A Survey of the Interaction of Calcium Ions with Mitochondria from Different Tissues and Species

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(Received 2 October 1970)

A survey was made of the capacity of mitochondria isolated from a number of different tissues and species to accumulate Ca^{2+} from the suspending medium during electron transport. The species examined included the rat, mouse, rabbit, hamster, guinea pig, cow, chicken, turtle, blowfly, yeast and *Neurospora crassa*. The tissues examined included vertebrate liver, kidney, brain, heart, spleen, thyroid and adrenal cortex, and the flight muscle of the blowfly. The mitochondria from all vertebrate tissues examined showed: (a) stimulation of State 4 respiration by added Ca^{2+} ($\text{Ca}^{2+}/\sim$ activation ratio about 2.0), accompanied by accumulation of Ca^{2+} and ejection of H^+ , with a $\text{H}^+/\text{Ca}^{2+}$ ratio about 1.0; (b) a requirement of phosphate for accumulation of large amounts of Ca^{2+} ; (c) respiration-independent high-affinity binding sites for Ca^{2+} ; (d) endogenous Ca^{2+} , which is largely released by uncoupling agents. However, mitochondria from yeast and blowfly flight muscle are unable to accumulate Ca^{2+} in a respiration-dependent process and possess no high-affinity Ca^{2+} -binding sites. These findings support the view that the high-affinity sites represent the ligand-binding sites of a specific Ca^{2+} 'permease' or transport system in the membrane. The relatively high affinity for Ca^{2+} , which equals or exceeds the affinity for ADP, and the generally uniform characteristics of Ca^{2+} transport in all the vertebrate mitochondria tested strongly suggest that respiration-linked Ca^{2+} accumulation plays a general and fundamental role in vertebrate cell physiology.

This paper reports some of the results of a comparative survey of the interaction between Ca^{2+} and mitochondria isolated from a number of different tissues. The major purposes of this investigation were to determine: (1) whether all types of mitochondria have the intrinsic capacity for respiration-coupled accumulation of Ca^{2+} ; (2) whether significant tissue or species differences exist that might be relevant to the biological role of mitochondria in Ca^{2+} transport; (3) whether mitochondria from some cells show significantly large deviations from the well-documented behaviour of rat liver mitochondria (reviewed by Lehninger, Carafoli & Rossi, 1967; Pressman, 1970; Lehninger, 1970) to render them useful for investigation of the relationship of ion movements to electron transport. In addition to the comparative results summarized in this paper, more detailed investigation of mitochondria from the yeasts *Saccharomyces cerevisiae* and *Torulopsis utilis* (Carafoli, Balcavage, Lehninger & Mattoon, 1970), blowfly flight muscle (Carafoli, Hansford,

Sacktor & Lehninger, 1971) and the flight muscle of the periodical cicada (Hansford, 1971) are described elsewhere.

EXPERIMENTAL

Isolation of mitochondria. Mitochondria were isolated from the liver and kidney cortex of different vertebrates with the conventional procedure of Schneider (1956), with 0.25 M-sucrose as the isolation medium. For preparation of heart mitochondria, the hearts were removed as rapidly as possible and placed in ice-cold isolation medium [100 mM-KCl, 10 mM-tris chloride (pH 7.4), 0.05 mM-EDTA (sodium salt), and 1% (w/v) bovine serum albumin]. They were opened and squeezed between layers of filter paper to remove the blood, and rinsed in several changes of the medium. Samples (2 g) freed of fat and connective tissue and finely minced with scissors, were homogenized with 30 ml of the isolation medium in a Polytron homogenizer (Bronwill Scientific, Rochester, N.Y., U.S.A.). The homogenization was carried out for 15 s, with the setting at 5.5. The cell debris, myofibrils, nuclei and other heavy components were removed by sedimentation at 900 g for 10 min. The mitochondria were then sedimented at 16000 g for 10 min and washed once with isolation

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medium from which EDTA was omitted. Rat and rabbit brain mitochondria were isolated according to Hamberger, Blomstrand & Lehninger (1970), *Neurospora crassa* mitochondria as described by Hall & Greenawalt (1967), *S. cerevisiae* mitochondria by the method of Beck, Mattoon, Hawthorne & Sherman (1968), and blowfly (*Phormia regina*) mitochondria by the method of Sacktor & Cochran (1958). Ox adrenal cortex mitochondria were isolated as follows: adrenal glands freshly obtained from the slaughter-house were placed in ice-cold 0.25M-sucrose containing 0.1% bovine serum albumin within 10–15 min after the death of the animal. The cortex was freed from fat and surrounding connective tissue and finely chopped with scissors. The mince was then homogenized in 10 vol. of the sucrose-bovine serum albumin medium in a Lucite Potter homogenizer; four passes of the pestle were usually employed. After the residue that sedimented at 800g for 10 min was discarded, the mitochondria were recovered at 14000g for 10 min. They were washed once with isolation medium not containing EDTA.

