THE ISOLATION AND COMPOSITION OF DENSE GRANULES FROM Ca^{++}-LOADED MITOCHONDRIA

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The mitochondrial uptake of Ca^{++} has been extensively studied since its first description by Vasington and Murphy (1961, 1962). It has been shown that the massive accumulation of Ca^{++} and phosphate (P_i) by respiring rat liver mitochondria is accompanied by the formation of electron-opaque granules within the mitochondrion. From a careful study of the stoichiometry of the Ca^{++} and P_i uptake, as well as from microincineration experiments, Greenawalt, Rossi and Lehninger (1964) suggested that the composition of these dense granules was calcium phosphate hydroxide (hydroxylapatite, $Ca_{10}(PO_4)_6(OH)_2$). However, their attempts to isolate the granules from mitochondria were unsuccessful.

In view of our previous experience with the isolation and characterization of calcareous corpuscles from the tapeworm, Taenia taeniaeformis (von Brand et al, 1960; Scott et al, 1962; von Brand and Weinbach, in press), it was of interest for us to attempt the isolation of the granules of mammalian mitochondria by similar procedures. Using simple techniques, we have readily obtained large quantities of dense granules from isolated rat liver mitochondria incubated under conditions favorable for massive accumulation of calcium and phosphate (Vasington and Murphy, 1962).

EXPERIMENTAL PROCEDURES

Incubation: Each reaction vessel contained 10 mM Tris-maleate buffer, pH 7.0; 4 mM P_i; 8 mM NaCl, 10 mM succinate; 10 mM $MgCl_2$; 3 mM ATP and 2.5 mM $CaCl_2$ in a final volume of 15 ml. One ml of rat liver mitochondria (Weinbach, 1962), containing 20 mg protein, was added and the suspension incubated at 30° for 20 minutes.

Isolation: The combined contents of 30 vessels were centrifuged at 10,000 x g for 10 minutes. The mitochondrial pellet was washed once with 0.25 M sucrose buffered at pH 7.0 with 5 mM Tris-maleate. Examination of these mitochondria by electron microscopy revealed an accumulation of electron dense granules. Isolation of these granules was accomplished by either of the following procedures. Method (A): The washed mitochondrial pellet was suspended in 3.0 ml of ethylenediamine and heated in a boiling water bath for 1.5 hours. During heating it was important frequently to redisperse the suspended material. The suspension was centrifuged at 16,000 x g for 5 minutes, and the precipitate succesively washed twice with water, once with absolute ethanol, and once with diethyl ether. The granules were air-dried overnight, then heated at 110° for 1 hour prior to weighing. Method (B): The dense granules were liberated by heating the mitochondrial pellet after resuspension in 3 ml of 10% KOH for 30 minutes at 100°. The granules were then collected by centrifugation and washed as described above. Method (C): The mitochondrial pellet was made into a smooth paste with a minimum amount of H_2O; 30 ml of 3% deoxycholate and 6 ml of 1 N NaOH added, and the volume adjusted to 60 ml with H_2O. To ensure complete solubilization of the mitochondria, the suspension was kept at room temperature for 30 minutes during which time it was intermittently shaken. The granules were then removed by centrifugation and washed as described above.

Analyses: Granules were incinerated at 600° for 18 hours, then dissolved in 3N HCl for chemical or radiochemical analyses. Ca^{++} was determined volumetrically after precipitation as the oxalate (Kolthoff and Sandell, 1952) isotopically with Ca^{45}; P_i, colorimetrically after wet ashing with

H_2SO_4-HNO_3-$HClO_4$ (LePage, 1948); Mg^{++}, by the methods of Orange and Rhein (1951), and McCann (1959). Carbonate was measured manometrically, and N by a micro Kjeldahl procedure. Trace elements were detected by emission spectroscopy (Melpar, Inc., Arlington, Va.).

RESULTS AND DISCUSSION

From a series of 18 large-scale experiments we have isolated 1.2 grams of dense granules. The average yields listed in Table I approximate the amounts of hydroxylapatite suggested by Greenawalt et al (1964) to be in mitochondria after massive Ca^{++}-loading. In addition to the major components listed in Table I, the granules when freshly isolated contained substantial amounts of organic material. Those isolated with ethylenediamine contained approximately 5% N; those isolated with deoxycholate, 1% N and those with KOH, 0.3% N. After incineration no N was detected in any of the samples. Emission spectroscopic analyses indicated trace amounts of other elements, including Fe, Cu, and Al.

Table I
Composition of Dense Granules

Method of Isolation	Yield*	Loss After Incineration**	Major Constituents***			
			Ca^{++}	P_i	Mg^{++}	$CO_3^{=}$
Ethylene-diamine	0.29	42.2	30.4	17.6	4.0	2.9
KOH	0.17	16.2	34.3	14.6	3.4	4.2
Deoxycholate	0.18	18.8	30.0	18.5	3.9	4.0

*Mg granules per mg of mitochondrial protein.
** Percent of total initial weight.
*** Determined after incineration and expressed as percent of total residual weight.

The granules as isolated are amorphous. In this respect they resemble tapeworm calcareous corpuscles which are amorphous in situ (von Brand et al, 1960). Incineration of the granules at 600° resulted in partial loss of $CO_3^{=}$, and induced crystallization. Supporting these results were electron microscopic studies which showed the presence of crystals only after incineration. X-ray diffraction analyses of the isolated granules

before and after incineration showed that the crystalline form contained only hydroxylapatite, or in some samples, hydroxylapatite and whitlockite (calcium orthophosphate, $Ca_3(PO_4)_2$) as major constituents.

The use of Ca^{45} showed that virtually all of this metal taken up by the mitochondria accumulated in the granules. Calculations based on uptake of the isotope confirmed the chemical analysis for Ca^{++}. Mitochondria incubated in the absence of ATP did not accumulate dense granules. Such tests served as convenient controls. Mitochondria after incubation with C^{14}-labeled ATP, yielded granules which contained 0.5% of the added nucleotide counts. This may be considered evidence that not only are adenine nucleotides taken up by respiring mitochondria during Ca^{++}-loading, but that significant amounts are associated with the dense granules. On the other hand, such findings must be interpreted with caution. It is quite possible that adenine nucleotides initially bound to mitochondrial membranes are released when these membranes are disrupted during isolation of the granules, and their binding to the granules is a secondary phenomenon. Significantly, it has been shown by Krane and Glimcher (1962) that synthetically prepared apatite crystals are capable of binding nucleoside di- and triphosphates.

Further work is in progress and full details of these experiments will be published elsewhere. We are indebted to Mr. C. Elwood Claggett for technical assistance, to Dr. H. Sheffield for the electron microscopic studies, and to Drs. D. B. Scott and M. U. Nylen for the X-ray diffraction analyses.

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