BIOCHEMICAL AND ULTRASTRUCTURAL ASPECTS OF Ca^{2+} TRANSPORT BY MITOCHONDRIA OF THE HEPATOPANCREAS OF THE BLUE CRAB *CALLINECTES SAPIDUS*

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ABSTRACT

Mitochondria isolated from the hepatopancreas of the blue crab *Callinectes sapidus* show up to 12-fold stimulation of respiration on addition of Ca^{2+}, which is accompanied by Ca^{2+} accumulation (Ca^{2+}:site = 1.9) and H^+ ejection (H^+:Ca^{2+} = 0.85). Sr^{2+} and Mn^{2+} are also accumulated; Mg^{2+} is not. A strongly hypertonic medium (383 mosM), Mg^{2+}, and phosphate are required for maximal Ca^{2+} uptake. Ca^{2+} uptake takes precedence over oxidative phosphorylation of ADP for respiratory energy. Once Ca^{2+} is accumulated by the crab mitochondria, it is stable and only very slowly released, even by uncoupling agents. ATP hydrolysis also supports Ca^{2+} uptake. Respiration-inhibited crab hepatopancreas mitochondria show both high-affinity and low-affinity Ca^{2+}-binding sites, which are inactive in the presence of uncoupling agents.

Crab hepatopancreas mitochondria have an enormous capacity for accumulation of Ca^{2+}, up to 5,500 ng-atoms Ca^{2+} per mg protein, with an equivalent amount of phosphate. Freshly isolated mitochondria contain very large amounts of Ca^{2+}, Mg^{2+}, phosphate, K^+, and Na^+; their high Ca^{2+} content is a reflection of the vary large amount of extramitochondrial Ca^{2+} in the whole tissue.

Electron microscopy of crab mitochondria loaded with Ca^{2+} and phosphate showed large electron-dense deposits, presumably of precipitated calcium phosphate. They consisted of bundles of needle-like crystals, whereas Ca^{2+}-loaded rat liver mitochondria show only amorphous deposits of calcium phosphate under similar conditions. The very pronounced capacity of crab hepatopancreas mitochondria for transport of Ca^{2+} appears to be adapted to a role in the storage and release of Ca^{2+} during the molting cycle of this crustacean.

Work in a number of laboratories (reviewed in references 1–3) has shown that mitochondria isolated from the liver and other tissues of vertebrates can transport and accumulate very large amounts of both Ca^{2+} and phosphate from the suspending medium at the expense of energy yielded from electron transport (4). As a consequence large electron-dense deposits of calcium phosphate appear in the mitochondrial matrix (5); these are crystallographically amorphous unless heated to

the transition temperature, after which they give the electron diffraction pattern of whitlockite (6). Mitochondria in intact vertebrate cells and tissues have also been observed to accumulate electron-dense deposits in the matrix under circumstances in which normal or induced deposition or lysis of bone is occurring (7). From these and other considerations it has been postulated that mitochondria may be the sites at which normal and abnormal biological calcification are initiated (2). It was further postulated that such deposits of calcium phosphate in the mitochondrial matrix may leave the mitochondria, possibly stabilized by some organic factor(s), and pass through the plasma membrane in membrane-surrounded form. Possibly relevant to this hypothesis are the extracellular, membrane-bounded "matrix vesicles" found by Anderson and his colleagues at the sites of earliest calcification in epiphyseal cartilage (8).

In an effort to find an easily accessible tissue known to participate in the transport and deposition of Ca^{2+} during a calcification process, our attention was drawn to the Crustacea. During their molting cycle some of the Crustacea, particularly species living on land or in fresh or brackish water, salvage Ca^{2+} from the old shell by reabsorbing and storing it in gastroliths and/or in soft tissues, from which Ca^{2+} is mobilized for calcification of the new shell. In this cycle of resorption, storage, and redeposition, very large quantities of Ca^{2+} are transported over a short period. Preliminary studies showed that the blue crab *Callinectes sapidus* from the low-salinity estuarine waters of the Chesapeake Bay, stores large amounts of mineral in its hepatopancreas (9). Therefore we chose to carry out a survey of Ca^{2+} transport in mitochondria isolated from this species and tissue, preparatory to study of the biochemical and morphological stages of calcification in Crustacea.

In a preceding paper (10) the isolation of mitochondria from the hepatopancreas of *C. sapidus* and their capacity for electron transport and oxidative phosphorylation in vitro were described. In this paper we report the respiration-coupled transport of Ca^{2+} by these mitochondria and the conditions leading to the formation of electron-dense granules of calcium phosphate in the mitochondrial matrix. The following paper (11) reports the size, ultrastructure, and chemical composition of cytoplasmic and extracellular calcium phosphate granules in crab hepatopancreas, as well as their possible role in calcium storage.

EXPERIMENTAL METHODS

Mitochondria were isolated from the crab hepatopancreas and suspended in the standard isolation medium (without EDTA) at a concentration of 50 mg of protein per ml, exactly as described earlier (10).

Respiration-coupled uptake of radioactive Ca^{2+} and other cations was measured by determining the cation remaining in the suspending medium after centrifugal removal of the mitochondria (1.5 min in a Coleman Microfuge [Coleman Instruments Div., Perkin Elmer Corp., Maywood, Ill.] at top speed or 4 min at 20,000 *g* in a Sorvall RC2-B ultracentrifuge [Ivan Sorvall, Newtown, Conn.]). Aliquots of the clear medium were counted in a Beckman model LS-100 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) to 1% precision. Binding of Ca^{2+} to respiration-inhibited mitochondria was measured according to Reynafarje and Lehninger (12). Oxygen uptake was measured polarographically with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) linked to a Sargent model SR recorder (Sargent-Welch Co., Skokie, Ill.). H^+ movements were recorded with a glass electrode and expanded-scale potentiometer (13). Inorganic phosphate was determined on neutralized perchloric acid extracts of mitochondria (10). The calcium and magnesium content of mitochondria was determined by atomic absorption spectrophotometry on 2 N HCl extracts, diluted to 0.5 N. Na^+ and K^+ were determined by flame photometry. Total amino acids and ninhydrin-positive material were determined by the method of Rosen (14) as modified by Grant (15).

Thin sections of tissues and isolated mitochondria were prepared for electron microscope examination by fixation with glutaraldehyde followed by osmium tetroxide or with glutaraldehyde alone as previously described, except that the final concentration of glutaraldehyde was 3% (16), dehydrated with acetone, and embedded in Epon 812 (17). The sections were stained with uranyl acetate and lead citrate or viewed unstained. Isolated mitochondria were also negatively stained with potassium phosphotungstate, pH 7.0. Specimens were observed in a Siemens Halske Elmiskop 1 A double-condenser electron microscope operated at 60 kV; micrographs were taken at magnifications ranging from 2,000 to 40,000 times.

