THE PHOSPHATE REQUIREMENT FOR Ca^{2+}-UPTAKE BY HEART AND LIVER MITOCHONDRIA

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1. Introduction

Although some years ago [1] the question of whether a permeant co-ion, usually phosphate, was a prerequisite for energised Ca^{2+} uptake by mitochondria was raised, the data at the time were equivocal because the preparations carry more or less endogenous phosphate. Recently [2], using *N*-ethylmaleimide to inhibit the movement of the residual endogenous P_i, the capacity of rat liver mitochondria for Ca^{2+} was given as 37 nmol/mg protein. It was there suggested that this could be a combination of binding to membrane material, with a contribution from CO_2 providing internal acidity. The accumulation of Ca^{2+} with carbonate has been demonstrated [3]. Most kinetic studies of Ca^{2+} uptake have been made in presence of added phosphate [4–6] though Reed and Bygrave [7] compared the behaviour with and without added P_i (or acetate), and noted that Ca^{2+} uptakes were limited without the addition. They proposed that with little penetrant co-anion the whole of the Ca^{2+} cycled in and out rapidly and continuously, and that added anion merely reduced the efflux. The kinetic consequence of this proposal is that the 'rate constant' determining the time course of approach to the steady state of the limited uptake is the sum of the separate constants for inward and outward movements (which we write as $k_{in} + k_{out}$) while the complete uptake obtained with added penetrant anion would proceed with rate constant k_{in}, and k_{out} would be negligible. Our observations provide evidence against the rapid cycling and favour the limitation of uptake being set by anion availability up to the onset of membrane damage.

Investigation of the essentiality of permeant anion for the Ca^{2+} uptake discloses one mechanism by which some agents inhibit the uptake, and it is on this account that agents such as Pb^{2+} [8] and Cd^{2+} are effective at concentrations lower than those which affect respiratory activity. The lanthanides appear to combine a phosphate scavenging effect with their generally accepted role of competition with Ca^{2+} for transporting sites. Another mechanism which also has the consequence of inhibiting Ca uptake, is seen with compounds which are inhibitory toward phosphate movement, as in the Brand et al. [2] application of *N*-ethylmaleimide. Besides with *N*-ethylmaleimide, 5,5'dithiobis(2-nitrobenzoic) acid and more effectively, mersalyl, we observe a similar effect with chlorpromazine.

2. Methods

Rat heart mitochondria were prepared from finely minced tissue (often 2 hearts) which was digested, with stirring at intervals, in about 10 ml of a medium having sucrose 200 mM, KCl 50 mM, EGTA 1 mM, BSA 0.1%, Tris–HCl 5 mM, pH 7.5 and 2.5 mg trypsin, for 15 min at 0–4°C. Other enzymes (nagarse or protease Type V (Sigma)) were used

Abbreviations: EGTA, ethylene glycol bis(2-aminoethyl) tetracetate; Hepes, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; DTNB, dithiobis(2-nitrobenzoic) acid.

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alternatively at similar w/v concentrations, but trypsin provided higher yields, which were about 30 mg protein from 1.5 g tissue. After the digestion, the volume was increased to 60–70 ml with 300 mM sucrose containing 1 mM EGTA and 0.1% BSA; the heavy material was removed by centrifugation up to 800 × *g* followed by switching the machine off and letting it coast to a stop. The supernatant was poured through a 0.2 mm mesh nylon gauze and the mitochondria were sedimented by centrifugation up to 9000 × *g* for 5 min. The pellet was resuspended in the sucrose medium supplemented with 5 mM KCl, and again sedimented. At this stage, any lighter material was removed by gentle swirling with medium; the major part of the pellet was then resuspended in buffered 300 mM sucrose (0.8 ml for the protein from 1.5 g tissue). Samples were taken for biuret reaction to measure the protein concentration and then 2 mg purified serum albumin was added to the suspension. This latter addition was necessary to maintain a stable performance. Liver mitochondria were prepared as before [9]. Respiratory rates were measured with a Clarke-type assembly.

