STUDIES ON THE TRANSPORT OF CALCIUM

BY RAT LIVER MITOCHONDRIA

by

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STATEMENT

The design, execution and interpretation of all experiments described in this thesis were my own except where specific acknowledgement of assistance is made in the text. In particular, Mr. M. McCuaig (Biochemistry, S.G.S., A.N.U.) conducted the electron microscopy, Mr. R. Ryall (Physical Biochemistry, J.C.S.M.R., A.N.U.) suggested the use of CaNTA as a suitable Ca$^{2+}$ buffer and computed the free Ca$^{2+}$ concentrations in this system, and Dr. J. Broomhead (Chemistry, S.G.S., A.N.U.) contributed the ruthenium complexes and his knowledge of their properties to the work described in the latter part of Section II.

The studies in this thesis were carried out during my employment as a Senior Demonstrator in the Department of Biochemistry, School of General Studies, Australian National University.

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PUBLICATIONS

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'Co-operative Interactions in Energy-dependent Accumulation of Ca²⁺ by Isolated Rat Liver Mitochondria'.

'Sigmoidal Kinetics Associated with Calcium Uptake and Related ATPase in Rat-liver Mitochondria'.

'An Oxygen Polarograph Designed for Undergraduate Use'.

'Binding of Calcium by Cellulose Membranes and Sephadex'.

'The Accumulation of Lanthanum by Rat Liver Mitochondria'.

'The Inhibition of Mitochondrial Calcium Transport by Lanthanides and Ruthenium Red'.

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'Kinetic Studies on the Calcium-induced ATPase of Rat Liver Mitochondria'.

'Kinetic and Binding Properties of the Mitochondrial Ca²⁺ Carrier'.
'A Kinetic Study of Mitochondrial Calcium Transport'.
Reed, K.C. & Bygrave, F.L.

'A Re-evaluation of Energy-independent Ca\textsuperscript{2+} Binding by Rat Liver Mitochondria'.
Reed, K.C. & Bygrave, F.L.

'On the Application of EGTA to \textit{in vivo} Studies of Ca\textsuperscript{2+} Transport.'
Reed, K.C. & Bygrave, F.L.

'A Low-molecular-weight Ruthenium Complex Inhibitory to Mitochondrial Ca\textsuperscript{2+} Transport'.
Reed, K.C. & Bygrave, F.L.
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SUMMARY

The interaction of Ca\textsuperscript{2+} with rat liver mitochondria has been examined by using the impermeant chelating agent ethanediolox-bis-(ethylamine)-tetraacetic acid (EGTA) to discriminate externally-bound Ca\textsuperscript{2+} from that which has been transported to the internal phase (matrix and internal surface of the inner membrane). This approach has made possible the first systematic kinetic analysis of energy-linked mitochondrial Ca\textsuperscript{2+} transport. The results are interpreted in relation to a working model for electrophoretic carrier-mediated Ca\textsuperscript{2+} transport.

The EGTA-quench technique was extended to studies of the binding of Ca\textsuperscript{2+} by respiration-inhibited mitochondria. In agreement with the model derived from kinetic studies, it was found that Ca\textsuperscript{2+} is still transported to the internal pool when mitochondria are inhibited or uncoupled. It has not been possible to demonstrate external carrier-specific Ca\textsuperscript{2+} binding.

The kinetic and binding analyses included a rigorous examination of the interaction of the specific inhibitors, La\textsuperscript{3+} and ruthenium red, with the Ca\textsuperscript{2+} carrier. In the second major section of this work, La\textsuperscript{3+} and ruthenium red were studied in their own right to more clearly identify their sites of interaction with mitochondria.

The major body of the thesis is preceded by a brief description of preliminary attempts to measure the kinetics of Ca\textsuperscript{2+} transport from which the later ideas and techniques evolved.
It would be nice to say that the concepts developed in this thesis arose directly from a recognition of the need for definitive kinetic and binding studies on the mitochondrial Ca\(^{2+}\) carrier, and that the necessary techniques and experimental approaches were then developed. Unfortunately this was not the case. The project was conceived slowly and laboriously, and its execution was worthy of an elephant (and almost as graceful); even now, it is barely in adolescence.

This chapter serves to introduce the main thesis by following the development both of my own appreciation of the mitochondrial Ca\(^{2+}\) transport system and of the concepts revealed through the work of other laboratories which have given the thesis a rational basis.

**PRELIMINARY OBSERVATIONS**

(a) The acute effects of Ca\(^{2+}\) on mitochondrial function

The studies to be described have their origin in an investigation of the effects of Ca\(^{2+}\) on the ATP-ADP exchange reaction of mitochondria (Bygrave and Leblanc, 1966). The observation had been made (Bygrave, unpublished work) that this reaction is inhibited by Ca\(^{2+}\), showing a sigmoidal dependence on its concentration (Fig. 1.9). It soon became evident that the dramatic inhibition between 50 and 150 nmoles Ca\(^{2+}\)/mg protein is paralleled by a stimulation of ATPase activity and by a loss of oxidative phosphorylation and acceptor control (Fig. 1.2; Bygrave and Reed, 1970).

The significance of the inhibitory effect of Ca\(^{2+}\) on the exchange reaction as an analytical system for studies on the mechanism of phosphorylation was justified by the results of further experiments in which the time course of response was followed. Since the above parameters altered simultaneously and thus could be assessed to be functionally related in their response to Ca\(^{2+}\), the temporal response of
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**PRELIMINARY EXPERIMENTS**

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The studies to be described had their origin in an investigation of the effects of Ca\textsuperscript{2+} on the ADP-ATP exchange reaction of mitochondria (Bygrave and Lehninger, 1966). The observation had been made (Bygrave, unpublished work) that this reaction is inhibited by Ca\textsuperscript{2+}, showing a sigmoidal dependence on its concentration (Fig. 1.1). It soon became evident that the dramatic inhibition between 50 and 150 nmoles Ca\textsuperscript{2+}/mg protein is paralleled by a stimulation of ATPase activity and by a loss of oxidative phosphorylation and acceptor control (Fig. 1.2; Bygrave and Reed, 1970).

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The reaction medium contained 80 mM-sucrose, 16 mM-tris-chloride (pH 7.4), 2 mM-(14C)-ADP (0.1 µCi), 0.2 mg mitochondrial protein and Ca^{2+} as indicated. After a 5 min preincubation, 6 mM-ATP was added and the mixtures incubated for a further 10 min. The incubation temperature was 25°C and the final volume 0.5 ml. Reactions were stopped with 0.2 ml of 2.5 M-perchloric acid, neutralized with 2.5 M-KOH and after centrifugation portions of the supernatant were assayed for inorganic phosphate or used for measurement of exchange activity. (Note: 'ngions' is used in this section synonymously with 'nmoles'.)

ATPase activity (O) was measured between 3 and 5 min after the addition of mitochondria by continuously monitoring H^{+} release in a medium containing 80 mM-sucrose, 16 mM-tris-chloride (pH 7.4), 2 mM-ADP, 6 mM-ATP and 1.15 mg mitochondrial protein in a final volume of 1.5 ml. Respiratory parameters were measured in a medium containing 80 mM-sucrose, 16 mM-tris-chloride (pH 7.4), 1 mM-phosphate, 7 mM-succinate, Ca^{2+} as indicated and 2.3 mg mitochondrial protein in a final volume of 1.5 ml at 25°C. Oxygen uptake in state 4 (■) was measured for 3 min prior to the addition of 1 mM-ADP and ^{32}P_{i} (0.25 µCi); following this addition oxygen uptake in state 3 (□) was measured for 2 min and an aliquot of the medium removed into perchloric acid for assay of esterified ^{32}P_{i}. Acceptor control ratios (▲) are the ratios of the state 3 to state 4 rates of oxygen uptake. The P/O ratios (△) were calculated from the total oxygen uptake from the time of the combined addition of ADP and ^{32}P_{i} until the reaction was stopped in perchloric acid.
any one of these should suffice to define that of all others. The parameter chosen for these studies was ATPase activity since it could be continuously recorded by measuring the ejection of protons with a glass electrode.

In these experiments, the earlier finding of Saris (1963) was 'rediscovered': the response of ATPase activity to $\text{Ca}^{2+}$ is triphasic (Fig. 1.3; Bygrave et al., 1971b). The apparently sigmoidal relation to $\text{Ca}^{2+}$ concentration (Figs. 1.1 and 1.2) is a consequence of the triphasic time course since it follows the same pattern at all concentrations (Fig. 1.4); in the earlier experiments (Figs. 1.1 and 1.2), ATPase activity had been measured over a set time interval.

Before discussing the effects of $\text{Ca}^{2+}$ concentration in more detail, additional features of the triphasic response should be considered. The initial rapid phase is associated with the active uptake of $\text{Ca}^{2+}$ by the mitochondria (Fig. 1.5; Saris, 1963; Bielawski and Lehninger, 1966); during the second 'resting' phase the $\text{Ca}^{2+}$ is maintained in the mitochondria, and in the final phase it is released concomitantly with the irreversible stimulation of ATPase (Fig. 1.5; Saris, 1963) and the loss of phosphorylation capacity. These movements of $\text{Ca}^{2+}$ are paralleled by those of phosphate hydrolysed from the $\gamma$-position of ATP (Fig. 1.6; cf. Bielawski and Lehninger, 1966). These experiments utilized ($\gamma$-32p)-ATP to enable the accurate measurement of both the distribution of inorganic phosphate arising from ATP hydrolysis and its stoichiometry to the amount of $\text{Ca}^{2+}$ accumulated. In agreement with previous work (Bielawski and Lehninger, 1966) it was found that the molar ratio ($\text{Ca}^{2+}$ accumulated/ATP hydrolysed) is $2.0 \pm 0.05$ while that of ($\text{Ca}^{2+}$ accumulated/phosphate accumulated) is 1.67. This latter ratio remains remarkably constant in spite of the slow but finite hydrolysis of ATP occurring during the second phase (Fig. 1.6).

A rationale for all of the third phase effects of $\text{Ca}^{2+}$ is provided by the observation that they are associated with large-amplitude mitochondrial swelling, measured in Fig. 1.7 on the basis of decreased absorbance at 520 nm (an estimate of light-scattering) and more directly in Fig. 1.8
Fig. 1.3. Influence of Ca\(^{2+}\) on ATPase activity: time course of phosphate and H\(^+\) release

The incubation contained 80 mM-sucrose, 16 mM-tris-chloride (pH 7.4), 6 mM-ATP and 30 mg mitochondrial protein in a total volume of 24 ml. At the time shown 5 µmoles Ca\(^{2+}\) was added. Proton release was continuously recorded; 1 ml aliquots of the solution were periodically removed into 0.4 ml of 2 M-HClO\(_4\) and subsequently assayed for inorganic phosphate. Incubation temperature was 26\(^\circ\)C.
Fig. 1.4. Influence of $\text{Ca}^{2+}$ on ATPase activity: concentration dependence of time course

$\text{Ca}^{2+}$ was added as shown (nmoles) to the standard medium (sucrose-tris-ATP as for Fig. 1.3) containing 1.3 mg mitochondrial protein in a total volume of 1.5 ml and the release of protons continuously recorded. Temperature 25°C.

Fig. 1.5. Uptake and release of $\text{Ca}^{2+}$ in ATP-supported system

To the standard incubation medium containing 3.7 mg mitochondrial protein was added 0.6 µmole $^{45}\text{Ca}^{2+}$ (0.1 µCi). 0.1 ml aliquots were filtered at the times shown and the filters counted for radioactivity. Proton release was continuously recorded. Both curves are the mean of two separate experiments. Total volume was 3 ml and temperature 25°C.
Fig. 1.6. Release and localization of phosphate during ATP-supported Ca\(^{2+}\) uptake

1.5 \(\mu\)mole Ca\(^{2+}\) was added (arrow) to the standard medium containing 20 mg mitochondrial protein and 0.4 \(\mu\)Ci (\(\gamma^{-32P}\))-ATP in a total volume of 4 ml at 25\(^\circ\)C. At the times shown, 0.2 ml aliquots were centrifuged (Eppendorf microcentrifuge: 2 min) and 0.1 ml of supernatant added to tubes containing 0.5 ml TCA (6 per cent w/v) and 50 mg charcoal. After standing on ice for 30 min the tubes were centrifuged and 0.25 ml of supernatant counted for radioactivity ((b)\(\bigcirc\)).

The mitochondrial pellets were rinsed, the tubes swabbed dry and the pellets suspended in 0.7 ml \(H_2O\). Protein was precipitated with 0.5 ml of 12 per cent TCA (w/v) and the tubes centrifuged. 0.25 ml aliquots of supernatant were counted for radioactivity ((a)\(\bigcirc\)) and to the remainder was added 100 mg charcoal. After standing on ice for 30 min the tubes were again centrifuged and 0.25 ml aliquots of supernatant counted ((a)\(\bullet\)). The \(32^P\)phosphate content of the mitochondria is not corrected for the sucrose-accessible space (see Fig. 1.8).

The upper curves in (b)\(\bullet\) were obtained by summing the mitochondrial \(\text{total}^{32}P_{i}\) and the supernatant \(\text{super}^{32}P_{i}\).
Fig. 1.7. Mitochondrial swelling following ATP-supported Ca\textsuperscript{2+} uptake: light scattering

1.2 \textmu mole Ca\textsuperscript{2+} was added to the standard medium containing 11.1 mg mitochondrial protein in a total volume of 9 ml. Fifteen sec after Ca\textsuperscript{2+} addition, stirring was discontinued and 3 ml was transferred to a cuvette and E\textsubscript{520} continuously recorded. Proton release was continuously monitored in the remaining solution. Temperature 25\textdegree{}C.
Fig. 1.8. Mitochondrial swelling following ATP-supported Ca\textsuperscript{2+} uptake: H\textsubscript{2}O spaces

Three simultaneous incubations were prepared, each containing the standard medium and 68 mg mitochondrial protein in a total volume of 6 ml. Temperature 25°C. They also included respectively 0.35 µCi (γ-\textsuperscript{32}P)-ATP, 7.5 µCi (\textsuperscript{3}H)-H\textsubscript{2}O, or 7.5 µCi (\textsuperscript{3}H)-H\textsubscript{2}O plus 3 µCi (\textsuperscript{14}C)-sucrose. 12 µmole Ca\textsuperscript{2+} was added to each at the time shown (arrow). 0.5 ml aliquots were periodically removed from the latter two incubations and centrifuged and the washed pellets were counted for \textsuperscript{3}H, or both \textsuperscript{3}H and \textsuperscript{14}C. Simultaneously, 0.2 ml aliquots were removed from the first incubation into 1 ml of 6 per cent TCA (w/v) containing 100 mg charcoal and \textsuperscript{32}P\textsubscript{i} estimated as previously described (Δ).

The volumes are total mitochondrial water (\textsuperscript{3}H)-H\textsubscript{2}O, mean of duplicates: ● ) and the sucrose-inaccessible space (\textsuperscript{3}H)-H\textsubscript{2}O minus (\textsuperscript{14}C)-sucrose: ○ ).
by the increase in mitochondrial water. It is interesting that the sucrose (as (14C)-sucrose) accessible space is identical to the total water space in the swollen state (Fig. 1.8), indicating that the inner membrane is permeable to sucrose in Ca2+-swollen mitochondria. Additional experiments showed that (14C)-inulin also can penetrate the inner membrane in this third phase.

Electron micrographs of samples taken at two min intervals over the entire triphasic response period (data not included) have shown that the mitochondria undergo the transition in conformational states 'condensed (phase 1) + twisted (phase 2) + orthodox (phase 2-3) + swollen, broken outer membrane (phase 3)' typical of the swelling response induced by a variety of agents (Myron and Connelly, 1971).

On the basis of the above observations it is reasonable to propose that the prime effect of Ca2+ in the third phase is to induce large-amplitude mitochondrial swelling. The inner (selectively permeable) membrane consequently becomes freely permeable to low molecular weight solutes including sucrose, phosphate, Ca2+, K+, Mg2+ (Lee et al., 1971a,b) and most significantly, H+. The loss of internal 'proton pressure' is then reflected in the loss of activities associated with coupled phosphorylation, comparable to the similar effect of classical uncouplers.

Of course this does not assist in establishing the mechanism of Ca2+-induced swelling, but it does clarify the interpretation of a wealth of previous data related to the uncoupling and swelling effects of Ca2+. It is beyond the scope of this thesis to discuss these effects in more detail. Nevertheless, recent work on the stabilization of mitochondrial structure by adenine nucleotides (Leblanc et al., 1970; Out et al., 1972; Klingenberg et al., 1971a; Stoner and Sirak, 1973a,b) and Mg2+ (Lee et al., 1971a,b; Binet et al., 1971; Binet and Volfin, 1971; Razin, 1972) offers exciting prospects for a clearer general understanding of cooperative 'subunit' interactions in membranes, and specifically of the protection afforded to mitochondria by these agents against the deleterious effects of Ca2+ under 'massive loading' conditions (Drahota et al., 1965).
(b) **The effect of Ca\textsuperscript{2+} concentration on the rate of ATPase activity**

The foregoing experiments, together with many others aimed at clarifying the mechanism of Ca\textsuperscript{2+}-induced swelling, led to a detailed examination of the effect of Ca\textsuperscript{2+} concentration on the three phases of the ATPase response.

The maximal rate of proton release during phase 3 ATPase activity shows the saturation behaviour typical of an enzyme-catalysed reaction (Fig. 1.9) with a maximum velocity of about 200 nequivs H\textsuperscript{+} released/min.mg protein and an apparent Km for Ca\textsuperscript{2+} (total) of about 100 µM, which is equivalent to a value for Ca\textsuperscript{2+} (free) of about 0.9 µM (Appendix). The lag time during the second (resting) phase is decreased with increasing Ca\textsuperscript{2+} concentration (Fig. 1.9) and is half-maximal at a concentration identical with the Km for phase 3 ATPase. It is probable that the third phase activity is dependent on the prior accumulation of at least a small amount of Ca\textsuperscript{2+} (cf. Chappell and Crofts, 1965) and it may thus be dependent on the internal concentration; it is measured here only as a function of external Ca\textsuperscript{2+}.

A further point of interest in these curves is the finding that the third phase of ATPase activity still occurs in the absence of exogenous Ca\textsuperscript{2+} (Fig. 1.9) and is in no way distinguishable in nature from that seen in its presence. The curve describing the concentration dependence of third phase ATPase extrapolates to (-)10 to 15 nmoles Ca\textsuperscript{2+}/mg protein, equivalent to the endogenous Ca\textsuperscript{2+} concentration in isolated rat liver mitochondria (Lehninger *et al*., 1967, after Thiers and Vallee, 1957). It is therefore suggested that 'spontaneous' mitochondrial swelling is identical to that induced by (the accumulation of) exogenous Ca\textsuperscript{2+}, being due to endogenous Ca\textsuperscript{2+}. It will be recalled further that isolation of rat liver mitochondria in a medium supplemented with EDTA both decreases their endogenous Ca\textsuperscript{2+} level and increases their resistance to 'ageing'.

Examination of the phase 1 activity showed that the response of the initial rate of proton release to Ca\textsuperscript{2+} is sigmoidal (Fig. 1.10a). The maximal activity is 280
Proton release was continuously monitored following the addition of Ca\(^{2+}\) (as shown) to the standard medium containing 1.31 mg mitochondrial protein in a total volume of 1.5 ml. Temperature 25°C. The lag time is measured from the time of Ca\(^{2+}\) addition to the extrapolated linear portions of phases 1 and 2.
Fig. 1.10. Concentration dependence of Ca$^{2+}$-induced ATPase: rate of phase 1 proton ejection

Proton release was continuously monitored before and immediately following the addition of Ca$^{2+}$ (as shown) to the standard medium containing 1.0 mg mitochondrial protein in 1.5 ml. The recorder chart speed was 80 mm/min; rates of proton release are corrected for the rate prior to Ca$^{2+}$ addition. (A) proton release vs [Ca$^{2+}$]; (B) double reciprocal plot of (A); (C) Hill plot of (A) using $V_{max}$ from (B). Temperature 25°C.
nequivs $H^+$ released/min.mg protein (Fig. 1.10b) and the $K_m$ about 650 $\mu$M total or about 6 $\mu$M free $Ca^{2+}$; the Hill coefficient, an index of the sigmoidicity, is 2.25. In view of the potential implications of the sigmoidal plot for the mechanism of $Ca^{2+}$ transport, the parameters involved in this initial response were studied more extensively.

