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Ca^{2+} Transport by Chondrocyte Mitochondria of the Epiphyseal Growth Plate

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Summary. In a study of the Ca^{2+} kinetics of mitochondria of chick epiphyseal chondrocytes, the rate of Ca^{2+} uptake was linear up to a medium Ca^{2+} concentration of 30 μM. The half maximal transport rate occurred at 34 μM Ca^{2+}. The Ca^{2+} uptake rate, expressed as a function of time, was 35 nmoles/mg protein/min; the presence of Mg^{2+} had little effect on Ca^{2+} accumulation. While these kinetic parameters did not differ significantly from mitochondria of cells of nonmineralizing tissues, the respiratory characteristics of the chondrocyte organelles exhibited functional differences. Thus, up to 350 nmoles Ca^{2+}/mg protein, chondrocyte mitochondria performed coupled oxidative phosphorylation. Calcium uptake was energy supported, while Ca^{2+} binding was low. Addition of respiratory inhibitors and uncouplers to these mitochondria resulted in a rapid loss of more than 80% of the total Ca^{2+}. The Ca/Pi ratio of the extrudate was very similar to the ratio of these ions in cartilage septum fluid. In the most mineralized zones of the epiphyseal plate, there was little change in the state 4 respiratory rate, but nonspecific Ca^{2+} binding was elevated and a high percentage of the total Ca^{2+} was in a nonextrudable form. The results indicate that in cells preparing for mineralization, much of the total mitochondrial Ca^{2+} is in a form that can be transported to the calcification front. In cells close to the calcification front, nonextrudable Ca^{2+} may form calcium phosphate granules described by other investigators.

In the process of endochondral bone formation, chondrocyte mitochondria accumulate Ca^{2+} (Arsenis, 1972; Shapiro & Lee, 1975*a*). The highest concentrations of Ca^{2+} are observed in the zone of provisional mineralization. Here, the mitochondrial Ca^{2+} content is over two hundred times greater than the values reported for mitochondria of cells of nonmineralizing tissues (Carafoli & Lehninger, 1971; Shapiro & Lee, 1975*b*). Brighton and Hunt (1974) have shown that a layer of cells in this cartilagenous zone loses Ca^{2+} from mitochondrial stores; coincidental with this event, calcification of extracellular matrix vesicles and collagen is seen. These findings lend support to the hypothesis that calcification is an ion transfer process that is directly controlled by mitochondrial activity (Shapiro & Greenspan, 1969; Lehninger, 1970).

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The effects of Ca^{2+} on mitochondrial function have been studied in two ways. The first approach has been to study the biochemical characteristics of soft tissue cells that contain elevated mitochondrial Ca^{2+} levels. Tissues included in this category are human myometrium (Malmström & Carafoli, 1977), vascular smooth muscle (Vallieres, Scarpa & Somlyo, 1975), dystrophic skeletal muscle (Mezon, Wrogemann & Blanehaer, 1974) and ascites tumor cells (Thorne & Bygrave, 1974). The Ca^{2+} concentration of these organelles is 20 times higher than other soft tissues mitochondria, but comparable only to the values reported for only the most superficial zone of growth plate cartilage (Shapiro & Lee, 1975*b*). A second approach to the study of the effects of Ca^{2+} on mitochondrial activity has been to load mitochondria of soft tissues with Ca^{2+} *in vitro* (Vasington & Murphy, 1962; Carafoli, Rossi & Lehninger, 1965; Greenawalt, Rossi & Lehninger, 1964). The results of the latter studies indicate that "massive" loading produces swelling, membrane damage, and organelle lysis (Lehninger, Carafoli & Rossi, 1967; Chappel & Crofts, 1965; Hackenbrook & Caplan, 1969; Slater & Cleland, 1953). In chondrocytes, while the mitochondrial Ca^{2+} concentrations are very high, surprisingly, observational studies indicate little evidence of pathological damage (Matthews *et al.*, 1970). Whether biochemical function is impaired at high Ca^{2+} levels has not been ascertained. The purpose of this investigation is to examine the relationship between mitochondrial Ca^{2+} load, Ca^{2+} uptake, and mitochondrial function. Using mitochondria obtained from selected regions of the mineralizing growth plate, the respiratory activities of mitochondria containing differing endogenous levels of Ca^{2+} are described, and kinetic parameters of Ca^{2+} uptake and release are reported.

