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A Re-evaluation of Energy-Independent Calcium-Ion Binding by Rat Liver Mitochondria

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The impermeability of the mitochondrial inner membrane to the chelator ethanedioxybis-(ethylamine)tetra-acetic acid permits discrimination between Ca^{2+} which has been transported to the internal (matrix) phase and Ca^{2+} which binds to the external surfaces of the mitochondrion. With this technique, it is shown that 'energy-independent high-affinity' binding is a measure of carrier-mediated active Ca^{2+} transport in respiration-inhibited mitochondria; the carrier also transports Ca^{2+} to the internal phase after treatment with carbonyl cyanide *m*-chlorophenylhydrazone, but in this case the active-transport component is inhibited. The Ca^{2+}-binding sites associated with the external membrane surfaces are similar in concentration and affinity for both inhibited and uncoupled mitochondria; it was not possible to measure external Ca^{2+} binding which could be identified as carrier specific. The results are discussed in relation to the mechanism of mitochondrial Ca^{2+} transport, and to previous studies of energy-independent Ca^{2+} binding.

Recent work in a number of laboratories has established beyond doubt that the energy-linked accumulation of Ca^{2+} by rat liver mitochondria is mediated by a specific membrane-associated carrier. This conclusion is based on the substrate saturability (Bygrave *et al.*, 1971; Spencer & Bygrave, 1973; Reed, 1974; Vinogradov & Scarpa, 1973), high affinity (Bygrave *et al.*, 1971; Spencer & Bygrave, 1973; Reed, 1974; Carafoli & Azzi, 1972), high specificity (Vainio *et al.*, 1970), specific inhibition (Mela, 1969; Vainio *et al.*, 1970; Moore, 1971; Vasington *et al.*, 1972; Reed & Bygrave, 1974*b*), genetic determination (Carafoli & Lehninger, 1971; Balcavage *et al.*, 1973; Carafoli *et al.*, 1971) and independence from respiration (Selwyn *et al.*, 1970; Scarpa & Azzone, 1970) of active Ca^{2+} transport.

Since the binding of Ca^{2+} to the carrier is an obligatory initial step in its transport, it might be possible to devise experimental conditions for measuring the concentration and dissociation constant of carrier-specific binding sites. These data are essential to a detailed analysis of the kinetics of Ca^{2+} transport (Reed, 1974; Reed & Bygrave, 1974*b*); in addition, they provide some of the criteria that must be met by any isolated protein purported to be the Ca^{2+} carrier (Lehninger, 1971; Gomez-Puyou *et al.*, 1972).

Studies on the energy-independent binding of Ca^{2+} to rat liver mitochondria have revealed a class of high-affinity binding sites with properties similar to those of the transport process itself (Lehninger *et al.*, 1969; Reynafarje & Lehninger, 1969; Carafoli & Lehninger, 1971) leading to a general equation of high-affinity energy-independent binding with carrier-specific binding. Serious doubts on the validity of this interpretation were raised by Mela & Chance (1969): whereas the number of high-affinity Ca^{2+}-binding sites has been reported variously at 0.6–8 nmol/mg of protein (Lehninger, 1969; Reynafarje & Lehninger, 1969; Carafoli & Lehninger, 1971), titration of the initial rate of Ca^{2+} transport with Ruthenium Red and lanthanides shows that these compounds inhibit the Ca^{2+} carrier by binding at sites numbering respectively 0.08 and $\leq$0.001 nmol of inhibitor/mg of protein (Reed & Bygrave, 1974*b*). Further, the striking sensitivity of high-affinity Ca^{2+} binding to the proton-conducting uncouplers 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone (Reynafarje & Lehninger, 1969) suggests that the site of such binding is not the Ca^{2+} carrier, since the carrier has been shown to function independently of metabolism and has been concluded to catalyse electrophoretic transport in response to a membrane potential (see Mitchell, 1968; Chance & Montal, 1971; Skulachev, 1971; Reed, 1974; and preceding references). Binding of Ca^{2+} to the carrier would thus be expected to be insensitive to uncoupling agents.

The present paper examines the energy-independent binding of Ca^{2+} to mitochondria by using the impermeability of the inner membrane to EGTA [ethanedioxybis(ethylamine)tetra-acetic acid)] (Reed &

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Bygrave, 1974*b*) as a topological criterion for distinguishing between external binding and carrier-mediated transport (see Azzone *et al.*, 1969). It is concluded that 'high-affinity energy-independent' binding represents Ca^{2+} which has been transported to the internal phase of the mitochondrion; a large proportion of 'low-affinity energy-independent' binding similarly is associated with the internal phase. There seems little possibility of measuring carrier-specific Ca^{2+} binding within the limitations of present methodology.

Experimental and Results

Mitochondria

Mitochondria were isolated and assayed for protein as described previously (Reed & Bygrave, 1974*a*).

Calcium binding

Mitochondria were incubated with $^{45}Ca^{2+}$ at 0°C in a standard medium of 250mM-sucrose and 2mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]–Tris (pH7.4) containing rotenone, antimycin and carbonyl cyanide *m*-chlorophenylhydrazone, as detailed in the legends to Figures. After the specified incubation period, they were separated from the medium by centrifugation (Eppendorf microcentrifuge: 2min at 12000*g*), and radioactivity was measured in the pellets or in samples (0.1ml) of the supernatant (Reed & Bygrave, 1974*b*). In all experiments except that of Fig. 2, the mitochondria were preincubated without Ca^{2+} under conditions identical with those of the succeeding incubation, then centrifuged (5min at 16000*g*) and resuspended in incubation medium which had been pretreated with Chelex-100 (see below).

Materials

EGTA, rotenone, oligomycin and antimycin were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.); antimycin solutions were standardized spectrophotometrically (Strong *et al.*, 1960). La^{3+} solutions were prepared and standardized as previously described (Reed & Bygrave, 1974*a*). Ruthenium Red was used as a filtered solution of the unpurified commercial product from Schmid and Co., Stuttgart, Germany (Reed & Bygrave, 1974*b*). Nupercaine hydrochloride was a generous gift of CIBA Pharmaceuticals (Crows Nest, N.S.W., Australia). Chelex-100 was supplied by Bio-Rad Laboratories (Calif., U.S.A.); columns for the removal of Ca^{2+} from sucrose solutions were prepared according to Bio-Rad Technical Bulletin no. 114. $^{45}Ca^{2+}$ was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