The release of protons during ATP-supported $Ca^{2+}$ uptake is a consequence not only of ATP hydrolysis (cf. phase 3 ATPase) but also of the uptake process itself, in which protons are ejected stoichiometrically with the $Ca^{2+}$ accumulated (Saris, 1963; Chance, 1965; Gear et al., 1967). The stoichiometry of this ejection varies considerably with the experimental conditions, and is further complicated in the present experiments by the retention of inorganic phosphate resulting from ATP hydrolysis during $Ca^{2+}$ transport. The ATPase activity and $Ca^{2+}$ uptake itself were therefore measured directly to more clearly define the origin of the sigmoidal relationship.

The initial rate of ATP hydrolysis was estimated by measuring the release of $^{32}P_i$ from $\gamma^{32}$P)-ATP in a 10 sec period immediately following the addition of $Ca^{2+}$. The results of a number of experiments similar to that of Fig. 1.11 indicate that the maximal rate of ATP hydrolysis is approximately 45 nmoles/10 sec.mg protein, equivalent to 270 nmoles/min.mg protein. At saturating concentrations of $Ca^{2+}$, there is thus a 1:1 stoichiometry between the rates of ATP hydrolysis and $H^+$ ejection (cf. Fig. 1.10b). In all experiments of this type, a sigmoidal relationship was again found between the initial rate of ATPase and $Ca^{2+}$ concentration. The mean Hill coefficient was 1.3, considerably less than that associated with the effect of $Ca^{2+}$ on the rate of $H^+$ ejection (cf. above), while the $K_m$ was approximately 800 $\mu$M total or about 8 $\mu$M free $Ca^{2+}$.

It seems clear that the $Ca^{2+}$-induced ATPase activity associated with $Ca^{2+}$ transport shows a sigmoidal dependence on $Ca^{2+}$ concentration, but is this an intrinsic property of the $Ca^{2+}$ transport system or solely of the ATPase?
Fig. 1.11. Concentration dependence of Ca\(^{2+}\)-induced ATPase: initial rate of (γ-\(^{32}\)P)-ATP hydrolysis

Ca\(^{2+}\) was added as shown (after a 1 min preincubation) to the standard medium containing 0.12 µCi (γ-\(^{32}\)P)-ATP, 0.5 µM-rotenone, 0.5 µM-antimycin and 3.4 mg mitochondrial protein in a total volume of 0.5 ml. Temperature 25°C. Following 10 sec incubation, 0.5 ml of cold 10 per cent TCA (w/v) was added and the quenched mixture transferred to a centrifuge tube containing 100 mg charcoal. After standing on ice for 60 min, the tubes were centrifuged and 0.5 ml aliquots of supernatant counted for radioactivity.

Fig. 1.12. ATP-supported Ca\(^{2+}\) uptake: time course

0.3 µmole \(^{45}\)Ca\(^{2+}\) (0.05 µCi) was added to the standard medium containing 1.85 mg mitochondrial protein in a total volume of 1.5 ml. Temperature 25°C. At 15 sec intervals 0.1 ml aliquots were filtered and the filters counted. Each point is the mean value of three separate experiments; the range of individual values was less than the diameter of these points. Fig. (B) is a first order (irreversible) plot of the data of (A).
answer to the latter question is indicated to be negative by the data of Drahota *et al.* (1965) and Chance (1965) which showed that the rate of State 4 respiration is not stimulated by concentrations of Ca$^{2+}$ below about 10 µM, suggesting that the sigmoidal response of the 'energy system' to Ca$^{2+}$ addition is independent of the energy source (ATP or respiratory substrate). The former question is considered in the following section.

(c) **The effect of Ca$^{2+}$ concentration on the rate of Ca$^{2+}$ transport**

The rate of Ca$^{2+}$ transport was measured by Millipore filtration of mitochondria which had been incubated with $^{45}$Ca$^{2+}$. Fig. 1.12 shows that the transport reaction follows irreversible first order kinetics with a half-time of about 30 sec. The initial rate of Ca$^{2+}$ uptake was measured in subsequent experiments by filtration at 10 sec, the shortest manageable period.

The dependence of the initial rate of Ca$^{2+}$ uptake on Ca$^{2+}$ concentration is shown in Fig. 1.13. The most interesting feature is again the sigmoidal relationship (10 sec data), indicating that the similar effect of Ca$^{2+}$ concentration on ATPase activity may be a reflection of a fundamental property of the Ca$^{2+}$ transport system itself (i.e. the Ca$^{2+}$ carrier: see below). The Hill coefficient of the initial rate curve of Fig. 1.13 is 1.34, although in a large number of experiments it varied between 1.3 and 1.7. The $K_m$ value was always of the order of 2-3 µM (free) Ca$^{2+}$.

Care was taken to ensure that the sigmoidal curve was not an artifact of the experimental conditions. In the first place, it is observed only when initial rates are measured (Fig. 1.13) so it is not a function of the filtration procedure. The possibilities of dilution by endogenous Ca$^{2+}$ or an isotopic exchange reaction were eliminated by measuring both $^{45}$Ca$^{2+}$ and total Ca$^{2+}$ remaining in the filtrate after a 10 sec incubation (Fig. 1.14). The measurements of total Ca$^{2+}$ were made by atomic absorption spectroscopy using an extremely sensitive instrument designed and built by Dr. J. David of CSIRO Soil Research, Canberra; although the
Fig. 1.13. ATP-supported Ca$^{2+}$ uptake: concentration dependence of initial rate

45Ca$^{2+}$ (sp.act. 0.4 µCi/µmole) was added as shown to the standard medium containing 1.0 mg mitochondrial protein in a total volume of 0.5 ml. Temperature 25°C. 0.1 ml samples were filtered 10 sec (O), 1 min (●) and 4 min (▲) after the addition of Ca$^{2+}$ and the filters counted for radioactivity. (A) uptake vs [Ca$^{2+}$]; (B) double reciprocal plot of 10 sec data of (A); (C) Hill plot of 10 sec data of (A) using $V_{\text{max}}$ from (B).
Fig. 1.14. ATP-supported Ca\textsuperscript{2+} uptake: concentration dependence of initial rate measured by atomic absorption and radioassay

Incubations contained 2.4 mg mitochondrial protein in the standard medium in a total volume of 2 ml. Temperature 25°C. Exactly 10 sec after the addition of \(^{45}\text{Ca}^{2+}\) (sp.act. 0.5 uCi/µmole), the incubation medium was expelled onto a Millipore filtration unit (filtration was virtually complete within 5 sec but to ensure uniformity the vacuum was maintained for exactly 10 sec) and the filtrate collected in small tubes. 50 µl was counted for radioactivity and the remainder used for atomic absorption analysis of total Ca\textsuperscript{2+}. 
Ca\(^{2+}\) concentration decreased only by about 10% during the 10 sec incubations it could nevertheless be measured with high accuracy.

Similar data to those of Fig. 1.13 have been obtained in experiments in which succinate oxidation provided the energy source for Ca\(^{2+}\) uptake, ATP hydrolysis being prevented by the inclusion of oligomycin (Bygrave et al., 1971a,b; Spencer and Bygrave, 1973).

This leads to a more detailed consideration of the chelating role of ATP in these experiments. The estimation of free Ca\(^{2+}\) concentration cited above is based on calculations using the stability constants of Ca\(^{2+}/ATP\) complexes and the acid dissociation constants of ATP (detailed calculations and results are included in the Appendix). From these data it is apparent firstly that the sigmoidal relationship with which we have been concerned is not due to a similar relationship between total and free Ca\(^{2+}\) concentrations, and secondly that the amount of Ca\(^{2+}\) accumulated within 10 sec at 25\(^\circ\)C greatly exceeds the initial free Ca\(^{2+}\) concentration. However, the fact that the Ca\(^{2+}/ATP\) system acts as a 'buffer', as shown by both the calculations and the first order kinetics, indicates that it is reasonably valid to treat the data in terms of initial rates.

The maximum velocity of Ca\(^{2+}\) transport (Fig. 1.13b) has not been discussed prior to this point because the value determined (80 nmoles Ca\(^{2+}/10\) sec.mg protein) includes not only the transported Ca\(^{2+}\) but also a fraction (of the order of 30 nmoles/mg protein) which is bound externally (see later). Correction for this background gives a value for V\(_{\text{max}}\) of about 300 nmoles Ca\(^{2+}/\)min.mg protein, comparable to the maximum velocity of ATP hydrolysis and H\(^+\) ejection. This raises the intriguing possibility that in the early stages of Ca\(^{2+}\) uptake, the molar stoichiometry of (Ca\(^{2+}\) accumulated: ATP hydrolysed) is 1:1 rather than 2:1 as is the case when uptake is complete.

The extremely high activity of the Ca\(^{2+}\) transport system at 25\(^\circ\)C prevents kinetic analyses in the absence of a Ca\(^{2+}\) buffer, since uptake greatly exceeds the free Ca\(^{2+}\) concentration within the 10 sec incubations. It was thought desirable to generate experimental conditions such that the rate of transport was sufficiently low that the
substrate-velocity relationship could be determined in the absence of a strong buffer. The first approach was simply to lower the incubation temperature to 3°C. A sigmoidal curve was again obtained, with a Hill coefficient of 1.4 (Bygrave et al., 1971a).

The second approach made use of Mela's finding (Mela, 1969a) that La$^{3+}$ inhibits the rate of Ca$^{2+}$ uptake. As in the low temperature experiments, ATP was omitted and succinate oxidation provided the energy source. The initial rate of Ca$^{2+}$ transport (10 sec incubation) was measured at 14 Ca$^{2+}$ concentrations both in the absence and in the presence of La$^{3+}$ at six different concentrations from 0.2 to 10 µM (Fig. 1.15). Some of the data has been omitted for the sake of clarity. As the concentration of La$^{3+}$ was increased, i.e. as the measurements more nearly approximated initial rates, the plots became more sigmoidal (Fig. 1.15a). The Hill coefficient extrapolated to 1.9-2.0 at infinite La$^{3+}$ concentration (Fig. 1.15c), suggesting this to be its actual value. The maximum velocity was approximately 100 nmoles Ca$^{2+}$ accumulated/10 sec.mg protein.

The inhibition by La$^{3+}$ was found to be competitive with respect to Ca$^{2+}$ (Fig. 1.15b) with a $K_i$ of the order of 0.67 µM (obtained from Dixon plots; Dixon, 1953). However, this value should not be taken as definitive since the data are not initial rate measurements; furthermore, La$^{3+}$ is itself accumulated by mitochondria (Reed and Bygrave, 1973a) so that its concentration decreased during the course of the preincubation.

The preceding data have established an important feature of mitochondrial Ca$^{2+}$ transport in the existence of a sigmoidal relationship between Ca$^{2+}$ concentration and its rate of transport. The techniques used are nevertheless too crude to permit a definitive analysis of the kinetic properties of this system. The problems inherent in such measurements and their solution are considered in the following chapter, but before discussing these the mitochondrial Ca$^{2+}$ transport system should be more clearly defined.
Incubations contained 250 mM-sucrose, 2 mM-succinate, 2 mM-tris-chloride (pH 7.4) and 0.7 mg mitochondrial protein in a total volume of 0.5 ml. Temperature 25°C. Mitochondria were preincubated with La³⁺ (as shown) for 1 min prior to the addition of 45Ca²⁺ (sp.act. 0.05 µCi/µmole); 0.1 ml aliquots were filtered 10 sec later and counted for radioactivity.

(a) Ca²⁺ uptake vs [Ca²⁺] at varying La³⁺ concentrations.
(b) Double reciprocal plots of (a).
(c) Variation of the Hill coefficient (n) with La³⁺ concentration.

Fig. 1.15. Succinate-supported Ca²⁺ uptake: concentration dependence of initial rate at varying La³⁺ concentrations.
THE INTERACTION OF Ca\textsuperscript{2+} WITH RAT LIVER MITOCHONDRIA

(a) Evidence for the existence of a specific carrier

Early observations on the gross effects of Ca\textsuperscript{2+} on mitochondrial function (Lehninger, 1949; Siekevitz and Potter, 1955; Slater and Cleland, 1953) were succeeded by a decade of detailed investigations of the factors involved in both the accumulation of Ca\textsuperscript{2+} and its subsequent uncoupling effect. The work of this period is too voluminous to be reviewed here and is not entirely relevant to the work of this thesis. However, an excellent review has been provided by Lehninger et al (1967).

Towards the close of the previous decade it became evident that the energy-linked accumulation of Ca\textsuperscript{2+} by mitochondria may be mediated by a specific membrane-associated carrier. The earliest evidence for this is inherent in the fact that the accumulation of Ca\textsuperscript{2+} is an energy-requiring process, deriving its energy either from substrate oxidation (oligomycin-insensitive) or from ATP hydrolysis (oligomycin-sensitive). It is difficult to envisage an active transport process which is carrier-independent.

Nevertheless, examples of such a process are provided by the accumulation of lipid-soluble cations by mitochondria (Skulachev et al, 1969a,b) and of lipid-soluble anions by sonicated submitochondrial particles (Liberman et al, 1969; Montal et al, 1970), but Ca\textsuperscript{2+} could not be similarly accumulated by electrophoretic diffusion through the bulk lipid phase of the mitochondrial membrane because of its inability to penetrate lipid bilayers (cf. Mueller et al, 1964).

Direct evidence along these lines lies in the species distribution of the ability of mitochondria to transport Ca\textsuperscript{2+}. To date, mitochondria isolated from all tissues and organisms have been found to have the capacity for Ca\textsuperscript{2+} transport with the exception of two strains of yeast, *Saccharomyces cerevisiae* and *Torulopsis utilis* (Carafoli and Lehninger, 1971; Carafoli et al, 1970; later work by Balcavage et al (1973) has shown that mitochondria from *S. cerevisiae* and *Candida utilis* are able to transport Ca\textsuperscript{2+}.
slowly, but the process has a low affinity for Ca\(^{2+}\) (K\(_m\) \approx 3 \text{ mM})). It seems then that the mitochondrial membrane itself is impermeable to Ca\(^{2+}\), and that an essential component of the Ca\(^{2+}\) transport system (not concerned with energy transduction) is genetically determined.

Blowfly flight muscle mitochondria provide another interesting exception to the otherwise uniform occurrence of energy-linked Ca\(^{2+}\) transport in that they can accumulate significant amounts of Ca\(^{2+}\) only in the presence of inorganic phosphate (Carafoli et al, 1971); in this case, Ca\(^{2+}\) transport may not be directly linked to the energy-transducing system but may occur rather in response to the active accumulation of phosphate (Hansford and Chappell, 1968), in contrast to other species where the converse situation obtains. Nevertheless, such Ca\(^{2+}\) transport is still sensitive to ruthenium red (Carafoli and Sacktor, 1972). Ca\(^{2+}\) transport per se is thus not obligatorily coupled to the reactions of oxidative phosphorylation, suggesting that it is mediated by a specific carrier which is independent of the immediate reactions of the energy-transducing system.

The data of Selwyn et al (1970) verify this conclusion directly for rat liver mitochondria. These workers have shown that in the presence of rotenone and antimycin, rat liver mitochondria swell when suspended in an isotonic solution of a Ca\(^{2+}\) salt, provided that the anion also is permeant (SCN\(^-\) or acetate + CCCP; Chappell and Haarhoff, 1967; Mitchell and Moyle, 1969a,b). The swelling, which is assumed to be a simple osmotic response to the superposition of the external salt concentration on the pre-existent mitochondrial osmolarity, is inhibited by low concentrations of Pr\(^{3+}\) implying that Ca\(^{2+}\) transport is mediated by the lanthanide-sensitive component discussed below.

Further direct evidence for the involvement of an ion-selective carrier is provided by the specificity of mitochondrial cation transport. Rat liver mitochondria can transport Ca\(^{2+}\), Sr\(^{2+}\), Mn\(^{2+}\) and Ba\(^{2+}\), the rate decreasing in that order (Vainio et al, 1970). Each of these ions inhibits the transport of the others, with the exception of Mn\(^{2+}\) whose transport is stimulated by Ca\(^{2+}\) (Chance and Mela, 1966a;
Mela and Chance, 1968; Vinogradov and Scarpa, 1973). The clear implication is that the ions interact at a common site which is an essential component of their transport. Furthermore, the smaller cation Mg$^{2+}$ is not transported by rat liver mitochondria (in contrast to heart mitochondria) although it does inhibit the transport of the other cations ($K_i \approx 2.5$ mM (Ba$^{2+}$); 5 mM (Ca$^{2+}$ and Sr$^{2+}$); 15 mM (Mn$^{2+}$); Vainio et al., 1970).

Inhibition of cation transport with far higher affinity is provided by the rare earth metals, the 15 trivalent cations between La$^{3+}$ and Lu$^{3+}$. The concentration required for 50% inhibition of Ca$^{2+}$ transport is less than 0.1 $\mu$M (Mela, 1969a; Vainio et al., 1970). Similar concentrations of lanthanides are effective in blocking cytochrome and bromothymol blue responses to Ca$^{2+}$ addition (Mela, 1967, 1968a,b). It has been concluded that the lanthanides inhibit Ca$^{2+}$ transport and the associated responses by binding to a Ca$^{2+}$-specific carrier with an affinity sufficiently high to enable a stoichiometric titration of the carrier (Mela and Chance, 1969). Ruthenium red also has been found to specifically inhibit Ca$^{2+}$ transport at relatively low concentrations (Moore, 1971; Vasington et al., 1972a,b), but the kinetic studies essential to the interpretation of its mode of action have not previously been undertaken.

A full discussion of the interaction of the lanthanides and ruthenium red with mitochondria has been deferred to later chapters, but it is sufficient at this stage to note that the effects of these highly specific and potent inhibitors constitute strong evidence for a Ca$^{2+}$ carrier.

A final piece of evidence for a mitochondrial Ca$^{2+}$ carrier is provided by the apparently high affinity of the transport process for Ca$^{2+}$. Measurements of the concentration dependence of respiratory stimulation by Ca$^{2+}$ (Chance, 1965; Chance and Schoener, 1966; Carafoli and Azzi, 1972) or of ATP hydrolysis (preceding data) associated with active Ca$^{2+}$ transport, and of the transport of Ca$^{2+}$ itself (preceding data; Vinogradov and Scarpa, 1973) indicate firstly that the transport process is saturable and secondly that half-maximal response occurs at $\mu$M concentrations of Ca$^{2+}$.
implying the involvement of a component with very high affinity for Ca\(^{2+}\) (for more detailed discussion, refer to later chapters). It must be stressed that estimates of the 'affinity' of the carrier system for Ca\(^{2+}\) can be derived only from kinetic and binding measurements. The final concentration of Ca\(^{2+}\) remaining outside the mitochondria at the cessation of active transport (<1 \(\mu\)M under optimal conditions: Drahota et al., 1965; Lehninger, 1970) is determined solely by the equilibrium distribution of Ca\(^{2+}\) across the membrane (which may be related to the rates of influx and efflux, or to the electrochemical potential across the membrane).

According to the criteria of substrate saturability, high affinity, high specificity, specific inhibition, genetic determination, and respiration-independent transport, it must be concluded that the transport of Ca\(^{2+}\) is mediated by a specific carrier, situated in the inner mitochondrial membrane.