Materials and Methods

Materials

BSA (bovine serum albumin), HEPES (N-2-hydroxyl-ethyl piperazine-N′-2-ethane sulfonic acid), ADP, ATP, succinate-Na_2, DNP (2,4-dinitrophenol), rotenone, antimycin A, ruthenium red, DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)), DTT (dithiothreitol), NTA (nitrilotriacetic acid), EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid) were all purchased from Sigma Chemical Co., St. Louis, Mo. CCCP (*m*-chloro-carbonylcyanide phenylhydrazone) was obtained from ICN Nutritional Biochemicals Corp., Cleveland, Ohio. $^{45}CaCl_2$ (1 mCi ^{45}Ca/μmole Ca^{2+}) was from New England Nuclear Corp.

Isolation of Mitochondria

White Rock chicks, 4–5 kg, 8–10 weeks old (West Jersey Biological Supply) were sacrificed and mitochondria were isolated from various regions of the epiphyseal plate of the long bones as described previously (Shapiro & Lee, 1975*a*). To decrease Ca^{2+} uptake by the mitochondria during the isolation procedure, the isolation medium contained 0.05 mM DTNB (Haugaard *et al.*, 1969*b*; Haugaard *et al.*, 1969*a*). The DTNB was removed by washing the mitochondria with 0.2 mM DTT.

Measurement of Oxygen Consumption

Mitochondria (1 mg protein/ml) were incubated with a medium (0.7 ml) containing 32 mM HEPES, 7.9 mM $MgCl_2$, 8.2 mM succinate and 2.6 mM inorganic phosphate. The osmolarity of the medium was adjusted to 250 mosmol with KCl, and the pH was 7.4. To induce state 3 respiration, 125 μM ADP was added to the mitochondrial suspension. Oxygen uptake by mitochondria was measured polarographically, at 26°, using a Clark oxygen electrode (No. 523, Yellow Springs Instruments, Yellow Springs, Ohio) linked to a 10-mV Sargent recorder (Model SRL). The respiratory control and P/O ratios were calculated as described by Chance (1959) and Estabrook (1967), respectively.

Measurement of Ca^{2+} Uptake

In all experiments net Ca^{2+} change was measured. The incubation medium contained 38 mM HEPES, 68 mM KCl, 2.0 mM inorganic phosphate, 8 mM succinate, and 3 μM rotenone. The Mg^{2+} concentration was varied from 0 to 7.5 mM. Calcium uptake was measured using the method described by Reed and Bygrave (1975). When the NTA-Ca buffer system was employed, $MgCl_2$ was omitted from the incubation medium. Aliquots (25 μl) of the mitochondrial suspension (containing about 1 mg protein/ml) were preincubated with 8 mM succinate and 3 μM rotenone for 1 min at 22°. The reaction was then started by the addition of appropriate amounts of $^{45}CaCl_2$ diluted with non-radioactive $CaCl_2$. The final volume was 250 μl. The time of incubation was varied according to the experiments: the actual time used in each experiment is indicated in the legends. Following incubation, 100-μl aliquots were removed and the reaction was terminated by the Millipore-quench technique of Reed and Bygrave (1974; 1975). The filter disc was dried and counted in a liquid scintillation counter. To measure Ca^{2+} uptake by mitochondria as a function of time, the experimental conditions were essentially the same as those indicated above, except that 100 μl of the incubation mixture was removed at different time intervals and counted. The extrusion of Ca^{2+} from chondrocyte mitochondria was measured in the presence of 0.5 μM CCCP, or 10 μM DNP, or 3 μM antimycin A plus 3 μM rotenone.