The medium used for study of Ca^{2+} uptake rates contained KCl 100 mM, Tris–HCl, 20 mM, pH 7.2 and Tris Hepes 10 mM; unless specified otherwise, succinate was used at 6 mM and rotenone at 1 μg/ml. The KCl used in the media was recrystallised and all sucrose and BSA solutions were passed through ion exchange columns to reduce their Ca^{2+} concentrations to less than μM.

The course of Ca^{2+} uptake was followed in a dual wavelength spectrophotometer (Chance-Aminco) using the colour change of the dye Arsenazo III [10] as monitored by difference in absorbance between the wavelengths 685 and 675 nm. About 0.5 mg protein was used per ml medium in the cuvette. The $CaCl_2$ addition was stirred in, which lost 1–3 s of the record whose course, however, could be estimated without difficulty. The cuvette contents were maintained at 22°C by a water jacket. The preparation of the dye solution was important because our sample of the solid appeared to contain a proportion of the Ca compound. By mixing about 10 mg with 0.4 g Dowex 50 W in the Tris form and 1 ml water, a stock solution was obtained from which an aliquot of 50 μl/25 ml medium provided a satisfactory measuring solution, having $A_{520\ nm}$ 1.6. The curvature of the absorbance change as well as the initial slope versus Ca relation depended on the dye concentration in the medium. Calibrations are attached to the illustrations.

3. Results

3.1. *Heart mitochondria*

The courses of uptake of an addition of Ca^{2+} to a suspension of heart mitochondria are compared in fig.1 with (A) added P_i (B) no addition, (C) dithiobis(2-nitrobenzoic) acid and (D) mersalyl. At the concentrations used, mersalyl completely blocks the uptake and DTNB almost completely blocks it. The uptake can be restored either by allowing the endogenous P_i to enter by adding a –SH compound, or by adding acetate, whose movement is unaffected by the inhibitors of P_i movement. A more rapid uptake after the addition of the –SH compound is also obtained on adding P_i.

This result indicates that Ca uptake by heart mitochondria is completely dependent upon the presence of penetrant anion. Low but variable concentrations of P_i present from endogenous sources in the successive preparations would account for the differing capacity for Ca^{2+} exhibited in the absence of added penetrant anion, as well as for the differing sensitivities of the Ca uptakes to cations which form insoluble phosphates (Pb^{2+}, Cd^{2+}, Y^{3+}). Figure 2 illustrates the progressive inhibition of Ca^{2+} uptake by Cd^{2+}; similar results were obtained with either Pb^{2+} or Y^{3+} (table 1A). With all three, the Ca uptake could be restored by an addition of acetate, as exemplified in fig.2, although there was a distinct slowing by Y^{3+} even with the acetate present (table 1B). This observation proves that the Ca^{2+} uptake had been limited by absence of penetrant anion and not by an interference of the foreign cation with energy production. Another procedure to bear out this point was to follow the Ca uptake from an acetate-containing medium to which the foreign cations were added. Table 1B summarises the rate constants holding for the Ca^{2+} uptakes which were complete and not limited to the amounts which would enter in the absence of acetate. The foreign cations are now seen to lessen the rate constants instead of the extent of uptake, consistent with the effects now being only on the availability of Ca-transporting sites. Of the three ions, Y^{3+} is the most effective.