Recent studies on the 'energy-independent' binding of Ca\(^{2+}\) to rat liver mitochondria (Lehninger et al., 1969; Reynafarje and Lehninger, 1969; Carafoli and Lehninger, 1971) have been cited frequently as providing further evidence for a carrier, since such studies indicate the presence of a class of high affinity binding sites (which have been equated with carrier binding sites) with properties similar to those of the transport process itself. For a number of reasons (see later) we have concluded that the criteria used in such experiments for defining binding are insufficient to permit measurement of carrier-specific binding. The results of such experiments must therefore be ignored in terms of providing evidence for a specific carrier. The high affinity Ca\(^{2+}\) binding protein in water extracts of rat liver mitochondria (Lehninger, 1971a,b; Gomez-Puyou et al., 1972) similarly constitutes no evidence for a carrier since it has not been shown to bear any relation to Ca\(^{2+}\) transport. On the contrary, the mitochondrial vesicles remaining after aqueous extraction still exhibit energy-linked Ca\(^{2+}\) transport (Vasington and Greenawalt, 1964; Greenawalt et al., 1965).
(b) The 'driving force' for active Ca\(^{2+}\) transport

The question of the basic driving force for mitochondrial Ca\(^{2+}\) transport is related closely to the mechanism of energy transduction in oxidative phosphorylation, a question which remains incompletely resolved. Mitochondria are capable of carrying out all of the reversible transductions possible between three equivalent energy forms represented by phosphate group transfer, redox, and electrochemical energy (Mueller and Rudin, 1969). Specifically, active Ca\(^{2+}\) transport may be supported by any one of these forms as has been established in experimental systems using ATP hydrolysis (Bielawski and Lehninger, 1966), substrate oxidation (Chance, 1965; Rossi and Lehninger, 1964), and the valinomycin-induced efflux of K\(^+\) in respiration-inhibited mitochondria (Rossi et al, 1967; Scarpa and Azzone, 1970) to support Ca\(^{2+}\) uptake.

The inter-convertibility of these three forms of energy necessitates the existence of a common intermediate energy form. Greville (1969) has summarized the three major hypotheses as to the nature of this intermediate in the following way:

(A) (Mitchell, 1968)

\[
\text{Respiration} \rightleftharpoons \text{H}^+ \text{ translocation} \rightleftharpoons \text{ATP synthesis} \rightleftharpoons \text{cation translocation}
\]

(B) (Chappell and Crofts, 1966)

\[
\text{Respiration} \rightleftharpoons X \rightleftharpoons \text{I} \rightleftharpoons \text{ATP synthesis} \downarrow (\text{proton pump}) \\
\text{H}^+ \text{ translocation} \rightleftharpoons \text{cation translocation}
\]

(C) (Slater, 1967)

\[
\text{Respiration} \rightleftharpoons X \rightleftharpoons \text{I} \rightleftharpoons \text{ATP synthesis} \downarrow (\text{cation pump}) \\
\text{cation translocation} \rightleftharpoons \text{H}^+ \text{ translocation}
\]
In relation to the immediate driving force for active cation transport, schemes (A) and (B) are similar in that both propose such transport to occur electrophoretically\(^1\) in response to an electrochemical gradient established by the outward (electrogenic) translocation of protons. On the other hand, scheme (C) requires a primary cation pump (electrogenic) driven directly by a high-energy intermediate (Rasmussen et al., 1965). A pre-existent membrane potential is not required by this scheme, and in fact is established by cation transport (positive inside). The available evidence suggests that scheme (C) is not tenable, and that \(\text{Ca}^{2+}\) transport in mitochondria is an electrophoretic process mediated by a carrier.

The arguments supporting an electrochemical gradient across the mitochondrial inner membrane (negative inside) have been detailed by Greville (1969), Chance and Montal (1971) and Skulachev (1971). The major points can be summarized as follows:

(i) mitochondria are able to transport a number of synthetic lipid-soluble cations\(^2\) into the osmotically active space against a large concentration gradient, accompanied by changes in mitochondrial function similar to those induced by the naturally-occurring metal cations, \(\text{Ca}^{2+}\) and \(\text{K}^{+}\) (+ valinomycin) (Skulachev et al., 1969a, b; Grinius et al., 1970; Bakeeva et al., 1970; Liberman et al., 1969; Montal et al., 1970). The postulate of a specific selective pump for each cation (scheme (C)) is impossible to reconcile with the transport of synthetic cations.

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\(^1\) The term 'electrophoretic transport' is used to denote ion transport driven by an electrochemical gradient (Pressman, 1970; Chance and Montal, 1971). 'Electrogenic transport' refers to active ion pumping which results in the establishment of such a gradient (Pressman, 1970).

\(^2\) The cations which have been studied are \(\text{DDA}^+\) (N,N-dibenzyl-N,N-dimethyl ammonium cation); \(\text{TPMP}^+\) (triphenyl methyl phosphonium cation); \(\text{TBA}^+\) (tetrabutyl ammonium cation); \(\text{TPAs}^+\) (tetraphenyl arsonium cation).
(ii) the membrane of submitochondrial vesicles prepared by sonication (SMP) has the reverse polarity to the inner membrane of intact mitochondria. This has been established structurally by electron microscopy of negatively-stained preparations which show the obverse localization of the inner membrane spheres (ATPase: coupling factor F₁) (Racker, 1969), functionally by the accessibility of the respiratory enzymes and F₁ to specific antibodies and proteolytic enzymes (Racker et al, 1970), and from extraction (Lenaz and MacLennan, 1966; MacLennan et al, 1966; Racker et al, 1969) and reconstitution studies (Lee, 1963; Racker et al, 1969). In a third approach, it has been shown that impermeant reagents which chemically modify surface components confirm the evidence of the other procedures (Schneider et al, 1972).

The picture which emerges is that in intact mitochondria (or in vesicles prepared by digitonin treatment), the coupling factors and most respiratory enzymes are located on the matrix surface of the inner membrane while cytochrome c is situated on its outer surface; cytochrome oxidase is accessible to reagents at both surfaces of the membrane. The localization of these components in SMP is the direct opposite. It would therefore be anticipated that an electrochemical gradient across the inner membrane which was generated by respiration or ATP hydrolysis would have the opposite polarity in SMP to that in intact mitochondria. Similar experiments to those described in (i) have been conducted with SMP, using instead synthetic lipid-permeable anions

1. It has been found that these particles accumulate such anions in an energy-linked process comparable to the accumulation of cations by intact mitochondria (Liberman et al, 1969; Grinius et al, 1970; Bakeeva et al, 1970; Montal et al, 1970). The anions are not accumulated by

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1 The anions which have been studied are: PCB⁻ (phenylidy-carbaundecaborane); TPB⁻ (tetraphenylboron); TNP⁻ (trinitrophenol; picrate).
intact mitochondria, and in fact mitochondria which have been preincubated with them in a de-energized state, actively extrude them on re-energization.

In all cases, movement of the cation or anion is compensated by counter-movement of $H^+$ or $OH^-$.

(iii) the neutral depsipeptide valinomycin acts as a specific carrier for $K^+$ in artificial lipid bilayer membranes (Lauger, 1972). It forms a 1:1 clathrate complex, bearing the single cation charge of $K^+$, which moves across the hydrophobic membrane core in response to an applied electric field. The well-documented effects of valinomycin/$K^+$ on mitochondria and SMP (see Greville, 1969; Chance and Montal, 1971; Skulachev, 1971; Rottenberg, 1973) are completely (and only) explicable in terms of a similar mechanism in these natural membranes.

(iv) the distribution of permeant anions across the mitochondrial membrane, mediated by the exchange-diffusion carriers (Klingenberg, 1970; Chappell, 1968) is governed by the pH gradient (Palmieri and Quagliariello, 1969; Quagliariello and Palmieri, 1970; McGivan and Klingenberg, 1971; Rottenberg, 1973); so too is the distribution of the carrier-independent anions acetate and oxalate (Palmieri et al., 1970; Rottenberg, 1973). In spite of the anions being co-transported with $K^+$ (in the presence of an ionophore) in some of these experiments, their concentration gradient is not related to the potassium gradient. The driving force for anion transport must therefore be the proton gradient.

There is much additional evidence for the occurrence of electrophoretic cation transport in intact mitochondria; the above examples have been chosen for their force and generality. Returning to the specific consideration of $Ca^{2+}$ transport, it can be concluded that an electrochemical gradient (negative inside) does exist across the inner membrane of energized mitochondria, that it is established by the outward translocation of protons (rather than inward pumping of cations), and that a specific carrier exists for
Ca$^{2+}$ transport which is not an integral component of the energy-transducing system (see (a) above). The proposal that respiration (or ATP)-supported Ca$^{2+}$ accumulation is electrophoretic is not a direct consequence of these data, but it is strongly implied by them and will be adopted throughout as the most logical interpretation.

(c) **Low affinity (non-specific) binding of Ca$^{2+}$**

The foregoing discussion of mitochondrial Ca$^{2+}$ transport is based on studies in which Ca$^{2+}$ is actively accumulated in the internal phase of mitochondria (matrix space and inner surface of the inner mitochondrial membrane). Additional to this interaction is the binding of a relatively small amount of Ca$^{2+}$ which remains associated with mitochondria supplemented with respiratory inhibitors in the absence of an energy source (Rossi et al., 1967; Reynafarje and Lehninger, 1969).

Quantitative measurements indicate that under these conditions mitochondria can bind 25-40 nmoles Ca$^{2+}$/mg protein with a dissociation constant of about 100 to 200 µM. There seems little doubt that these sites are membrane phospholipids (Scarpa and Azzone, 1969). It is generally assumed, however, that they are situated exclusively on the outer membrane and the external surface of the inner membrane, an assumption based on the criterion that the carrier is unable to transport Ca$^{2+}$ in the absence of an energy source. The evidence of Selwyn et al. (1970) cited above, together with that of this thesis, shows this to be invalid: the absence of energy does not suffice as a topographical criterion (see also Azzone et al., 1969).

Nevertheless, these low affinity binding sites are an inseparable feature of the interaction of Ca$^{2+}$ with mitochondria, and a large proportion of them are undoubtedly situated externally to the inner membrane. Their nature and location are discussed more fully in a later section, but they have been introduced at this point to enable a full appreciation of the factors influencing measurements of Ca$^{2+}$ transport.
SCOPE OF THESIS

The main work described in this thesis is an extension of the preliminary kinetic experiments on the Ca\(^{2+}\)-induced ATPase. The thesis has been presented in two broad sections, the first dealing with the development and application of a technique which, for the first time, permits a systematic kinetic analysis of mitochondrial Ca\(^{2+}\) transport. The second section is an outgrowth of this work concerned with the interactions of specific inhibitors of Ca\(^{2+}\) transport with mitochondria. The relation between these two sections is symbiotic, in that the kinetic studies of Ca\(^{2+}\) transport provided the means of identifying the inhibitor mechanisms, while a knowledge of the interaction of the inhibitors in turn was essential to understanding the kinetic and Ca\(^{2+}\) binding properties of the carrier.

Both aspects of the work were studied concurrently, of necessity, but neither is yet complete. This thesis must therefore be considered as an attempt to define the lines of approach which will be most fruitful in understanding the mechanism of mitochondrial Ca\(^{2+}\) transport. The emphasis throughout is placed on the Ca\(^{2+}\) carrier (and Ca\(^{2+}\) binding sites) *per se*, rather than on the interaction of Ca\(^{2+}\) with the energy-transducing system which has been the approach to this problem commonly adopted in previous studies.
KINETIC AND BINDING STUDIES OF THE
INTERACTION OF Ca²⁺ WITH RAT LIVER MITOCHONDRIA

The overall features of active Ca²⁺ transport are well documented with respect to the anaerobiosis of counter-ion movements and the interaction of Ca²⁺ with the respiratory chain. On the other hand, the realization that Ca²⁺ transport is mediated by a specific carrier which operates independently of the energy-conserving system is a comparatively recent development. Partly for this reason, but largely for those discussed below, little attempt has been made to analyze the kinetic properties of the carrier in common with all hormone transport systems (and with conventional transport reactions in general).

The first difficulty encountered in attempting to measure the rate of Ca²⁺ transport is its extremely high activity, necessitating the use of techniques with correspondingly high time resolution. Mela and Chance (1966) have successfully developed a spectrophotometric method based on the absorbance difference between nitrato and its Ca²⁺ complex at 540-510 nm. However, this method is limited technically by the double requirement for rapid cycling and ultraviolet wavelength spectrophotometry, in addition to the purely theoretical restrictions imposed by the use of an internal indicator. The effect of any variation of experimental conditions (e.g., ionic strength, temperature, inhibitors, etc.) on Ca²⁺ transport must be clearly distinguished from its effect on the interaction of Ca²⁺ by nitrato and on the extinction of the free and existing forms; it is further possible that the binding of nitrato itself to mitochondria may vary with experimental conditions.

The alternative approach to measurement of Ca²⁺ transport is to separate the mitochondria from their suspending medium (by centrifugation or microfiltration) at specific time intervals after the addition of the radioactive "⁴⁴Ca²⁺" and to assay the radioactive associated with...
DEVELOPMENT OF A METHOD FOR KINETIC STUDIES OF Ca\textsuperscript{2+} TRANSPORT

Experimental difficulties

The overall features of active Ca\textsuperscript{2+} transport are well documented with respect to the stoichiometry of counter-ion movements and the interaction of Ca\textsuperscript{2+} with the respiratory chain. On the other hand, the realization that Ca\textsuperscript{2+} transport is mediated by a specific carrier which operates independently of the energy-coupling system is a comparatively recent development. Partly for this reason, but largely for those discussed below, little attempt has been made to analyze the kinetic properties of the carrier. In common with all membrane transport systems (and with conventional enzymes) the development of a rational mechanism depends on a thorough analysis of its kinetic and binding properties.

The first difficulty encountered in attempting to measure the rate of Ca\textsuperscript{2+} transport is its extremely high activity, necessitating the use of techniques with correspondingly high time resolution. Mela and Chance (1968) have successfully developed a spectrophotometric method based on the absorbance difference between murexide and its Ca\textsuperscript{2+} complex at 540-510 nm. However, this method is limited technically by the double requirement for rapid mixing and dual-wavelength spectrophotometry, in addition to the purely theoretical restrictions imposed by the use of an internal indicator. The effect of any variation of experimental conditions (pH, ionic strength, temperature, inhibitors etc.) on Ca\textsuperscript{2+} transport must be clearly distinguished from its effect on the chelation of Ca\textsuperscript{2+} by murexide and on the extinction of the free and chelating forms; it is further possible that the binding of murexide itself to mitochondria may vary with experimental conditions.

The alternative approach to measurement of Ca\textsuperscript{2+} transport is to separate the mitochondria from their suspending medium (by centrifugation or microfiltration) at specific time intervals after the addition of the radioisotope \textsuperscript{45}Ca\textsuperscript{2+}, and to assay the radioactivity associated with
them. As has been pointed out by Mela and Chance (1968) the time resolution of this technique is unsatisfactory for kinetic studies, prompting these authors to state with regard to their own newly-developed murexide technique: "We stress its unique position as the only kinetic method now available for measurements of divalent cation uptake in mitochondria." However, the $^{45}\text{Ca}^{2+}$ technique should be satisfactory at temperatures sufficiently low to ensure that 10 sec incubations permit initial rate measurements. The kinetics of the adenine nucleotide translocase and the phosphate and substrate carriers of mitochondria have been studied systematically under such conditions.

Nevertheless, both techniques suffer from a second fundamental limitation: their inability to discriminate between externally bound and transported (internal) $\text{Ca}^{2+}$ since both measure total mitochondrial (or free) $\text{Ca}^{2+}$. Only the internal fraction is relevant to kinetic studies of $\text{Ca}^{2+}$ transport. The externally-bound fraction, which may be roughly equated with the energy-independent low affinity binding mentioned earlier, is of the order of 30 nmoles $\text{Ca}^{2+}$/mg protein at saturation. Measurements of the rate of $\text{Ca}^{2+}$ transport made with the standard techniques thus include this component as a background, the magnitude of which is a function of the $\text{Ca}^{2+}$ concentration used. A reasonable estimate of the initial rate of transport can be obtained at saturating concentrations of $\text{Ca}^{2+}$ provided that the rate of decrease of free $\text{Ca}^{2+}$ is measured (as with the murexide techniques), or the rate of increase in mitochondrial $\text{Ca}^{2+}$ is corrected for the external binding contribution.

However, the dependence of the initial rate of transport on $\text{Ca}^{2+}$ concentration cannot be measured with certainty, since both the rate of transport and the background of external binding vary with $\text{Ca}^{2+}$ concentration. It is studies of precisely this nature which are most valuable in analyzing the mechanism of the carrier. To date, they have not been carried out due to the lack of a suitable experimental technique which discriminates between transport and binding.
The final problems associated with kinetic studies of Ca\(^{2+}\) transport are similarly critical in substrate velocity measurements. On the one hand, it is impossible to ensure that incubation systems are completely free of Ca\(^{2+}\) since the chemicals, distilled water and the mitochondria themselves contain small amounts which cause significant dilution errors when exogenous \(^{45}\text{Ca}^{2+}\) concentrations of less than about 20 \(\mu\text{M}\) are being used. On the other hand, Ca\(^{2+}\) is chelated (albeit weakly) by a large variety of organic materials including dicarboxylic acids (respiratory substrates) and phosphate. The only means of ensuring accurately known concentrations of \(^{45}\text{Ca}^{2+}\) in the micromolar range is to use a Ca\(^{2+}\) 'buffer' system. In the preliminary experiments described above, ATP/CaATP served precisely this role in addition to providing the energy source, but in those experiments the rate of Ca\(^{2+}\) transport was still superimposed on the background of its external low affinity binding.

**Lines of approach**

The problems inherent in making kinetic measurements of mitochondrial Ca\(^{2+}\) transport resolve essentially into the elimination of external binding since the speed of the process can be offset by using low temperatures; the question of Ca\(^{2+}\) buffers is a separate issue independent of the basic method.

It was initially decided that the most promising technique would be one involving the assay of \(^{45}\text{Ca}^{2+}\) in mitochondria after their separation from the medium by filtration or centrifugation, rather than continuous measurements based on, for example, murexide absorbance changes. The reasons for this preference are clear from the preceding comments, but additional factors are the greater sensitivity afforded by radioassay and the inability to use Ca\(^{2+}\) buffers in techniques dependent on measuring the free Ca\(^{2+}\) concentration.

Two lines of approach are available: the first is to include in the incubations a compound which specifically inhibits the low affinity external binding without affecting the Ca\(^{2+}\) carrier. The attraction of this approach is that it permits study of the interaction of Ca\(^{2+}\) with the carrier.
alone, but it would be extremely difficult to prove that a particular compound has the desired specificity. Thus Na\(^+\), K\(^+\), Mg\(^{2+}\) (Vainio et al., 1970; Scarpa and Azzone, 1968; Azzone et al., 1969), lanthanides and ruthenium red (Reed and Bygrave, 1973b) all inhibit both the carrier and low affinity binding (although to differing degrees). The most promising candidates in this respect appear to be UO\(_2\)\(^{2+}\) (see later) and certain local anaesthetics (Scarpa and Azzi, 1968; Mela, 1969a); the specificity of these compounds for the low affinity sites over the carrier resides not so much in their ability to generally substitute for Ca\(^{2+}\), but rather in their preference for binding to phospholipid matrices. However, any compound which inhibits the external binding will do so competitively with respect to Ca\(^{2+}\) so that estimates of \(V_{\text{max}}\) will still include the binding contribution.

The alternative approach is to quench reactions with an inhibitor specific for the Ca\(^{2+}\) carrier and to subsequently remove the externally bound fraction prior to separation of the mitochondria. On theoretical grounds alone, this presents fewer ambiguities than the former approach. Its practical advantages include the possibility of very short, well-defined incubation times, but more importantly it provides a means of clearly discriminating between three pools of Ca\(^{2+}\)-internal, externally bound, and free - under any set of experimental conditions. This general approach was therefore developed.