Chemical Analysis

Protein was determined using the method described by Lowry *et al.* (1951). Total Ca^{2+} was measured by atomic absorption spectroscopy (Perkin-Elmer, Model 103 and 360). Inorganic phosphate was assayed by the method of Fiske and Subbarow (1925).

Results and Discussion

This is the first report of the kinetics of Ca^{2+} accumulation by mitochondria of cells that are concerned with the initiation of the mineralization process. The velocity of Ca^{2+} uptake by mitochondria of the resting-proliferating (78 nmoles Ca^{2+}/mg protein), proliferating-hypertrophic (300 nmoles Ca^{2+}/mg protein), and hypertrophic-calcifying (850 nmoles Ca^{2+}/mg protein) zones, respectively, expressed as a function of the medium Ca^{2+} concentration is shown in Fig. 1. Using the NTA-Ca buffer system, the rates of Ca^{2+} uptake by mitochondria of the two least calcified zones are linear up to a medium Ca^{2+} level of about 30 μM. Half maximal transport rates for these mitochondria occurs at about 34 μM Ca^{2+}. No attempt was made to measure the initial velocity of Ca^{2+} uptake. The high endogenous Ca^{2+} level of normal epiphyseal plate mitochondria (*see* Table 1) and even rachitic chick mitochondria (Shapiro, Burke & Lee, 1976) interferes with this measurement. The most appropriate parameters for expressing Ca^{2+} uptake are therefore the half maximal transport rate and the maximal velocity (V_{max}). In

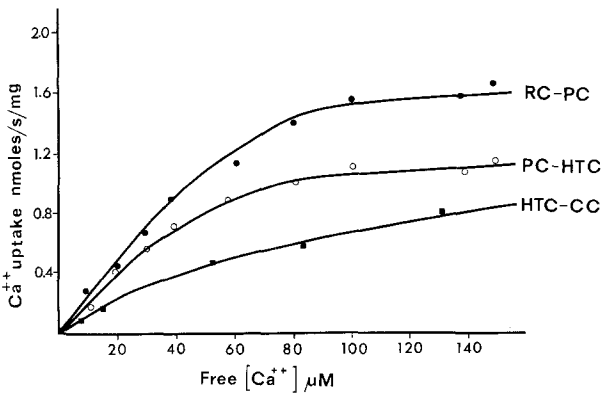

Fig. 1. Mitochondrial Ca^{2+} accumulation measured as a function of medium Ca^{2+} concentration. Prior to measuring Ca^{2+} uptake, 25 μl of the mitochondrial suspension (about 50 μg protein) was preincubated for 1 min with a medium which contained 8 mM succinate and 3 μM rotenone. The reaction medium contained 38 mM HEPES, 68 mM KCl, 3.8 mM P_i and 3 μM rotenone. The reaction was started by the addition of an appropriate amount of $^{45}CaCl_2$, diluted with NTA-Ca buffer, to the mitochondrial suspension. After 10 sec, 100 μl of the suspension was removed, mixed with 0.1 ml of quench medium (2 mM EGTA plus 3 μM ruthenium red) and filtered through a 0.45 μM Millipore filter. The filter was washed with 10 ml of ice cold sucrose containing the quench solution, dried and counted. Endogenous Ca^{2+} contents: 78 nmoles Ca^{2+}/mg protein (●—●); 300 nmoles/mg protein (○—○); 850 nmoles Ca^{2+}/mg protein (■—■)

Table 1. Effects of mitochondrial Ca^{2+} on oxidative phosphorylation

Mitochondrial Ca^{2+} content (nmoles Ca^{2+}/ mg protein)	O_2 consumption (μatoms O/min/mg protein)		ADP/O	RCR
	State 3	State 4		
166	0.030	0.008	1.70	3.6
184	0.048	0.015	1.65	3.2
215	0.045	0.013	1.63	3.5
336	0.045	0.014	1.50	3.3
342	0.043	0.013	1.60	3.2
397	0.035	0.014	–	–
436	0.032	0.013	–	–
1593	0.025	0.012	–	–
2686	0.012	0.012	–	–