ADP/O ratios, respiratory control, stimulation of respiration by Ca^{2+} and Ca^{2+} -induced H^+ movements. Measurements of oxygen uptake were carried out at 25°C with a Clark oxygen electrode (Kielley & Bronk, 1958) whose chamber was designed to prevent the diffusion of oxygen from the atmosphere into the reaction medium. Substrates, phosphate, adenine nucleotides and other additions were made in the form of potassium salts. The standard medium (vol. 2.70 ml) for measurements of the respiratory response of liver, kidney, brain, and *Neurospora* mitochondria to ADP (65 nmol/mg of protein) or Ca^{2+} (80 ng-atoms/mg of protein) contained 80 mM-KCl, 10 mM-tris chloride, pH 7.4, 10 mM-MgCl₂, and 10–20 mM-succinate (or a mixture of pyruvate and L-malate) as respiratory substrate. Mitochondria were added at 5.0 mg of protein, and phosphate, added as indicated, was present at 5.0 mM. For mitochondria from heart and adrenal cortex, the medium was 225 mM-mannitol, 55 mM-sucrose, 10 mM-tris chloride, pH 7.4, and 0.2% bovine serum albumin; the substrate was pyruvate-malate for heart and succinate for adrenal cortex. For yeast mitochondria, the medium contained 0.6 M-mannitol, 10 mM-phosphate buffer, pH 6.5, 16 mM-succinate and 0.3–0.6 mg of mitochondrial protein. The medium for blowfly flight-muscle mitochondria consisted of 0.25 M-sucrose, 20 mM-phosphate buffer (pH 7.3), 50 mM-MgCl₂, 10 mM-glycerol phosphate (or 10 mM-pyruvate plus 5 mM-proline) as respiratory substrate, and mitochondria (1.8 mg of protein). The measurements of the movements of H^+ were carried out in media containing 80 mM-KCl, 5.0 mM-succinate (liver, kidney), 5.0 mM-pyruvate plus 1.0 mM-malate (heart and adrenal cortex), 5.0 mM-pyruvate plus 5.0 mM-proline (blowfly muscle), 5.0 mM-tris chloride, pH 7.4, 5.0 mg of mitochondrial protein (liver, kidney, heart, adrenal cortex), 1.0 mg of mitochondrial protein (blowfly muscle) in a final volume of 1.8 ml. The medium and the final volume for *S. cerevisiae* was the same as described for the studies of O_2 uptake. The movements of H^+ were monitored on a Beckman Expandomatic pH meter, linked to a strip-chart recorder.

Uptake of Ca^{2+} and phosphate: high- and low-affinity Ca^{2+} binding. The respiration-supported uptake of $^{45}\text{Ca}^{2+}$ was determined in the same media used to study the stimulation of respiration by Ca^{2+} , scaled up to permit

the withdrawal of several samples. The mitochondria were separated from the medium by filtration through Millipore filters (HAWP 01300; pore diam. 0.45 μm), and the Ca^{2+} uptake was determined by counting the radioactivity remaining in the clear filtrates in a Beckman LS100 or a Packard model 2002 Tri-Carb scintillation counter. Accumulation of non-isotopic Ca^{2+} was determined with a Beckman or Perkin-Elmer model 303 atomic absorption spectrophotometer. For this purpose the mitochondrial pellets were extracted with boiling 0.5 M-HCl (10 min). The measurements were carried out in 0.5 M-HCl containing 1% $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ to eliminate interference by phosphate. Net uptake of P_i by blowfly flight-muscle mitochondria was measured on trichloroacetic acid extracts of mitochondrial pellets. Mitochondria were spun down from the reaction medium at 20000g for 5 min at 0°C, and washed once with cold 0.25 M-sucrose. High- and low-affinity binding of $^{45}\text{Ca}^{2+}$ were determined as described by Reynafarje & Lehninger (1969).

Endogenous Ca^{2+} in mitochondria. Endogenous Ca^{2+} was determined on HCl extracts of the mitochondrial pellets, as described above. The discharge of endogenous Ca^{2+} by 2,4-dinitrophenol was followed at 25°C in 0.25 M-sucrose, in the presence of 0.1 mM-2,4-dinitrophenol. Samples (2.0 ml) were withdrawn at the required times and centrifuged at 20000g for 5 min at 0°C. The Ca^{2+} content of the pellets was measured as described above.