EGTA quenching

(a) Preliminary considerations

The dual requirements for quenching transport and removing externally bound Ca\(^{2+}\) could be met simultaneously by using a powerful impermeant chelating agent, the obvious choice being EGTA. Each of the criteria required of it have been examined in detail before it was used as a quenching agent.
The first of these is its ability to chelate Ca\textsuperscript{2+}. From the calculations in the Appendix, it is evident that a five-fold excess of EGTA over Ca\textsuperscript{2+} decreases the concentration of 'free' Ca\textsuperscript{2+} to about 10\textsuperscript{-8}M. Since the apparent K\textsubscript{m} for Ca\textsuperscript{2+} transport is of the order of 10\textsuperscript{-6} M (Introduction) and the apparent K\textsubscript{d} for low affinity binding is about 10\textsuperscript{-4} M (Reynafarje and Lehninger, 1969), treatment of mitochondria with a five-fold or greater excess of EGTA should completely inhibit both the transport and external binding of Ca\textsuperscript{2+}.

Secondly, neither EGTA nor CaEGTA should be able to penetrate the inner membrane through which Ca\textsuperscript{2+} is transported. Experiments with the passive swelling technique of Chappell and Haarhoff (1967) have shown that no mitochondrial swelling occurs in iso-osmotic solutions of NH\textsubscript{4}EGTA or NH\textsubscript{4}EDTA in either the presence or absence of phosphate and malate (data not shown), indicating that neither EGTA nor EDTA can enter the osmotically active space. Harris and van Dam (1968) have also reported that the mitochondrial spaces accessible to \textsuperscript{14}C-sucrose and \textsuperscript{14}C-EDTA are identical; similar isotopic studies have not been made with EGTA. However, work in Chappell's laboratory (Chappell et al, 1963; Chappell and Crofts, 1965) shows that EGTA cannot chelate Ca\textsuperscript{2+} or Mn\textsuperscript{2+} which has been accumulated by mitochondria in an energy-linked process. More recent work by Puskin and Gunter (1973) confirms the inaccessibility of accumulated Mn\textsuperscript{2+} to EDTA.

The final requirement is that it must not directly affect any mitochondrial functions. Both EGTA and EDTA have been used routinely in studies of mitochondria for many years with no evidence of effects other than those associated with removal of endogenous Ca\textsuperscript{2+}.

These criteria were then tested directly under the experimental conditions required for kinetic studies. It must be stressed at the outset that the validity of the EGTA technique can only be established empirically since there is no real basis for comparison between the initial rates of Ca\textsuperscript{2+} transport obtained under the conditions used in this work and those of previous studies. This applies equally
to the use of EGTA in Ca\(^{2+}\) binding as to the kinetics of transport. The arguments used are to a certain extent cyclic, but they are nevertheless reinforced by the foregoing evidence.

(b) **Experimental conditions**

The standard technique consisted of pre-incubating the mitochondria for one min in the reaction medium, then incubating for a few sec with \(^{45}\)Ca\(^{2+}\) and quenching with, generally, a ten to twenty-fold excess of EGTA. The incubation time used in initial rate studies was usually five or 10 sec; occasionally, longer times were used in conjunction with very low concentrations of mitochondria. Since starting and quenching operations were done manually, the timing error was probably \(\pm 10\) per cent (\(\pm 0.25\) sec for 5 sec incubations). Better resolution (required for experiments at higher temperatures) and accuracy should be obtained with an automated injection apparatus (Heldt and Klingenberg, 1968) or with continuous-flow systems.

In the first variation of the standard technique, incubations were carried out in a stirred, thermostatted glass vessel; a small aliquot of the quenched suspension was filtered on a 13 mm Millipore filter (0.45 µm pore diameter) and the mitochondria washed with 2 ml of ice-cold medium containing 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4), 2 mM-succinate-tris and 0.01 mM-EGTA-tris. The filter was placed in a glass vial with 10 ml of methyl cellosolve scintillation cocktail and counted for \(^{45}\)Ca\(^{2+}\).

In the second variation, each incubation was performed in a separate disposable centrifuge tube (Eppendorf). After each addition, the tubes were mixed vigorously. Immediately after quenching, each tube was centrifuged, the supernatant removed and the mitochondrial pellet dissolved in 0.1 ml Soluene at 60°C. This solution was quantitatively transferred to a scintillation vial with 10 ml of methyl cellosolve cocktail.
While the basic technique described above appears to be quite suitable for measuring initial rates of Ca\(^{2+}\) transport, the use of EGTA in studies of both the later stages of transport (in the absence of a permeant anion) and in energy-independent binding is complicated by its induction of a relatively slow and limited efflux. From the results of experiments described in following chapters, it is concluded that the most satisfactory quenching conditions are obtained by adding a mixture of EGTA (ten to twenty-fold excess) and ruthenium red (about 1 to 2 nmoles/mg protein). Fortunately, EGTA does not chelate ruthenium red (in contrast to the lanthanides, the alternative high-affinity specific inhibitors of Ca\(^{2+}\) transport) and the inhibition by ruthenium red is non-competitive and is not released with time (see later).

Separation by centrifugation was generally used in experiments carried out at 0°C since vacuum microfiltration of small aliquots is ineffective at low temperatures due to the high viscosity of sucrose. The radioactivity of the pellets was corrected for \(^{45}\)Ca\(^{2+}\) trapped in the extra-mitochondrial space, which was measured in identical experiments in which EGTA had been added to the mitochondria before \(^{45}\)Ca\(^{2+}\). This background was minimized in some cases by diluting the quenched incubation prior to centrifugation. Temperature control in the experiments carried out at 0°C was achieved by maintaining the tubes in an ice-water bath and carrying out all operations in a 4°C cold room.

---

\[ K_d = \frac{E \times R}{E \times R} \]  
\[ (E = \text{concentration of carrier binding sites for ruthenium red (R))} \]

Expressing this in terms of total ruthenium red (\(R_T\)) and carrier site (\(E_T\)) concentrations

\[ K_d = \frac{(E_T - E_R)(R_T - E_R)}{E_R} \]

At 99 per cent saturation, \(ER = 0.99E_T\) so that

\[ R_T = 99(K_d) + 0.01(E_T) \] where \(K_d = 0.03 \mu M\) and \(E_T = \text{the } \mu M\) equivalent of 0.08 nmoles/mg.protein (see later)

\[ = (2.97 + 0.01.E_T) \mu M. \]
The standard incubation medium used throughout these studies contained sucrose (250 mM) and generally 5 mM-hepes-tris and 2 mM-succinate-tris. The neutralization of acidic components with tris avoided the necessity for metal cations, which are known to inhibit Ca\(^{2+}\) transport, while simultaneously ensuring a reasonably strong buffer at low ionic strength. It is conceivable that tris inhibits Ca\(^{2+}\) transport to a similar extent to Na\(^+\) and K\(^+\), but the presence of a cation of some kind is obviously unavoidable.

The pH of the stock incubation medium was always adjusted to 7.4 at the desired temperature immediately before use. Later experiments showed this to be a very poor choice since it is in a region of marked variation of transport rate with pH; the ideal value is about 8.5.

The optimal experimental conditions were but gradually refined, so that the technique described above as being most suitable was not used in many of the experiments. It has not been possible to repeat all of the work defining the kinetic properties of Ca\(^{2+}\) transport, nor is it necessary to do so in most cases. It should nevertheless be borne in mind that some discrepancies exist in the data as a consequence of using sub-optimal quenching conditions.

(c) Experimental demonstration of validity

Fig. 2.1 shows the effect of EGTA concentration on the amount of Ca\(^{2+}\) retained by mitochondria after a 5 sec incubation at 10° C. The non-removable fraction reaches a minimum when a four to five-fold excess of EGTA is used, in agreement with calculations (see Appendix). The absence of further effects at higher concentrations indicates that EGTA is acting solely as an impermeant chelating agent. The second important feature of this data is the independence from quenching time of the non-removable fraction; thus, neither Ca\(^{2+}\) nor CaEGTA can enter or leave the space inaccessible to EGTA. This conclusion is reinforced by experiments in which EGTA was added to mitochondria before \(^{45}\)Ca\(^{2+}\); in this case, the radioactivity associated with the mitochondria remains at background level for at least 10 min.
Fig. 2.1. Initial rate of Ca\textsuperscript{2+} transport using EGTA quenching: effect of EGTA concentration and quenching time.

Incubations contained 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4) and 2 mM-succinate-tris in a total volume of 0.5 ml. Mitochondria (1 mg protein) were incubated for 1 min prior to the addition of 200 µM-\textsuperscript{45}Ca\textsuperscript{2+} (0.05 µCi) and then for 5 sec before being quenched by the addition of EGTA-tris at the concentrations shown. Aliquots of 50 µl were filtered 10 sec (●) or 3 min (○) after quenching, washed and assayed for radioactivity. Incubation temperature was 10°C.

Fig. 2.2. Time course of Ca\textsuperscript{2+} transport using EGTA quenching

The incubation contained 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4), 1 mM-phosphate-tris and 2 mM-succinate-tris in a total volume of 4.0 ml. Mitochondria (13 mg protein) were preincubated for 1 min prior to the addition of 325 µM-\textsuperscript{45}Ca\textsuperscript{2+}. At the times shown, 100 µl aliquots were removed into 160 µl of ice-cold quench medium (250 mM-sucrose, 2 mM-hepes-tris (pH 7.4), 2 mM-EGTA-tris) and mixed. At the end of the experiment, all tubes were centrifuged (4 min at 12,000 x g) and 100 µl samples of the supernatants were assayed for radioactivity. Incubation temperature was 10°C.
Fig. 2.2 shows that the pool of EGTA-inaccessible Ca\textsuperscript{2+} increases with time as would be expected if it represents transported Ca\textsuperscript{2+}. It should be noted that the initial rate in this experiment is faster than in that of Fig. 2.1, due to the inclusion of phosphate (Chance and Yoshioka, 1966). After about 2 min, virtually no Ca\textsuperscript{2+} remains accessible to EGTA.

By themselves, these experiments do not provide conclusive evidence that the EGTA-inaccessible pool of Ca\textsuperscript{2+} is equivalent to the internal space of mitochondria to which Ca\textsuperscript{2+} is actively transported, but they are completely consistent with this proposition. The combined evidence of the above data with those of the following chapters leaves little doubt that EGTA fulfils the criteria required of it.

(d) Additional applications of EGTA quenching

The properties of EGTA which provide the basis for its application in kinetic studies further ensure that it can be used to examine the kinetics and extent of carrier-mediated Ca\textsuperscript{2+} efflux from pre-loaded mitochondria. Preliminary experiments of this nature are included in the following chapter.

Its use in yet another context is described in the subsequent chapter, which is concerned with measurements of energy-independent Ca\textsuperscript{2+} binding based on the topographical criterion provided by EGTA.

Finally, it can (and should) be used to determine stoichiometric relationships of counter-ion movement and energy expenditure during energy-linked Ca\textsuperscript{2+} transport, since such relationships become meaningful only when the internal and externally-bound pools of Ca\textsuperscript{2+} are known.

This last application has not been utilized, but it is of obvious value in assessing the super-stoichiometry and oscillatory phenomena associated with Ca\textsuperscript{2+} transport under certain experimental conditions (Carafoli \textit{et al}, 1966, 1967; Rossi and Azzone, 1968; Azzone \textit{et al}, 1969).
Finally, some comment is required on the choice of a compound suited for the chelate component of a Ca$^{2+}$ buffer. The most commonly used buffer system is Ca/CaEGTA (or EDTA), but this is quite unsatisfactory for use in studies of mitochondrial Ca$^{2+}$ transport. The apparent Km for the Ca$^{2+}$ carrier system is within the range of $10^{-6}$ to $10^{-5}$ M, so a suitable buffer must generate stable concentrations of free Ca$^{2+}$ within this range. The detailed calculations included in the Appendix show that with EGTA at pH 7.4 there is a nearly linear relation (slope = 1) between total and free Ca$^{2+}$ concentrations only when the free concentration is less than $10^{-8}$ M. In fact when free Ca$^{2+}$ is in the region of $10^{-6}$ M, it changes 50 times as rapidly as does the total concentration. The Ca$^{2+}$/EGTA system is analogous to a strong acid/strong base titration, the equivalence point being at $10^{-6}$ M (pCa = 6). The situation with EDTA is quite similar. The only means of generating the required conditions with these chelates is to include a second cation (eg. Mg$^{2+}$) or to use lower pH values. Neither of these solutions is satisfactory.

The two most suitable compounds appear to be ATP (discussed in "Introduction") and nitrilotriacetic acid (NTA), both of which 'buffer' in the region of $10^{-6}$ to $10^{-4}$ M free Ca$^{2+}$ (Appendix). The use of ATP, while desirable in some respects, is limited by the fact that it cannot be used successfully in studies of mitochondrial Ca$^{2+}$ transport in the absence of a permeant anion. Both the permeation and hydrolysis of ATP by mitochondria can be prevented by the use of appropriate inhibitors (attractyloside or bongkrekic acid, and oligomycin, respectively), but there is usually a small amount of inorganic phosphate associated with preparations of ATP. It is preferable to use a completely inert chelate, and NTA seems to meet this requirement. There are no previous data relating to its use in mitochondrial systems.
Considerable emphasis has so far been placed on the necessity for strong buffers in establishing concentration velocity relationships in kinetic studies of Ca\(^{2+}\) transport, but they have not been used in much of the work described in this thesis since many experiments were carried out with 'saturating' concentrations of Ca\(^{2+}\) where a buffer is no longer required.

The use of a buffer generates additional experimental problems in that the total concentration of Ca\(^{2+}\) is approximately 100 times greater than its free concentration; for valid kinetic studies with initial rate measurements, the decrease in free Ca\(^{2+}\) concentration during an incubation should be no more than about 5-10 per cent. Thus the total Ca\(^{2+}\) concentration is approximately 1,000 to 2,000-fold higher than the concentration removed into the mitochondria. If the mitochondria are simply separated by micro-centrifugation after quenching, the background radioactivity of \(^{45}\)Ca\(^{2+}\) trapped in the pellet and adherent fluid will be intolerably high. The ideal separatory technique is therefore Millipore filtration followed by extensive washing of the residue. However, provided that the quenched incubation mixture is diluted extensively, that high-speed centrifugation is used to give a compact pellet, and that the tube and pellet are rinsed, centrifugation can be (and has been) used successfully.
A model for the mechanism of mitochondrial Ca\(^{2+}\) transport can be formulated diagramatically as follows:

\[ (I) \]
\[
\begin{array}{c}
\text{(out)} + \text{inner} \\
\text{membrane} \\
\text{(in)} \\
E \xleftrightarrow{\text{E,Ca}^{2+}} E \xleftrightarrow{\text{E,Ca}^{2+}} (E = \text{Ca}^{2+} \text{ carrier})
\end{array}
\]

The basic postulate of a charged carrier transporting Ca\(^{2+}\) in response to an electric field has been developed in the Introduction. It has been further assumed that the carrier is mobile in the lipid phase (cf. valinomycin/K\(^{+}\)), physically transporting Ca\(^{2+}\) which remains bound to its initial binding site. There is no evidence to support this; the assumption is included mainly as an aid to visualizing the processes involved.

This chapter attempts to define the properties of Ca\(^{2+}\) transport in terms of the above mechanism by using conventional steady-state kinetic theory to interpret substrate/velocity relationships for the overall transport process. For this purpose, the reaction can be considered analogous to a classical enzyme-catalysed conversion:

\[ (II) \]
\[
\begin{array}{c}
\text{E} \xrightarrow{k_1} \text{EA} \xrightarrow{k_3} \text{EP} \xrightarrow{k_5} \text{E} + \text{A (out)} \\
\text{E} \xrightarrow{k_2} \text{E} + \text{P (in)} \xrightarrow{k_4} \text{E} \xrightarrow{k_6} \text{E} \xrightarrow{k_7} \text{E} \xrightarrow{k_8} \text{E}
\end{array}
\]

where A = external \(\text{Ca}^{2+}\)

\[ P = \text{internal Ca}^{2+}\]

The relation between Ca\(^{2+}\) concentration and the initial rate is of the standard form (see below), but \(V\) and \(K_m\) are
defined by seven different rate constants. There is little to be gained by using such a complex expression since many (or most) of the reverse rate constants involved will be so small and some of the forward rate constants so large that the kinetics will be mainly determined by the others. A more practical model is that represented by the Briggs-Haldane approximation:

\[
\begin{align*}
\text{E} + A & \quad \xrightleftharpoons[k_2]{k_1} \quad \text{EA} \\
\text{(out)} & \quad \xrightarrow{k_3} \quad \text{E} + P \\
\text{(in)} & 
\end{align*}
\]

The equation describing the effect of Ca\(^{2+}\) concentration (A) on the initial rate of transport (v) is

\[
v = \frac{V \cdot A}{K_m + A} , \quad \text{where} \\
V \quad \text{(maximum velocity)} = k_3 \cdot E_T \\
\left( E_T \quad \text{is total carrier concentration} \right) \\
\text{and} \quad K_m \quad \text{(measured substrate concentration at } \frac{V}{2}) = \frac{k_2 + k_3}{k_1}
\]

This model (III) will be used throughout this Chapter, but it should be borne in mind that its accuracy depends on the assumption of a single forward rate constant (k_3) being so small that it alone determines the kinetics. Both this and the complex model ignore the possibility of a sigmoidal

\[1\] For the complex model (II):

\[
v = \frac{V \cdot A}{K_m + A} \\
\text{where} \quad V = \frac{k_3 k_5 k_7 \cdot E_T}{k_3 k_6 + k_3 k_6 + k_4 k_6 + k_3 k_7 + k_4 k_7 + k_5 k_7} \\
\text{and} \quad K_m = \frac{k_2 k_4 k_6 + k_2 k_4 k_7 + k_2 k_5 k_7 + k_3 k_5 k_7}{k_1 (k_3 k_5 + k_3 k_6 + k_4 k_6 + k_3 k_7 + k_4 k_7 + k_5 k_7)}
\]
relation between \([\text{Ca}^{2+}]\) and velocity which was indicated by the data presented in the Introduction. However, for most purposes the simplified model is a good approximation\(^1\).

The two independent variables which can be expected to change as a function of altered experimental conditions are \(V\), the zero-order rate constant at infinitely high substrate concentration which is a function 'solely' of the rate-limiting step(s) and \(\frac{V}{K_m}\), the first-order rate constant at infinitely low substrate concentration which is a function 'solely' of the rate constants for formation of the enzyme-substrate complex. The third parameter which is of value is \(K_m\); although it is not a completely independent variable, it can nevertheless provide useful information.

The steady-state kinetic analysis depends critically on the measurement of initial rates. Many of the experiments to be described were therefore done with two different incubation times. The data for both times gave very similar results, indicating that true initial rates were being measured.