The mitochondrial suspensions were incubated at 26° with 0.7 ml of reaction mixture containing 32 mM HEPES, 7.9 mM $MgCl_2$, 8.2 mM succinate, 2.6 mM P_i and 0.125 mM ADP. The mitochondrial protein concentration was 0.8 mg/ml.

this study V_{max} occurs at about 80 μM, but differences are seen between the mitochondrial preparations. Thus, mitochondria containing 78 nmoles Ca^{2+}/mg protein exhibit a higher V_{max} (1.6 nmoles Ca^{2+}/sec/mg protein) than organelles containing 300 nmoles Ca^{2+}/mg protein ($V_{max} = 1.1$ nmoles Ca^{2+}/sec/mg protein). For these mitochondria, the V_{max} is 2–3 times greater than the values reported for myometrium ($V_{max} = 0.55$ nmoles Ca^{2+}/mg protein/sec, Ca^{2+} concentration 113 ± 50 nmoles/mg protein) (Malmström & Carafoli, 1977), but considerably lower than vascular tissue (4–12 nmoles/mg protein, Ca^{2+} concentration 92–251 nmoles/mg protein) (Vallieres, Scarpa & Somylo, 1975). These results suggest that, while the mitochondrial Ca^{2+} level of these soft tissues and the resting-proliferating cartilage are similar, the kinetics of Ca^{2+} accumulation are not due simply to differences in the endogenous Ca^{2+} concentration, but reflect specific differences in mitochondrial transport activity and cell function. It was not possible to obtain values for V_{max} and K_m for the mitochondria of the hypertrophic-calcifying zone. This was probably due to the high concentration of intramitochondrial Ca^{2+} and the low respiratory rate of these mitochondria (Table 1). The V_{max} values obtained for mitochondria of the resting-proliferating and proliferating-hypertrophic zones are within the range reported by Mela and Chance (1968) and Spencer and Bygrave (1973) for liver mitochondria.