RESULTS

Stimulation of Respiration and H^+ Ejection by Ca^{2+}

The optimal medium for Ca^{2+} uptake contained 200 mM mannitol, 83 mM sucrose, 10 mM Mg^{2+}, 6 mM phosphate, and 0.67 mg/ml bovine serum

albumin (BSA).[1] The addition of Ca^{2+} to crab hepatopancreas mitochondria respiring on succinate in this medium stimulated the oxygen consumption to greater rates than those given by ADP (Fig. 1). The ratio of the Ca^{2+}-stimulated to the state 4 respiration was in the range 5.5–12.5, higher than in virtually all other animal mitochondria tested (cf. reference 3) and also higher than the ratio evoked by ADP, namely, 4.5–8.5 (10). After the stimulation produced by Ca^{2+}, the rate of respiration at 25°C returned to the initial state 4 rate within 1 min. The Ca^{2+}:site respiratory activation ratio (number of Ca^{2+} ions required per pair of electrons to activate each energy-conserving site) with succinate as substrate was normal, between 1.9 and 2.0. Similar jumps of oxygen uptake were evoked by pulses of Ca^{2+} with the substrates pyruvate plus proline, malate, ascorbate plus *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), α-ketoglutarate, and glutamate; the Ca^{2+}:site respiratory activation ratio was uniformly in the range 1.80–1.90. Successive pulses of Ca^{2+} yielded a succession of jumps in oxygen uptake, with essentially complete return of the rate of oxygen consumption to the original state 4 rate and with essentially constant Ca^{2+}:site stoichiometry (Fig. 1).

Activation of respiration by Ca^{2+} was accompanied by accumulation of Ca^{2+} from the medium (Fig. 1). The Ca^{2+}:site accumulation ratio (number of Ca^{2+} ions accumulated per pair of electrons per site) in succinate-supported systems was in the range 1.79–1.95, approximately the same as in rat liver mitochondria (18). After the completion of the jump in oxygen uptake induced by addition of 100 μM Ca^{2+} (100 ng-atoms per mg protein), with return of oxygen consumption to the state 4 rate, the concentration of free Ca^{2+} remaining in the medium was about 1.0 μM; after addition of 1,000 μM Ca^{2+}, about 50 μM Ca^{2+} remained. Thus the fraction of the added Ca^{2+} accumulated during such respiratory jumps, uncorrected for the small losses of Ca^{2+} probably occurring during recovery of the mitochondria from the medium, ranges from at least 95 to 99%.

Ejection of H^+ occurred during the activation of respiration and the uptake of Ca^{2+}, as in mammalian mitochondria (1–3). The ratio nanogram-atoms H^+ ejected to nanogram-atoms Ca^{2+} accumulated was about 1.0 with succinate as substrate in the absence of phosphate. The presence of a low concentration of phosphate (0.25 mM) has no effect on this stoichiometry.

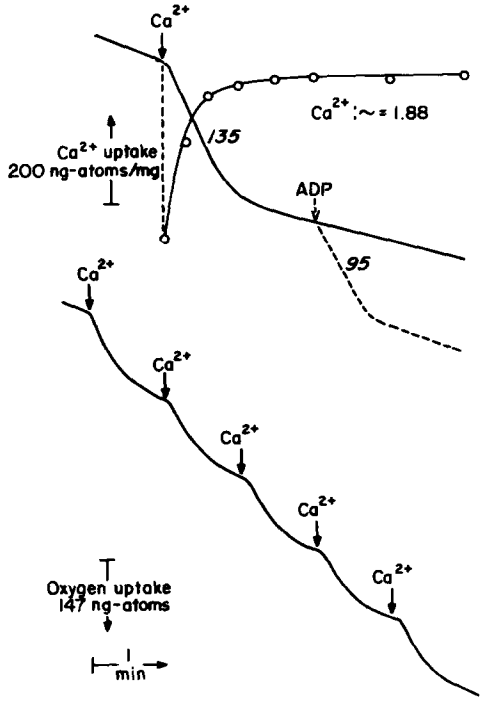


FIGURE 1 Activation of oxygen consumption and Ca^{2+} uptake. The incubation medium (3 ml) consisted of 200 mM mannitol, 83 mM sucrose, 15 mM Tris-succinate, 10 mM $MgCl_2$, 6 mM P_i, 10 mM Tris-chloride, and 0.67 mg/ml BSA, pH 7.2, 25°C. Mitochondrial protein was added at 1.0 mg/ml. When added, Ca^{2+} was 400 (upper trace) and 150 (lower trace) ng-atoms per mg protein and ADP was 133 μM. The rate of oxygen consumption was expressed as nanogram-atoms O per minute per milligram protein.

Requirements for Respiration-Dependent Accumulation of Ca^{2+}

For maximal rates of Ca^{2+}-stimulated oxygen uptake, Mg^{2+} was required in the medium at a level of at least 6 mM (data not shown). When Mg^{2+} was absent, Ca^{2+} stimulated the oxygen uptake to about 75% of the maximal rates but no return to the state 4 rate ensued. In the absence of Mg^{2+}, ADP evoked no stimulation of respiration (10).

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DNP, 2,4-dinitrophenyl; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

Maximal Ca^{2+} uptake was given in media containing mannitol (200 mM) and sucrose (83 mM); the total osmolarity of the system, including all components, was 380 mosM. When mannitol plus sucrose was replaced by 100 mM sucrose or by 100 mM NaCl or KCl, Ca^{2+} additions evoked little or no stimulation of oxygen uptake in the absence of Mg^{2+} and about 75% of the maximal oxygen uptake rate in the presence of 10 mM Mg^{2+}. BSA was necessary in the medium for maximal Ca^{2+} uptake as well as maximal oxidative phosphorylation (10).

The crab hepatopancreas mitochondria showed exceptional capacity to accumulate Ca^{2+} in the absence of phosphate. After addition of 400 ng-atoms of Ca^{2+} per mg protein, more than half was bound in the absence of phosphate, with normal stimulation of oxygen consumption. Binding of at least 600 ng-atoms of Ca^{2+} per mg protein occurred when Ca^{2+} was added at 4,000 ng-atoms per mg protein (Table I). The crab mitochondria thus have 7–15 times the capacity of rat liver mitochondria (1, 2) to bind Ca^{2+} in the absence of added phosphate in a respiration-dependent process. In no case was a state 6 type of respiratory inhibition (19) observed on adding excess Ca^{2+} in the absence of permeant anions. Phosphate was, however, required for maximal amounts and rates of Ca^{2+} uptake; about 6.0 mM phosphate was optimal. Higher concentrations of phosphate caused precipitation of calcium phosphate from the test medium in the absence of mitochondria.

ADP or ATP was not required and had no significant effect on the rate or stoichiometry of Ca^{2+} uptake by crab mitochondria up to or in excess of 150 ng-atoms Ca^{2+} per mg protein. Only at much higher Ca^{2+} loads did the supportive effects of ATP or ADP (18, 20) become apparent (see below).

Respiration-supported Ca^{2+} uptake by crab hepatopancreas mitochondria was inhibited by the respiratory inhibitor antimycin A and also by the uncoupling agent carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), but not by oligomycin.

TABLE I

Massive Accumulation of Divalent Cations

	Conditions	Divalent cation accumulation
		ng-atoms/mg protein
Exp. 1	Complete	3,599
	−ATP	1,913
	$-P_i$	1,488
	−ATP, $-P_i$	635
	+Oligomycin	3,425
Exp. 2	Complete (Ca^{2+})	4,415
	Complete (Sr^{2+})	2,392

The experimental conditions were as in Fig. 1 except that 4 mM ATP was present in the complete system. Oligomycin was added at 20 μg/mg protein. In exp. 1 the Ca^{2+} was added at 4,000 ng-atoms/mg protein and the system incubated for 40 min. In exp. 2 Ca^{2+} and Sr^{2+} were added at 6,000 ng-atoms/mg and phosphate and ADP at 6 mM each.