Relation of 'enzyme' and substrate concentrations to the rate of \(\text{Ca}^{2+}\) transport

Fig. 3.1 shows that, under the conditions used in the subsequent experiments, a linear relation exists between the concentration of mitochondria and the initial rate of \(\text{Ca}^{2+}\) transport (as measured by EGTA quenching at 10°C). This is a criterion which must be satisfied in any study of enzyme kinetics; in the present case it is complicated by the fact

\(^1\)The general equation for 'n' infinitely co-operative binding sites is:

\[
v = \frac{V_0 A^n}{K + A^n}, \text{ derived from the model }
\]

\[
(IV) \quad A + E \xrightleftharpoons[k_2]{k_1} EA + A \xrightleftharpoons[k_4]{k_3} EA_2 \ldots + A \xrightleftharpoons[k_y]{k_x} EA_n \xrightarrow[k_z]{k_y} E + P
\]

where \(k_x\) greatly exceeds all other forward rate constants. Non-integral values of \(n\) can be used in the equation to approximate less than infinite co-operativity. The Hill plot (see later) is based on the logarithmic form of this general equation:

\[
\log \frac{v}{v-V} = n \log A - \log K
\]
Fig. 3.1. **Initial rate of Ca\(^{2+}\) uptake as a function of mitochondria concentration**

Incubations contained 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4) and 2 mM-succinate-tris in a final volume of 0.5 ml. Mitochondria (at the concentrations shown) were preincubated for 1 min prior to the addition of 200 µM-\(^{45}\)Ca\(^{2+}\) (sp.act. 0.5 µCi/mole) and then incubated for 5 sec. Incubations were quenched with 1 mM-EGTA-tris and the mitochondria were then separated from a 100 µl aliquot by filtration or centrifugation. The data with three separate preparations of mitochondria (as shown by the symbols) are combined on a normalized scale where 100 = 35 nmoles Ca\(^{2+}\) in the mitochondria at 5 sec (mean of the three experiments). The temperature was 10°C.
that mitochondria are involved in the 'product' (i.e., transported Ca\(^{2+}\)) in addition to providing the 'enzyme'. The linearity of the plot shows that neither the catalytic activity of the carrier nor the total capacity of mitochondria for Ca\(^{2+}\) is exceeded under these conditions.

The energy requirement for respiration-supported Ca\(^{2+}\) transport has been examined in the experiments of Fig. 3.2 and 3.3. With succinate as the respiratory substrate (Fig. 3.2), the rate of transport is maximally stimulated to a level of 50 per cent above that supported by endogenous substrate oxidation. At concentrations of succinate higher than 2 mM the rate is inhibited, probably due to the chelation of Ca\(^{2+}\) (Sillén and Martel, 1964).

The nature of the exogenous respiratory substrate has no significant effect on the rate of Ca\(^{2+}\) transport, as seen in Fig. 3.3. The four substrates used in these experiments provide energy coupling through one, two, three and four sites respectively. The rate of Ca\(^{2+}\) transport, as distinct from the rate of Ca\(^{2+}\)-stimulated respiration (data not shown), is apparently independent of the number of coupling sites involved (the slightly greater rate seen with ascorbate is probably due to its weaker chelation of Ca\(^{2+}\) as compared with the other substrates).

These experiments were carried out in order to establish conditions for the measurement of initial rates of Ca\(^{2+}\) transport in which the provision of energy is not rate-limiting. More detailed studies of the role of the respiratory substrate in Ca\(^{2+}\) transport should obviously include a strong Ca\(^{2+}\) buffer to overcome the chelating effect of substrate anions, and they should be carried out under conditions in which the oxidation of endogenous substrates is suppressed (e.g., rotenone/succinate, antimycin/ascorbate). In fact, it may be preferable to study the kinetics of Ca\(^{2+}\) transport in an experimental system which is completely independent of respiration so that no confusion could exist as to the rate-limiting process (i.e., transport itself, or respiration). Such systems include the influx of Ca\(^{2+}\) in response to valinomycin-induced K\(^+\) efflux (Scarpa and Azzone, 1970) and the influx of Ca\(^{2+}\) with a permeant anion (SCN\(^-\)) in response to an osmotic gradient (Selwyn et al., 1970). However, these
**Fig. 3.2. Effect of succinate concentration on rate of Ca\(^{2+}\) transport**

Incubations contained 1 mg mitochondrial protein in 250 mM-sucrose, 10 mM-hepes-tris (pH 7.4) and succinate-tris at the concentrations shown in a final volume of 0.5 ml. Incubations were carried out at 10°C for 5 sec with 200 µM-\(^{45}\)Ca\(^{2+}\) (0.1 µCi) and were quenched with 1 mM-EGTA-tris. 50 µl aliquots were filtered and the filters washed and counted for radioactivity. Each point is the mean of two separate experiments.

**Fig. 3.3. Effect of varying the respiratory substrate on rate of Ca\(^{2+}\) transport**

Incubation conditions were identical to those of Fig. 3.2 except that the respiratory substrate was varied as shown. Concentrations were 5 mM-ascorbate-tris, 50 µM-TMPD, 5 mM-succinate-tris, 5 mM-\(\beta\)-hydroxybutyrate (Na-D,L), 5 mM-\(\alpha\)-oxoglutarate-tris, 5 mM-malonate-tris, 0.5 µM-rotenone, 0.5 µM-antimycin and 0.5 µM-CCCP. Data are the means of two separate experiments.
would be less satisfactory from other points of view, the former because of the limit imposed on the magnitude and rate of establishment of the electrochemical gradient by the endogenous K⁺ content (and the uncertainty of this factor) and by the ambiguity which would accompany the use of permeant anions; the latter by the fact that the external concentration of Ca²⁺ would not only directly affect the carrier but would also determine the magnitude of the driving force, in this case osmotic pressure.

Thus, a respiration-supported system has been used throughout. It has been assumed that under all conditions tested in subsequent experiments, a steady-state electrochemical gradient exists across the mitochondrial membrane, the magnitude of which does not alter sufficiently for this to become the rate-limiting factor provided that saturating concentrations of respiratory substrate are used. This assumption implies that the electrophoretic movement of E.Ca²⁺ across the membrane core is not rate-limiting, a concept that will be developed subsequently in more detail.

The final two experiments of Fig. 3.3 show that the abolition of energy production by respiratory inhibitors or uncouplers, strongly inhibits, but does not completely prevent, the movement of a small amount of Ca²⁺ into a pool inaccessible to EGTA. This is considered more fully in the following chapter.

Relation between Ca²⁺ concentration and its initial rate of transport

The effect of Ca²⁺ concentration on its rate of transport is shown in Fig. 3.4. The data fit a linear double-reciprocal plot and give a $K_m$ of 50 µM-Ca²⁺, in marked contrast to the earlier results (Introduction) which showed a sigmoidal substrate-velocity relationship and a $K_m$ of 2-3 µM. The main difference in the experimental conditions under which these two sets of data were obtained are firstly the temperature (0°C cf. 25°C) and secondly, the omission of a strong Ca²⁺ buffer from the present experiments (cf. the use of ATP in the earlier experiments).
Incubations contained 0.25 mg mitochondrial protein and 250 mM-sucrose, 2 mM-succinate-tris and 5 mM-hepes-tris (pH 7.4) in a final volume of 0.52 ml. Temperature was 0°C. Incubations were carried out for 10 sec with $^{45}$Ca$^{2+}$ (sp.act. either 6 µCi/µmole (1-20 µM) or 0.2 µCi/µmole (45 to 350 µM)) as shown and quenched with a ten-fold excess of EGTA-tris. The tubes were centrifuged immediately and the pellets counted for radioactivity. Each point is the mean of four separate experiments.
In a very recent report, Vinogradov and Scarpa (1973) have presented the results of experiments in which the initial rate of succinate-supported \( \text{Ca}^{2+} \) transport by liver mitochondria was measured as a function of its concentration by continuously recording murexide absorbance changes in a dual-wavelength spectrophotometer using rapid-mixing stopped-flow techniques. The experiments were carried out in a medium containing 2 mM-Mg\(^{2+}\) and 75 mM-K\(^+\) to inhibit low-affinity external binding (see previous chapter). The results showed a sigmoidal relation between \([\text{Ca}^{2+}]\) and velocity (Hill coefficient 1.63) with an extrapolated maximum rate very similar to that found in the ATP-supported system described earlier (when uncorrected for external binding); \( \text{viz.} \) 480(+) mmoles \( \text{Ca}^{2+}/\text{min.mg} \) protein. Contrary to the authors' interpretation, this figure includes the external low-affinity binding component since its inhibition by K\(^+\) and Mg\(^{2+}\) is competitive (Scarpa and Azzi, 1968).

The \( K_m \) for \( \text{Ca}^{2+} \) reported in that work is about 60 \( \mu \text{M} \), which the authors conclude to be the correct value rather than 2 \( \mu \text{M} \) as indicated by the data of Bygrave \( \text{et al.} \) (1971a,b). However, in drawing this conclusion Vinogradov and Scarpa (1973) have completely ignored the chelation of \( \text{Ca}^{2+} \) by components of their reaction system which included 35-60 \( \mu \text{M} \)-murexide, 7 mM-succinate and 5 mM-phosphate. The free concentration of \( \text{Ca}^{2+} \) at half-saturation must be far less than the 60 \( \mu \text{M} \) total.

Related to this question of the actual \( K_m \) is the determination by Carafoli and Azzi (1972) of the concentration of \( \text{Ca}^{2+} \) required for half-maximal oxidation of cytochrome \( \text{b} \) in respiring rat liver mitochondria. A \( \text{Ca}^{2+}/\text{EGTA} \) buffer system was used by these authors such that the total \( \text{Ca}^{2+} \) concentration was about 3,000-fold higher than its free concentration. The half-maximal effect was induced by 2-3 \( \mu \text{M-Ca}^{2+} \) (free) and the dose-response relation was again sigmoidal.

It cannot be emphasized too strongly that the reason for using a strong \( \text{Ca}^{2+} \) buffer is not primarily to maintain the concentration of \( \text{Ca}^{2+} \) at a 'constant' level during initial rate measurements (and indeed it is not used in this context in the present work), but rather to ensure that a known concentration of free \( \text{Ca}^{2+} \) is used. Vinogradov and Scarpa (1973) have missed this point completely. When initial rates
of Ca\(^{2+}\) uptake or Ca\(^{2+}\)-induced respiratory responses are measured as a function of total Ca\(^{2+}\) in a medium containing dicarboxylic acids, phosphate and/or murexide, the apparent \(K_m\) is 50-150 µM-total-Ca\(^{2+}\) (Chance, 1965; Chance and Schoener, 1966; Scarpa and Azzone, 1970; Vinogradov and Scarpa, 1973; Fig. 3.4). Calculation of the free Ca\(^{2+}\) concentration from the stability contents for the various complexes shows it to be less than 10 µM at half-saturation in each of these cases.

It is quite obvious that [Ca\(^{2+}\)] must be expressed in terms of its free concentration and this can best be achieved by using a Ca\(^{2+}\) buffer sufficiently strong that calculated free concentrations are not significantly affected by other weak chelators. The experiment of Fig. 3.4 has therefore been repeated using a Ca\(^{2+}\)/NTA buffer to generate known, stable concentrations of free Ca\(^{2+}\). The results (Fig. 3.5) show that the [Ca\(^{2+}\)]/velocity curve is sigmoidal (Hill coefficient 1.7) and that the \(K_m^1\) is 4 µM. The fact that similar data have been obtained under a wide variety of experimental conditions indicates that the sigmoidicity and low \(K_m\) are fundamental properties of the Ca\(^{2+}\) carrier.

The reason for the hyperbolic relationship in the experiment of Fig. 3.4 is not known with certainty, but it may be due partially to isotope dilution by endogenous Ca\(^{2+}\) and partly to a non-linear relation between total and free [Ca\(^{2+}\)] in the weak Ca\(^{2+}\)-buffer system of this experiment (2 mM-succinate + unknown components). The possibility had been considered that the sigmoidal curve could be an artifact arising from the use of mitochondria at concentrations so high in relation to the \(K_m\) that their binding of Ca\(^{2+}\) (to both carrier and non-specific external sites) would be sufficient to significantly deplete the concentration of free Ca\(^{2+}\) (Cha, 1970). However, this possibility is eliminated by using a strong Ca\(^{2+}\) buffer, and further experiments have shown that the shape of the [Ca\(^{2+}\)]/velocity curve is independent of the concentration of mitochondria (e.g., Fig. 3.5).

\(^1K_m\) has been used to denote the Ca\(^{2+}\) concentration at half-maximal velocity; i.e. when \(\log \frac{v}{V_v} = 0\).

It is perhaps preferable to denote this by the symbol \(K_{0.5}\) (Koshland, 1970).
Fig. 3.5. Influence of Ca$^{2+}$ concentration (buffered with NTA) on rate of Ca$^{2+}$ transport

Incubations contained 250 mM-sucrose, 2 mM-succinate-tris, and 10 mM-NTA-tris (pH 7.4) in a total volume of 0.54 ml. Data are the means of three separate experiments carried out with 0.125, 0.25 and 0.5 mg mitochondrial protein for incubation periods of 40, 20 and 10 sec respectively (there was no systematic variation between the three experiments). Transport was initiated by the addition of $^{45}$Ca$^{2+}$ (1 µCi/µmole) and quenched with a mixture of 2 µM-ruthenium red and 30 mM-EGTA. The quenched mixture was diluted with 5 ml of ice-cold sucrose/hepes/succinate and filtered on a 1 inch Gelman filter (0.45 µm pore size). The incubation tube and filter were rinsed with a further 5 ml of this solution and the filter was counted for radioactivity. Temperature was 0°C.

(a) Transport rate as a function of free Ca$^{2+}$ concentration. The total concentration of Ca$^{2+}$ was varied between 0.046 and 1.85 mM; 'free' concentrations were obtained from the data in the Appendix.

(b) Double reciprocal plot of (a). $v = \text{n moles Ca}^{2+} \text{transported/5 sec.mg protein; the Ca}^{2+} \text{concentration is 'µM free'}.\n
(c) Hill plot of (a). The slope = n (Hill coefficient) and the abscissa intercept = log $K_m$. 
Effect of pH on the initial rate of Ca\textsuperscript{2+} transport

The variation of the initial velocity of an enzymic reaction with pH can be (and has most frequently been) used simply to establish optimal conditions for assay, but considerable information can be obtained about the groups involved in substrate binding and in the rate-limiting step of catalysis by studying the effect of pH on the independent rate constants defined at the beginning of this chapter (Cleland, 1970).

The first-order rate constant \( \frac{v}{K_m} \) was measured as the reciprocal of the slope of the linear double-reciprocal plots of initial velocity \( \text{versus total Ca}^{2+} \) concentration in an 'unbuffered' medium. The values for \( K_m \) are therefore approximately ten-fold too high. It has been assumed that the ratio of free to total \([\text{Ca}^{2+}]\) at the \( K_m \) remains constant over the entire pH range since the stability constant of the major chelating component, succinate, does not change significantly over this range (pKa's = 4.0, 5.3). This assumption would be weakened if other components of the medium with pKa's between six and nine cause significant chelation of \( \text{Ca}^{2+} \) (NTA was not used since its stability constant varies markedly over the required pH range).

In all incubations, a composite pH buffer of mes, hepes and tris (pKa's = 6.15, 7.55 and 8.3 respectively at 20°C; Good \textit{et al}, 1966) was used so that the solution was buffered (for H\textsuperscript{+}) over the entire range. The pH of all solutions was initially adjusted to 8.5 with KOH and titrated to the desired value with HCl to ensure identity of cationic composition.

The results indicated that there is little significant variation in \( V \) with pH. The true dependence of \( V \) on pH could be very difficult to establish since the rate-limiting step (reflected in \( V \)) occurs in a compartment physically separated from the external phase and hence from the external pH.

The plot of \( \log \frac{V}{K_m} \text{versus pH} \) (Fig. 3.6) asymptotes to a slope of one at \( \text{low pH} \) and to a slope of zero at high pH, with the two asymptotes intersecting at pH 7.8. The data are interpreted as showing that a group(s) involved in the initial binding of \( \text{Ca}^{2+} \) to the carrier has a pKa of 7.8 and is active in its dissociated state. The pKa can generally be assigned...
A stock incubation solution was prepared at 0°C containing 250 mM-sucrose, 3 mM-succinate-tris, 3 mM-mes-tris and 3 mM-hepes-tris, and its pH was adjusted to 8.5 with KOH (the final concentration of K⁺ was about 8 mM). Aliquots of this solution were adjusted to the pH values shown with HCl. Incubations were carried out for 10 sec with 1 mg mitochondrial protein and five different concentrations of ⁴⁵Ca²⁺ (sp.act. 0.1 μCi/μmole) from 40 to 400 μM; quenching was done with a ten-fold excess of EGTA-tris. Incubation volume was 0.53 ml and the temperature was 0°C. The plot shows the effect of pH on the first order rate constant $V$ ($V = \text{nmoles Ca}^{2+}/10 \text{ sec.mg protein}$; $K_m = \mu M$).
quite unambiguously to the initial binding step (Cleland, 1970), but the reservation regarding possible variations in the measured values of \( K_m \) must be considered. For this reason, it is concluded that the initial binding site contains a group with a pKa of \( 7.5 \pm 0.5 \) at \( 0^\circ C \) (which allows for a ten-fold variation in the ratio of free/total \( Ca^{2+} \) at the \( K_m \)).

In attempting to identify the nature of this binding site, two other pieces of information are available; since the apparent steady-state dissociation constant for the \( Ca^{2+} \)-carrier complex is of the order of 4 \( \mu M \) (\( K_m \) at \( pH \) 7.4 and \( 0^\circ C \)) the site involved has a high affinity for \( Ca^{2+} \). Further, the specificity of the transport process requires that the relative affinity of the site for divalent cations be in the order \( Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+} > Mg^{2+} \) (Vainio et al., 1970).

The requirements for high affinity and specificity are satisfied only by a \( Ca^{2+} \) binding site involving chelation by ionized oxygens (see Williams, 1970). A complete search of the available data on pKa's and stability constants of organic compounds (Sillén and Martel, 1964; Sillén et al., 1971) has shown that no single functional group has the desired properties. The compounds which best approximate the requirements are the multidentate ligands of the NTA/EDTA/EGTA type in which the cation is chelated by three carboxyl residues in reasonably close proximity to a tertiary nitrogen (compare also with phospholipids: Dawson and Hauser, 1970). Such compounds have a high affinity for \( Ca^{2+} \) although their absolute stability constants, their relative affinity for other divalent cations, and the pKa of the nitrogen ionizing near neutrality vary with the structure of the molecule and hence with the chelate geometry. Thus, it is tentatively proposed that the binding site of the mitochondrial carrier involves (possibly) three carboxylate residues in close proximity to a tertiary nitrogen which has a pKa close to 7; the nitrogen would be that of the imidazole ring in a histidine residue (or the primary amine of an N-terminal).

This suggestion is amenable to preliminary testing by treating the mitochondria with reagents relatively specific for histidine (Westhead, 1972) or carboxylate residues (Carraway and Koshland, 1972). The most appropriate
experimental assay for subsequent inhibition of $\text{Ca}^{2+}$ transport is the passive swelling technique of Selwyn et al. (1970) which obviates the need for energy transduction.

To date the tertiary structure of a $\text{Ca}^{2+}$ binding site is known for two proteins, a carp myogen (Nockolds et al., 1972) and the extracellular nuclease of *Staphylococcus aureus* (Arnone et al., 1971). In both proteins the $\text{Ca}^{2+}$ is strongly coordinated to the carboxylate groups of two aspartate and one glutamate residue. The myogen binding site in addition contains a more weakly coordinating aspartate residue which may in part be responsible for its having a higher affinity for $\text{Ca}^{2+}$ than the nuclease (Pechère et al., 1971; cf. Cuatrecasas et al., 1967a,b).

A final implication of the data on the pH dependence of $\text{Ca}^{2+}$ transport concerns the proton efflux which has been observed to accompany $\text{Ca}^{2+}$ transport. Under the usual experimental conditions in which $\text{Ca}^{2+}$ accumulation is measured (250 mM-sucrose or 80 mM-NaCl plus buffer (pH 7.4) and respiratory substrate) there is an approximate 1:1 stoichiometry between $\text{H}^+$ efflux and $\text{Ca}^{2+}$ accumulation (Saris, 1963; Drahota et al., 1965; Rossi et al., 1966; Gear et al., 1967). Since the pKa of the $\text{Ca}^{2+}$ binding site measured in this work is close to the pH of the internal phase of the mitochondrion (Mitchell and Moyle, 1969a; Addanki et al., 1968; Gear et al., 1967), the proton efflux could be mediated by protonation of the $\text{Ca}^{2+}$ binding site during the 'recovery' phase of the carrier.