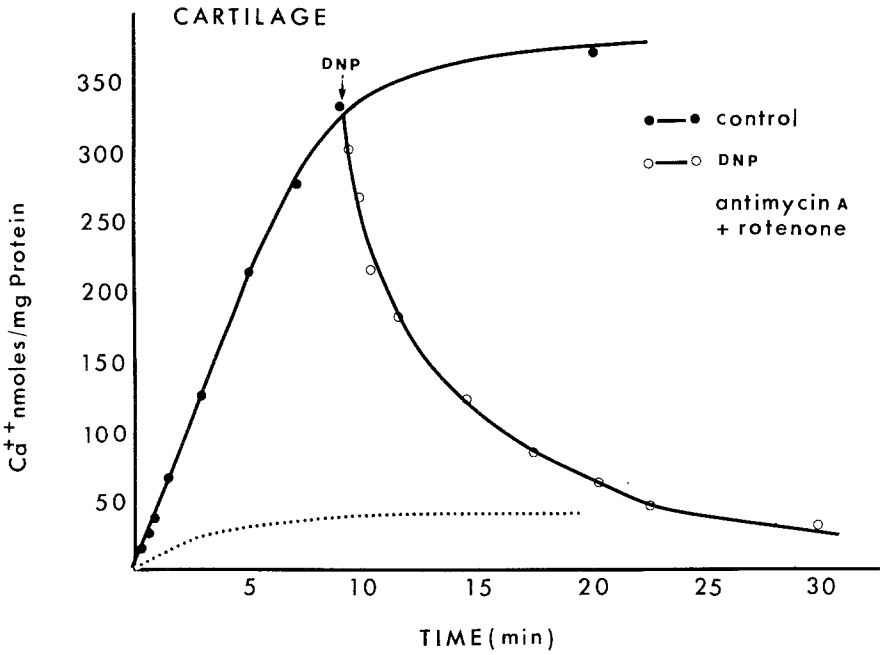

Fig. 2. Net Ca^{2+} uptake and efflux by mitochondria. Chondrocyte mitochondria were incubated with a reaction mixture as described in *Materials and Methods*. At different time intervals, 100 µl of the suspension was removed, filtered, and counted. To terminate the reaction, the quench and Millipore filtration technique was employed as described in Fig. 1. To measure nonenergy supported Ca^{2+} uptake, antimycin A and rotenone were present in the reaction mixture. At 10 min, Ca^{2+} efflux was studied by adding 0.01 mM DNP to the medium and measuring the mitochondrial Ca^{2+} concentration. The Ca^{2+} concentration of mitochondria was about 100 nmoles/mg protein. In all experiments, the mitochondrial protein concentration was 200 µg/ml

The uptake of Ca^{2+} by chondrocyte mitochondria, expressed as a function of time, is shown in Fig. 2. In the presence of succinate, mitochondria accumulate over 300 nmoles of Ca^{2+} within the first 10 min. During this time, the rate of Ca^{2+} uptake is approximately 35 nmoles/mg protein/min. After 10 min, there is only a slight increase in Ca^{2+} uptake. The rate of Ca^{2+} accumulation by this preparation is similar to the values noted by Drahota *et al.* (1965) for rat liver mitochondria during state 4 respiration (40 nmoles/min/mg protein). From this result and the Ca^{2+} uptake data, it is clear that the energy supported Ca^{2+} uptake characteristics of chondrocyte mitochondria are similar to the values reported for mitochondria of cells that are not concerned with the calcification phenomenon.

While the kinetic data show that similarities exist in the ability of both hard and soft tissue mitochondria to accumulate Ca^{2+}, previous studies suggest that differences exist in the respiratory characteristics of these two cell types (Shapiro & Lee, 1975*b*). That differences do exist is apparent from the data presented in Table 1. Thus, at mitochondrial Ca^{2+} levels greater than 350 nmoles/mg protein, there is a loss of coupling activities, but little change in the state 3 respiratory rate. Indeed, the respiratory rate is not altered until the endogenous mitochondrial Ca^{2+} concentration is above 1,000 nmoles/mg protein. In soft tissues, mitochondrial Ca^{2+} levels greater than 100–210 nmoles/mg protein result in total loss of receptor control and mitochondrial cation release (Lehninger, Carafoli & Rossi, 1967; Bygrave & Reed, 1970; Bygrave, Reed & Spencer, 1971). However, if soft tissue mitochondria that have elevated Ca^{2+} levels are compared with resting cartilage, considerable differences in their respiratory characteristics are seen. Thus, ADP stimulates state 4 respiration in vascular tissue (Vallieres, Scarpa & Somlyo, 1975) and cartilage mitochondria, but this nucleotide has only a slight effect on uterine muscle (Malmström & Carafoli, 1977). These tissue-specific differences lend credence to the view that the Ca^{2+} load reflects the physiological function of the tissue or cell and by itself does not govern mitochondrial activity.

The effect of Mg^{2+} on mitochondrial Ca^{2+} uptake is shown in Fig. 3. At Mg^{2+} concentrations of 0, 3.5 and 7.5 mM, Ca^{2+} uptake is not affected by the presence of Mg^{2+} in the medium. This result contrasts with the findings of Crompton *et al.* (1976) who have shown that Mg^{2+} inhibits Ca^{2+} uptake by heart mitochondria. It is of interest to note that Mg^{2+} inhibition of Ca^{2+} transport appears to vary with cell type. For example, Jacobus *et al.* (1975) found that Mg^{2+} had only a slight effect on Ca^{2+} accumulation by liver mitochondria, while Chen, Greenawalt & Lehninger (1974) used as much as 10 mM Mg^{2+} in Ca^{2+} transport studies of mitochondria of the calcifying hepatopancreas of the blue crab.