Maximum Rate and Affinity for Ca^{2+}

The effect of Ca^{2+} concentration on the initial rate of the stimulated oxygen uptake is given in the form of a Lineweaver-Burk plot (Fig. 2). The initial rate was taken as the amount of extra oxygen uptake in the first 15 s after Ca^{2+} addition, well beyond the lag time of the Teflon-coated oxygen electrode (2 s). The maximal stimulated rate of oxygen uptake (succinate) was about 150 ng-atoms oxygen per min per mg protein, corresponding to a rate of Ca^{2+} uptake of about 600 ng-atoms Ca^{2+} per min per mg. The apparent K_m for added Ca^{2+} in the respiratory activation was about 87 μM, about that for some vertebrate mito-

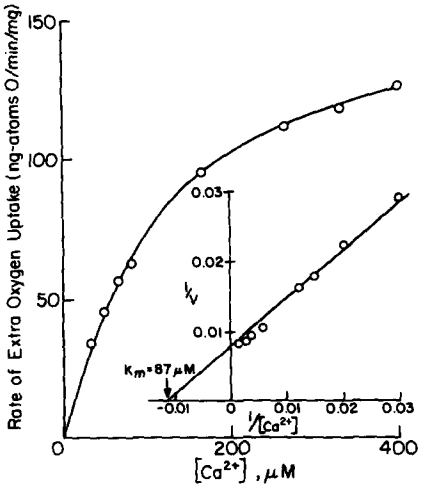


FIGURE 2 Effect of Ca^{2+} concentration on rate of stimulated oxygen consumption. The *inset* shows a Lineweaver-Burk plot of the data. Details as in Fig. 1. Ca^{2+} was added in the concentrations shown.

chondria. This value is uncorrected for complexation of Ca^{2+} by succinate and phosphate; presumably the true K_m for Ca^{2+} is much lower.

Precedence of Ca^{2+} Uptake over Oxidative Phosphorylation

In mitochondria of most vertebrate tissues Ca^{2+} uptake takes precedence over ADP phosphorylation for energy generated by respiration (18). Crab hepatopancreas mitochondria show the same properties (Fig. 3). When Ca^{2+} and ADP were added simultaneously to mitochondria respiring in state 4, the total extra oxygen uptake evoked was equal to the sum of the extra oxygen uptakes after separate, consecutive additions of ADP and Ca^{2+}. The oxygen uptake trace showed a point of inflection corresponding to the completion of the Ca^{2+}-activated respiration and the onset of the ADP-activated respiration. Ca^{2+} uptake measurements showed that the presence of ADP did not retard the rate or amount of Ca^{2+}

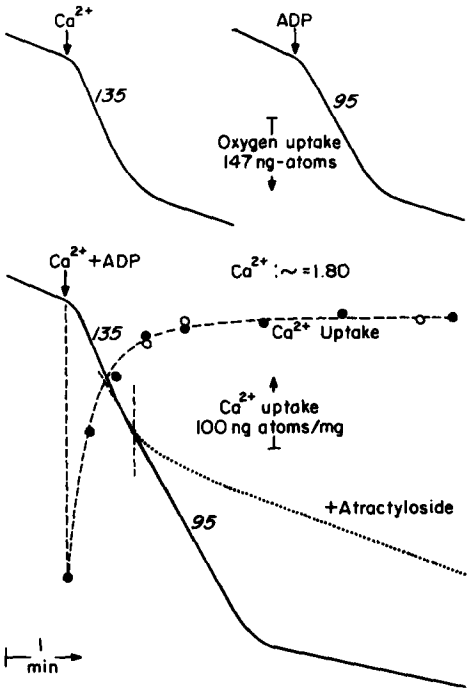


FIGURE 3 Ca^{2+}-induced and ADP-induced oxygen uptake rates and the dominance of Ca^{2+} uptake over phosphorylation. Details as in Fig. 1. When added, Ca^{2+} was 400 ng-atoms per mg protein and ADP was 180 μM (upper trace) or 240 μM (lower trace). Atractyloside (dotted trace) was added at 5 μM.

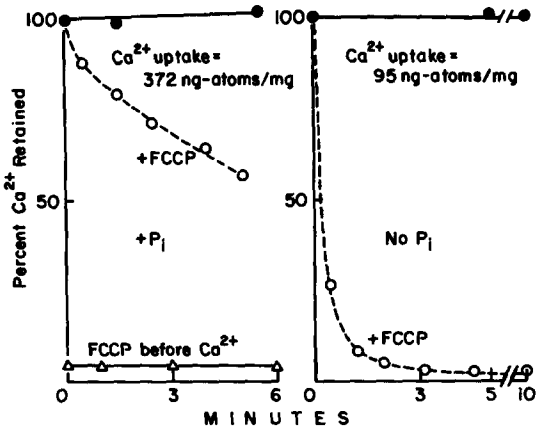


FIGURE 4 Effect of FCCP on Ca^{2+} uptake and release in the presence and absence of phosphate. Details as in Fig. 1 except that Mg^{2+} was 3 mM. Ca^{2+} was added at 400 (left) and 100 (right) ng-atoms per mg protein, in the presence and absence of 6 mM phosphate, as shown. FCCP, 0.75 μM, was added after Ca^{2+} had been incubated with the mitochondria for 5 min. When FCCP was present before Ca^{2+} addition, it was added at 0.5 μM.

uptake. These conclusions were supported by a similar experiment in which atractyloside was present; after the Ca^{2+} was accumulated the oxygen consumption returned to the state 4 rate, as would be expected when phosphorylation of external ADP is inhibited (Fig. 3).

Retention and Release of Ca^{2+}

Once Ca^{2+} is accumulated by crab mitochondria, whether in the absence or presence of phosphate, it is rather tenaciously held with no loss for 40 min or longer at 23°C (Fig. 4). The uncoupling agent FCCP yielded only a very slow, partial release of Ca^{2+} after loading with phosphate. Presumably the Ca^{2+} and phosphate are deposited in a stable insoluble form (see below).

ATP-Supported Ca^{2+} Uptake

Ca^{2+} uptake by crab hepatopancreas mitochondria is also supported by the hydrolysis of ATP (Fig. 5). In this case no phosphate addition is required, ample phosphate evidently being available from the hydrolysis of ATP. Only a small fraction of the Ca^{2+} uptake so observed is due to endogenous respiration since antimycin A or cyanide gave very little inhibition. ATP-supported

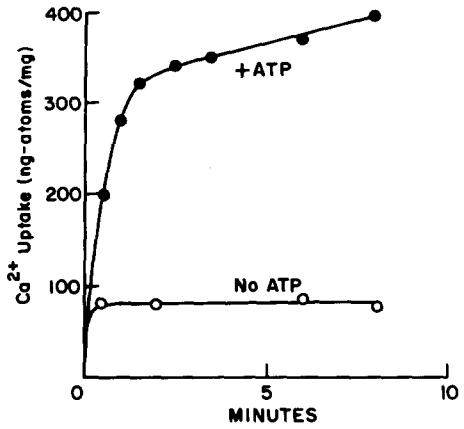


FIGURE 5 Support of Ca^{2+} accumulation by ATP. Details as in Fig. 1, except that succinate was replaced by 8 mM ATP, Mg^{2+} was 3 mM, and phosphate was omitted. Ca^{2+} was added at 400 ng-atoms per mg protein.