Electrophoretic $\text{Ca}^{2+}$ transport would preclude double protonation of a single $\text{Ca}^{2+}$ binding site of the carrier in its 'free' state. It should be recalled in this context that carrier-mediated $\text{Ca}^{2+}$ transport in mitochondria is comparable
to valinomycin-mediated $K^+$ transport (an electrophoretic process in which valinomycin does not undergo a change in ionization state: Läuger, 1972; Pressman et al., 1967) rather than to the non-electrophoretic $M^+/H^+$ exchange mediated by nigericin (Mueller and Rudin, 1967; Pressman et al., 1967; Pressman, 1968).

The effect of permeant 'anions':

(a) Experimental observations

The inclusion of phosphate (Rossi and Lehninger, 1964; Chance and Yoshioka, 1966) or acetate (Rasmussen et al., 1965; Mela and Chance, 1968) in the incubation medium increases both the rate and extent of $Ca^{2+}$ uptake by mitochondria. The latter effect is well documented but there has been no substantial attempt to analyse the kinetics of $Ca^{2+}$ transport in the presence of these anions. Such an approach should be useful in determining the mechanism of their stimulation and hence the basic mechanism of the $Ca^{2+}$ carrier.

Initial rates were measured in the first instance in a medium unbuffered for $Ca^{2+}$ (Fig. 3.7). The double-reciprocal plots of initial velocity as a function of total $[Ca^{2+}]$ are linear (Fig. 3.7a; cf. Fig. 3.4). From this data it is obvious that phosphate increases the maximum velocity of $Ca^{2+}$ transport, but its effect on the $K_m$ is not clear. The abscissa intercepts decrease with increasing phosphate concentration but they are sufficiently close to suggest that the $K_m$ may remain constant; the apparent increase in $K_m$ may be due to a 'pseudo-competitive' inhibition associated with the chelation of $Ca^{2+}$ by phosphate.

This is more obvious in the double-reciprocal plots of the initial velocity of $Ca^{2+}$ transport as a function of phosphate concentration at various (total) concentrations of $Ca^{2+}$ (Fig. 3.7b). Each curve has three separate components: a plateau at high $\frac{1}{F_1}$ which asymptotes to the velocity of $Ca^{2+}$ transport in the absence of phosphate; a descending portion which represents the usual substrate/velocity relation; and a vertical asymptote to the ordinate typical of substrate inhibition.

This data bears out the earlier comments regarding the
Fig. 3.7. Influence of phosphate on the rate of $Ca^{2+}$ transport

Incubations contained 0.2 mg mitochondrial protein and 250 mM-sucrose, 2 mM-succinate-tris, 5 mM-hepes-tris and phosphate-tris at the concentrations shown in a final volume of 0.28 ml. The pH was adjusted to 7.37 with HCl. Incubations were carried out for 5 sec with $^{45}Ca^{2+}$ (sp. act. 1 µCi/µmole) as shown and quenched with a mixture of 6 mM-EGTA and 1 µM-ruthenium red; the quenched mixtures were diluted with 1 ml of cold incubation medium and immediately centrifuged. The supernatants were aspirated and the pellets were washed with 1 ml of incubation medium and recentrifuged before being dissolved in Soluene for scintillation counting. Temperature was 0°C. All data are the means of two separate experiments.

(a) double-reciprocal plots of $Ca^{2+}$ transport as a function of $[Ca^{2+}](µM)$: ○, no phosphate; ●, 0.04 mM-phosphate; △, 0.1 mM; ▲, 0.5 mM.

(b) double-reciprocal plots of $Ca^{2+}$ transport as a function of $[Pi]$: ●, 18 µM-$Ca^{2+}$; ○, 36 µM-$Ca^{2+}$; △, 89 µM-$Ca^{2+}$; ▲, 179 µM-$Ca^{2+}$; ■, 357 µM-$Ca^{2+}$. 
chelation of Ca\(^{2+}\) by phosphate. They are not particularly useful, so the experiment has been repeated with a CaNTA buffer to overcome this effect (Fig. 3.8). The increase in maximum velocity is quite evident in the double reciprocal plots (Fig. 3.8b). The more interesting features of this data are the retention of the sigmoidal relationship between [Ca\(^{2+}\)] and its rate of transport in the presence of phosphate (Hill coefficient again = 1.7) and the identity of the \(K_m\) both with and without phosphate (Fig. 3.8c).

Similar experiments carried out with 10 mM-acetate show that it increases the maximum velocity more than does 2 mM-phosphate (Fig. 3.9a; cf. Fig. 3.8b). In addition it slightly increases the 'sigmoidicity' (Hill coefficient = 1.8) and increases the \(K_m\) to about 6 \(\mu\)M (Fig. 3b).

An alternative means of studying the effect of the permeant acids is to examine the time course of Ca\(^{2+}\) transport. Fig. 3.10a shows that in the presence of phosphate the data fit the irreversible first-order rate equation \(k't = \ln \frac{A_0}{A}\), where \(A_0\) is the initial Ca\(^{2+}\) concentration and \(A\) its concentration at \(t\) min. The rate constant \(k'\) agrees well with independent measurement of \(\frac{V}{K_m}\) from initial rates\(^1\). In the absence of a permeant acid, this equation cannot be used since the reaction does not go to completion. The data have therefore been treated as a reversible first-order reaction \(((k' + k'')t = \ln \frac{A_0 - A_{eq}}{A - A_{eq}})\) where \(k'\) and \(k''\) are the rate constants for the forward and reverse reactions and \(A_{eq}\) is the equilibrium concentration of Ca\(^{2+}\) outside the mitochondria. \(k'\) can be estimated from the initial slope of the irreversible plot (Fig. 3.10a) when the reverse reaction is negligible.

\(^1\) The integrated Michaelis equation is

\[
\frac{V}{K_m} \cdot t = \ln \frac{A_0}{A} + \frac{(A_0 - A)}{K_m}
\]

A plot of \(\frac{1}{t} \cdot \ln \frac{A_0}{A} \text{ versus } \frac{(A_0 - A)}{t}\) should be linear with a slope of \(\frac{1}{K_m}\) and an intercept of \(V\). It is not clear why the present data are described by the first-order expression alone.
Fig. 3.8. Influence of phosphate on the rate of Ca$^{2+}$ transport (NTA-buffered Ca$^{2+}$)

The incubation procedure was similar to that described for Fig. 3.5 using 0.25 mg mitochondrial protein in 20 sec incubations.

(a) Transport rate as a function of free Ca$^{2+}$ concentration in the presence of 2 mM- (●) or 0.5 mM-phosphate-tris (○) and in its absence (△).

(b) Double-reciprocal plots of (a). $v = \text{nmol Ca}^{2+}$ transported/20 sec.mg protein. Ca = WM free Ca$^{2+}$.

(c) Hill plots of the data of (a) for the experiments with phosphate present. The line is the best fit to the data of Fig. 3.5c; i.e. in the absence of phosphate.
Fig. 3.9. Influence of acetate on the rate of $\text{Ca}^{2+}$ transport (NTA-buffered $\text{Ca}^{2+}$)

The incubation procedure was similar to that described for Fig. 3.8.

(a) Double reciprocal plot of the initial rate of $\text{Ca}^{2+}$ transport as a function of the free $\text{Ca}^{2+}$ concentration ($\mu$M) in the presence (■) or absence (O) of 10 mM-acetate-tris. $v = \text{nmoles Ca}^{2+} \text{ transported/20 sec.mg protein}$.

(b) Hill plot of the data of (a) with acetate present. The line of best fit for Figs. 3.5c and 3.8c has been included for comparison.
Incubations contained 250 mM-sucrose, 2 mM-succinate-tris, 2 mM-hepes-tris (pH 7.4) (Δ), and in addition either 10 mM-acetate-tris (○) or 2 mM-phosphate-tris (●) in a total volume of 2 ml. Temperature 0°C. Mitochondria (4.0 mg protein) were preincubated for 1 min prior to the addition of 175 μM-45Ca2+ (sp.act. 0.2 μCi/μmole). At the times shown thereafter, 0.1 ml aliquots were removed into 0.1 ml of ice-cold incubation medium containing in addition 2 mM-EGTA-tris and 1.8 μM-ruthenium red. At the end of the experiment, all tubes were centrifuged (Eppendorf: 2 min at 12000 x g) and 0.1 ml aliquots of supernatant were counted for radioactivity. The parameters of the plot are derived from the irreversible first-order rate equation

\[ t = \frac{2.303}{k'} \cdot \log \frac{A_0}{A} \text{ (min)} \]

(b) Reversible first-order plot of Ca₂⁺ transport in the absence of a permeant acid

The data of (a) (Δ) replotted according to the reversible first-order rate equation

\[ t = \frac{2.303}{(k'+k'')} \cdot \log \frac{A_0 - A_{eq}}{A - A_{eq}} \text{ (min)} \]
The linearity of the plot (Fig. 3.10b) suggests that the reaction is reversible first-order. Normally one could evaluate the equilibrium constant for the reaction from this data \( K_{eq} = \frac{k'}{k''} \) but it must be noted that in deriving the rate constants, the concentration of internal (transported) \( Ca^{2+} \) has been obtained simply by subtracting the external concentration at time \( t \) from the initial concentration: it thus included both bound and free internal \( Ca^{2+} \) with the former probably constituting the major fraction. The calculation of \( k'' \) assumes the transported \( Ca^{2+} \) to be completely free in a volume equivalent to the total incubation volume.

The data obtained with acetate (Fig. 3.10a) also deviate from the linear first-order reversible plot, but to a lesser degree (this has been verified in additional experiments). The reversibility of the \( Ca^{2+} \) carrier is seen directly in the data of Fig. 3.11 which show the EGTA-induced efflux of \( Ca^{2+} \) from pre-loaded mitochondria. In the absence of a permeant acid, approximately 20 per cent of the internal \( Ca^{2+} \) is removed in 20 min at \( 0^\circ C \). With acetate, the rate of efflux is much slower while with phosphate no detectable efflux occurs in the same time period.

The efflux occurs more rapidly at higher temperatures (Fig. 3.12) and goes to near completion in about 60 min at \( 25^\circ C \) (data not shown). It is inhibited by a very low concentration of ruthenium red and is therefore assumed to be carrier-mediated (see later).

(b) Interpretation

When the foregoing data are considered in conjunction with the known characteristics of mitochondrial \( Ca^{2+} \) transport, a rational picture of the carrier mechanism begins to emerge. The first point is trivial, but nonetheless important to the design of these experiments: the fact that the maximum velocity can be increased simply by including permeant acids which by themselves have no effect on energy generation or maintenance shows that the energy source is not the rate-limiting component in \( Ca^{2+} \) transport.
Fig. 3.11. **EGTA-induced efflux of Ca\(^{2+}\) from pre-loaded mitochondria (0°C)**

Incubations contained 4.1 mg mitochondrial protein in 250 mM-sucrose, 2 mM-succinate-tris, 5 mM-hepes-tris (pH 7.4) and where shown 10 mM-acetate-tris or 2 mM phosphate-tris, in a total volume of 2.1 ml. Mitochondria were pre-loaded with 400 nmoles \(^{45}\)Ca\(^{2+}\) (0.1 µCi/µmole) and at the time shown (arrow) 2 mM-EGTA-tris (○) or a mixture of 2 mM-EGTA and 2 µM-ruthenium red (●) were added to the incubation mixture. 200 µl samples were centrifuged (Eppendorf: 2 min at 12,000 x g) at the indicated times and 100 µl aliquots of supernatant were counted for radioactivity. The temperature was 0°C.
Fig. 3.12. EGTA-induced efflux of Ca$^{2+}$ from pre-loaded mitochondria (25°C)

The procedure was exactly as for Fig. 3.11 except that the temperature was 25°C.
The lack of effect of up to 2 mM-phosphate on the $K_m$ for $\text{Ca}^{2+}$ further shows that $k_3$, the rate constant for the limiting step in $\text{Ca}^{2+}$ transport, is insignificant compared with $k_2$ (and $k_1$) (refer to model III above) and it remains rate-limiting in the presence of phosphate. A corollary is that the $K_m$ can be regarded as the (steady-state) dissociation constant $\frac{k_2}{k_1}$ of the $\text{Ca}^{2+}$-carrier complex.

Conversely, the increase in $K_m$ induced by 10 mM-acetate, together with the higher maximum velocity, suggests that under these conditions the rate constant for the (formerly) limiting step no longer is the sole determinant of the velocity of transport.

The next question of course is what is the rate-limiting step? Considering first the inward movement of the loaded carrier, it has been concluded both here (Introduction) and elsewhere (Lehninger, 1970; Skulachev, 1971; Chance and Montal, 1971; Greville, 1969) that this is an electrophoretic movement in response to a membrane potential. This could conceivably be rate-limiting (cf. $K^+/\text{monactin}$; Läuger, 1972), but if so it would not be accelerated by co-transport of the electrically neutral species, acetic acid and 'phosphoric acid' ($\text{H}_2\text{PO}_4^-/\text{H}^+$) (see later). The same argument can be extended to the final recovery step of the carrier. The possibility of a direct interaction of these compounds with the carrier is ruled out by the constancy of the $K_m$ and Hill coefficient during phosphate stimulation and by the lack of effect of phosphate when its transport is inhibited by mersalyl (Tyler, 1968) or DTNB (Haugaard et al, 1969).

It is therefore concluded that the rate-limiting step in $\text{Ca}^{2+}$ transport is its dissociation from the carrier in the internal phase of the mitochondrion (cf. $K^+/\text{valinomycin}$: Läuger, 1972). The means by which acetate and phosphate could increase the dissociation rate is apparent from the pH dependence of the rate constant for $\text{Ca}^{2+}$ binding (above) and the known interaction of these anions with mitochondria. The latter aspect will now be discussed in more detail.
Phosphate penetrates the inner mitochondrial membrane in a carrier-mediated reaction, its final distribution in the inner and outer phases being governed by the $\Delta p\text{H}$ across the membrane (Chappell and Haarhoff, 1967; Tyler, 1968; Mitchell and Moyle, 1969b; Palmieri et al, 1970; McGivan and Klingenberg, 1971).

At all external $p\text{H}$ values between six and eight, the counter-movement of $\text{OH}^-$ (or inward movement of $\text{H}^+$) fully compensates the anionic charge of phosphate (McGivan and Klingenberg, 1971). From this fact, from the quantitative dependence of its distribution on both $\Delta p\text{H}$ and the external $p\text{H}$, and from the sensitivity of anion uptake to uncouplers (which increase the permeability of the membrane to $\text{H}^+$: Liberman et al, 1969) it has been concluded that the net uptake process is equivalent to transport of the electrically-neutral species phosphoric acid. In real terms, this is achieved by coupling $\text{H}_2\text{PO}_4^-$ uptake to simultaneous $\text{H}^+$ uptake or $\text{OH}^-$ ejection (McGivan and Klingenberg, 1971).

The uptake and equilibrium distribution of acetate is accompanied and governed by similar relationships (Chappell and Haarhoff, 1967; Mitchell and Moyle, 1969b; Palmieri and Quagliariello, 1969; Palmieri et al, 1970; McGivan and Klingenberg, 1971), but its mode of entry to the internal phase is probably by diffusion of the associated acid.

These two processes can be represented as follows:
The net result of the transport of both phosphate and acetate is to decrease the internal pH\(^1\) (cf. McGivan and Klingenberg, 1971) (although the extent to which this occurs depends on their internal concentration which in turn is a function of their external concentration and of the initial pH).

The data of Fig. 3.6 show that the external binding site of the Ca\(^{2+}\) carrier has a pKa near 7.5 and that the rate constant for Ca\(^{2+}\) binding is largest at high pH. Conversely, the rate constant for Ca\(^{2+}\) release is highest at low pH. If Ca\(^{2+}\) remains bound to the same (or a similar) site during its transport, the same may be said of its release to the internal phase which is proposed to be the rate-limiting step. The stimulation of the maximum velocity of Ca\(^{2+}\) transport by phosphate and acetate is thus ascribed quite simply to their causing a decrease in pH of the internal phase. From the relation between pH and the first-order rate constant for Ca\(^{2+}\) binding (Fig. 3.6) it can be calculated that a fall in internal pH of 0.3 units in the region of 7.4 would double the rate constant for release and consequently double the maximum velocity. Measurements of the intramitochondrial pH of heart mitochondria by Addanki et al. (1968), while not strictly comparable, suggest that a decrease of this magnitude may be induced by phosphate and acetate. The intramitochondrial pH should obviously be studied in detail under the conditions of the present experiments.

The concept that the anions discussed above stimulate the initial rate of Ca\(^{2+}\) transport by virtue of their being permeant is closely allied to their role in increasing the extent of Ca\(^{2+}\) uptake. It has long been known that the uptake of Ca\(^{2+}\) in the absence of such an anion is accompanied by ejection of protons into the external medium (Saris, 1963) and by a corresponding alkalinization of the internal phase.

\(^1\)The non-stoichiometric removal of protons from the outer phase (0.7 H\(^+\)/H\(_2\)PO\(_4^-\) transported) represents the approximate ratio of \([\text{HPO}_4^{2-}]\)/\([\text{HPO}_4^{2-}] + [\text{H}_2\text{PO}_4^-] + [\text{H}_3\text{PO}_4] \) at pH 7.4 (McGivan and Klingenberg, 1971).
(Addanki et al, 1968; Gear et al, 1967; Chance and Mela, 1966b,c) which is probably associated with the ionization of membrane components (Chance and Mela, 1966b,c; Gear et al, 1967). Under such conditions, both Ca\(^{2+}\) uptake and the respiratory stimulation associated with it cease when approximately 100 nmoles Ca\(^{2+}\)/mg protein have been accumulated (Chance, 1965; Rossi and Lehninger, 1964); at this stage, respiration is actually inhibited and the respiratory carriers enter a unique state (Chance, 1965) which has been designated state 6 (Chance and Schoener, 1966). The inhibition may be due directly to the increased internal alkalinity associated with Ca\(^{2+}\) accumulation or it may be a consequence of strong binding of Ca\(^{2+}\) to ionized respiratory components (cf. Vinogradov et al, 1972).

The internal alkalinization and respiratory inhibition associated with state 6 can be reversed or prevented by the addition (and subsequent uptake) of phosphate (Chance, 1965; Rossi and Lehninger, 1964) or acetate (Rasmussen et al, 1965; Mela and Chance, 1968; Chance and Yoshioka, 1966) as would be anticipated from the dependence of their distribution ratio on \(\Delta\text{pH}\). Lehninger et al (1967) mention that the permeant acids propionate and arsenate have similar effects (see also Chappell et al, 1963; Chappell and Haarhoff, 1967). A recent report by Elder and Lehninger (1973) provides striking evidence of the necessity for the penetration of the acidic form and its subsequent dissociation inside the mitochondria, since they were able to show that CO\(_2\) is the permeant species in bicarbonate-stimulated Ca\(^{2+}\) uptake but it must then be converted to HCO\(_3^-\) (by an intramitochondrial carbonic anhydrase) for it to have an effect on Ca\(^{2+}\) uptake.

This emphasizes the point made above: the requirement for relief of state 6 is for a permeant acid, not for a permeant anion. The effects of such agents are due to neutralization of the internal alkalinity resulting from proton efflux during Ca\(^{2+}\) uptake, not to charge neutralization by co-transport of the anion with Ca\(^{2+}\). These facts have been obvious for many years. It is to be hoped that the misleading concept of permeant anions influencing Ca\(^{2+}\) uptake will be discarded.
These remarks are best illustrated in the diagram of Fig. 3.13 in which the measured stoichiometries of H\(^+\) ejection and permeant acid uptake to Ca\(^{2+}\) uptake are accommodated. The data is derived from Rasmussen et al (1965), Gear et al (1967) and Rossi and Lehninger (1963) for a low K\(^+\) medium at pH 7.4. It should be noted that in each case the transport of one Ca\(^{2+}\) ion results in a net increase of one positive charge in the internal phase. This must obviously be compensated for, but the nature of the counter-ion(s) is not known (Lehninger et al, 1967). It is assumed to be similar in all cases and has been omitted for clarity.