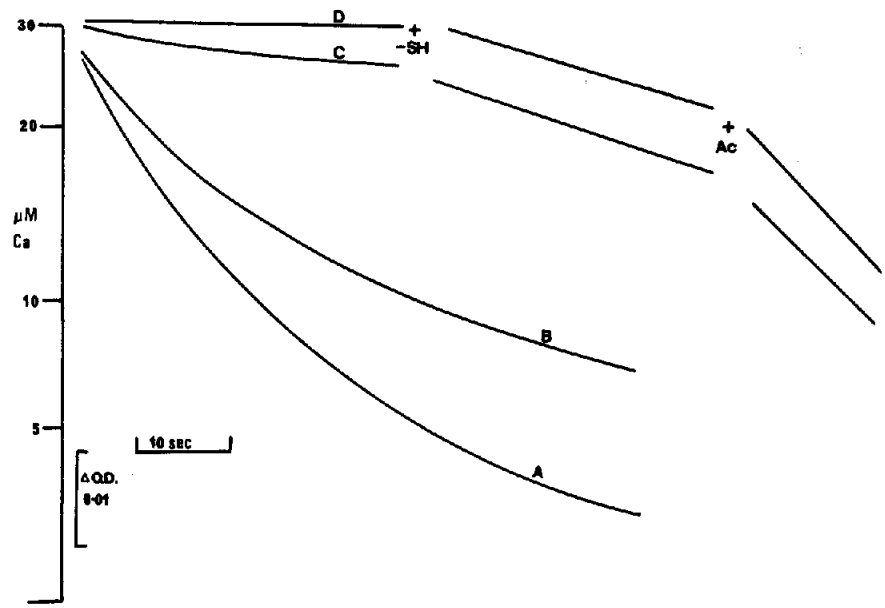

Fig.1. The anion requirement for uptake of Ca by heart mitochondria. A comparison between the uptakes in presence of: Added phosphate (P_i) at 3 mM (A); With no addition, endogenous P_i about 10 μM (B); With dithiobis(2-nitrobenzoic) acid, at 200 μM (C); with mersalyl, at 40 μM (D). The two latter agents impede phosphate movement, their effect is removed by Cleland's reagent added at 2 mM (marked −SH) and a slow resumption of Ca movement proceeds on account of endogenous phosphate. Addition of acetate (10 mM) at Ac accelerates the Ca uptake. A similar effect (example in fig.4) was obtainable with P_i added after the Cleland's reagent. Conditions as in Methods with protein at 0.56 mg.ml^{-1}.

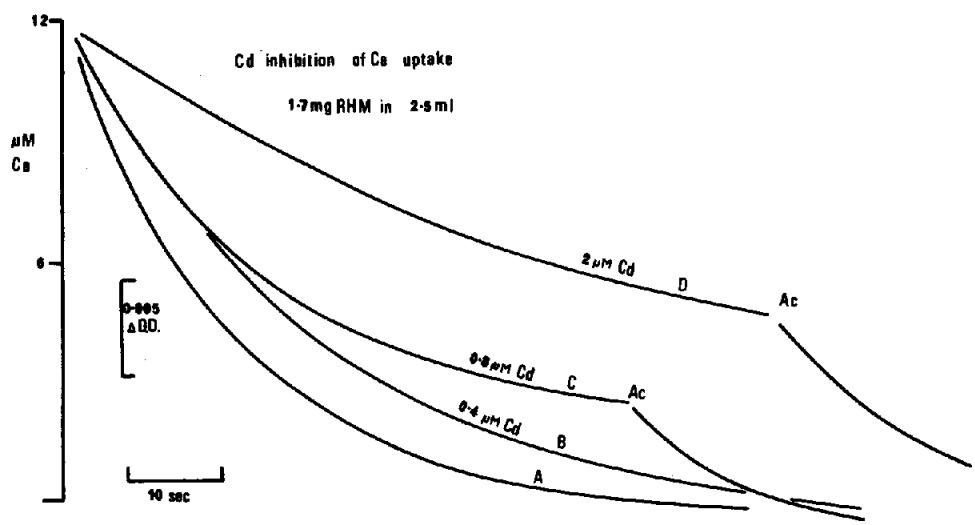

Fig.2. The effect of Cd ions on the Ca uptake by heart mitochondria unsupplemented with penetrant anion. The control (curve A) shows the uptake which is attributed to endogenous phosphate. Curves B, C and D are for mixtures to which $CdCl_2$ was added before the $CaCl_2$ at concentrations of 0.4 μM, 0.8 μM and 2 μM. That the Cd^{2+} is acting by removing penetrant anion and not by blocking Ca uptake sites, or inhibiting energy supply, is shown by the resumption of Ca uptake obtainable on adding acetate (10 mM) at Ac. Conditions as in Methods with protein at 0.68 mg.ml^{-1}.