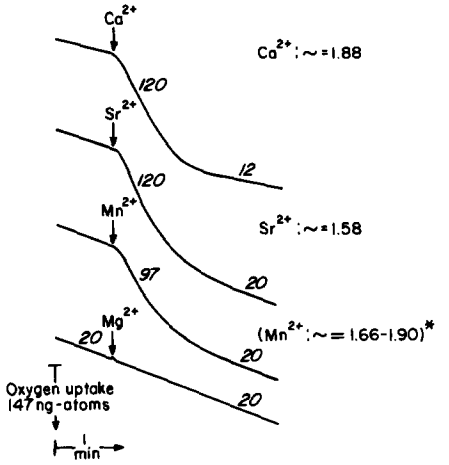


FIGURE 6 Activation of respiration by Ca^{2+}, Sr^{2+} and Mn^{2+}. Details as in Fig. 1. When added, Ca^{2+} and Sr^{2+} were 400 μM and Mn^{2+} was 333 μM. Mg^{2+} was added at 20 mM in addition to that amount (10 mM) originally present. * The stoichiometry was estimated by assuming 70–80% of the Mn^{2+} added was accumulated.

Ca^{2+} uptake was inhibited by oligomycin and atractyloside, as expected.

Respiratory Activation by Other Divalent Cations

Both Sr^{2+}- and Mn^{2+}-stimulated state 4 respiration of crab hepatopancreas mitochondria (Fig. 6) with normal return to state 4 rates, when tested at levels of 300–400 ng-atoms per mg protein. The Sr^{2+}:site activation ratio was 1.6 with 15 mM succinate and 6 mM phosphate in the medium. Sr^{2+} showed about the same activity as Ca^{2+}. Unlike vertebrate mitochondria, in which the response to Mn^{2+} addition is relatively sluggish (21, 22), Mn^{2+} gave a good stimulation (Fig. 6). Under these conditions, Mg^{2+} did not stimulate oxygen consumption.

Respiration-Independent Ca^{2+} Binding

Mitochondria of vertebrate tissues show two classes of respiration-independent Ca^{2+}-binding sites differing in affinity for Ca^{2+} (3, 12); the high-affinity sites are relatively few in number compared to the low-affinity sites. The Scatchard plot of Ca^{2+} binding to respiration-inhibited crab hepatopancreas mitochondria shown in Fig. 7 also exhibits biphasic character. In such experiments the mitochondria were first titrated with antimycin A and KCN to ascertain the concentrations required to completely inhibit oxygen uptake. The dissociation constant K_D of the high-affinity Ca^{2+}-binding sites was 0.25–0.37 μM; that of the low-affinity sites was more difficult to extrapolate accurately but probably exceeds 31 μM. There are about 4–6 ng-atoms of high-affinity and 95–170 ng-atoms of low-affinity Ca^{2+}-binding sites per mg protein in the crab mitochondria. The uncoupler FCCP strongly inhibited Ca^{2+} binding at both the high-affinity and low-affinity sites. Mg^{2+}, at 10 mM, was as effective as 0.5 μM FCCP with respect to the inhibition of respiration-independent Ca^{2+}-binding activity.

Massive Loading of Ca^{2+} and Phosphate

Data in Table I show that upon addition of 4,000 ng-atoms Ca^{2+} per mg protein to crab mitochondria supplemented with succinate, phosphate, and ADP, very large amounts of Ca^{2+} are taken up, about 3,600 ng-atoms per mg protein, which greatly exceeds the maximum amounts of Ca^{2+} uptake observed in mammalian mitochondria (1,500–2,500 ng-atoms per mg [4, 23]). Both phosphate and ADP are required; oligomycin produced little inhibition. When succinate was omitted and the ATP was increased to 15 mM, over 2,900 ng-atoms of Ca^{2+} were accumulated per mg protein. Up to 4,500 ng-atoms Ca^{2+} per mg protein was accumulated during oxidation of succinate when Ca^{2+} was added at 6,000 ng-atoms per mg.

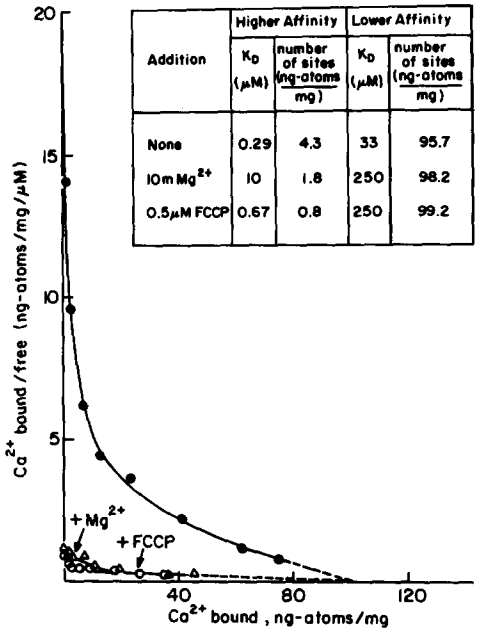


FIGURE 7 Scatchard plot of Ca^{2+} binding to respiration-inhibited crab mitochondria. The incubation medium contained 200 mM mannitol, 83 mM sucrose, 0.225 μM antimycin A, 100 μM KCN, and 2 mM Tris-Cl, pH 7.2, 0°C. The experimental procedures are described in Methods. When added, mitochondrial protein was 1.0 mg/ml; Ca^{2+} in the range 1.0–200 μM; FCCP, 0.5 μM; and Mg^{2+}, 10 mM. The open circles are the experimental points in the presence of FCCP and the open triangles the points in the presence of Mg^{2+}.

The data in Fig. 8 show that phosphate is accumulated from the medium together with Ca^{2+}. When the value for P_i is taken as the sum of endogenous content and that accumulated the atomic ratio $Ca:P_i$ = 1.60 is between that of amorphous tricalcium phosphate (1.50) and that of hydroxyapatite (1.67).

The maximum amounts of Ca^{2+} and phosphate that may be loaded by mitochondria from the crab hepatopancreas are actually substantially larger than those given in Table I, since crab mitochondria as freshly isolated already contain up to 900 ng-atoms each of Ca^{2+} and phosphate per mg protein (Table II), whereas freshly isolated rat liver mitochondria contain only 5–10 ng-atoms Ca^{2+} and about 10 nmol phosphate per mg protein. Thus the largest Ca^{2+} loads observed in crab mitochondria exceeded 5,500 ng-atoms of Ca^{2+} per mg protein, or over 33% of their dry weight.