Theoretical considerations

Kinetic studies of mitochondrial Ca^{2+} transport have revealed a sigmoidal dependence of transport velocity on Ca^{2+} concentration (Bygrave *et al.*, 1971; Spencer & Bygrave, 1973; Vinogradov & Scarpa, 1973; Reed, 1974), the Hill coefficient being approx. 1.7. Half-maximal velocity is attained at a concentration of 4μM ($K_{0.5}$) at 0°C and pH7.4 (Reed, 1974). This value has been concluded to represent the steady-state dissociation constant of the Ca^{2+}–carrier complex (Reed, 1974), and as such it is probably quite similar to the dissociation constant for Ca^{2+} binding (K_d'). Assuming that the Hill coefficient reflects cooperative binding at two interacting Ca^{2+}-binding sites (Koshland, 1970), the measured $K_{0.5}$ is a combined expression for the dissociation constants of two binding steps:

$$E + A \rightleftharpoons EA \qquad \text{and} \qquad EA + A \rightleftharpoons EA_2$$

$$K_{d_1} = \frac{E \cdot A}{EA} \qquad K_{d_2} = \frac{EA \cdot A}{EA_2}$$

where E is the free carrier binding sites, A is the free Ca^{2+} and EA_2 is the carrier-bound Ca^{2+}. Then:

$$K_{0.5} \approx K_d' = K_{d_1} \cdot K_{d_2} = \frac{E \cdot A^2}{EA_2}$$

By rearrangement, the expected relation between free and carrier-bound Ca^{2+} is:

$$EA_2 = \frac{E_T \cdot A^2}{K_d' + A^2}$$

where E_T is the total concentration of carrier binding sites.

It must be stressed that this treatment is an approximation in that it assumes two infinitely cooperative binding sites; the real number may be higher and the extent of interaction between sites is certainly less than infinite.

Since the approximate value of K_d' is known, EA_2 (bound Ca^{2+}) may be calculated for any value of A (free Ca^{2+}). Fig. 1(*a*) shows simulated Scatchard plots (Scatchard *et al.*, 1957) calculated from the above expression where $K_d' = 4 \times 10^{-6}$M. Curves were constructed with the total concentration of binding sites (E_T) at 0.01, 0.1 and 1.0nmol/mg of protein, with the resultant alteration in scales described in the legend.

The difficulties in making experimental measurements of such binding are virtually insurmountable, for even if the total concentration of Ca^{2+}-binding sites is as high as 1nmol/mg of protein, the concentration of Ca^{2+} bound is at best less than one-thousandth of its free concentration. To obtain a valid extrapolation to the total concentration of binding sites requires accurate measurements under even less favourable conditions.

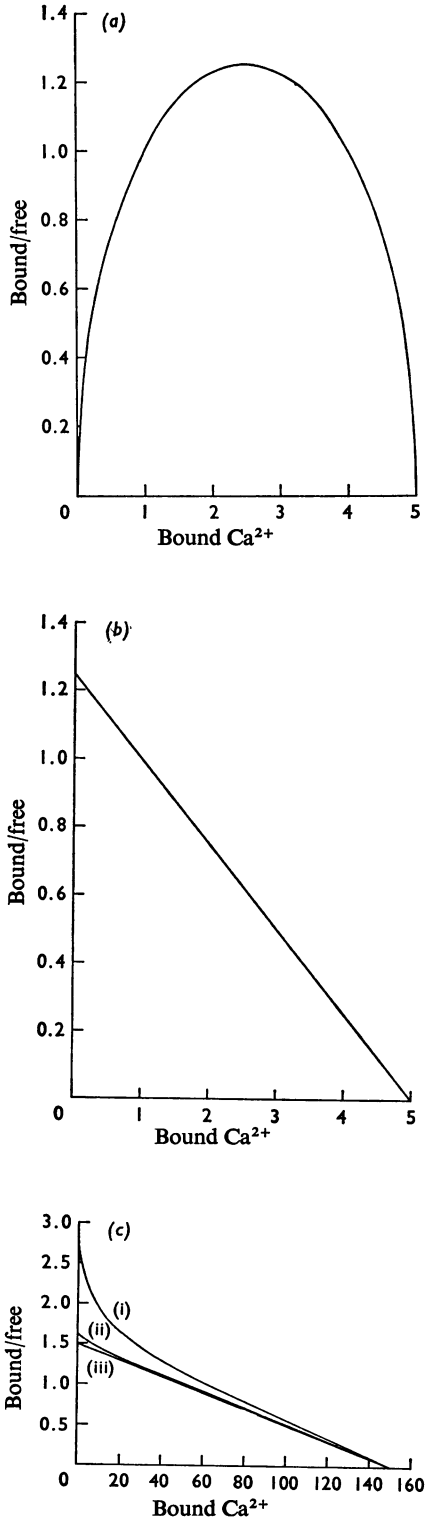


Fig. 1(*b*) shows the theoretical Scatchard plot calculated from identical data with the exception that binding occurs at non-interacting sites (Hill coefficient = 1). If this represents the true situation, analysis of carrier-specific binding is obviously more feasible, but the sigmoidal substrate–velocity relation in Ca^{2+} transport then requires an alternative interpretation.

There remain two further theoretical difficulties in the analysis of mitochondrial Ca^{2+} binding. First is the existence of a large number of sites which bind Ca^{2+} with relatively low affinity (Chappell *et al.*, 1963; Rossi *et al.*, 1967; Scarpa & Azzi, 1968; Scarpa & Azzone, 1969; Lehninger, 1969; Reynafarje & Lehninger, 1969; Carafoli & Lehninger, 1971). There can be little doubt that these sites are preponderantly, if not entirely, phospholipids (Scarpa & Azzone, 1969; Lehninger *et al.*, 1969).

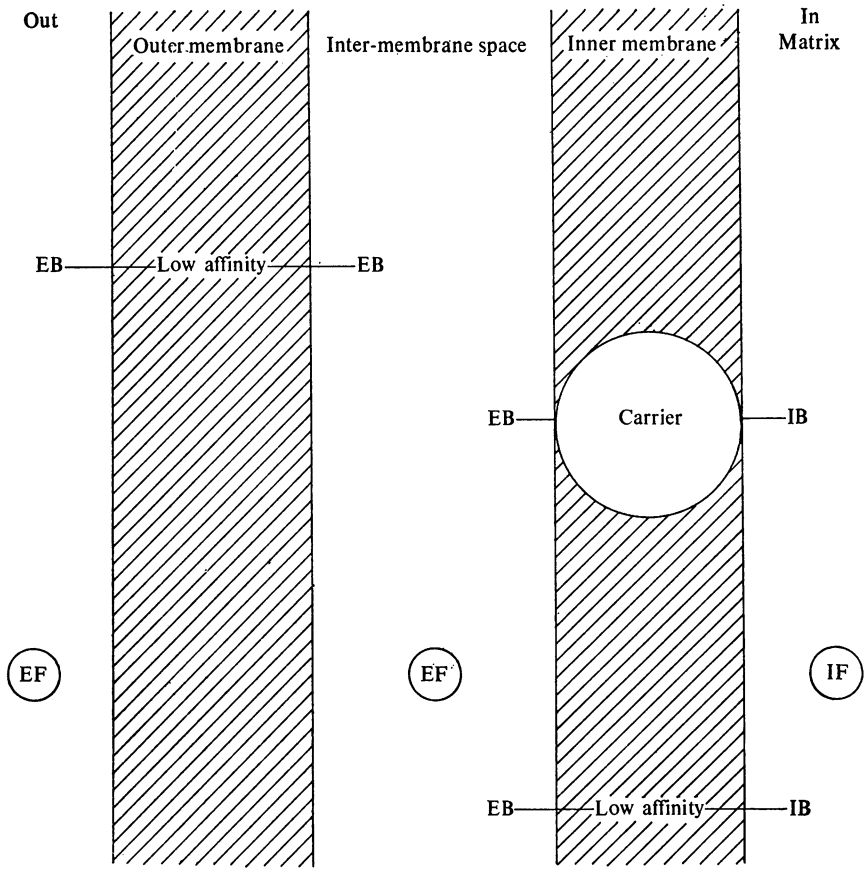
The binding of Ca^{2+} at the low-affinity sites imparts a very high background to attempted measurements of carrier-specific binding. Fig. 1(*c*) shows simulated Scatchard plots of the simultaneous binding of Ca^{2+} to such low-affinity sites (total concentration = 30nmol/mg of protein; $K_d = 100\mu M$; data from above references) and to the non-cooperative 'carrier' sites of Fig. 1(*b*) [the bound/free ratio for cooperative carrier sites (Fig. 1*a*) is too low to affect the low-affinity Scatchard plot]. It is obvious that carrier-specific binding could be measured with reasonable accuracy in the presence of simultaneous low-affinity binding only if it is non-cooperative and if the