From the foregoing discussion, one could predict that a permeant anion would have no effect on either the extent or the rate of Ca\(^{2+}\) uptake. Thiocyanate provides a means of testing this since it has been shown to penetrate the inner membrane as the dissociated species SCN\(^-\) (Mitchell and Moyle, 1969b; Palmieri et al, 1970; Selwyn et al, 1970). The oxygen polarograph tracings of Fig. 3.14 compare the respiratory stimulation induced by Ca\(^{2+}\) in the absence of penetrant species, in the presence of the permeant acids acetate, phosphate and CO\(_2\) and in the presence of SCN\(^-\).

The stimulation of the rate and extent of the Ca\(^{2+}\) response by the acids is in agreement with previous work (Chance, 1965; Rasmussen et al, 1965; Elder and Lehninger, 1973). On the other hand, SCN\(^-\) has no apparent effect on either parameter.

The final tracing shows the result of a similar experiment in which the mitochondria were supplemented with the permeant base NH\(_3\) (as NH\(_4^+\)) (Chappell and Haarhoff, 1967). It was anticipated that this would inhibit the Ca\(^{2+}\) response due to an increase in internal alkalinity of the mitochondria, but it too had no effect. It seems likely that the distribution of NH\(_4^+\) across the membrane is governed by \(\Delta pH\) as with the permeant acids, and that under the conditions of the experiment the distribution ratio was unfavourable (cf. Chance and Mela, 1966c).

The lack of effect of SCN\(^-\) (and NH\(_4^+\)) is seen quite clearly in Fig. 3.15 which shows the time course of Ca\(^{2+}\) uptake. These results eliminate the possibility of a stimulation of Ca\(^{2+}\) transport by co-transport of a counterion.
Fig. 3.13. Stoichiometry of Ca\(^{2+}\), proton and anion movements during respiration-supported Ca\(^{2+}\) uptake

The quantitative relationships are derived from Rasmussen et al (1965), Gear et al (1967), Rossi and Lehninger (1963) and McGivan and Klingenberg (1971).

(a) No permeant acid
Net: \(-1 \text{Ca}^{2+} + 1 \text{H}^+\)

(b) Acetate (50 mM)
Net: \(-1 \text{Ca}^{2+} - 0.8 \text{Ac}^- + 0.2 \text{H}^+\)

(c) Phosphate
Net: \(-1.7 \text{Ca}^{2+} - 1 \text{Pi} + 0.7 \text{H}^+\)
But calcium phosphate precipitates inside the mitochondria (under the appropriate conditions) with net acidification. The product is probably hydroxylapatite (Rossi and Lehninger, 1963; Greenawalt et al., 1964; Thomas and Greenawalt, 1968):

\[
\begin{align*}
\text{Net:} & \quad -1.7 \text{ Ca}^{2+} \\
& - 1 \text{ Pi} \\
& + 1.7 \text{ H}^+
\end{align*}
\]

The $\text{H}^+/\text{Ca}^{2+}$ stoichiometry is less in a phosphate-supplemented medium than in a medium lacking a permeant acid (Saris, 1963; Chance, 1965; Gear et al., 1967). The extent of the depression (generally to $\sim0.8$) would be a function of the internal pH and of the stability and solubility constants of various calcium phosphate species.
Fig. 3.14. Effect of various permeant species on Ca\(^{2+}\)-stimulated respiration

Mitochondria (5.5 mg protein) were added (arrow) to the vessel of an oxygen polarograph containing 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4) and 5 mM-succinate-tris in a total volume of 3.1 ml. The incubations also contained 5 mM-acetate-tris (pH 7.4), 4 mM-phosphate-tris (pH 7.4), 0.5 ml of CO\(_2\)-saturated water (acidic), 5 mM-KSCN or 10 mM-NH\(_4\)Cl as shown. Further additions were 322 \(\mu\)M-Ca\(^{2+}\) and 1 mM-acetate-tris. Temperature 30°C.

Fig. 3.15. Time course of Ca\(^{2+}\) uptake in the presence of SCN\(^-\) and NH\(_4\)\(^+\)

Incubations contained 250 mM-sucrose, 2 mM-succinate-tris, 5 mM-hepes-tris (pH 7.4) (O), and in addition either 10 mM-KSCN (●) or 10 mM-NH\(_4\)Cl (△) in a total volume of 2.08 ml. Temperature 0°C. Mitochondria (4.1 mg protein) were preincubated for 1 min prior to the addition of 200 \(\mu\)M-\(^{45}\)Ca\(^{2+}\) (sp.act. 0.1 \(\mu\)Ci/\(\mu\)mole). At the times shown thereafter, 0.1 ml aliquots were removed into 0.1 ml of ice-cold incubation medium containing in addition 2 mM-EGTA and 2 \(\mu\)M-ruthenium red. At the end of the experiment, all tubes were centrifuged (Eppendorf: 2 min at 12,000 \(\times\) g) and 0.1 ml aliquots of supernatant were counted for radioactivity.
The electrophoretic nature of Ca\textsuperscript{2+} transport (see Introduction) implies that the total extent of Ca\textsuperscript{2+} uptake under all experimental conditions is determined solely by the equilibrium distribution ratio of free Ca\textsuperscript{2+} across the membrane. This in turn is a function of the membrane electrochemical potential. The constancy of the distribution ratio of K\textsuperscript{+} in coupled mitochondria under a variety of conditions has been well demonstrated by Rottenberg (1973). It is impossible at this stage to determine the equilibrium ratios of free Ca\textsuperscript{2+} since a large fraction of the internal Ca\textsuperscript{2+} is bound (Gear et al, 1967) but it would be anticipated that this too would be constant in the steady states at the cessation of net Ca\textsuperscript{2+} uptake in the absence and in the presence of acetate and phosphate\textsuperscript{1}. It should be noted that the greater net uptake of Ca\textsuperscript{2+} in the presence of acetate is compensated (probably quantitatively) by the increased volume of the matrix space (Rasmussen et al, 1965; See also Rottenberg, 1973). In the case of phosphate-stimulated transport, the internal concentration of free Ca\textsuperscript{2+} remains very low due to calcium phosphate precipitation (Greenawalt et al, 1964; Thomas and Greenawalt, 1964) ensuring that uptake in this case goes virtually to completion.

The reversibility of the carrier implicit in this discussion is shown by both analyses of the time course of Ca\textsuperscript{2+} uptake (Figs. 3.10a,b) and by the efflux induced in loaded mitochondria by EGTA (Figs. 3.11,12). The efflux is interpreted as an attempt to re-establish the steady-state distribution ratio of free Ca\textsuperscript{2+}. Its very slow rate confirms the accuracy of the simple kinetic model III for the Ca\textsuperscript{2+} carrier in which the rate of the overall reverse reaction is assumed to be negligible.

Comparison of the rates of efflux in the presence and absence of acetate again demonstrates the importance of pH

\textsuperscript{1}Since the alkalinity (and increased internal binding of Ca\textsuperscript{2+}) of state 6 causes an inhibition of respiration, the membrane potential may be less than in the non-inhibited steady state. If this is the case, the distribution ratio of Ca\textsuperscript{2+} across the membrane would be less in state 6.
in controlling the rate of $\text{Ca}^{2+}$ transport. The major differences between the experimental conditions in these two cases is the pH of the internal and external phases, it being lower inside and higher outside in the presence of acetate in the steady-state following $\text{Ca}^{2+}$ accumulation (refer above). In terms of the pH dependence of $\text{Ca}^{2+}$ binding to the carrier, both these factors would tend to decrease the rate of efflux in the acetate-supplemented mitochondria by decreasing the rates of both internal binding and external release. The relative importance of these two factors could be assessed by varying the external pH by altering the pH of the EGTA solution used to initiate efflux.

The most fruitful studies of the efflux would require a knowledge of the internal free $\text{Ca}^{2+}$ concentration. Until there is some means of measuring this, estimates of the steady-state distribution ratio of $\text{Ca}^{2+}$ and of the kinetics of efflux must remain qualitative.

Inhibition by lanthanides and ruthenium red

The earliest evidence for the involvement of a specific carrier in mitochondrial $\text{Ca}^{2+}$ transport was the demonstration by Mela (1967, 1968a,b) of the marked inhibition by $\text{La}^{3+}$ of $\text{Ca}^{2+}$-associated responses. This was later extended to show that $\text{Ca}^{2+}$ transport itself (measured with the murexide technique) is inhibited by all rare earth cations at concentrations extrapolating to complete inhibition at less than 0.1 nmoles lanthanide/mg protein (Mela, 1969a; Vainio et al., 1970; Scarpa and Azzone, 1970), a figure which has been used as a maximum estimate of the concentration of carrier sites in the mitochondrion (Mela and Chance, 1969).

Moore (1971) has reported that ruthenium red also is a potent inhibitor of respiration-supported $\text{Ca}^{2+}$ uptake, and detailed studies by Vasington et al. (1972a,b) have established that, at low concentrations, the inhibition by ruthenium red is specific for reactions associated with $\text{Ca}^{2+}$ transport. However, there has been no attempt to define its mechanism of inhibition in terms of a kinetic analysis of the inhibition of $\text{Ca}^{2+}$ transport.
In previous studies with ruthenium red, a number of features have been overlooked. Thus Moore (1971) used a molecular weight of roughly one-third the correct value (Fletcher et al., 1961) based on the data of Morgan and Burstall (1936), and neither in that work nor in the subsequent studies of Vasington et al. (1972a,b) is there any indication that recrystallised ruthenium red was used. Since commercial preparations generally contain less than 20 per cent ruthenium red (Luft, 1971; see Appendix), it must be assumed that their data apply to such a crude mixture and that the molar concentrations cited have little relevance to the actual amount of ruthenium red present. These points are discussed in detail in a later chapter but it should be noted that the ruthenium red used in all experiments reported in this chapter had been recrystallised to spectroscopic purity (Fletcher et al., 1961; Luft, 1971).

The development of the EGTA-quench technique provides the means of determining the mechanism of carrier inhibition by the lanthanides and ruthenium red by an analysis of the kinetics of Ca\(^{2+}\) transport. Before such studies were undertaken, the gross characteristics of their inhibition were determined by examining their effect on Ca\(^{2+}\)-stimulated respiration. Acetate was included in these experiments to ensure a constant high rate of Ca\(^{2+}\) uptake for a time sufficient to permit accurate measurement of the rate of oxygen consumption.

When La\(^{3+}\) is added a few seconds before Ca\(^{2+}\) (Fig. 3.16a), the normal response is modified; respiration is still stimulated by Ca\(^{2+}\), but to a lesser extent, and only after a lag period which is due to the initial strong inhibition of Ca\(^{2+}\) transport (Mela, 1969a; Lehninger and Carafoli, 1971). The potency of the inhibition is not obvious from these experiments, since 1.8 nmoles La\(^{3+}\)/mg protein inhibited the maximally-stimulated rate only by about 50 per cent (cf. Mela, 1969a; Vainio et al., 1970).

The final trace of Fig. 3.16a shows that both the initial lag period and the subsequent partial inhibition of
Fig. 3.16. Effect of preincubation time on the inhibition of Ca\textsuperscript{2+}-stimulated respiration by La\textsuperscript{3+} and ruthenium red

(a) The incubations contained 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 5 mM-succinate-tris, 10 mM acetate-tris, 0.33 µM-rotenone and 5.4 mg mitochondrial protein (RLM) in a final volume of 3.1 ml; the temperature was 30\textdegree C. Additions of Ca\textsuperscript{2+} (323 µM) and La\textsuperscript{3+} (3.1 µM) were made at the times shown.

(b) Incubation conditions were similar to those of (a) except that 4.3 mg mitochondrial protein was used. Ruthenium red (RR) was added as shown at a concentration of 0.265 µM. Numbers below the traces are rates of oxygen uptake in ng atoms of oxygen/min.mg protein.
respiratory stimulation are abolished by preincubating the mitochondria with La^{3+} for about five min prior to Ca^{2+} addition. The recovery of the respiratory response with increased incubation time, both in the presence and absence of Ca^{2+}, is due to the accumulation of La^{3+} by mitochondria (Reed and Bygrave, 1973a) as had been suggested by Mela (1968a, 1969b).

Fig. 3.16b shows the results of similar experiments with ruthenium red. The inhibition of Ca^{2+}-stimulated respiration by this compound remains constant over long periods (Fig. 3.16b, final trace) and is more complete, even at very low concentrations. Ruthenium red thus remains bound to the Ca^{2+} carrier for a considerable time, in contrast to La^{3+}.

When the rate of oxygen uptake immediately following Ca^{2+} addition is plotted as a function of ruthenium red concentration, a sigmoidal curve is obtained (Fig. 3.17a). The cause of the sigmoidal shape is not clear; it is not seen in the inhibition of Ca^{2+} uptake itself (see below). Virtually complete inhibition of the respiratory response is attained with 0.15 nmoles ruthenium red/mg protein.

The effect of ruthenium brown (the structurally similar oxidised form of ruthenium red; Fletcher et al, 1961) is indistinguishable from that of ruthenium red both in these experiments (Fig. 3.17a) and in those concerned with the initial rate of Ca^{2+} transport. Spectroscopic studies showed that the brown complex is immediately reduced to ruthenium red on its addition to a mitochondrial suspension. It is also reduced in the incubation medium alone, but in a comparatively slow reaction.

A crude filtered solution containing less than 30 per cent ruthenium red (see later) had inhibitory properties qualitatively similar to those of the pure complex (Fig. 3.17b). However, it was far more potent in terms of the amount of ruthenium red required for complete inhibition (about 0.02 nmoles/mg protein). This value refers to the concentration of ruthenium red itself in the crude solution, calculated from $E_{534}$ (Luft, 1971); obviously some low molecular weight
Fig. 3.17. Inhibition of Ca\(^{2+}\)-stimulated respiration by ruthenium complexes

(a) Pure ruthenium red and ruthenium brown. Incubation conditions were identical to those of Fig. 3.16b. Mitochondria were preincubated with the indicated concentration of ruthenium red (●) or ruthenium brown (○) for 15 sec prior to the addition of 323 µM-Ca\(^{2+}\).

(b) Crude ruthenium red. Incubations contained 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 5 mM-succinate-tris (■) and either 2 mM-phosphate-tris (○) or 10 mM-acetate-tris (●), with 4.2 mg mitochondrial protein in a final volume of 2 ml; the temperature was 25°C. Mitochondria were preincubated for 15 sec with the indicated concentration of crude ruthenium red (see 'Appendix') prior to the addition of 500 µM-Ca\(^{2+}\).
component(s) of the unpurified solution possess inhibitory properties similar to those of ruthenium red (see later).

In the experiment of Fig. 3.18, the EGTA quenching method was used to examine the inhibition of $Ca^{2+}$ transport by $Nd^{3+}$, a rare earth cation which has effects similar to those of $La^{3+}$ (see also Fig. 3.19 below, and Mela, 1969a). This experiment was done at $10^\circ C$ with a 60 sec preincubation of the mitochondria with $Nd^{3+}$, the two sets of data being obtained with immediate filtration on the one hand, and with a three min delay prior to filtration on the other. The agreement between the results is confirmatory evidence for the validity of the quench technique.

Under the conditions of this experiment, the inhibition of $Ca^{2+}$ uptake is sigmoidal. It seemed possible that this was a consequence of $Nd^{3+}$ uptake during the preincubation (Reed and Bygrave, 1973a) so in subsequent experiments a shorter preincubation time and lower temperature were used. The results of such an experiment with $La^{3+}$ and $Nd^{3+}$ as the inhibitors, carried out at $0^\circ C$ with a five sec preincubation are shown in Fig. 3.19a. Comparison with Fig. 3.18 shows that inhibition occurs at lower concentrations, and under these conditions it is not sigmoidal; analysis of the effects of lanthanides on $Ca^{2+}$ transport is thus critically dependent on the incubation conditions.

The initial slope of inhibition curves similar to that shown in Fig. 3.19a has, in previous reports (e.g. Mela and Chance, 1969), been extrapolated to the abscissa to give an estimate of the number of lanthanide binding sites associated with the $Ca^{2+}$ carrier. The incompleteness of inhibition at higher concentrations has been assumed to indicate the presence of lanthanide-insensitive $Ca^{2+}$-binding sites on the carrier (Mela, 1969a; Vainio et al, 1970). However, it is obvious from the high potency of the lanthanides that they must be considered as 'tightly-bound' inhibitors and the kinetic data should thus be analysed according to treatments designed specifically for this class (Straus and Goldstein, 1943; Morrison, 1969; Henderson, 1972).
Fig. 3.18. Inhibition of the initial rate of Ca$^{2+}$ transport by Nd$^{3+}$ (1 min preincubation).

Incubations contained 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4) and 2 mM-succinate-tris in a total volume of 0.5 ml. Mitochondria (1 mg protein) were preincubated with Nd$^{3+}$ for 1 min prior to the addition of 200 μM-$^{45}$Ca$^{2+}$ (0.05 μCi); incubation was continued for 5 sec and quenched by the addition of 1 mM-EGTA-tris. Aliquots of 50 μl were filtered 10 sec (○) or 3 min (○) after quenching, washed and assayed for radioactivity. Incubation temperature was 10°C.
Inhibition of the initial rate of Ca$^{2+}$ transport by lanthanides (5 sec preincubation)

(a) Incubations contained 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4) and 2 mM-succinate-tris in a total volume of 0.54 ml in Eppendorf microcentrifuge tubes. Mitochondria (1.0 mg protein) were preincubated for 1 min without inhibitors then for 5 sec with Nd$^{3+}$ (○) or La$^{3+}$ (□). Each incubation was started by the addition of 100 nmoles $^{45}$Ca$^{2+}$ (0.01 µCi) and was continued for 10 sec before being quenched with 1 µmole EGTA-tris. The tube was immediately centrifuged (2 min at 12,000 x g), the supernatant was removed and the pellet was prepared for scintillation counting by dissolution in 0.1 ml Soluene. Incubation temperature was 0°C.

(b) Dixon plot. Experimental conditions were similar to those of (a) except that 0.93 mg protein was present and the incubation time was 5 sec. The concentrations of $^{45}$Ca$^{2+}$ and EGTA-tris used for starting and stopping incubations were 185 µM and 1.85 mM (○) or 46 µM and 0.46 mM (□) respectively. The ordinate is $\frac{1}{v}$ where $v$ is the initial rate of Ca$^{2+}$ transport expressed as nmoles Ca$^{2+}$ in mitochondria/5 sec.mg protein. The inhibitor used was La$^{3+}$.
The most informative derived plot for such data is that of \( \frac{1}{1-\alpha} \) against \( \frac{1}{\alpha} \), where \( \alpha = \frac{v_i}{v_0} \), the ratio of the inhibited to the uninhibited rate at the same concentration of substrate when the total inhibitor concentration is \( i_t \) (Henderson, 1972). This plot is linear, and from it both the concentration of inhibitor binding sites (\( E_t \)) and the dissociation constant for the inhibitor (\( K_i \)) can be obtained, provided that certain conditions are met. The basic condition which determines the nature of the plots is the value of the ratio \( \frac{E_t}{K_i} \).