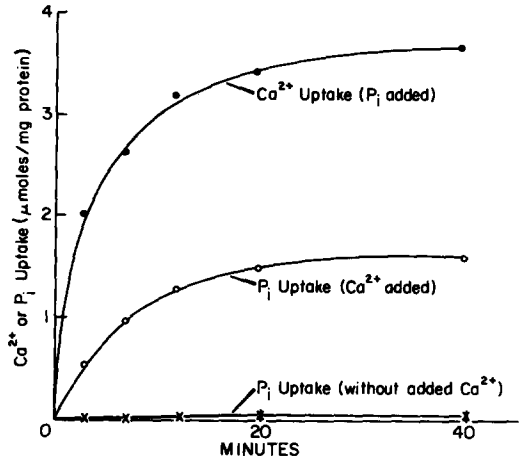


FIGURE 8 Uptake of Ca^{2+} and phosphate during massive loading. Details as in Fig. 1, except that P_i was 4 mM, and Mg^{2+} was 3 mM. ATP (6 mM) was also present. Ca^{2+} was added at 4,000 ng-atoms per mg protein. The P_i accumulation was measured isotopically by determining the ^{32}P remaining in the medium after the mitochondria were removed by centrifugation.

Mineral Content of Freshly Isolated Crab Mitochondria

The Ca^{2+}, Mg^{2+}, Na^+, K^+, and phosphate content of mitochondria freshly isolated from crab hepatopancreas is given in Table II. The Ca^{2+} content ranged from 55 to nearly 900 ng-atoms Ca^{2+} per mg protein, vastly in excess of the amounts found in freshly isolated liver mitochondria (5–10 ng-atoms per mg [3]). The large variability in the Ca^{2+} content of the crab mitochondria is probably due to the fact that the animals were sampled in different seasons and may have been in different periods of intermolt, although all appeared to have hard, well-filled shells. Crabs caught in the fall months showed the highest content of Ca^{2+}.

A substantial fraction of the Ca^{2+} found in freshly isolated crab mitochondria was accumulated from the cytoplasmic contents during homogenization of the tissue and the subsequent fractionation procedure. Direct measurements showed the soluble supernatant fraction of crab hepatopancreas homogenate, after centrifugal removal of the mitochondria, to contain about 1.8–2.1 mM Ca^{2+}. Moreover, addition to the homogenization medium of 25 μM ruthenium red, which inhibits respiration-dependent Ca^{2+} uptake by mitochondria (24), reduced the Ca^{2+} content of the

TABLE II

The Mineral Content of Freshly Isolated Crab Mitochondria

Date of preparation	Content (ng-atoms/mg protein)				
	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	P_i
Summer crabs					
7/7 to 8/25/1971	86.2 (55–224)	13.8 (7.0–35.2)	56.8 (48–73)	462 (403–571)	
Fall crabs					
10/13 to 12/8/1971	661 (320–896)	36.0 (21–48.8)			821 (610–920)

See text for details. The data were obtained from several mitochondrial preparations on different dates and reported as mean values, with the ranges shown in parentheses.

mitochondria subsequently isolated by 70%; the Mg^{2+} content was unaffected (Table III). Evidently sufficient respiration occurs during homogenization and fractionation to cause considerable Ca^{2+} to be accumulated by the mitochondria from the large amount present in the homogenate whole organ (25).

The K^{+} and Na^{+} contents of the crab mitochondria are several times higher than in mammalian mitochondria; the Mg^{2+} content is also high in most cases. The phosphate content, from 600 to 900 nmol/mg protein, is some 40- to 60-fold higher than in rat liver mitochondria. Since more phosphate is present than required as counteranion for the Ca^{2+} and Mg^{2+} it may also serve as the major counteranion for the very high K^{+} and Na^{+} content.

Crab hepatopancreas homogenates have been found to contain large amounts ($\sim$181 μmol/g wet tissue) of soluble ninhydrin-positive material, probably free amino acids and peptides, which increased during fractionation at the expense of the total protein content, presumably because of intense proteolytic activity. These amino acids may represent a major respiratory substrate for the crab mitochondria, particularly during isolation.

TABLE III

Effect of Inhibitors on Ca^{2+} and Mg^{2+} Content of Freshly Isolated Crab Mitochondria

Additions to isolation medium	Divalent cation content of mitochondria (ng-atoms/mg protein)	
	Ca^{2+}	Mg^{2+}
None	591 ± 18	139 ± 6
5 mM EDTA	583 ± 45	123 ± 0.1
25 μM ruthenium red (RR)	184 ± 9	133 ± 0.6
RR + EDTA	145 ± 8	136 ± 0.2

The additions shown were made to a crab hepatopancreas homogenate immediately after preparation but before fractionation. Data are means of two experiments.

Ultrastructure of Mitochondria of Crab Hepatopancreas

Fig. 9 shows that mitochondria in intact intermolt hepatopancreas cells are not greatly different from mitochondria of rat liver, with typical spacing of outer and inner membranes and with relatively sparse and irregularly disposed cristae. Both spherical and ellipsoidal profiles are observed. As shown in Fig. 9 most of the mitochondria were devoid of electron-dense deposits, even in cells that contain large cytoplasmic granules such as those shown in the following paper (11).

Fig. 10 is a low-power micrograph of mitochondria freshly isolated from crab hepatopancreas. Most of the mitochondria contain electron-dense deposits, some of which appear as small clusters of needles (*n*). In addition, within the matrices of the mitochondria many nonstaining regions (*c*) are seen which contain very fine needle-like precipitates in their centers or at their edges. These sites appear somewhat similar to the "hollow" centers of the granular deposits seen in massively loaded mitochondria isolated from crab hepatopancreas (Fig. 11) or rat liver and even more like the sites remaining after discharge of