Fig. 1. *Simulated Scatchard plots of carrier-specific mitochondrial Ca^{2+} binding*

In all the plots, bound and free Ca^{2+} concentrations are expressed as mol/litre (M) in a solution containing 5mg of mitochondrial protein/ml. The dissociation constant for carrier-specific binding (K_d') is 4×10^{-6}M. (*a*) $EA_2 = E_T \cdot A^2/(K_d'+A^2)$. The concentration of bound Ca^{2+} (EA_2) was calculated over a wide range of free Ca^{2+} concentrations (A) for E_T values of 0.01, 0.1 and 1.0 nmol/mg of protein. The ordinate scale is multiplied by 10^5, 10^4 and 10^3 respectively for the three values of E_T, and the abscissa scale by 10^8, 10^7 and 10^6. (*b*) $EA = E_T \cdot A/(K_d'+A)$. The ordinate scale is multiplied by 10^2, 10 and 1, the abscissa scale by 10^8, 10^7 and 10^6 respectively for E_T values of 0.01, 0.1 and 1.0nmol/mg of protein.

(*c*) $$EA = \frac{E_T' \cdot A}{K_d'+A} + \frac{E_T'' \cdot A}{K_d''+A}$$

where $E_T' = 1.0$nmol/mg of protein (i), 0.1nmol/mg of protein (ii) or 0 (iii). $K_d' = 4\times10^{-6}$M; $E_T'' = 30$nmol/mg of protein; $K_d'' = 100\times10^{-6}$M. When E_T' is 0.01nmol/mg of protein, or when its binding is expressed by the relation of (*a*), the total binding curve is not significantly different from that for $E_T' = 0$. The abscissa scale is multiplied by 10^6.



Scheme 1. *Mitochondrial pools available to* Ca^{2+}

A gross distinction was made, first between the pools separated by the selectively permeable inner membrane denoted by E (external) and I (internal), and secondly between bound (B) and free (F) Ca^{2+}.

concentration of binding sites approaches 1 nmol/mg of protein. Since the inhibition of Ca^{2+} transport by La^{3+} indicates less than 0.001 nmol of carrier/mg of protein (Reed & Bygrave, 1974*b*) each carrier molecule must then have about 1000 independent Ca^{2+}-binding sites, each with high specificity and high affinity for Ca^{2+}. This is impossible to reconcile with reasonable estimates of protein size and tertiary structure.

The second difficulty is the obvious necessity to discriminate between bound Ca^{2+} and Ca^{2+} that has been transported by the carrier and released from it to the internal phase. The magnitude of this critical problem has not been recognized previously. An explicit definition is required of the mitochondrial pools available to Ca^{2+} (Scheme 1). Clearly, the only way that transported Ca^{2+} can be distinguished from binding which occurs independently of transport is by distinguishing between internal and external pools. This topological definition is more useful in quantifying Ca^{2+}-binding sites than is the 'energetic' criterion applied in previous studies [compare Reynafarje & Lehninger (1969) with Rossi *et al.* (1967) and Scarpa & Azzone (1970)].

Experimental considerations

The most sensitive means of assaying Ca^{2+} binding is to use $^{45}Ca^{2+}$ but, as with kinetic studies, one is faced immediately with the dual problems of dilution by endogenous Ca^{2+} (Mela & Chance, 1969) and chelation by incubation components, both of which cause over-estimation of the dissociation constant. Endogenous Ca^{2+} can be removed by pretreatment of all solutions with a chelating resin, but there seems no acceptable means of completely overcoming the

latter problem since the use of a strong buffer to generate known free Ca^{2+} concentrations makes the accurate measurement of bound Ca^{2+} very difficult. A further requirement is to generate conditions where externally bound Ca^{2+} is a large proportion of total mitochondrial Ca^{2+}. The inclusion of respiratory inhibitors and uncouplers to increase the ratio of externally bound Ca^{2+}/total mitochondrial Ca^{2+} (and thus permit more accurate measurement of external binding) exaggerates the problem associated with dilution of $^{45}Ca^{2+}$ since both agents induce a release of endogenous mitochondrial Ca^{2+} (Carafoli, 1967; Reynafarje & Lehninger, 1969; Chance *et al.*, 1969).

A final point relevant to the experimental criteria defining external binding is that it should be rapidly reversible. Analysis of binding data by Scatchard plots (Scatchard *et al.*, 1957) requires that binding be reversible, but this fundamental requirement was not considered by Reynafarje & Lehninger (1969) in their analysis of high- and low-affinity energy-independent binding.

Measurement of external binding

A simple method for discriminating between Ca^{2+} bound reversibly to external sites and Ca^{2+} in the internal pools is to treat the mitochondria with excess of EGTA after incubation with $^{45}Ca^{2+}$. Since EGTA cannot penetrate to the internal pools (Scheme 1) (Reed & Bygrave, 1974*b*), the decrease in bound $^{45}Ca^{2+}$ after such treatment equals the fraction that was reversibly bound at sites external to the permeability barrier, whereas the remaining fraction is equivalent to irreversibly bound $^{45}Ca^{2+}$, presumably identical with the internal pools.

Fig. 2 shows the results of such an experiment with 1nmol of $^{45}Ca^{2+}$/mg of protein. The incubation conditions were similar to those of Reynafarje & Lehninger (1969) except that the temperature was 0°C and duplicate samples were analysed for free Ca^{2+}, one being centrifuged and the other treated with EGTA before centrifugation. Although there appears to be little significant change in external free Ca^{2+} with time (Reynafarje & Lehninger, 1969), probably due to continued Ca^{2+} transfer during centrifugation, there is a very large increase in the EGTA-inaccessible fraction.