Protein was measured with a biuret reaction or according to Murphy & Kies (1960). The $^{45}\text{CaCl}_2$ was obtained from Nuclear Science and Engineering Corp., Pittsburgh, Pa., U.S.A. or from New England Nuclear Corp., Boston, Mass., U.S.A.

RESULTS

Stimulation of State 4 respiration in different mitochondria by Ca^{2+} . Table 1 shows the results of representative experiments on the rates of oxygen consumption before and after addition of small amounts of Ca^{2+} (80 ng-atoms of Ca^{2+} /mg of protein) to various types of mitochondria, respiring in the absence or presence of phosphate. Under both conditions the mitochondria from all vertebrate tissues tested showed behaviour qualitatively similar to that given by rat liver mitochondria, namely stimulation of oxygen uptake upon addition of Ca^{2+} to mitochondria respiring in State 4. In all cases the rate of respiration returned to the State 4 rate fairly sharply after the Ca^{2+} -induced respiratory jump. The rate of the Ca^{2+} -stimulated respiration ranged from about 3 to nearly 11 times the State 4 rate; in most cases, the addition of Ca^{2+} produced a higher rate of oxygen consumption than the addition of ADP. None of the vertebrate mitochondria tested showed inhibition of respiration by Ca^{2+} (i.e. the State 6 of Chance & Schoener, 1966) at the concentrations of Ca^{2+} added (80 ng-atoms/mg of mitochondrial protein).

Mitochondria from invertebrate species gave somewhat different results on addition of Ca^{2+} (Table 1). Those from *Neurospora* showed only a

Table 1. *Effects of Ca^{2+} on the respiration of different mitochondria*

The conditions of incubation and the media are as described in the Experimental section. The respiratory substrates were succinate for liver, brain, rabbit kidney, *Neurospora* and *S. cerevisiae*, pyruvate-proline for blowfly muscle, pyruvate-malate for heart and adrenal cortex, glutamate-malate for rat kidney. The rate results are given in ng-atoms of oxygen consumed/min per mg of mitochondrial protein at 25°C.

Source of mitochondria	No phosphate added				Phosphate added			
	State 4 rate	After Ca^{2+}	Respiratory control ratio	$\text{Ca}^{2+}/\sim$ ratio	State 4 rate	After Ca^{2+}	Respiratory control ratio	$\text{Ca}^{2+}/\sim$ ratio
Liver								
Rat	12	46	3.8	2.03	15	47	3.1	1.95
Mouse	23	50	2.2	2.22	19	69	3.6	2.00
Rabbit	12	36	3.0	2.00	16	59	3.7	1.92
Guinea pig	10	32	3.2	2.00	7	65	9.3	1.82
Chicken	18	26	1.5	2.54	20.0	34	1.7	2.38
Turtle	14	19	1.3	2.53	19.0	36	1.9	1.64
Kidney								
Rat	27	67	2.5	2.10	39	94	2.4	2.00
Rabbit	21	36	1.7	2.13	34	141	4.1	1.82
Heart								
Rat	18	38	2.1	2.35	21	60	2.9	1.96
Rabbit	16	40	2.5	2.08	27	90	3.3	1.90
Rat brain	19	39	2.0	2.41				
Ox adrenal cortex	20	130	6.5	1.98	25	133	5.3	1.90
<i>Neurospora</i>	74	98	1.3	—	—	—	—	—
<i>S. cerevisiae</i>	94	101	1.1	—	—	—	—	—
Blowfly flight muscle	27	27	1.0	—	—	—	—	—

very small respiratory stimulation on addition of Ca^{2+} , less than that given by ADP; the Ca^{2+} -stimulated respiration failed to return to the State 4 rate. Mitochondria from *S. cerevisiae* and from blowfly muscle yielded no detectable respiratory stimulation on addition of 80ng-atoms of Ca^{2+} /mg of mitochondrial protein.

Table 1 also shows that, in all the vertebrate mitochondria tested, the number of Ca^{2+} ions required to activate each energy-conserving site (the $\text{Ca}^{2+}/\sim$ activation ratio) is slightly above 2.0 in the absence of phosphate and slightly less in its presence, in agreement with earlier work on rat liver mitochondria (Rossi & Lehninger, 1964).

Ca^{2+} -induced H^+ ejection. Table 2 shows that in all vertebrate mitochondria tested Ca^{2+} addition caused H^+ ejection; the molar ratio H^+ ejected/ Ca^{2+} added was about 1.0 in the absence of phosphate and about 0.8–0.9 in the presence of phosphate. No detectable movements of H^+ were induced by Ca^{2+} in blowfly muscle and *S. cerevisiae* mitochondria.