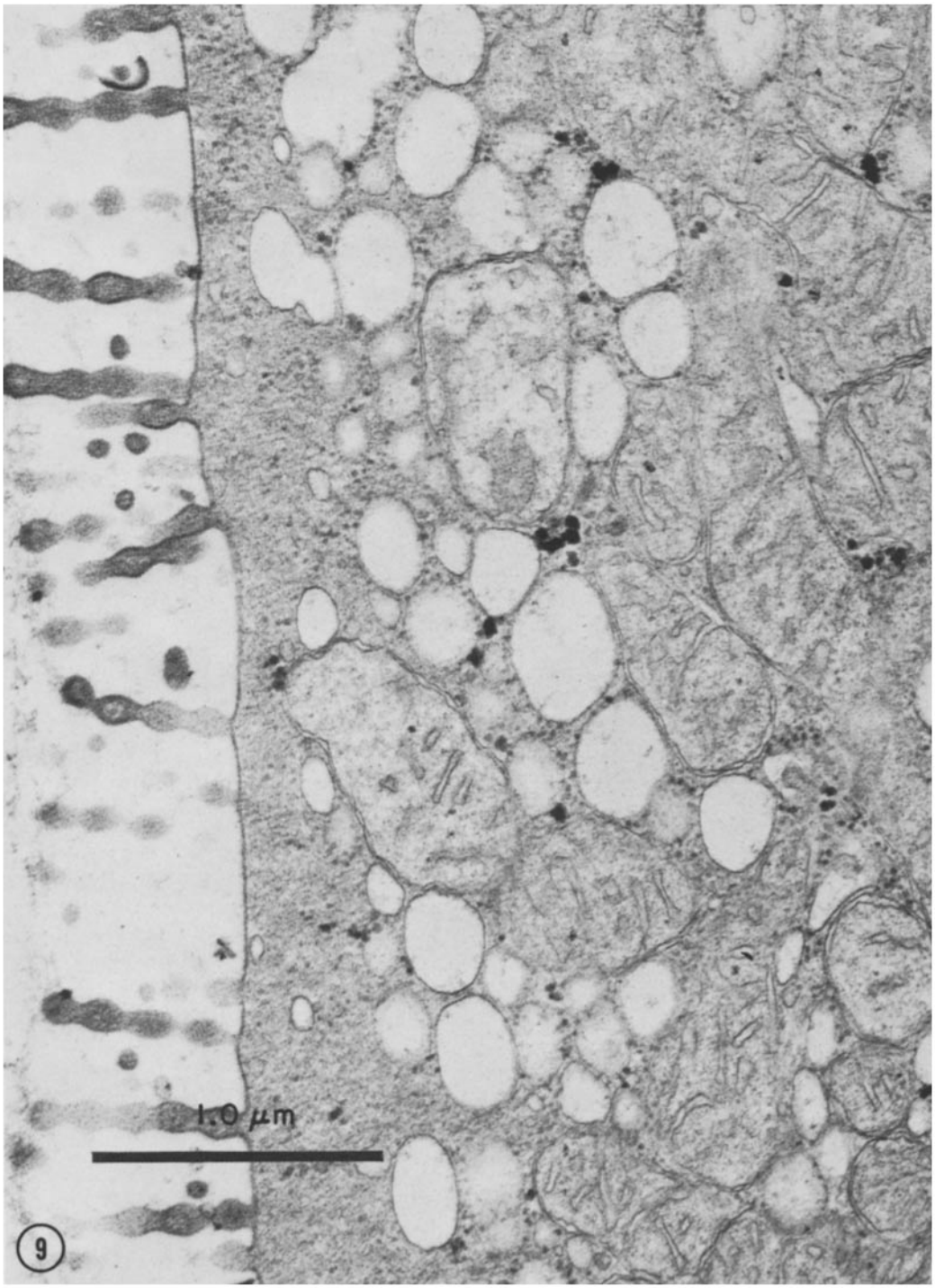
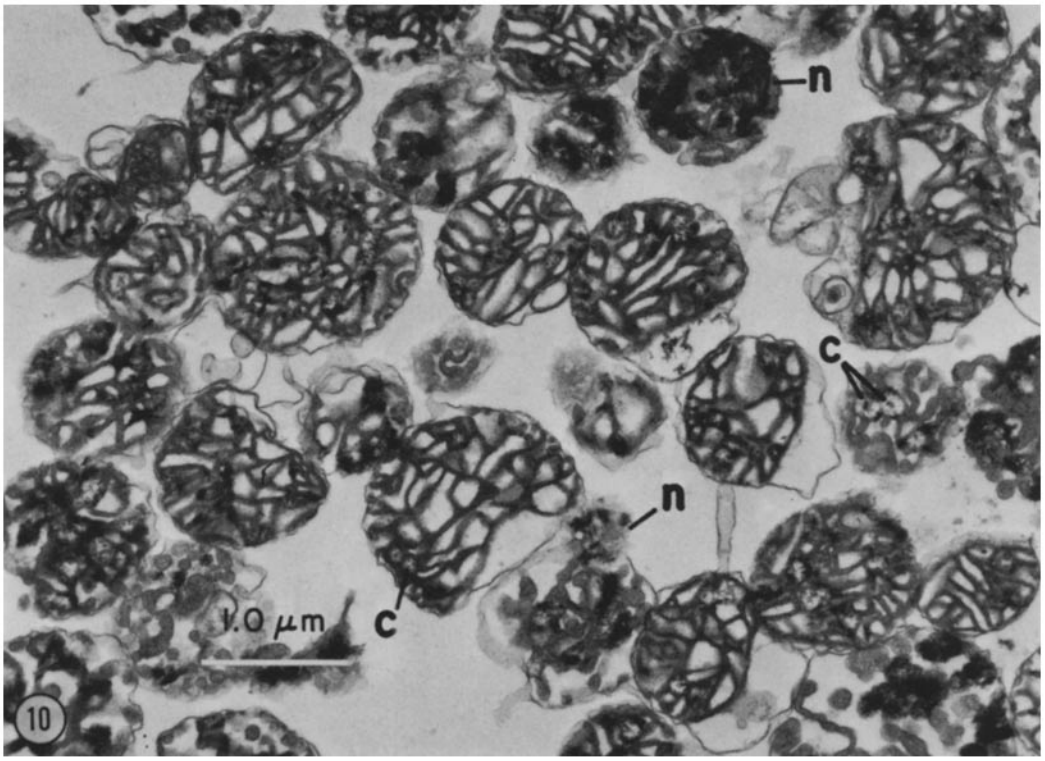


FIGURE 9 Thin section of epithelial cell of crab hepatopancreas. Microvilli, plasma membrane, mitochondria, and membrane-enclosed vesicles are shown. No prominent electron-dense deposits can be seen in the mitochondrial profiles. Fixed with glutaraldehyde and OsO_4. The sections were stained with uranyl acetate and lead. × 43,000.



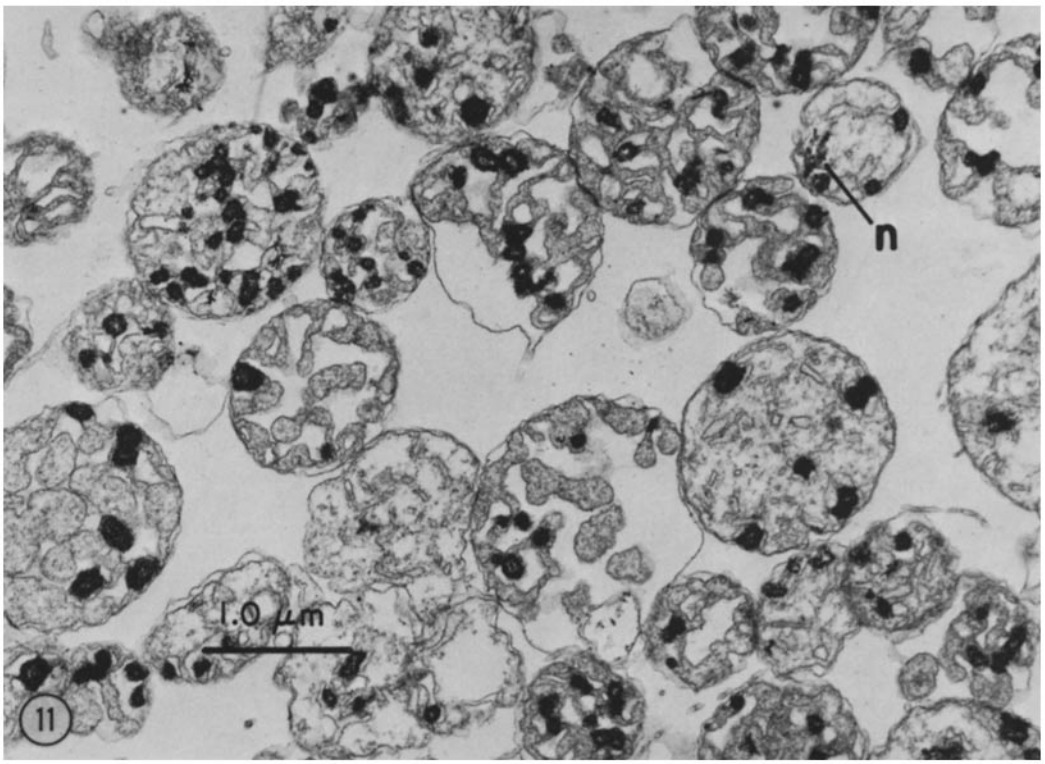


FIGURE 10 Freshly isolated hepatopancreas mitochondria. Electron-dense deposits, presumably Ca^{2+} and phosphate, are visible. Some are present as fine needle-like deposits (*n*) and others appear to have nonstaining centers (*c*) containing fine needles. Fixed and stained as described in Fig. 9. × 21,000.