The two distinct pools of mitochondrial Ca^{2+} revealed with EGTA were then examined in some detail. It must be mentioned at the outset that the EGTA-inaccessible pool decreases with time after EGTA addition (Fig. 11), so the proportion of external (EGTA-removable) Ca^{2+}/total mitochondrial Ca^{2+} is over-estimated in most of the data.

In all subsequent experiments the mitochondria initially were preincubated at the desired concentration in the presence of antimycin, rotenone (and carbonyl cyanide *m*-chlorophenylhydrazone when used), centrifuged, and were then resuspended in

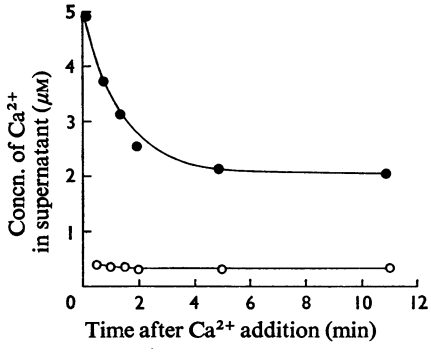


Fig. 2. *Reversible and irreversible components of 'high-affinity energy-independent' Ca^{2+} binding*

Mitochondria (30mg of protein) were preincubated for 2min with 1μM-rotenone and 1μM-antimycin in a total volume of 6ml. $^{45}Ca^{2+}$ (30nmol; 0.1μCi) was then added and samples (0.5ml) were removed into microcentrifuge tubes. At the times shown, the contents of a tube were either centrifuged (○) or treated with 0.1mM-EGTA–Tris (●) and centrifuged. Samples of all supernatants were counted for radioactivity. For further details see the text.

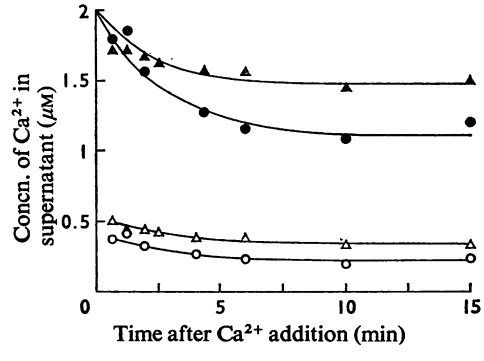


Fig. 3. *Effect of carbonyl cyanide m-chlorophenylhydrazone on components of 'high-affinity energy-independent' Ca^{2+} binding*

Pretreated mitochondria (12mg of protein) were incubated with 1μM-rotenone, 1μM-antimycin (○, ●) and where indicated, 1μM-carbonyl cyanide *m*-chlorophenylhydrazone (△, ▲) in a total volume of 6ml. $^{45}Ca^{2+}$ (12nmol; 0.05μCi) was added and then samples (0.25ml) were removed into microcentrifuge tubes. At the times shown, the contents of a tube were either centrifuged (○, △) or treated with 0.4mM-EGTA–Tris and centrifuged (●, ▲). Samples of all supernatants were counted for radioactivity. For further details see the text.

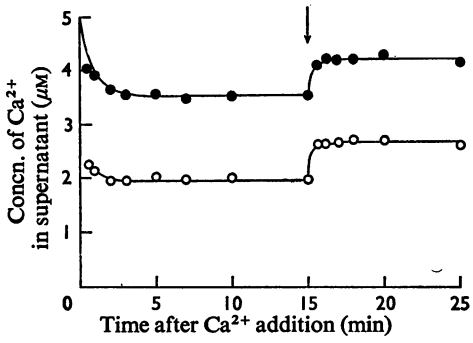


Fig. 4. *Carbonyl cyanide m-chlorophenylhydrazone-induced efflux of Ca^{2+} from respiration-inhibited mitochondria*

The incubation procedure was similar to that of Fig. 3 except that the concentration of mitochondria was 5mg of protein/ml and of $^{45}Ca^{2+}$, 5μM. Antimycin and rotenone were each present at 2.5μM; 5μM-carbonyl cyanide *m*-chlorophenylhydrazone was added at the time shown by the arrow.

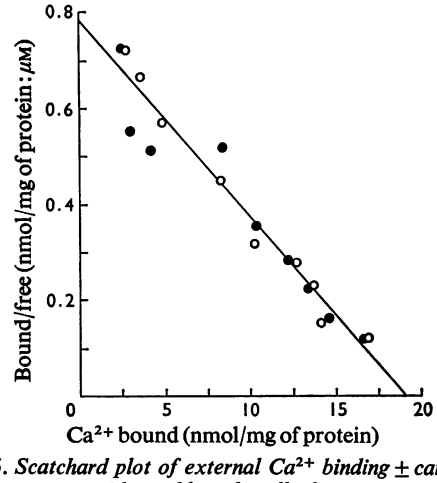


Fig. 5. *Scatchard plot of external Ca^{2+} binding ± carbonyl cyanide m-chlorophenylhydrazone*

Mitochondria (2mg of protein/ml) were pretreated as described in Fig. 3. Samples (0.5ml) of the suspension were transferred to nine microcentrifuge tubes containing from 4 to 100nmol of $^{45}Ca^{2+}$ (sp. radioactivity 2μCi/μmol) in 0.1ml of incubation solution. The tubes were mixed and samples (0.25ml) of each were transferred to a second series of tubes. Exactly 10min after the addition of mitochondria, the first set of tubes was centrifuged, and 0.8mM-EGTA–Tris was added simultaneously to the second set and these too were centrifuged. Radioactivity was measured in samples of all supernatants. The concentration of free Ca^{2+} is calculated from the radioactivity of the untreated supernatants, whereas the externally bound Ca^{2+} is calculated from the difference between the EGTA-treated and untreated supernatants. ○, 1μM-Carbonyl cyanide *m*-chlorophenylhydrazone; ●, no carbonyl cyanide *m*-chlorophenylhydrazone.

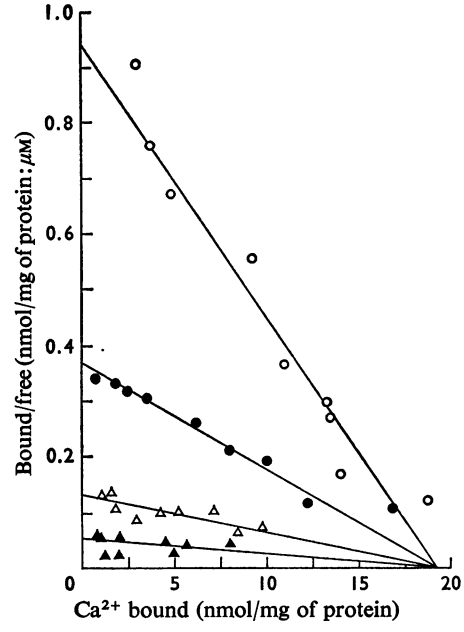


Fig. 6. *Inhibition of external Ca^{2+} binding by nupercaine, K^+ and Na^+*

The experimental procedure was similar to that of Fig. 5 except that carbonyl cyanide *m*-chlorophenylhydrazone was included in all incubations (similar data were obtained in all cases when carbonyl cyanide *m*-chlorophenylhydrazone was omitted). The pretreated mitochondria were resuspended, before incubation with $^{45}Ca^{2+}$, in buffered 250mM-sucrose (pH7.4) (○), 250mM-sucrose containing 83μM-nupercaine (●), 125mM-KCl (△) or 125mM-NaCl (▲). Other conditions were as described in Fig. 5.

