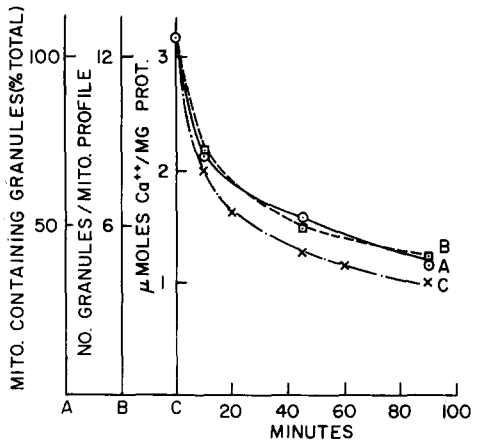

FIGURE 17 Correlation between content of calcium phosphate, the number of mitochondria containing dense granules, and the number of granules per mitochondrial profile during discharge of Ca^{++} and phosphate from loaded mitochondria by 2,4-dinitrophenol. Details as in text.

DISCUSSION

The correlated biochemical and biophysical findings reported in this paper leave no doubt that respiration-linked accumulation of Ca^{++} and phosphate ions from the medium by isolated rat liver mitochondria is accompanied by the condensation or precipitation of calcium phosphate in sharply localized, electron-opaque deposits in the mitochondria. The evidence may be summarized: (*a*) The dense granules form when Ca^{++} and phosphate are taken up from the medium. (*b*) Dense granules do not form and phosphate is not accumulated if Ca^{++} is omitted from the medium. (*c*) Dense granules do not form if ATP is omitted from the medium, a circumstance which also fails to support uptake of Ca^{++} and P_i. (*d*) The specific gravity of the mitochondrion increases as Ca^{++} and phosphate are accumulated. (*e*) The dense granules decrease in number on incubating the loaded mitochondria with 2,4-dinitrophenol, a condition which leads to discharge of Ca^{++} and phosphate from the mitochondria.

These findings are consistent with our earlier calculations that the amounts of Ca^{++} and P_i accumulated by rat liver mitochondria, if distributed wholly in the intramitochondrial water, would very greatly exceed the solubility products of simple calcium phosphate salts at neutral pH and would therefore exist as insoluble deposits. Furthermore the findings are consistent with the fact that the deposited salt has the Ca:P ratio of hydroxyapatite, as reported earlier (3–5).

It is also clear that in Ca^{++}-loaded mitochondria the dense granules possess intrinsic electron-opacity, since they are electron-opaque following fixation of mitochondria with formaldehyde in the absence of heavy metal stains or fixatives. On the other hand, it is possible that the electron-opaque osmium tetroxide and lead actually stain some other molecular components of the granules instead of or in addition to the calcium phosphate, among which may be those molecular groups which serve as the nidus or nucleus for the growth of the calcium phosphate deposits.

It is significant that both isopycnic centrifugation methods and electron microscopy reveal that some 20 per cent or more of normal, freshly isolated mitochondria, after incubation in the medium favoring maximal accumulation of Ca^{++}, fail to show accumulation of dense granules; these mitochondria are observed to be rather swollen.

FIGURE 15 Loaded mitochondria containing dense granules (*dg*). Note association of granules with the cristae. × 56,000.

FIGURE 16 Loaded mitochondrion after discharge of Ca^{++} and P_i with DNP for 90 minutes. Only vestiges of the large electron-opaque granules can be seen (*vg*). The membranes associated with the granules (*m*) and small dense particles within the vestigial granules are distinct (arrows). × 56,000.

It appears possible that that part of the mitochondrial population which fails to show granular deposits may correspond to the lighter and larger mitochondria found by Werkheiser and Bartley (23) in freshly isolated rat liver mitochondria obtained from sucrose homogenates, in contrast with the smaller, denser type of mitochondria rich in K^+ and phosphate which predominate; the latter presumably have retained capacity for ion transport. Another possibility, already mentioned, is that the mechanism regulating ion uptake and accumulation is destroyed in some mitochondria during isolation or incubation.

A number of observations suggest that the deposition of Ca^{++} is a graded process of accretion at specific mitochondrial sites favorable to localization of the calcium phosphate. First, there is a wide spectrum of sizes of the electron-opaque granules. A considerable portion of loaded mitochondria were found to contain granules about 500 to 1000 A in diameter, which are larger than the normal dense granules found in freshly isolated mitochondria, but much smaller than the maximal size observed (<3000 A), suggesting that formation of large granules may occur by growth of smaller granules. Furthermore the finding that the dense granules are in turn made up of much smaller particles is also consistent with growth by accretion of small units. The occurrence of a gradual process of granule formation and growth during loading is further supported by the experiments on isopycnic centrifugation of partially loaded mitochondria, which showed formation of mitochondria with intermediate specific gravities.