FIGURE 11 Crab hepatopancreas mitochondria massively loaded in vitro, and fixed with glutaraldehyde and OsO_4 as in Fig. 9. Almost every mitochondrion contains several large, discrete electron-dense deposits, most of which have nonstaining centers. Relatively few needle-like deposits (*n*) are seen. Sections were stained with uranyl acetate and lead. × 21,000.

Ca^{2+} and P_i from massively loaded rat liver mitochondria by 2,4-dinitrophenyl (DNP) (5). Since only a small minority of the mitochondria visualized in intact cells of crab hepatopancreas contain such deposits, it is tentatively concluded that the crab mitochondria accumulate Ca^{2+} and phosphate from the extramitochondrial phase during homogenization and isolation procedures, presumably as the result of respiration-coupled active transport (Table II).

The ultrastructure of isolated crab mitochondria after massive loading in vitro is shown in Fig. 11. Levels of about 4,500 ng-atoms of Ca^{2+} per mg protein were accumulated over 20 min at 25°C. The large electron-dense deposits of Ca^{2+} and phosphate can readily be seen in the matrix of almost every mitochondrion; frequently these granules appear to have less densely staining centers. Similar nonstaining regions are seen in the freshly isolated mitochondria (Fig. 10). Small indistinct needles are only occasionally seen around the nonstaining centers (*c*) of the granules in the loaded mitochondria or as individual deposits.

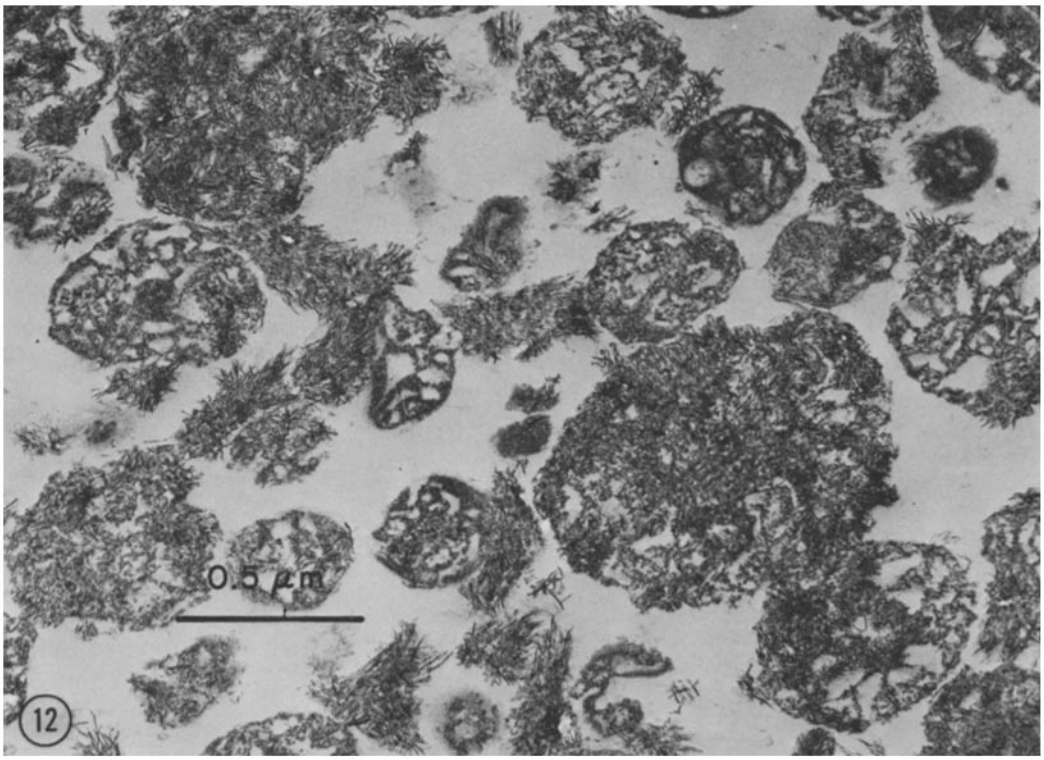
If the massively loaded mitochondria are fixed only with glutaraldehyde, rather than with both glutaraldehyde and OsO_4, the appearance of the mitochondria and the accumulated Ca^{2+} and phosphate is grossly different. As shown in Fig. 12 the mitochondria (which do not give clearly defined membrane images) contain masses of rather uniformly distributed needles. Since it has been shown that fixation of Ca^{2+}-loaded mitochondria with OsO_4 extracts as much as 50% of the accumulated Ca^{2+} (J. W. Greenawalt, unpublished observations), it seems likely that the localization of the granules seen in Figs. 10 and 11 results, at least in part, from the extraction of some of the matrix Ca^{2+} and phosphate. This effect was observed in earlier experiments with Sr^{2+} accumulation in rat liver mitochondria (26). The needle-like appearance of the deposits in glutaraldehyde-fixed mitochondria can be seen clearly at higher magnification in Fig. 13.

The inner membrane of isolated crab mitochondria, prepared for negative contrast with potassium phosphotungstate, was found to be covered with closely packed, projecting particles (inner membrane spheres) similar to those described for mitochondria from a wide variety of tissues (27–29).

DISCUSSION

The Ca^{2+} transport activity of mitochondria isolated from the hepatopancreas of the blue crab resembles in many respects that of mitochondria from such vertebrate tissues as rat liver, but in other respects there are striking differences. Among the important points of similarity are (*a*) the stimulation of respiration by Ca^{2+} with concurrent accumulation of Ca^{2+}, followed by a return to normal state 4 respiration, (*b*) similar stoichiometric relationships in the activation of electron transport and in Ca^{2+} accumulation, with a Ca^{2+}:site ratio of approximately 2, (*c*) stimulation of respiration by Ca^{2+}, Sr^{2+}, and Mn^{2+}, but not by Mg^{2+}, (*d*) the requirement of phosphate for maximum loading of Ca^{2+}, (*e*) the formation of electron-dense deposits of calcium phosphate visualized with the electron microscope, (*f*) the respiration-dependent uptake of Ca^{2+} in the absence of phosphate, i.e., membrane loading, (*g*) the presence of both low-affinity and high-affinity respiration-independent binding sites for Ca^{2+}. In all these respects crab hepatopancreas mitochondria qualitatively resemble mitochondria isolated from various vertebrate tissues as reported earlier (3). However they differ from mitochondria isolated from blowfly flight muscle (30), from many plant tissues (31), and from yeast (32), which have only a very limited activity in Ca^{2+} transport.