Chelex-treated sucrose–Hepes–Tris buffer to minimize the dilution of isotopic label by endogenous Ca^{2+} released by the inhibitors.

Fig. 3 compares the time-course of distribution of a low concentration of $^{45}Ca^{2+}$ between the internal and external pools in the presence and absence of carbonyl cyanide *m*-chlorophenylhydrazone. Transfer between pools occurs in both cases, but the internal pool reaches a lower steady-state value in the presence of carbonyl cyanide *m*-chlorophenylhydrazone. The addition of carbonyl cyanide *m*-chlorophenylhydrazone to respiration-inhibited mitochondria pre-incubated with $^{45}Ca^{2+}$ induces an efflux from the internal pool as shown in Fig. 4 (the higher concentration of free Ca^{2+} in this experiment is due to the use of a relatively high concentration of antimycin, see below). The rate of efflux is considerably faster than the initial rate of entry of Ca^{2+} into the internal pool (cf. Figs. 2 and 3).

Both the total number of external binding sites (19nmol of Ca^{2+}/mg of protein) and their dissociation

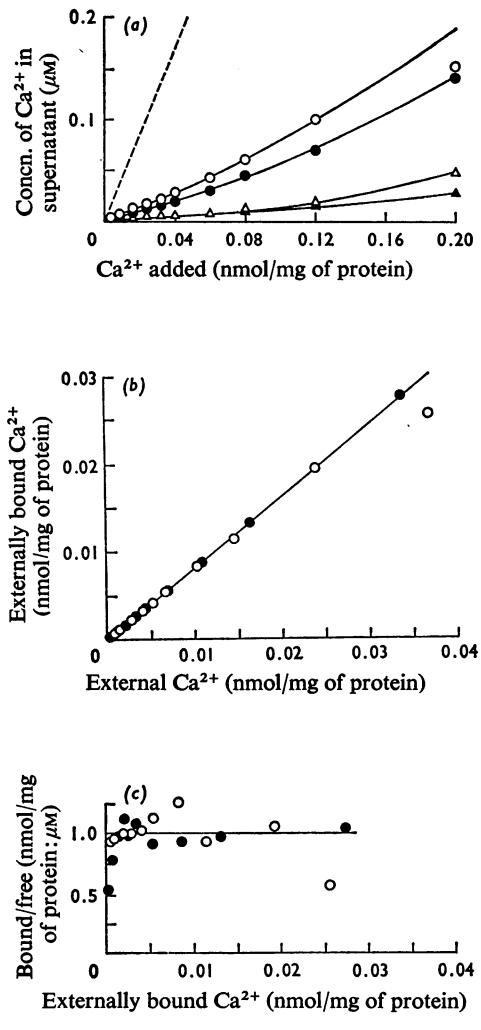


Fig. 7. *External binding at very low concentrations of* Ca^{2+}

The experimental procedure was similar to that of Fig. 5 except that carbonyl cyanide *m*-chlorophenylhydrazone was omitted and the concentration of mitochondria was 5mg of protein/ml. The concentration of antimycin used was insufficient for complete inhibition of succinate oxidation to ensure that no significant uncoupling occurred (see Fig. 8). The rotenone concentration was sufficient to block β-hydroxybutyrate oxidation. The closed and open symbols refer to two separate experiments, in each of which duplicate samples of supernatant were counted for radioactivity (sp. radioactivity of $^{45}Ca^{2+}$ was 0.3μCi/nmol). (*a*) △, ▲, $^{45}Ca^{2+}$ free in supernatant; ○, ●, $^{45}Ca^{2+}$ in supernatant after EGTA treatment. The dashed line shows the relation between 'μM' and 'nmol/mg of protein'. (*b*) External Ca^{2+} is that in the EGTA supernatants; externally bound Ca^{2+} is the difference between that value and free Ca^{2+} in the untreated supernatants. (*c*) Scatchard plot of external binding.

constant (25μM) are identical with and without carbonyl cyanide *m*-chlorophenylhydrazone (Fig. 5). In this and in subsequent experiments involving the concentration dependence of binding, measurements were made after a 10–15min incubation with $^{45}Ca^{2+}$ at 0°C, i.e. at the completion of the time-dependent transfer of Ca^{2+} between mitochondrial pools (Figs. 2, 3 and 4).

The external binding sites are competitively inhibited by K^+, Na^+ and the local anaesthetic nupercaine (Fig. 6); the inhibition constants (K_i) are 17mM, 6.6mM and 54μM respectively, calculated from the 'apparent K_d' values.

Additional experiments concentrated on measuring the binding of very low concentrations of Ca^{2+} in the hope of detecting a class of external sites which could be identified with the Ca^{2+} carrier from its known kinetic properties. Fig. 7 shows data from an experiment in which the total Ca^{2+} concentration was varied from 0.05 to 0.2nmol/mg of protein. The Scatchard plot (Fig. 7*c*) is horizontal and can be extrapolated to the ordinate at a value not significantly different from those of Figs. 5 and 6 where higher concentrations of Ca^{2+} were used. Similar data are obtained when the mitochondria are pretreated with carbonyl cyanide *m*-chlorophenylhydrazone, although the internal pool is much smaller in this case. These experiments were repeated in a medium containing 125mM-NaCl to determine whether inhibition by Na^+ of the numerous external Ca^{2+}-binding sites would permit identification of sites with higher affinity. However, the ordinate intercept of Scatchard plots from these experiments was indistinguishable from that of the appropriate experiment in Fig. 6.

The concentration of antimycin used in experiments of this type has a marked effect on the equilibrium distribution of $^{45}Ca^{2+}$, although it does not affect the external binding parameters. Fig. 8 shows the results of experiments in which internal, externally bound and free Ca^{2+} were measured as a function of antimycin concentration in the presence and absence of rotenone. In both cases, approx. 88% of the Ca^{2+} is inaccessible to EGTA at concentrations of antimycin below 0.1nmol/mg of protein, the approximate concentration required for complete inhibition of respiration (Estabrook, 1962; Ernster *et al.*, 1963). At higher concentrations of antimycin, the size of the internal pool decreases and both the external and free pools increase. Rotenone exaggerates these effects, presumably since the combination of inhibitors provides more complete respiratory inhibition than is afforded by antimycin alone. It is apparent that absolute measurements of each pool size vary with the concentration of antimycin used.