The progressive increase in Ca^{2+} levels by mitochondria of cells of the epiphyseal growth plate suggests that chondrocytes continue to accumulate Ca^{2+} *in vivo*, despite a loss in respiratory control. To study the relative Ca^{2+} affinities of these mitochondria, Ca^{2+} uptake was measured by mitochondria that were coupled (>300 nmoles/mg protein), and in organelles that exhibited no respiratory control (>500 nmoles/mg protein). At low endogenous Ca^{2+} loads (135–250 nmoles/mg protein), nonenergy supported binding is very low (Table 2) and similar to the

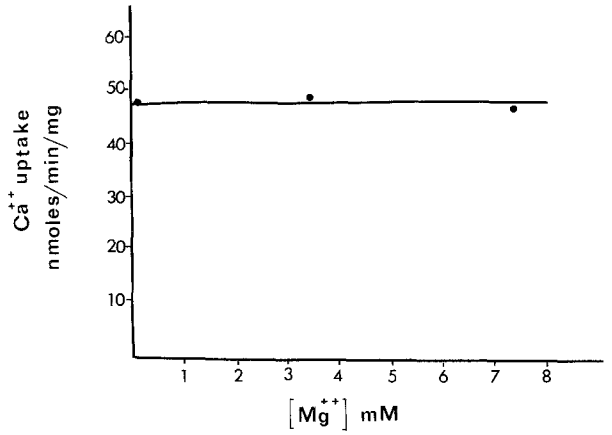

Fig. 3. Effect of Mg^{2+} concentration on Ca^{2+} uptake by chondrocyte mitochondria. Mitochondria were incubated in the medium described in *Materials and Methods*, which was supplemented with Mg^{2+} (0–7 mM) as indicated. Free Ca^{2+} concentration was 100 μM in the reaction medium

Table 2. Effects of ATP and succinate on Ca^{2+} uptake by cartilage mitochondria

Addition	Ca^{2+} uptake (nmoles Ca^{2+}/min/mg protein)	
	Endogenous Ca^{2+} [a] 135–250	Endogenous Ca^{2+} [a] 550–1500
3 μM antimycin A + 3 μM rotenone	0.6±0.1 (5)	9.4±0.8 (4)
8 mM succinate	28.0±7.5 (5)	13.7±4.2 (4)
4 mM ATP	55.8±7.5 (5)	38.0±6.8 (5)
8 mM succinate + 4 mM ATP	74.9±10.1 (4)	46.0 (2)

The reaction medium utilized was the same as described in the text. Mitochondria were incubated with this medium in a final volume of 0.25 ml for 1 min at 26°. After 1 min, 0.1 ml of the aliquot was added to 0.1 ml of a quench medium containing 2 mM EGTA plus 3 μM ruthenium red and was filtered. The protein content was 0.6 mg/ml. The values shown are the means±SE. The number of experiments is shown in parentheses.

[a] Endogenous Ca^{2+} concentration is expressed as nmoles Ca^{2+}/mg protein.

levels reported in nonmineralizing tissues. However, at high Ca^{2+} loads, in the presence of antimycin and rotenone, Ca^{2+} binding is elevated. This finding supports an earlier observation that chondrocyte mitochondria have a high degree of nonspecific Ca^{2+} binding (Shapiro & Lee, 1975*a*); when the organelles were washed with medium containing EGTA or ruthenium red, the Ca^{2+} level was not decreased. This type of binding may be related to the presence of glycoproteins of high Ca^{2+} affinity in calcifying cartilage (Vittur & Bernard, 1973; Bernard & Vittur, 1973).

With mitochondria that contain low endogenous Ca^{2+} loads, the presence of succinate results in a 50-fold increase in Ca^{2+} uptake (Table 2). As shown in Table 1, the stimulatory effect of this substrate on cation accumulation is directly dependent on the endogenous Ca^{2+} concentration. The additive and stimulatory effects of ATP on both types of loaded mitochondria is also indicated in Table 2, and the values are in good agreement with previously published data concerning chondrocyte cation accumulation (Shapiro & Lee, 1975*b*).