Energy-linked uptake of Ca^{2+} in the absence of phosphate. The capacity of mitochondria from various sources to accumulate Ca^{2+} during respiration was examined under two sets of conditions. In the first series of experiments neither phosphate

nor ADP was present in the medium; under these conditions only limited amounts of Ca^{2+} can be taken up by rat liver mitochondria, presumably because of the unavailability of permeant anions or acids (Lehninger *et al.* 1967). To initiate the reaction $^{45}\text{CaCl}_2$ was added at 80ng-atoms/mg of mitochondrial protein. Exactly 1.0min after addition of Ca^{2+} , the time required for the completion of the Ca^{2+} -induced jump of oxygen uptake, the mitochondria were quickly filtered off and the amount of $^{45}\text{Ca}^{2+}$ remaining in the filtrate was determined as described in the Experimental section. Parallel vessels contained 0.1mm-2,4-dinitrophenol.

The results in Table 3 show that all the vertebrate mitochondria tested, as well as those from *Neurospora*, showed uptake of $^{45}\text{Ca}^{2+}$. In each case Ca^{2+} uptake was inhibited by 2,4-dinitrophenol, although the extent of inhibition varied somewhat. Mitochondria from neither *S. cerevisiae* nor blowfly muscle showed dinitrophenol-sensitive binding of Ca^{2+} , in consonance with their failure to yield respiratory stimulation by Ca^{2+} .

Energy-linked uptake of Ca^{2+} in the presence of phosphate. When phosphate is present in the medium together with ADP and Mg^{2+} , rat liver and kidney mitochondria can accumulate Ca^{2+} in

Table 2. Ca^{2+} -Induced movements of H^+ in mitochondria

The conditions of incubation and the media are as described in the Experimental section. The respiratory substrates were succinate for liver, kidney and *S. cerevisiae*, pyruvate-malate for heart and adrenal cortex, pyruvate-proline for blowfly muscle. The results are given in ng-atoms/test system. The temperature was 25°C.

Source of mitochondria	No phosphate added			Phosphate added		
	Ca^{2+} added	H^+ ejected	$\text{H}^+/\text{Ca}^{2+}$ ratio	Ca^{2+} added	H^+ ejected	$\text{H}^+/\text{Ca}^{2+}$ ratio
Rat liver	400	412	1.03	400	345	0.86
Rabbit kidney	400	405	1.01	400	340	0.85
Rat heart	400	365	0.91	400	335	0.84
Rabbit heart				400	360	0.90
Chicken liver	400	240	0.6	400	280	0.70
Turtle liver	400	388	0.97	400	280	0.70
Ox adrenal cortex	400	375	0.94	400	335	0.84
<i>S. cerevisiae</i>	200-400	0	—	200-400	0	—
Blowfly flight muscle	200	0	—	200	0	—

Table 3. Energy-linked Ca^{2+} accumulation by mitochondria

Source of mitochondria	Ca^{2+} uptake (ng-atoms/mg of protein)	
	No 2,4-dinitro-phenol added	2,4-Dinitro-phenol added
Liver		
Rat	96.6	3.3
Rabbit	83.3	4.2
Mouse	89.9	6.7
Guinea pig	79.1	5.0
Turtle (<i>Pseudemys dorsibignyi</i>)	70.2	3.8
Chicken	76.0	4.3
Kidney		
Rat	97.1	8.3
Rabbit	66.6	5.0
Heart		
Rat	66.6	27.5
Rabbit	76.6	25.0
Brain		
Rat	84.9	8.3
Adrenal cortex (cow)	150.0	9.2
Flight muscle (blowfly)	16.6	15.0
<i>Neurospora</i>	58.3	0.8
<i>S. cerevisiae</i>	75.0	72.5

amounts up to and exceeding 2500 ng-atoms/mg of protein ('massive loading'), (Vasington & Murphy, 1962; Rossi & Lehninger, 1963; Lehninger *et al.* 1967). Under these conditions mitochondria isolated from rat and rabbit heart, from rat and guinea-pig liver, from rat, rabbit and guinea-pig kidney, from ox adrenal cortex and from rat and rabbit brain were also found to accumulate massive amounts of Ca^{2+} . The results were very similar within this group, with accumulations up to 1200 ng-atoms of Ca^{2+} /mg of mitochondrial protein

under the conditions used (results are not shown). However, the unexpected finding was made that blowfly muscle mitochondria, which were found above to be unable to carry out energy-linked accumulation of Ca^{2+} in the absence of phosphate, accumulated Ca^{2+} in the presence of phosphate (see Fig. 2). This behaviour thus resembles the case of mitochondria from maize seedlings, which have also been shown to have an absolute requirement of phosphate for Ca^{2+} uptake (Hodges & Hanson, 1965).