The effect of antimycin at higher concentrations is similar to that of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (Fig. 3). Löw & Vallin (1963) and Haas (1964) have described an uncoupling

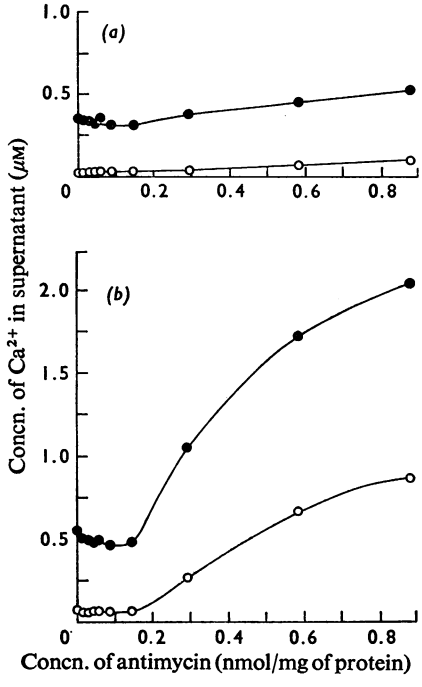


Fig. 8. *Effect of antimycin concentration on components of 'high-affinity energy-independent' Ca^{2+} binding*

Mitochondria (5mg of protein/ml) were pretreated in the absence of respiratory inhibitors. Samples (0.5ml) were added to tubes containing 0.1ml of incubation solution plus antimycin at the final concentrations shown [identical volumes (6μl) of ethanol, the antimycin solvent, were included in all tubes]. The mitochondria were preincubated for 5min before the addition of 1nmol of $^{45}Ca^{2+}$/mg of protein (sp. radioactivity 10μCi/μmol), and then incubated for 10min. After this time, radioactivity was measured in duplicate samples of supernatant (○), and supernatant after EGTA treatment (●). (*a*) No rotenone; (*b*) plus rotenone (0.2nmol/mg of protein).

effect of antimycin on bovine heart mitochondria (and derived particles) when concentrations higher than those required for respiratory inhibition are used.

Table 1 shows the effect of oligomycin on the distribution of 1nmol of Ca^{2+}/mg of protein between the three pools. It causes a measurable but relatively slight decrease in the internal fraction only when antimycin and rotenone are present. It can be concluded that the contribution of endogenous ATP to the maintenance of this pool is very slight.

The preceding experiments give no indication of carrier-specific Ca^{2+} binding, but there remains the possibility of detecting it by using the specific inhibitors La^{3+} and Ruthenium Red (Reed & Bygrave, 1974*b*). These inhibitors were added at various concentrations after 10min incubation of the mitochondria with $^{45}Ca^{2+}$. They have no significant effect on the concentration of free Ca^{2+}, but they cause a marked increase in the EGTA-inaccessible pool at concentrations similar to those which inhibit active transport (Fig. 9). Each time these experiments were carried out the effect of Ruthenium Red was more pronounced than that of La^{3+}.

A possible interpretation of these data is that the mechanism of transport inhibition by these compounds could be due to their causing a large increase in affinity of the carrier for Ca^{2+} and/or forcing and 'locking' the Ca^{2+}-loaded carrier in such a position that its binding sites are internally located (cf. the mechanism of inhibition of the adenine nucleotide translocase by bongkrekic acid; Erdelt *et al.*, 1972).

However, a simpler interpretation proved to be correct. The lack of effect of the inhibitors on the concentration of free Ca^{2+} (Fig. 9) is incompatible with the above interpretation. Further, Fig. 10 shows that the increase in internal Ca^{2+} due to Ruthenium Red addition exceeds 1nmol/mg of protein, which is far too high to be due to carrier-specific binding (see above). In fact the apparent increase is due to

Table 1. *Effect of respiratory and energy-transfer inhibitors on components of 'high-affinity' Ca^{2+} binding*

The experimental procedure was similar to that described in Fig. 8 except that the inhibitors were varied as shown (total Ca^{2+} added = 1nmol/mg of protein, or 4.17μM).

	Concentration of Ca^{2+} in appropriate pool		
Additions	Free (μM)	Externally bound (nmol/mg of protein)	Internal (nmol/mg of protein)
Nil	0.021	0.102	0.893
Oligomycin (1μg/mg of protein)	0.022	0.081	0.914
Antimycin (0.15nmol/mg of protein)	0.052	0.091	0.896
Antimycin+oligomycin	0.059	0.091	0.895
Antimycin+rotenone (0.2nmol/mg of protein)	0.382	0.212	0.696
Antimycin+rotenone+oligomycin	0.585	0.248	0.611
Antimycin+rotenone+oligomycin+carbonyl cyanide *m*-chlorophenylhydrazone (5μM)	2.085	0.379	0.120

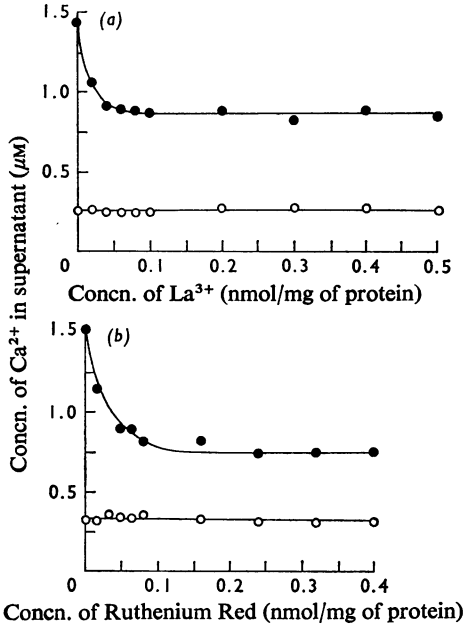


Fig. 9. *Effects of La^{3+} and Ruthenium Red on components of 'high-affinity energy-independent' Ca^{2+} binding*

Mitochondria (2mg of protein/ml) were pretreated as described in Fig. 3 (minus carbonyl cyanide *m*-chlorophenylhydrazone). $^{45}Ca^{2+}$ (2μM; sp. radioactivity 5μCi/μmol) was added, and after 10min incubation samples (0.5ml) were transferred to tubes containing (in 50μl) either La^{3+} (*a*) or Ruthenium Red (*b*) at the final concentrations shown. These were mixed immediately and samples (0.2ml) were treated with 0.25mM-EGTA and immediately centrifuged; the remaining suspensions also were centrifuged. Radioactivity was measured in all supernatants (total concentration of $^{45}Ca^{2+} = 1.82\mu$M). ○, Untreated supernatants; ●, supernatants after EGTA treatment. The Ruthenium Red solution used in this and subsequent experiments was a crude filtered solution (see the text). Its concentration is expressed as 'inhibitory equivalents' of Ruthenium Red, as determined from its inhibition of respiration-supported Ca^{2+} uptake.