Table 1

(A) The diminished quantity of Ca removed from an addition providing 18 nmol/mg protein caused by the prior addition of either Cd^{2+} (fig.2), Pb^{2+} or Y^{3+}

Cation added	Concentration (μM)	Ca removed from the 18 nmol/mg added. (nmol/mg)	Rate constant of Ca removal (s^{-1}) (calculated for 1 mg protein/ml)
None	–	18	0.064
Cd^{2+}	0.4	18	0.058
	0.6	14.5	0.072
	0.8	13	0.074
	1.6	11.5	0.074
None	–	18	0.070
Pb^{2+}	1.0	15.5	0.080
	2.0	11.5	0.082
	3.0	8.3	0.077
Y^{3+}	0.2	14.3	0.081
	0.36	6.0	0.071
	0.50	2.3	0.070

Conditions as in Methods with protein 0.68 $mg.ml^{-1}$

No penetrant anion was added. Note that the rate constant determining the equilibrium process is little affected.

(B) The reduction in rate of Ca uptake caused by Cd^{2+}, Pb^{2+} and Y^{3+} when these ions are applied in presence of 10 mM acetate

Cation added	Concentration (μM)	Rate constant of Ca withdrawal (s^{-1}) (calculated for 1 mg protein.ml^{-1})
None	–	0.073
Cd^{2+}	2	0.073
	4	0.055
Pb^{2+}	2	0.060
	4	0.047
None	–	0.061
Y^{3+}	2	0.014
	3	0.0097
	4	0.0055

Conditions as in Methods with protein 0.52 $mg.ml^{-1}$

The Ca added was to 38 nmol/mg protein. Uptakes proceeded to completion.