To detect more subtle differences in the behaviour of various types of mitochondria with respect to the effect of phosphate on Ca^{2+} uptake, a second series of experiments was carried out in which an intermediate amount of Ca^{2+} (160 ng-atoms/mg of mitochondrial protein) was added. Typical results are given in Fig. 1, which shows that the dependence of vertebrate mitochondria on phosphate for accumulation of large amounts of Ca^{2+} increases in the order liver, kidney and spleen.

The most critical dependence of Ca^{2+} uptake on phosphate was shown by blowfly mitochondria (Fig. 1). In this case the requirement for phosphate was found to be independent of the amounts of Ca^{2+} added, down to concentrations of $1 \mu\text{M}$ - Ca^{2+} . Data reported elsewhere indicate that acetate can replace phosphate in supporting Ca^{2+} uptake by blowfly mitochondria (Carafoli *et al.* 1971).

Endogenous Ca^{2+} content of mitochondria: effect of 2,4-dinitrophenol. Table 4 gives the total calcium content of freshly isolated mitochondria from various cell types, measured by atomic-absorption spectrometry. In all cases the mitochondria were isolated from homogenates of the tissue in 0.25 M-sucrose; no EDTA or other Ca^{2+} -sequestering agent was added. Mitochondria from rat liver contained from 10 to 15 ng-atoms of Ca^{2+} /mg of mitochondrial protein (cf. Lehninger *et al.* 1967;

Fig. 1. Effect of phosphate on respiration-linked accumulation of Ca^{2+} in mitochondria. Source of mitochondria: (a) guinea-pig liver; (b) rabbit kidney; (c) rabbit spleen; (d) blowfly flight muscle. O, P_i added; Δ , no P_i added.

Carafoli, 1967; Reynafarje & Lehninger, 1969). Most other types of mitochondria contained much more Ca^{2+} , particularly those from rat kidney, rat heart and ox adrenal cortex. On the other hand, mitochondria from yeast (see also Carafoli *et al.* 1970) showed relatively little endogenous Ca^{2+} .

When EDTA was added to the 0.25M-sucrose medium employed for homogenization of the tissue and washing of the mitochondria, the amount of

endogenous Ca^{2+} in the mitochondria was decreased to one-third or less. The remaining endogenous Ca^{2+} was not removed by washing with EDTA, even at very high concentrations.

Experiments summarized in Fig. 2 show that 2,4-dinitrophenol very rapidly discharges half or more of the endogenous Ca^{2+} from liver, heart, brain and kidney mitochondria. Most of the Ca^{2+} discharge occurred before the first measurement could be made (30s). Discharge of Ca^{2+} in this

manner is accompanied by stoichiometric H^+ uptake (Carafoli, 1967). These results are consistent with earlier observations on rat liver mitochondria (Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965; Carafoli, 1967; Lehninger, Rossi, Carafoli & Reynafarje, 1968). Dinitrophenol discharged Ca^{2+} from blowfly mitochondria only very slowly and had no effect on the endogenous Ca^{2+} of yeast mitochondria.

Table 4. Ca^{2+} content of freshly isolated mitochondria

The isolation medium contained no EDTA.

Source of mitochondria	Ca^{2+} content (ng-atoms/mg of protein)
Rat liver	10-14
Rat kidney	35-55
Rat heart	30-40
Rabbit heart	24-39
Rabbit brain	16
Ox adrenal cortex	36-89
<i>Neurospora</i>	20
<i>S. cerevisiae</i>	8-9
Blowfly muscle	10-60
Turtle liver	48-50
Chicken liver	30-35

Low-affinity Ca^{2+} binding. Various types of mitochondria were examined for their capacity for low-affinity Ca^{2+} binding, by determining the amount of Ca^{2+} bound on adding increasing concentrations of free Ca^{2+} in the presence of respiration inhibitors. The binding results are presented as Scatchard plots (Fig. 3). As was shown earlier (Reynafarje & Lehninger, 1969), rat liver mitochondria yield a biphasic Scatchard plot for Ca^{2+} binding, the horizontal rectilinear leg corresponding to low-affinity and the vertical leg to high-affinity Ca^{2+} binding. All mitochondria except those for blowfly muscle also showed biphasic Scatchard plots (Fig. 3). Table 5 shows the number of low-affinity Ca^{2+} -binding sites, which in most vertebrate mitochondria fall within the range 20-60 ng-atoms/mg of mitochondrial protein. Rat liver mitochondria were near the bottom of the range and showed the smallest number (20-28 ng-atoms/mg of protein) whereas rabbit heart mitochondria showed the largest (57-61 ng-atoms/mg of protein), in proportion to the relative surface area of their inner membrane systems.