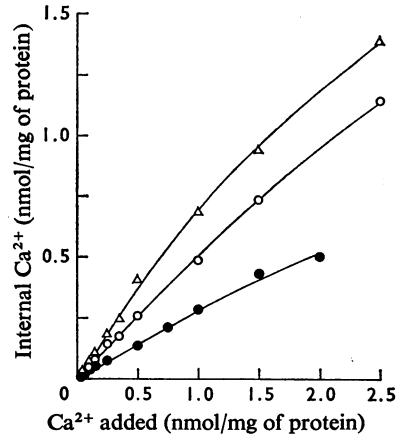


Fig. 10. *Apparent stimulation of internal energy-independent Ca^{2+} binding by La^{3+} and Ruthenium Red*

Mitochondria (2mg of protein/ml) were pretreated as described in Fig. 3 (minus carbonyl cyanide *m*-chlorophenylhydrazone) and samples (0.5ml) were transferred to microcentrifuge tubes containing 0.1ml of $^{45}Ca^{2+}$ (sp. radioactivity 0.25μCi/nmol) as shown. After 10min incubation, 0.016nmol of Ruthenium Red (△), 0.02nmol of La^{3+} (○) or nil (●) was added and the tubes were incubated for a further 5min. Samples were then centrifuged before and after treatment with 0.4mM-EGTA and all supernatants were counted for radioactivity. The Figure shows the amount of $^{45}Ca^{2+}$ not removed by EGTA (i.e. internal Ca^{2+}).

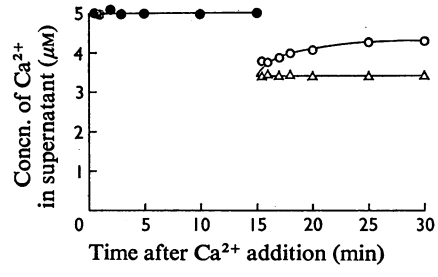


Fig. 11. *Time-dependent removal of internal Ca^{2+} by EGTA*

The preparation of mitochondria was identical with that in Fig. 4. In each experiment, 10nmol of $^{45}Ca^{2+}$ was added to 2ml of the suspension. This either was preceded immediately by the addition of 1mM-EGTA (●), or was followed after 15min by the addition of EGTA (○) or 1.5μM-Ruthenium Red and EGTA (△).

inhibition of EGTA-induced efflux as seen in Fig. 11. Further experiments in which Ruthenium Red was found to inhibit the entry of Ca^{2+} into the internal (EGTA-inaccessible) pool (K. C. Reed, unpublished work) have confirmed this interpretation.

These effects of Ruthenium Red and La^{3+} strongly support the assumption that the EGTA-inaccessible fraction of respiration-inhibited mitochondria is due to Ca^{2+} which has been transported to the internal pool by the Ca^{2+} carrier. The lesser effect of La^{3+} on carrier-mediated efflux is due probably to a combination of the release of its inhibition with time (a consequence of its being transported; Reed & Bygrave, 1974*a*) and the difference in inhibitory mechanisms of La^{3+} and Ruthenium Red (Reed & Bygrave, 1974*b*).

Fig. 12 shows the results of an experiment in which Ruthenium Red was included with the EGTA-quenching solution to prevent EGTA-induced efflux. This technique was used in conjunction with pretreatment of the mitochondria with both carbonyl cyanide *m*-chlorophenylhydrazone and EGTA to ensure maximum removal of endogenous Ca^{2+}. Again, Ruthenium Red does not alter the total concentration of mitochondrial Ca^{2+} (cf. Fig. 9), but it does decrease the measured values of external binding. The Scatchard plots of external binding (Fig. 12*c*) show that the correct value for the concentration of external Ca^{2+}-binding sites (measured with Ruthenium Red–EGTA quenching) is approx. 15nmol/mg of protein, which is 25% less than their concentration measured with EGTA alone (Figs. 5, 6 and 12). Since the slope of the plot is identical in both cases, the over-estimation of external binding in the latter case is due to an over-estimate of the total number of binding sites rather than an effect on the dissociation constant ($K_d = -1/\text{slope}$). The dissociation constant itself (approx. 140μM) was far higher in this experiment than in previous ones. The reason for this is not clear, but it may be related to the extensive depletion of endogenous bivalent cations during pretreatment of the mitochondria.

In similar experiments carried out at very low concentrations of $^{45}Ca^{2+}$, the ordinate intercepts of the Scatchard plots were identical with those of Fig. 12(*c*). In no case has there been an indication of a small number of external binding sites with a higher affinity for Ca^{2+} than those numbering about 15nmol/mg of protein. It might eventually prove possible to identify carrier-specific Ca^{2+}-binding sites by pretreating mitochondria with sufficient Ruthenium Red to inhibit transport completely, provided that Ruthenium Red does not inhibit the

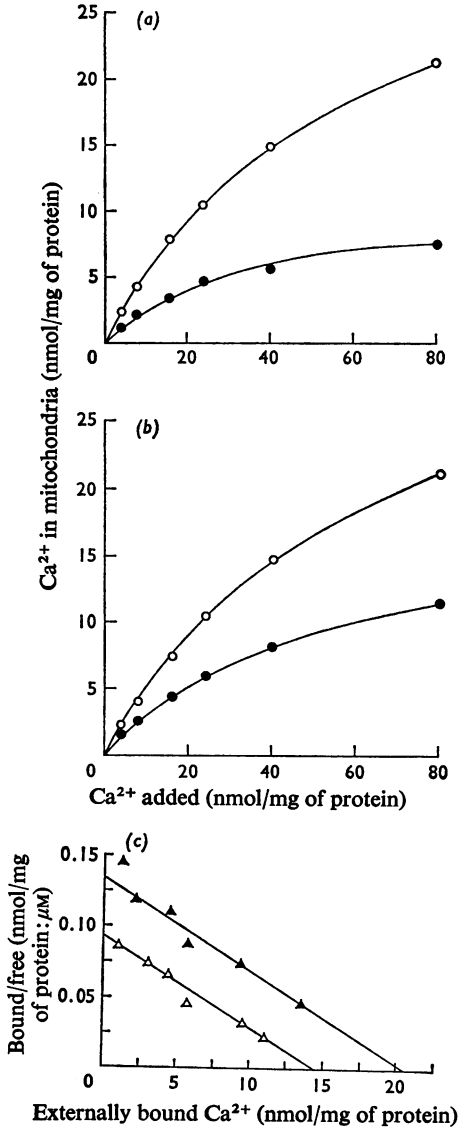


Fig. 12. *Energy-independent Ca^{2+} binding: titration of Ca^{2+}-depleted mitochondria in the presence and absence of Ruthenium Red*

Mitochondria were suspended at 2.5mg of protein/ml in 250mM-sucrose, 2mM-Hepes–Tris (pH7.2), 0.5mM-EGTA–Tris and 10μM-carbonyl cyanide *m*-chlorophenylhydrazone at 0°C. After 15min, the suspension was centrifuged (16000*g* for 5min) and the pellet was resuspended and re-centrifuged twice more in 250mM-sucrose (Chelex treated), 2mM-Hepes–Tris (pH7.2), and then finally suspended in the same medium at 5mg of protein/ml. (The mitochondria remained completely uncoupled as shown by the lack of effect of extra carbonyl cyanide *m*-chlorophenylhydrazone on succinate-supported respiration.) $^{45}Ca^{2+}$ (sp. radioactivity 0.3μCi/μmol) was added to 1ml of this suspension at the concentrations shown; 0.2ml samples were then transferred to four separate microcentrifuge tubes. Exactly 15min later, 1μM-Ruthenium Red was added to two of these tubes, followed immediately by 1mM-EGTA to one of these and to one of the remaining two tubes. All four tubes were immediately centrifuged and the supernatants were removed completely. The pellets were counted for radioactivity. (*a*) Total $^{45}Ca^{2+}$ in mitochondria before (○) and after (●) EGTA treatment. (*b*) Total $^{45}Ca^{2+}$ in mitochondria after the addition of Ruthenium Red before (○) and after (●) EGTA treatment. (*c*) Scatchard plots of external binding with (△) and without (▲) Ruthenium Red.

binding of Ca^{2+} to the carrier (as suggested by its non-competitive inhibition; Reed & Bygrave, 1974*b*).