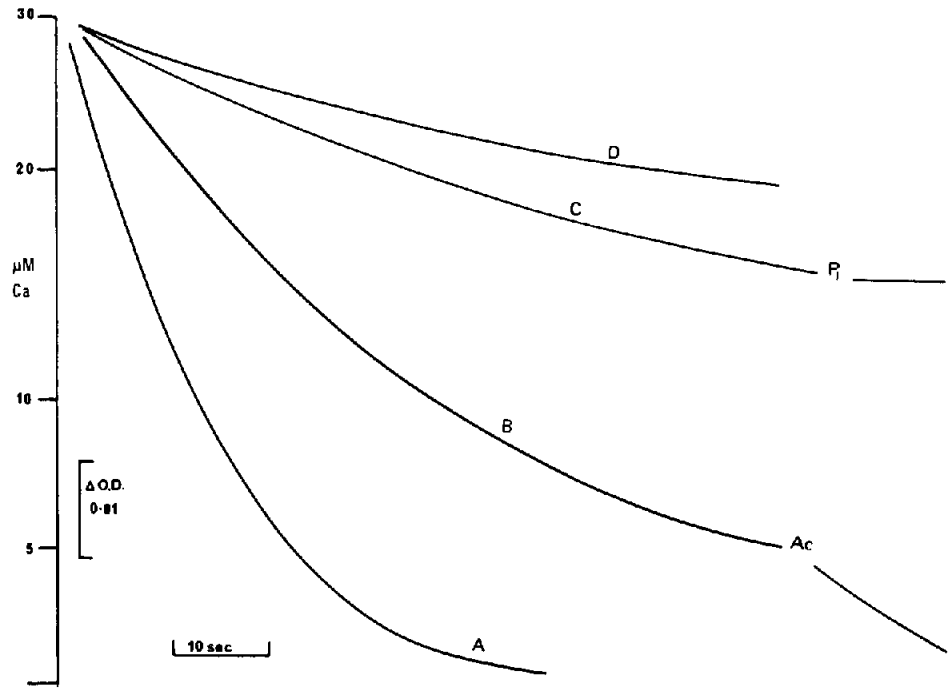

Fig.3. The effect of chlorpromazine on the Ca uptake by heart mitochondria unsupplemented with penetrant anion. The control curve (A) is without chlorpromazine and in B, C and D respectively the concentrations added before the $CaCl_2$ addition were 30 μM, 60 μM and 90 μM. As in fig.2, the uptake of Ca (B) was restored on adding acetate (at Ac) to 10 mM, but not by P_i. Conditions as in Methods with protein at 0.54 $mg.ml^{-1}$.

A likely example is afforded by chlorpromazine of an agent one of whose effects upon Ca uptake is on account of interference with phosphate transport. This drug progressively lessens the extent of Ca uptake like the phosphate scavenging metal ions and at the lower concentrations used its effect was lessened by acetate (fig.3, curve B), though at the higher concentrations acetate was no longer effective. The latter observation is indicative of an additional effect, which might be on the energy production.

If we conclude that a limited Ca uptake is determined by the availability of penetrant anion, the question is whether such Ca as has entered is rapidly turning over with that outside. Alternatively, the internal Ca may be relatively static. Arguments in favour of the latter can be based on both kinetic data and on observations of the respiratory energy demand after a Ca uptake. We see in table 1A that the rate constant of the approach to equilibrium between internal and external Ca remains the same whether all or only part of the total Ca moves into the particles when phosphate supply is limited. The addition of phosphate increases the rate constant (table 2). Hence it is unlikely that without the anion there was an efflux contributing k_{out} (see Introduction) to the total turnover rate, because adding anion would much reduce k_{out} and slow equilibration, contrary to observation. Also, if k_{out} contributed significantly to the total turnover rate, then stopping the inward component involving k_{in} by adding ruthenium red would lead to an efflux at a rate comparable with that of influx; for example, if Ca uptake ceased when half the external Ca had been removed, the dynamic situation would require that $k_{in} = k_{out}$. However, in agreement with Rossi et al. [11] and Sordahl [4], the efflux seen when ruthenium red was added was slow, for example 0.5 μM only induced a release rate of 0.5 $nmol.min^{-1}.mg^{-1}$, about 1% of the uptake rate from the concentration of Ca used for loading. Chance [12] notes that the respiratory rate after a limited

Table 2
The consequence of adding penetrant anion to the rates of uptake of Ca by heart mitochondria

Additions Ca (nmol/mg)	Anion	Rate constant of Ca withdrawal (s^{-1}) (calculated for 1 mg protein.ml^{-1})
6	None	0.056
20	None	0.070
6	1.6 mM P_i	0.080
20	1.6 mM P_i	0.083
6	3 mM P_i	0.079
20	3 mM P_i	0.091
6	5 mM acetate	0.077
20	5 mM acetate	0.068

Conditions as in Methods with protein at 0.67 mg.ml^{-1}

Two successive additions were made in each experiment. The uptakes proceeded to completion.

uptake of Ca obtained without added penetrant anion is low, despite the presence of external Ca, and Stucki and Ineichen [13], using Ruthenium Red and liver mitochondria, concluded that only 20% of the resting respiration was accountable to Ca cycling. If referred to Ca-stimulated respiration the proportion would be less, say 2–4%, and this could reasonably be the ratio of k_{out}/k_{in}.