The dissociation constants of the low-affinity Ca^{2+} -binding sites are also given in Table 5. The

Fig. 2. Discharge of endogenous Ca^{2+} from mitochondria by 2,4-dinitrophenol. Source of mitochondria: (a) rat kidney; (b) rat heart; (c) ox adrenal cortex; (d) rabbit brain; (e) blowfly muscle; (f) yeast. O, 2,4-Dinitrophenol added; ●, no 2,4-dinitrophenol added.

Fig. 3. Scatchard plots of Ca²⁺ binding by mitochondria. Source of mitochondria: (a) rat liver; (b) rabbit liver; (c) guinea-pig liver; (d) rabbit kidney; (e) rat kidney; (f) rabbit heart; (g) rat heart; (h) rat brain; (i) rabbit brain; (j) ox adrenal cortex; (k) yeast; (l) *Neurospora*; (m) blowfly muscle.

Table 5. Low-affinity Ca²⁺ binding in different mitochondria

Source of mitochondria	Number of sites (ng-atoms/mg of protein)	K _m (μM)
Liver		
Rat	20–28	38–52
Mouse	43	10.4
Rabbit	22	20
Guinea pig	52	9.4
Chicken	32	42
Turtle	19	35
Kidney		
Rat	24–32	12–30
Rabbit	50	62.5
Heart		
Rat	50–64	40–62
Rabbit	53–66	55–59
Brain		
Rat	14	67
Rabbit	14–46	37–66
Ox adrenal cortex	57–61	70–129
<i>Neurospora</i>	46	20
<i>S. cerevisiae</i>	40–50	11–14
<i>T. utilis</i>	39	39
Blowfly muscle	0	0

affinity of these sites is highest in rat kidney mitochondria, but relatively low in ox kidney-cortex mitochondria. It is noteworthy that yeast mitochondria, which show no energy-linked accumulation of Ca²⁺, have only a single class of binding sites for Ca²⁺ with an affinity intermediate between those of the low- and high-affinity sites of rat liver mitochondria (Carafoli *et al.* 1970).

High-affinity Ca²⁺ binding. Table 6 shows the number and dissociation constants of the high-affinity Ca²⁺-binding sites extrapolated from the Scatchard plots shown in Fig. 3. The number of these sites was least in rat brain mitochondria (0.2–0.8 ng-atom/mg of protein) and greatest in ox adrenal-cortex mitochondria (9–11 ng-atoms/mg of protein). Their dissociation constants indicated that the affinity of these sites for Ca²⁺ was highest in rat liver mitochondria (K_m 0.4–1.6 μM) and lowest in mitochondria from rabbit brain and ox adrenal cortex (K_m 1.2–9.7 μM). The dissociation constants of the high-affinity Ca²⁺-binding sites extrapolated from the Scatchard plots tend to be overestimated; the values given thus represent upper limits.

Table 6. *High-affinity Ca²⁺ binding in different mitochondria*

Source of mitochondria	Number of sites (ng-atoms/mg of protein)	K_m (μM)
Liver		
Rat	1.2-8	0.4-1.6
Mouse	4.4	1.21
Rabbit	3.9	0.67
Guinea pig	5.5	0.10
Chicken	2.9	0.6
Turtle	3.8	3.40
Kidney		
Rat	4-6	0.4-1.7
Rabbit	18	0.72
Heart		
Rat	2-4.5	0.4-1.4
Rabbit	3.5-6.5	0.7-1.2
Brain		
Rat	2	0.8
Rabbit	0.2-0.8	1.2-5.0
Ox adrenal cortex	9-11	1.2-9.7
<i>Neurospora</i>	None	—
<i>S. cerevisiae</i>	None	—
Blowfly muscle	None	—

DISCUSSION

Mitochondria from vertebrate tissues. The results reported in this paper, together with other observations on pigeon heart (Chance, 1963) and ox heart (Brierley, Murer & Bachmann, 1964), show that mitochondria from the tissues of all vertebrate species tested are capable of the stoichiometric coupling of Ca²⁺ accumulation to electron transport, with very similar properties. Since respiration-coupled Ca²⁺ transport was found to occur in mitochondria from all vertebrate tissues tested (liver, heart, brain, kidney, adrenal cortex and thyroid), derived from three classes of vertebrates (mammals, birds, and reptiles), it would appear that the capacity for respiration-linked Ca²⁺ uptake may be a general property of mitochondria in all cells of vertebrates and thus as characteristic a mitochondrial feature as oxidative phosphorylation. The only noteworthy difference observed among the various vertebrate mitochondria was some variation in the requirement for phosphate for Ca²⁺ uptake.