These findings, as well as the failure to find evidence of crystallinity in the calcium phosphate deposits, may be related to the observations of Watson and Robinson (26), who reported that the non-biological, *in vitro* formation of apatite crystals at room temperature proceeds through three stages: in Stage 1 there is a rapid formation of a clumped precipitate of calcium phosphate; in Stage 2, a structural rearrangement of the deposits into a membrane-like structure; and finally, in Stage 3, formation of definite apatite crystals. Only in the latter two stages can characteristic electron diffraction patterns be discerned; in fact, clear patterns of apatite were obtained only at Stage 3. It was also noted by these workers that human rib bone crystals do not develop beyond Stage 2, whereas enamel crystals go to Stage 3. Furthermore, they found that the formation of such crystals is critically dependent on pH. If, after Stage 1, the suspension is neutralized with NaOH from pH 5.0 to pH 6.8, the crystal growth stops at Stage 2. The fact that neither x-ray nor electron diffraction patterns characteristic of crystallinity could be obtained from calcium phosphate–loaded mitochondria could therefore also be a reflection of the pH and buffering capacity of the intramitochondrial milieu. It is also possible that, under the conditions we investigated, the deposited calcium phosphate does not proceed beyond Stage 1 of apatite formation, as defined by Watson and Robinson. It is of interest that the many small particles making up the large dense granules in Ca^{++}-loaded mitochondria resemble, superficially at least, the fine granular precipitate typical of Stage 1 in the growth of synthetic apatite crystals as described by Watson and Robinson (26).

The experiments on isopycnic centrifugation of maximally and partially loaded mitochondria indicate that this method may be a valuable supplement to electron microscopy in analyzing the dynamics of growth of the deposits as well as in distringuishing the behavior of individual portions of the mitochondrial population. While only limited effort was made in this study to utilize continuous and less steep density gradients to separate mitochondria of intermediate specific gravity, it is obvious that the method potentially could yield data of value in following the transition from small granules to larger ones and determining whether this change proceeds in synchrony in all parts of the mitochondrial population.

Dense granules present as clusters in mitochondria incubated in the absence of Ca^{++} (Fig. 7) are also seen occasionally in freshly isolated mitochondria suspended in sucrose. The seeming absence of dense granules of any type in mitochondria incubated in the absence of ATP (Fig. 8) complements the chemical evidence showing that this compound is required for ion accumulation. It appears that ATP is essential for retention of calcium phosphate and the granular structure, even when the respiratory chain and coupled phosphorylation are intact.

A possible role of "normal" mitochondrial dense granules in ion transport has been postulated by Weiss (27) and often speculated on by others. Although the findings reported here are consistent with this picture, they do not answer the question conclusively. It is possible that the

massive amounts of calcium phosphate accumulated in maximally loaded mitochondria may mask the normal dense granules, which are much smaller (<500 A). It is interesting in this regard, and in the light of Peachey's recent report (32), that granules of about the same size and electron density as normal dense granules can sometimes be seen in mitochondria from which accumulated Ca^{++} and phosphate have been discharged with DNP. In some cases these appear near vestiges of the large dense granules. However, the frequent association of the large dense granules with the cristae in Ca^{++}-loaded mitochondria does not have a parallel in the case of the normal dense granules, which characteristically are located in the matrix. Since the respiratory enzymes and the high energy intermediates which drive accumulation of calcium phosphate are present in the membranes, one would expect the calcium phosphate to accumulate on or near the membranes. Fig. 16 suggests that the calcium phosphate is not discharged uniformly from the large dense granules by the action of DNP but rather that certain sites bind the ions more tenaciously than others. This is indicated by the small dense particles present within the vestigial granules. In any case, the function and chemical identity of the normal dense granules so often seen in sections of intact tissues or in freshly isolated mitochondria are matters of great importance. Further evidence will be required to substantiate the hypothesis that they are nuclei for the growth of the much larger deposits of calcium phosphate described in this paper.

We have made some attempts to disrupt the membranes of Ca^{++}-loaded mitochondria and to recover the large dense granules, by differential centrifugation procedures, from either aqueous or non-aqueous suspensions. However, no success has been as yet achieved; the granules usually disintegrate or dissolve when the mitochondria are ruptured. Direct chemical and enzymatic analysis of the dense granules should, of course, give important information regarding the composition and mechanism of formation of the granules.

The dense granules of calcium phosphate seen in the isolated calcium-loaded rat liver mitochondria may be equivalent to electron-opaque granules seen in mitochondrial profiles in thin sections of osteoclasts in healing fractures of bone, as described by Gonzales and Karnovsky (28). They also may prove to have some relationship to the so called "calcareous corpuscles" of tapeworms which have been isolated and studied by von Brand and his colleagues (29, 30). Thus the deposition of the massive amounts of calcium phosphate by isolated mitochondria as reported in this study may be representative of a wide-spread biological capability elicited by specific physiological conditions. The extensive studies of Vallee and his colleagues (*cf.* reference 10) have shown that Ca^{++} accumulation occurs in mitochondria of the liver during carbon tetrachloride poisoning. More recently, Reynolds (31) has reported that mitochondria observed in thin sections of the liver of rats poisoned with carbon tetrachloride show dense granules similar to those described here.

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Some of the data in this paper have already been briefly communicated: LEHNINGER, A. L., ROSSI, C. S., and GREENAWALT, J. W., *Fed. Proc.*, 1963, **22,** 526; and GREENAWALT, J. W., LEHNINGER, A. L., and ROSSI, C. S., *J. Appl. Physics*, 1963, **34,** 2504, abstract.

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