3.2. *Liver mitochondria*

The phenomena which have been described came to light in the course of a more general study of the uptake and retention of Ca by heart mitochondria. However, it seemed worth trying to find how complete is the anion dependence of Ca uptake by liver mitochondria. Comparing the limited Ca uptakes obtained alternatively with *N*-ethylmaleimide at

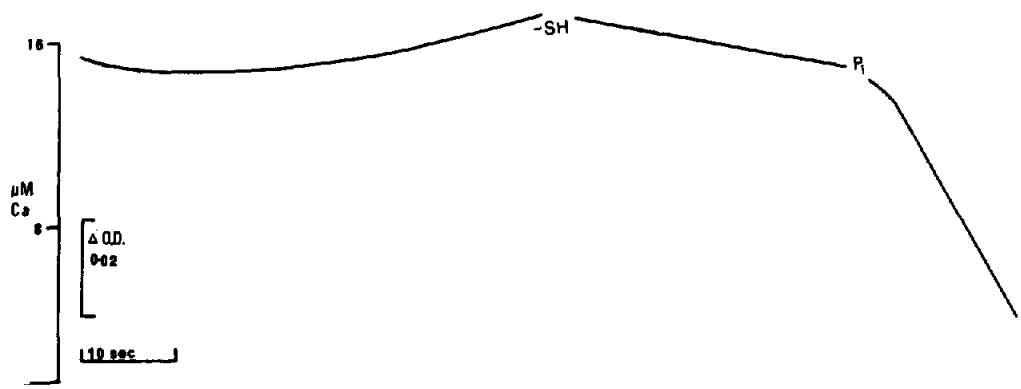

Fig.4. The blocking of Ca uptake by liver mitochondria by the combination of mersalyl at 80 µM and oligomycin (5 µg/mg protein). The mersalyl inhibits movement of phosphate and the oligomycin inhibits ATPase activity which would generate internal phosphate. The inhibition is relieved by Cleland's reagent (added at −SH) which allows endogenous phosphate to penetrate, and uptake is accelerated on providing more phosphate (to 3 mM) at P_i. Conditions as in Methods with protein at 0.63 mg.ml^{-1}.

0.8 mM and mersalyl at 80 μM, the latter was found the more effective. When oligomycin at 5 μg/mg protein was also added before the Ca to suppress ATPase activity and so prevent generation of internal phosphate in response to traces of Ca entering, then Ca uptake was almost suppressed (fig.4). That the mersalyl had not prevented energy generation is shown by the restoration of Ca uptake on adding phosphate (fig.4).

4. Discussion

Our observations show that the presence of a penetrant anion is a prerequisite if Ca is to be taken up in quantities exceeding 1 nmol/mg protein by liver or heart mitochondria. The suspensions themselves usually carry sufficient phosphate to permit considerable Ca uptake (for example, up to 60 nmol/mg protein). To show the anion requirement, it is necessary either to remove or block the entry of phosphate and, with liver mitochondria, also to prevent its internal generation.

The kinetic data and the lack of rapid release by Ruthenium Red both point to there being only a slow release of Ca, even when uptake remains incomplete due to insufficiency of permeant anion. The conclusion is opposite to that reached by Reed and Bygrave [7], but their kinetic constants, like ours, correspond to a faster equilibration when uptake is completed in presence of anion than when it is incomplete owing to an anion deficiency. An important corollary to the anion requirement, which is normally met by phosphate, whether added or not, is that the proton concentration shift which normally accompanies uptake of Ca [12,14] can be attributed properly to the change of ionisation of the phosphate anion as it passes to the more alkaline interior from the medium. The shift leaves about 1 H^+/Ca entering with the exact value depending upon the external pH [15]. Such an accumulation of phosphate accompanying Ca uptake provides internal anion to exchange further with certain other anions. It is notable that adding acetate or other weak monobasic permeant anion lessens and can nearly abolish the pH shift [16]. The ionisation of these acids does not appreciably alter on entry because their pK (4–5) is so much less than the physiological pH. Ca uptake obtainable in presence of inhibitors of phosphate movement or after removal of phosphate with precipitants can be a useful means of following anion penetration; preliminary trials show, for example, that β-hydroxybutyrate at 20 mM restores a slow Ca movement into heart mitochondria. This result resembles that obtained by direct measurement of entry of this acid along with Ca into liver mitochondria [2].

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