All the vertebrate mitochondria have a very high affinity for Ca²⁺, as reflected by the low dissociation constants of the high-affinity Ca²⁺-binding sites, by the functional response of the respiratory chain to low concentrations of Ca²⁺, and by the fact that Ca²⁺ stimulates respiration to higher rates than ADP. All the vertebrate mitochondria tested thus resemble rat liver mitochondria, in which Ca²⁺ accumulation has been shown to take primacy over ADP phosphorylation under conditions not

far removed from intracellular (Rossi & Lehninger, 1964; Lehninger, 1970). These observations strongly suggest that the concentration of Ca²⁺ in the cytoplasm is very critical in cell function and that mitochondria may play an important role in regulating the concentration of free Ca²⁺ in the cytoplasm by their very high affinity for Ca²⁺ and their capacity to segregate Ca²⁺ very rapidly and, presumably, reversibly. The role of mitochondria in segregation and release of Ca²⁺ in the contraction-relaxation cycle of red muscles and in the formation of amorphous calcium phosphate deposits in the early steps of biological calcification have been reviewed by Lehninger (1970). Other aspects of the role of Ca²⁺ in regulatory processes have been discussed by Bygrave (1967) and Rasmussen (1970).

Mitochondria from insect flight muscle. Although mitochondria from the tissues of vertebrates appear to be very similar in their interaction with Ca²⁺, the capacity for rapid respiration-linked accumulation of Ca²⁺ does not appear to be universal in mitochondria from various insect and plant species. Flight-muscle mitochondria from the blowfly show some exceptional differences from those of mammalian muscle, indeed from all vertebrate mitochondria. They show no respiratory stimulation by Ca²⁺, no uptake of Ca²⁺ in the absence of phosphate, and no high-affinity Ca²⁺-binding sites. The foremost conclusion from these observations and others reported elsewhere (Carafoli *et al.* 1971) is that blowfly mitochondria lack a specific carrier for Ca²⁺, which can therefore enter mitochondria only very slowly, presumably by simple non-mediated physical diffusion. At low concentrations of free Ca²⁺, such as are presumed to exist in the intact blowfly muscle cell, the rate of Ca²⁺ accumulation and the Ca²⁺/~ accumulation ratio have been found to be negligibly small. It thus appears very unlikely that segregation of Ca²⁺ by mitochondria is an important element in contraction-relaxation cycles in blowfly muscle. On the other hand Ca²⁺ is required to activate the adenosine triphosphatase activity of the myofibrils and to activate respiration and glycogen breakdown in this tissue.

A parallel study of the mitochondria from the flight muscle of the periodical cicada of the southeastern United States (Hansford, 1971) showed them to have a profoundly different pattern of behaviour. The cicada mitochondria showed a high rate of Ca²⁺ accumulation, and a high affinity for Ca²⁺. These and other significant differences suggest that the asynchronous flight muscles of the blowfly may have fundamentally different mechanisms for excitation-contraction coupling and/or allosteric control of respiration than the presumably synchronous flight muscle of the periodical cicada.

Plant mitochondria. The available information on the behaviour of mitochondria from plant cells is limited and allows no broad generalizations. As shown in our results and the results of Carafoli *et al.* (1970), the yeasts *S. cerevisiae* and *T. utilis* lack the ability to accumulate Ca^{2+} under conditions in which mammalian mitochondria show high activity. Moreover, mitochondria from *S. cerevisiae* were shown to have no response to low concentrations of Ca^{2+} , either in the rate of respiration or in the oxidation-reduction state of cytochrome *b*, a very sensitive indicator of the interaction of Ca^{2+} with the respiratory chain (Chance, 1965). Since they also lack high-affinity binding sites for Ca^{2+} , it may be concluded that they possess no specific Ca^{2+} carrier.

Because mitochondria from *N. crassa* also show limited ability to accumulate Ca^{2+} and lack high-affinity Ca^{2+} -binding sites, they may also lack a specific Ca^{2+} carrier. The slow respiration-dependent Ca^{2+} accumulation shown by *Neurospora* mitochondria is probably the result of damage to their membranes caused by the methods required to disrupt the cell wall (Hall & Greenawalt, 1967).