On the other hand, there are a number of significant differences in the behavior of the Ca^{2+} transport system of crab hepatopancreas mitochondria compared with those from rat liver. The crab hepatopancreas mitochondria show exceptionally high degrees of respiratory stimulation, up to 12-fold, in contrast to mitochondria from most vertebrate tissues, which usually give four- to six-fold stimulation under the same conditions. The crab mitochondria also show exceptionally high rates of Ca^{2+} transport, a reflection of their relatively high respiratory rates. The crab mitochondria also show a substantially greater capacity for respiration-dependent accumulation of Ca^{2+} in the absence of phosphate or other permeant anions, several times greater than that of rat liver mitochondria, indicating that they have a very much larger number of membrane sites participating in membrane loading of Ca^{2+} (33). They also have a much larger capacity for accumulation of Ca^{2+} and phosphate in the matrix, up to 5,500 ng-atoms Ca^{2+} per mg protein, or four to five times the maximum capacity of rat liver mitochondria.



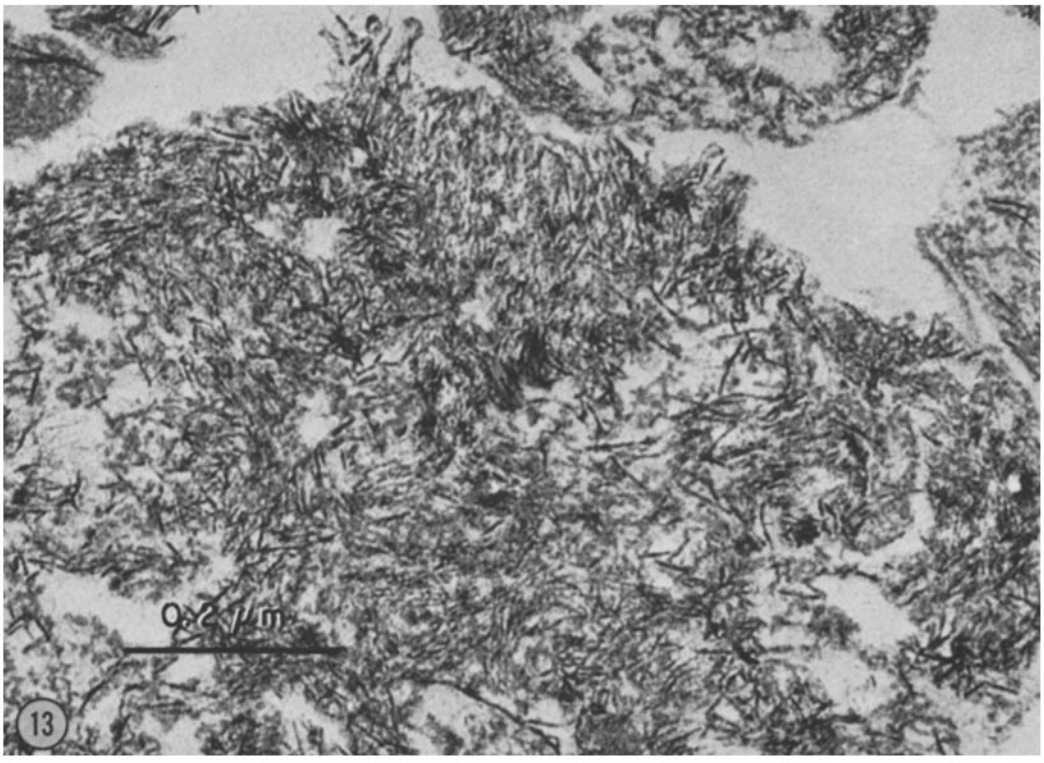


FIGURE 12 Crab hepatopancreas mitochondria massively loaded in vitro but fixed with glutaraldehyde only. Membranes are indistinct; fine needle-like deposits are present throughout the mitochondrial matrices. Sections were stained with uranyl acetate and lead. × 52,500.

FIGURE 13 Higher magnification of preparation shown in Fig. 12, showing needle-like appearance of the deposits. Section stained with uranyl acetate and lead. × 150,000.

All these properties support a possible role of mitochondria in the storage of Ca^{2+} in the hepatopancreas of the crab.

An especially striking feature in the Ca^{2+} transport behavior of the crab hepatopancreas mitochondria is the crystalline appearance of the calcium phosphate deposits in the matrix after in vitro accumulation, as detected by electron microscopy. Matrix deposits of Ca^{2+} and phosphate in rat liver mitochondria loaded in vitro under the same conditions are definitely amorphous, as indicated by X-ray (5) and electron (6) diffraction analysis. It has been postulated (2) that in vertebrate tissues amorphous calcium phosphate in mitochondria is kept from being transformed into hydroxyapatite, an otherwise rapid and spontaneous process, by the presence of specific inhibitors, such as those participating in the prevention of calcium phosphate stones in the urinary system (34). Detailed physical analyses, including X-ray and electron diffraction studies, will be required to determine whether or not the deposits of Ca^{2+} and phosphate in crab mitochondria are, in fact, crystalline. If it is confirmed by these determinations that the calcium phosphate in the loaded mitochondria is crystalline, they may lack the crystallization inhibitors apparently present in rat liver mitochondria.

Another striking feature of isolated crab hepatopancreas mitochondria is their capacity to retain loads of calcium phosphate tenaciously, even in the presence of uncoupling agents, which normally cause rapid discharge of Ca^{2+} from rat liver mitochondria (3, 5). The tenacious retention of Ca^{2+} by the isolated crab mitochondria stands in sharp contrast to the fact that in thin sections of intact crab hepatopancreas most of the mitochondria are completely devoid of electron-dense deposits, although the cytoplasm and extracellular spaces contain very large amounts of Ca^{2+} in the form of large granules (11). When the hepatopancreas is homogenized and the mitochondria recovered by centrifugation, a process taking place at 0°C over about 30 min, the resulting mitochondria are found to be heavily loaded with calcium phosphate. Thus it appears that mitochondria in the intact hepatopancreas cell are either kept from acquiring huge loads of Ca^{2+}, or the loading and release of Ca^{2+} are in dynamic balance, to yield a steady-state in which relatively little Ca^{2+} is present in the matrix. These observations suggest that the crab mitochondria may be useful in study of the stimulus and mechanism for Ca^{2+} release by mitochondria in the intact cell.

The very large content of Ca^{2+} in crab hepatopancreas, much of which is present in large cytoplasmic and extracellular granules as shown in the following paper (11), and the exceptional ability of crab mitochondria to transport and accumulate Ca^{2+} in a crystalline form, presumably hydroxyapatite, are consistent with the known ability of some of the visceral tissues to store Ca^{2+} in various crustacean species and with the increasing evidence (33) that mitochondria participate in the buffering of cytoplasmic Ca^{2+} concentration by reversible segregation of Ca^{2+} in the mitochondrial matrix (1, 2, 35). The data reported in this and the following paper (11) suggest the possibility that the mitochondria of crab hepatopancreas, because of their unusual activity, participate in the storage of large amounts of Ca^{2+} and phosphate, possibly by the initial segregation of small insoluble deposits in the matrix, followed by their emergence into the cytoplasm, to form cytoplasmic granules which ultimately become extracellular.

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