Discussion

Mechanism of Ca^{2+} transport

The present report shows unequivocally that the interaction of Ca^{2+} with respiration-inhibited mitochondria is more complex than has been assumed previously. At least two distinct pools of mitochondrial Ca^{2+} can be distinguished. One of these does not involve reversible binding in that Ca^{2+} is not removed from it by the strong chelator EGTA. It is assumed that this represents Ca^{2+} which has been transported to the internal phase by the Ca^{2+} carrier, on the basis of its inaccessibility to EGTA, its increase with time, its sensitivity to very low concentrations of Ruthenium Red and La^{3+} in both the forward and reverse directions, and its sensitivity to carbonyl-cyanide *m*-chlorophenylhydrazone. A similar conclusion was reached by Selwyn *et al.* (1970) from their studies of Ca^{2+} transport with the more simple technique of passive swelling.

These findings are entirely consistent with the proposition that the carrier transports Ca^{2+} electrophoretically across the inner membrane in response to an electrochemical potential (negative inside) until the equilibrium distribution ratio of free Ca^{2+} appropriate to the energy status of the mitochondrion is attained. When respiration is blocked with antimycin and rotenone, this ratio favours the internal phase to the extent that net uptake occurs. The extent of uptake under these conditions, which previously has been termed 'high-affinity energy-independent binding' (Reynafarje & Lehninger, 1969), provides what is probably the most accurate assessment of the 'energy reserve' of inhibited mitochondria (see Chance *et al.*, 1969).

Mitochondria uncoupled with carbonyl cyanide *m*-chlorophenylhydrazone also can transport $^{45}Ca^{2+}$ to the internal phase in a carrier-mediated reaction. This reaction is not simply a radioisotopic exchange with internal Ca^{2+} (although this undoubtedly occurs) since the internal pool is saturated at $^{45}Ca^{2+}$ concentrations of about 20nmol/mg of protein (Fig. 12), whereas the total concentration of Ca^{2+} in mitochondria treated with carbonyl cyanide *m*-chlorophenylhydrazone is approx. 6nmol/mg of protein (Carafoli, 1967; Reynafarje & Lehninger, 1969). The pretreatment procedures of the present experiments probably decrease this still further.

Of equal significance to the proposed mechanism of the Ca^{2+} carrier (see above) is the finding that Ca^{2+} transport in inhibited and uncoupled mitochondria is reversible, as shown by the sensitivity of EGTA-induced efflux to Ruthenium Red (Figs. 11 and 12). Previous work in this laboratory (Reed, 1974) has shown a similar reversal of carrier-mediated transport in respiring mitochondria.

'High-affinity' binding

The numerous inconsistencies which arise in equating 'energy-independent high-affinity' binding of Ca^{2+} with carrier-specific binding are rationalized by the data of this report, which show it to be due to limited active transport. It is unfortunate that 'high-affinity' binding has been used so frequently as a diagnostic for carrier-specific Ca^{2+} binding.

Both theoretical considerations, based on the kinetic properties of the carrier, and experimental measurements of external binding point to the futility of attempting to measure carrier-specific binding. There seems no way of obtaining accurate data for concentrations of bound and free Ca^{2+} at the very low values required for such measurements. The binding of radioactive lanthanides (competitive inhibitors of Ca^{2+} transport; Reed & Bygrave, 1974*b*) presents fewer problems in that isotope dilution and exchange are not involved, and the carrier has an affinity two orders of magnitude higher for lanthanides than for Ca^{2+} (Reed & Bygrave, 1974*b*). However, previous attempts to measure carrier-specific binding of $^{140}La^{3+}$ (Lehninger & Carafoli, 1971) have suffered from defects similar to those discussed above (see Reed & Bygrave, 1974*a*).

'Low-affinity' binding

The second pool of mitochondrial Ca^{2+} defined in the present experiments is one which can be removed by EGTA, and is therefore a reversibly-bound pool in rapid equilibrium with free external Ca^{2+}. It is concluded that the binding sites are located on surfaces external to the inner phase, i.e. in the outer membrane and external surface of the inner membrane (Scheme 1). The linearity of Scatchard plots suggests a single class of site which binds approx. 15nmol of Ca^{2+}/mg of protein (Fig. 12) with K_d of approx. 20–25μM (Figs. 5–7). The number of external sites represents about half the total Ca^{2+} associated with respiration-inhibited or uncoupled mitochondria (see Fig. 12 and under 'Theoretical considerations'). The K_d found in the present work is considerably lower than the values previously determined for 'low-affinity' binding, a finding which can be ascribed to the precautions taken to minimize isotope dilution by endogenous Ca^{2+} in the present experiments.

The sensitivity of externally bound Ca^{2+} to Na^+ and K^+, and more particularly to nupercaine, suggests that the binding sites are the polar head-groups of membrane phospholipids (Dawson & Hauser, 1970; Papahadjopoulos, 1972; Seeman, 1972; see also Reed & Bygrave, 1974*b*). Previous studies of 'energy-independent low-affinity' binding are not strictly

comparable, but Scarpa & Azzi (1968) and Scarpa & Azzone (1969) have demonstrated qualitatively similar effects.

Perhaps the major conclusion to be drawn from the present paper is that there is little value in studying the interaction of Ca^{2+} with mitochondria by measuring total mitochondrial binding. Experimental conditions are now available, and must be used, for discriminating between internal and externally bound Ca^{2+}. It is only in this way that the processes involved in both binding and transport can be understood. In particular, the effect of potential inhibitors on both the carrier and external binding can be measured independently with the techniques described in this and previous reports (Reed, 1974; Reed & Bygrave, 1974*b*).

Further refinement depends on the ability to measure separately the bound and free internal pools, for which the best methods presently available involve the use of paramagnetic Ca^{2+} analogues, i.e. Mn^{2+} (Chappell *et al.*, 1963; Puskin & Gunter, 1973) and some lanthanides (see Williams, 1970; Reed & Bygrave, 1974*a*).

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