Although Bonner & Pressman (1965) have briefly reported that mitochondria from mung-bean seedlings show no respiratory stimulation by Ca^{2+} , those from maize seedlings do accumulate Ca^{2+} , as shown in the detailed studies of Hanson and his group (Hodges & Hanson, 1965; Kenefick & Hanson, 1966; Stoner & Hanson, 1966). Unpublished work in this laboratory (C. H. Chen) indicates that mitochondria from the white potato and the sweet potato also accumulate Ca^{2+} during respiration.

Mechanism of Ca^{2+} transport by mitochondria. The comparative biological results reported here also provide some evidence regarding the mechanism of the coupling of Ca^{2+} movements to electron transport. They strongly support the view that Ca^{2+} is transported by a specific carrier system, whose occurrence is evidently genetically determined. There are two alternative hypotheses as to the nature of the Ca^{2+} carrier. One view is that the three energy-coupled electron carriers of the respiratory chain actually react with and cause the transport of Ca^{2+} into the matrix (Chance, 1965). The alternative hypothesis is that the Ca^{2+} carrier is not a member of the chain, but an independent molecular system, one which is, however, responsive to electrochemical gradients generated by electron transport (Reynafarje & Lehninger, 1969; Lehninger & Carafoli, 1970; Lehninger, 1970). Since the Ca^{2+} -transport system is apparently absent in mitochondria of blowfly muscle, yeast and *Neurospora*, which otherwise are capable of coupling phosphorylation to electron transport, it does not appear likely that the electron-carrier molecules *per se* function directly as Ca^{2+} -carrier systems.

However, further examination of the spectra and other properties of the electron carriers in these organisms is required before this possibility can be dismissed. The finding that the Ca^{2+} carrier is not present in some species of mitochondria is analogous to the reported distribution of the carriers specific for tricarboxylic acid-cycle intermediates, which are present in rat liver and kidney mitochondria, but are absent in mitochondria of some insect flight muscles (Chappell, 1968; Hansford, 1971). Such comparative information will be useful in further chemical characterization of the Ca^{2+} -binding factor, which has been extracted from rat liver mitochondria in soluble form (Lehninger, 1970, 1971).

We consider it likely that all mitochondria, whatever the cell type, possess the electrochemical capacity for moving Ca^{2+} across the membrane. This capacity cannot be expressed, however, unless a pathway for trans-membrane movement of Ca^{2+} is available, either through the occurrence of a specific Ca^{2+} carrier system or through simple physical permeability of the mitochondrial membrane to Ca^{2+} . This view is supported by the observation that respiring yeast mitochondria, which have no Ca^{2+} carrier, will slowly accumulate Ca^{2+} in a dinitrophenol-sensitive manner when very high external concentrations of Ca^{2+} (5 mM) are added, presumably as the result of slow inward leakage of external Ca^{2+} through the membrane (W. X. Balcavage, unpublished work). Moreover, yeast mitochondria will respond to the addition of K^{+} in the presence of valinomycin with a stimulation of oxygen uptake (Carafoli *et al.* 1970).

The results reported here are relevant to another fundamental question regarding the mechanism of mitochondrial ion-transport processes. Hanson & Miller (1967) have postulated, on the basis of the requirement of phosphate in accumulation of Ca^{2+} by maize mitochondria, that respiration-coupled phosphate uptake is an obligatory prerequisite for Ca^{2+} uptake, which is suggested to follow secondarily, in contrast with the more generally held view that any anion may accompany Ca^{2+} uptake provided it (or its corresponding acid) can penetrate the membrane (for discussion see Rossi & Lehninger, 1963, 1964; Millard, Wiskich & Robertson, 1964; Rasmussen, Chance & Ogata, 1965; Mitchell, 1966; Lehninger *et al.* 1967; Chappell, 1968). The results reported in this paper show that mitochondria from most vertebrate tissues can accumulate at least 80 ng-atoms of Ca^{2+} /mg of protein in a respiration-dependent process in the absence of added phosphate in the medium. Although blowfly mitochondria show dependence on phosphate for Ca^{2+} uptake, the phosphate may be replaced by acetate (Carafoli *et al.* 1971), which is known to enter blowfly mitochondria readily

(Hansford & Chappell, 1968). Nevertheless, there still remains the problem of accounting for the balance of electrical charges during respiration-linked Ca^{2+} uptake by vertebrate mitochondria in the absence of phosphate, which is accompanied by ejection of only 1.0H^+ ion per Ca^{2+} ion accumulated, thus leaving unidentified the manner in which about one-half of the electrical charge of the entering Ca^{2+} is compensated.

The work was supported by grants from the U.S. Public Health Service (GM-05919) and National Science Foundation (GB-7032). The authors are indebted to Mrs Paulette Riley for technical assistance.

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