As the cells of the growth plate approach the calcification front, the rise in mitochondrial Ca^{2+} is accompanied by a loss of respiratory control. Table 3 shows that this process is marked by an overall increase in the total quantity of extrudable Ca^{2+}, while the total percentage of extrudable Ca^{2+} is decreased. Thus, at low endogenous Ca^{2+} loads (208–420 nmoles/mg protein), the respiratory inhibitors and CCCP both cause extrusion of more than 80% of the total mitochondrial Ca^{2+}. Figure 2 shows that the rate of extrusion is rapid for the first 2 min and then falls off rapidly. At 10 min, maintenance of mitochondrial Ca^{2+} levels in the absence of DNP indicates that there is no net change in the mitochondrial and medium Ca^{2+} concentration. This finding suggests that Ca^{2+}-stimulated Ca^{2+} efflux does not occur. The percentage extrusion at high endogenous Ca^{2+} loads is similar to the values reported by Chen, Greenawalt & Lehninger (1974) for the Ca^{2+}-loaded mitochon-

Table 3. The effects of respiratory inhibitors and uncoupler on Ca^{2+} extrusion by chondrocyte mitochondria

Endogenous Ca^{2+} (nmoles/mg protein)	Antimycin A + rotenone		CCCP	
	Net Ca^{2+} extrusion (nmoles/mg protein)	% Extrusion	Net Ca^{2+} extrusion (nmoles/mg protein)	% Extrusion
208–480	166–365	77–88	171–370	82–90
1925–2399	458–912	20–38	384–963	16–50

Mitochondria from different regions of cartilage were incubated with a reaction mixture containing 3 μM antimycin A + 3 μM rotenone or 0.5 μM CCCP for 10 min at 26°. Following incubation, the mitochondria were centrifuged at 15,000 × *g* for 10 min. The Ca^{2+} content of the pellets was analyzed by atomic absorption spectroscopy. The final volume of the incubation mixture was 0.5 ml and the protein concentration was 0.7 mg/ml. In the control experiments, mitochondria were incubated with a reaction mixture containing 10 μl of ethyl alcohol in place of the inhibitors.

Table 4. Ca^{2+} and P_i analysis of mitochondrial extrudates following exposure to DNP

Ca^{2+} (μg/mg protein)	P_i	Ca/P_i ratio
8.90	4.50	1.98
11.68	6.87	1.70
15.66	8.70	1.80
16.57	8.10	2.06
25.68	14.93	1.72

Mitochondria, from different regions of cartilage, were incubated with a reaction mixture containing 10 μM DNP for 5 min at 26°. After 5 min, 1.5 ml of the incubation mixture (0.6 mg protein/ml) was filtered through a 0.45 μm Millipore filter and the filtrate was collected.

dria of the blue crab. In addition, the results obtained in this study corroborate the observations by Dziak and Brand (1974) that about 50% of bone cell Ca^{2+} is in an extrudable form.

It has been speculated that in mineralizing tissues there exists a number of physiologically important Ca^{2+} pools (Borle, 1972). While no attempt was made to define such pools, this study indicates that the total quantity of Ca^{2+} in cells preparing for mineralization is elevated, and a high percentage of the total Ca^{2+} content is in an extrudable form. Furthermore, as the total Ca^{2+} load of these cells rises, there is a dramatic increase in the total quantity of extrudable and nonextrudable Ca^{2+}. The nonextrudable Ca^{2+} "pool" probably corresponds to the "granules" described by Martin and Matthews (1969, 1970). The Ca^{2+}/P_i ratio of the fluid extruded from the mitochondria in the presence of DNP (Table 4) is similar to the values reported by Greenawalt *et al.* (1964); the Ca^{2+} and P_i composition of the extrudate is almost identical to the extracellular fluid of the longitudinal septa of the growth plate (Howell *et al.*, 1968). Whether mitochondria control the calcification of vesicles and collagen by modulating the level of Ca^{2+} and P_i in this fluid phase awaits further investigation.

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