Fig. 3.20, taken from Henderson's paper on the kinetics of tight-binding inhibitors (Henderson, 1972), illustrates the three zones of kinetic behaviour (Straus and Goldstein, 1943) as \( \frac{E_t}{K_i} \) varies. At very high values of \( \frac{E_t}{K_i} \) the dose-response plot is linear (a) and the concentration of inhibitor binding sites can be obtained directly by extrapolation; however, in this case the \( K_i \) (dissociation constant for the enzyme-inhibitor constant) can not be evaluated since the slope of the derived plot is zero (b). At very low values of \( \frac{E_t}{K_i} \) the dose-response plot is independent of enzyme concentration (e) and the derived plot extrapolates to the origin (f). This represents the limiting situation where most of the inhibitor is free, and hence the conditions under which the Michaelis-Menten equation is valid. The \( K_i \) can be evaluated by the usual procedures (e.g., Dixon, 1953) but the concentration of inhibitor binding sites, \( E_t \), cannot.

At intermediate values of \( \frac{E_t}{K_i} \) (0.01 to 100), both \( K_i \) and \( E_t \) can be determined from the derived plot (d) from the slope and from the ordinate intercept respectively.

The data from a large number of experiments using \( \text{La}^{3+} \), \( \text{Nd}^{3+} \), \( \text{Eu}^{3+} \) and \( \text{Tb}^{3+} \) have been replotted in the derived form outlined above. In all cases the plots have a positive slope that extrapolates to the origin. It is concluded that under the particular conditions used in these experiments \( \frac{E_t}{K_i} \) is \( \ll 0.01 \) (Henderson, 1972). Most of the inhibitor is therefore
values of the parameters are $K_i$, 100.0; $A_t$, 100.0; $E_t$, 0.2, 0.4, 0.6, 0.8 and 1.0; $K_i$, 0.0001 (a and b), 0.10 (c and d) and 100.0 (e and f); $I_t$ was varied as indicated in the diagrams; a unit of concentration for these parameters is omitted because the shapes of the curves depend only on the ratio of $A_t/K_i$, $I_t/K_i$, $E_t/A_t$, and $E_t/K_i$. In (c) and (f) the same line was obtained at all values of $I_t$.

**Fig. 3.20.** Relationship of dose-response curves and replots to changes in enzyme concentration at different affinities of enzyme for inhibitor.

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Abbreviations: $v_0$ = uninhibited rate; $v_I$ = rate at total inhibitor concentration $I_t$; $K_a$ = apparent Michaelis constant; $A_t$ = total substrate concentration; $E_t$ = total enzyme concentration; $K_i$ = dissociation constant for inhibitor.
free, and the data can be analysed with the classical Michaelis-Menten treatment. Consequently, the estimation of binding sites by extrapolation of the primary plot (Fig. 3.19a) is invalid (contrast with Mela and Chance, 1969), since such an analysis requires that most of the inhibitor be bound (Henderson, 1972; Morrison, 1969).

The high proportion of free inhibitor is substantiated by the linearity of the Dixon plots (Dixon, 1953) obtained with varying La$^{3+}$ concentrations at two different concentrations of Ca$^{2+}$ (Fig. 3.19b). The extrapolated lines intersect above the abscissa showing competitive inhibition (verified by double-reciprocal plots) with a $K_i$ of $2 \times 10^{-8}$ M. Scarpa and Azzone (1970) similarly have found competitive inhibition of mitochondrial Ca$^{2+}$ uptake by La$^{3+}$ using the murexide technique, but in earlier experiments with the same technique Mela (1969a) found non-competitive inhibition by Pr$^{3+}$; both groups obtained linear Dixon plots and a value for $K_i$ of $5 \times 10^{-8}$ M.

The concentration of La$^{3+}$ binding sites cannot be determined from the present data, but it has an approximate upper limit of $2 \times 10^{-9}$ M, or 0.001 nmoles/mg protein, based on the upper limit of the ratio $\frac{E_t}{K_i}$. A precise estimate would require incubations containing mitochondria at very high concentrations, but then secondary effects would probably intervene.

The effect of ruthenium red on the initial rate of Ca$^{2+}$ transport at $0^\circ$C is shown in Fig. 3.21a. The derived plots again have a positive slope but in this case they extrapolate to intersect the ordinate above the origin (Fig. 3.21b). The degree of scatter in such a plot is inevitably high (cf. Henderson, 1972) but is more so than usual in these experiments because of the relatively large timing errors involved. It has been found that the scatter is always greater in titrations with ruthenium red than with the lanthanides. The accuracy of the values obtained for the inhibitor constants has been increased by combining the data of five separate experiments (Fig. 3.21b).
Fig. 3.21. Inhibition of the initial rate of Ca$^{2+}$ transport by ruthenium red

(a) Experimental conditions were similar to those of Fig. 3.19 except that 0.95 mg protein was present and the incubation time was 5 sec. Each point is the mean of duplicate incubations.

(b) The data of 5 separate experiments similar to (a) replotted in the form discussed in the text. The abscissa is $1 - \alpha$ where $\alpha = \frac{v_i}{v_0}$ (the ratio of the inhibited rate to the uninhibited rate at the same substrate concentration) and the ordinate is $\frac{i_t}{1-\alpha}$ where $i_t$ is the total concentration of ruthenium red (nmoles/mg protein). The intercept on the ordinate gives the concentration of inhibitor binding sites ($E_t$) and the intercept on the abscissa is $-\frac{E_t}{K_i}$. 
The concentration of ruthenium red binding sites is 0.08 ± 0.01 nmoles/mg protein. However, in contrast to the binding with La\[^{3+}\], the slope of the plot is not affected by varying the concentration of Ca\[^{2+}\]. The inhibition by ruthenium red is therefore non-competitive and the slope gives $K_i$ directly, about $3 \times 10^{-8}$ M, a value similar to that for La\[^{3+}\]. There are two assumptions implicit in the above treatment of the kinetic data. The first is that the binding of lanthanides and ruthenium red to the carrier is reversible. This appears to be so for the lanthanides (Reed and Bygrave, 1973a), but has not been demonstrated for ruthenium red\(^1\). Secondly, the rate-limiting step in Ca\[^{2+}\] transport is taken to be that equivalent to substrate conversion or product release in classical enzyme theory; i.e. transport or release, rather than initial binding. The preceding data have established that this is the case.

The competitive inhibition by La\[^{3+}\] is typical of the ability of the rare earth cations to substitute for Ca\[^{2+}\] in biological systems. Both cations bind electrostatically to ionized oxygen groups although the lanthanides do so with far greater affinity by virtue of their higher charge density and similar ionic radius (Williams, 1970). However, the inhibition by ruthenium red provides no information on the nature of the Ca\[^{2+}\] binding sites since, by the definition of a non-competitive inhibitor, it does not bind at the substrate site.

A comparison is made in Table 1 between the concentrations of inhibitor binding sites associated with two mitochondrial carrier systems (the Ca\[^{2+}\] carrier, and the adenine nucleotide translocase) and the ATPase, and the concentration of 'insoluble' cytochromes. All of these membrane-associated components

---

\(^1\)The reversibility of binding of ruthenium red to the carrier could be established by measuring the loss of inhibition after passage of a fully inhibited preparation of mitochondria through a small column of Sephadex G-25. Partial reversal of inhibition would also be expected on dilution of an inhibited preparation, but the high affinity of binding would make this less sensitive.
### TABLE 3.1. The concentration of high-affinity inhibitor binding sites and membrane-bound cytochromes in rat liver mitochondria

<table>
<thead>
<tr>
<th>Membrane component</th>
<th>Ligand</th>
<th>Number of binding sites (nmoles/mg protein) (± s.d.)</th>
<th>Method used for estimation</th>
<th>Source of data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) carrier</td>
<td>Lanthanides</td>
<td>0.001</td>
<td>Inhibition of Ca(^{2+}) transport</td>
<td>Present work</td>
</tr>
<tr>
<td>Ca(^{2+}) carrier</td>
<td>Ruthenium red</td>
<td>0.08±0.01</td>
<td>Inhibition of Ca(^{2+}) transport</td>
<td>Present work</td>
</tr>
<tr>
<td>Adenine nucleotide translocase</td>
<td>ADP</td>
<td>0.14</td>
<td>(^{14})C-ADP binding (attractyloside-sensitive)</td>
<td>Weidemann et al., 1970</td>
</tr>
<tr>
<td>Adenine nucleotide translocase</td>
<td>Atractyloside</td>
<td>0.12 to 0.16</td>
<td>(^{35})S-attractyloside binding</td>
<td>Klingenberg et al, 1971b</td>
</tr>
<tr>
<td>Adenine nucleotide translocase</td>
<td>Bongkrekic acid</td>
<td>0.41 to 0.50</td>
<td>Inhibition of ATPase</td>
<td>Henderson, 1972</td>
</tr>
<tr>
<td>ATPase</td>
<td>Rutamycin</td>
<td>0.11 to 0.17</td>
<td>Inhibition of ATPase</td>
<td>Henderson, 1972</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>Antimycin</td>
<td>0.04</td>
<td>Inhibition of succinate oxidation</td>
<td>Present work</td>
</tr>
<tr>
<td>Cytochrome b (total)</td>
<td></td>
<td>0.28±0.005</td>
<td>Spectroscopy</td>
<td>Williams, 1968</td>
</tr>
<tr>
<td>Cytochrome a + a(_{3}) (total)</td>
<td></td>
<td>0.28±0.005</td>
<td>Spectroscopy</td>
<td>Williams, 1968</td>
</tr>
<tr>
<td>Cytochrome a + a(_{3}) (total)</td>
<td></td>
<td>0.26±0.007</td>
<td>Spectroscopy</td>
<td>Present work</td>
</tr>
</tbody>
</table>
are intimately associated with the energy-conserving reactions of mitochondrial respiration and the data indicate that all are present in roughly similar concentrations with the notable exception of the Ca\textsuperscript{2+} carrier. The extremely low number of La\textsuperscript{3+} binding sites is so small as to suggest that some additional factor(s) may be involved which invalidate the kinetic analysis. If the concentration of this carrier is of the order of 0.001 nmoles/mg protein, its molecular activity (single-site carrier) would be as high as 1,000-1,400 sec\(^{-1}\) at 0\textdegree C. It is perhaps relevant that Läuger (1972) has calculated that the valinomycin/K\textsuperscript{+} complex has an over-all transport rate of 2 x 10\(^{4}\) sec\(^{-1}\) in a phosphatidyl-inositol bilayer at 25\textdegree C.

However, it is unlikely that the data pertaining to lanthanide inhibition of the Ca\textsuperscript{2+} carrier are quantitatively correct. The chelation of Ca\textsuperscript{2+} by components of the incubation solution was discussed in some detail in an earlier section. Since La\textsuperscript{3+} is chelated by the same components (low affinity external binding sites of mitochondria, succinate, etc.) but approximately 100 to 1,000-fold more strongly (Sillén and Martel, 1964; Sillén et al., 1971), its free concentration also will be considerably less than its total concentration, although this will be offset to some degree by the far higher concentration of Ca\textsuperscript{2+} present. The calculated value of \(K_i\) for La\textsuperscript{3+} is probably an over-estimate, which in turn means that the concentration of La\textsuperscript{3+} binding sites on the Ca\textsuperscript{2+} carrier is less than the upper limit ascribed above.

We therefore suggest that the Ca\textsuperscript{2+} carrier has a number of Ca\textsuperscript{2+} binding sites, but the (competitive) binding of La\textsuperscript{3+} to only one of these is sufficient to cause inhibition of Ca\textsuperscript{2+} transport by virtue of its very high affinity for the carrier and its consequent slow rate of release to the internal phase (Reed and Bygrave, 1973a). The lanthanides may be able to bind to all the Ca\textsuperscript{2+} sites, but in so doing they would not further enhance their inhibition of the initial rate of Ca\textsuperscript{2+} transport. In this context, previous evidence suggests a
co-operative interaction between at least two sites is required to explain the sigmoidal relation between $Ca^{2+}$ concentration and its rate of transport (Bygrave et al., 1971a, b; Vinogradov and Scarpa, 1973; Fig. 3.6) and the stimulation of $Mn^{2+}$ transport by low concentrations of $Ca^{2+}$ (Chance and Mela, 1966a; Mela and Chance, 1968; Vinogradov and Scarpa, 1973) or lanthanides (Vainio et al., 1970).

The mechanism of the non-competitive inhibition by ruthenium red is not clear. One possibility is that it prevents the actual transport step in the carrier cycle without affecting $Ca^{2+}$ binding. Regardless of its precise mechanism, it is clear that inhibition by ruthenium red requires its binding at sites apparently 100-fold more numerous than those required for $La^{3+}$ inhibition.

**General discussion**

Although the present results are still somewhat limited they represent the first attempt at a rational study of the mechanism of the $Ca^{2+}$ carrier. A number of conclusions can be inferred from its kinetic properties, summarized in Table 3.2, and most of these have been developed throughout the preceding discussion.

The implications of the sigmoidal substrate-velocity relationship are not so clearly resolved. Such curves are usually interpreted in terms of positive homotropic co-operativity between substrate binding sites (Koshland, 1970). However, the quantitative treatment of co-operativity is predicated on the measurement of a parameter which is directly proportional to the degree of saturation, and it thus may not be possible to draw firm conclusions from velocity measurements. The reasons for this are firstly, conformational transitions prior to the rate-limiting step may not all be rapid and reversible; secondly, the rate-limiting step may not be identical for all enzyme-substrate complexes. In spite of these limitations, in almost every case of a co-operative enzyme the Hill coefficient calculated from substrate/velocity data has given an accurate value for the
TABLE 3.2. Properties of the mitochondrial $\text{Ca}^{2+}$ carrier

A summary of the features of the $\text{Ca}^{2+}$ carrier obtained from kinetic studies at $0^\circ\text{C}$ and pH 7.4.

<table>
<thead>
<tr>
<th></th>
<th>nil</th>
<th>+2 mM-phosphate</th>
<th>+10 mM-acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill coefficient</td>
<td>1.7</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>4</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>$V$ (nmoles/sec.mg protein)</td>
<td>0.4 - 0.7</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>pKa of binding group(s)</td>
<td>7.5 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor binding sites (nmoles/mg protein):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>La$^{3+}$</td>
<td>$\leq 0.001$ (competitive)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ruthenium red</td>
<td>0.08 ± 0.01 (non-competitive)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
maximum number of interacting binding sites (Koshland, 1970). We might therefore infer that the Ca\textsuperscript{2+} carrier has a minimum of two interacting binding sites for Ca\textsuperscript{2+}. A further complicating factor in the case of the Ca\textsuperscript{2+} carrier is the probability of similar co-operative interactions during the release of Ca\textsuperscript{2+} to the internal phase.

The simplest model consistent with the data of this report and with known features of the carrier is shown in the final figure (Fig. 3.22). The rate-limiting step at saturating concentrations of Ca\textsuperscript{2+} is implied to be that determined by \( k_7 \). Some other probable relationships and the bases for making them are listed in the figure.

There obviously remain many features whose quantitative significance is unknown. In particular, the nature of the second rate-limiting step which becomes apparent when Ca\textsuperscript{2+} transport is measured in the presence of high concentrations of permeant acid can only be guessed at; it may be \( k_9 \). A more positive assignment would require a knowledge of the extent to which pH influences the co-operativity of Ca\textsuperscript{2+} binding, in addition to its effect on the combined rate constants for binding.

The inhibition of lanthanides and ruthenium red could also be extremely valuable in clarifying the co-operative interactions involved in Ca\textsuperscript{2+} binding. Since La\textsuperscript{3+} binds at the same site(s) as does Ca\textsuperscript{2+} but with many times higher affinity, it would be expected to have a marked effect on the degree of co-operativity between the interacting binding sites. Studies along these lines would require NTA buffers (see earlier) with dual calculations of both free Ca\textsuperscript{2+} and free La\textsuperscript{3+} concentrations, since both ions are chelated by NTA. This approach would simultaneously permit a more accurate measurement of the \( K_i \) for lanthanides. Similar studies with ruthenium red (which is not chelated by NTA or EGTA) would assist in clarifying the basis of its non-competitive inhibition.
**Notes:**

(i) $E = \text{Ca}^{2+}$ carrier.

(ii) Degree of protonation of $\text{Ca}^{2+}$ binding sites is dependent on pH.

(iii) Dehydration/hydration of $\text{Ca}^{2+}$ and $H^+$ at membrane interfaces has been omitted: it is obviously of importance in determining interaction energies.

(iv) The following relationships between the magnitude of rate constants can be inferred:

$$
\begin{align*}
    k_3 &> > k_1, & \text{from co-operativity of } \text{Ca}^{2+} \text{ binding.} \\
    k_9 &> > k_7, & \\
    k_5 &> > k_6, & \text{from electrophoretic transport, and} \\
    k_{13} &> > k_{14}, & \text{from efflux measurements.} \\
    k_5 &> > k_{13}, & \\
    k_1 k_3 &> > k_2 k_4, & \text{from low } K_m.
\end{align*}
$$

$k_3$ and $k_5 > > k_7$ from the effect of permeant acids.

$k_1$ and $k_3$ are higher when external pH $> 7.5$ from pH dependence of binding rate.

$k_7$ and $k_9$ are higher when internal pH $< 7.5$. 

---

**Fig. 3.22. Model for mitochondrial $\text{Ca}^{2+}$ transport**
The studies carried out in Chance's laboratory (Chance 1965; Mela, 1968a; Vainio et al., 1970; Chance and Mela, 1966b,c; Chance and Yoshioka, 1966; Chance, 1967; Mela and Chance, 1968) on the kinetics of response of respiratory components and bromothymol blue (BTB) to the addition of 'saturating' concentrations of Ca\(^{2+}\) are quite consistent with the model of Fig. 3.22. In particular the oxidation of cytochrome b is most illuminating because of its location at the energy-coupling site through which the crossover of redox state occurs during Ca\(^{2+}\) uptake (Chance, 1965). The half-time for the initial oxidation of cytochrome b is 30-50 msec (Mela and Chance, 1968; Chance et al., 1969), but the effect of phosphate and acetate on its oxidation rate is not known. Its initial oxidation is followed by a slower reduction cycle to a steady state which is maintained throughout the accumulation of Ca\(^{2+}\) (Chance and Schoener, 1966).

The initial oxidation of cytochrome b might be expected to be the result of Ca\(^{2+}\) appearing at the inner surface of the membrane and hence decreasing the membrane potential. The redox state of cytochrome b would thus reflect the concentration of the intermediate E(Ca\(^{2+}\))\(^2\) at the inner surface of the inner membrane. If this interpretation is correct, the slowest of the three rate constants, \(k_1 k_3 k_5\) (Fig. 3.22), has a half-time of about 0.04 sec equivalent to a first order rate constant of 17 sec\(^{-1}\) at 15-20\(^{\circ}\)C. The first order constant for the rate of breakdown of E(Ca\(^{2+}\))\(^2\) could also be calculated from the cyclic oxidation of cytochrome b (see Chance, 1965), but comparison of these values with the measured maximum velocity of Ca\(^{2+}\) transport at a similar temperature requires a knowledge of the concentration of the carrier and of its Ca\(^{2+}\) binding sites.

The relation of the redox changes in respiratory components to the steps involved in Ca\(^{2+}\) transport could be better defined by comparing the data of Vainio et al. (1970) for the rate of cytochrome b oxidation induced by various divalent cations with kinetic studies of the transport of these cations similar to those of Ca\(^{2+}\) in the present work. Such kinetic data is insufficient at present to permit firm conclusions.
The response of BTB (in the absence of a permeant acid) is much slower than that of the cytochromes; indeed its half-time of alkalization is longer than that for complete Ca\(^{2+}\) uptake (Mela and Chance, 1968). It has been suggested that BTB remains associated with the cristal membrane and that pH changes indicated by its absorbance occur in a region of that membrane in contact with the inner phase (Chance and Mela, 1966b,c). The alkalinity measured by BTB during unaccompanied Ca\(^{2+}\) uptake is certainly in qualitative agreement with independent measurements (Gear \textit{et al}, 1967; Addanki \textit{et al}, 1968), but there is some doubt as to its quantitative significance. In particular the distribution of BTB in the inner and outer phases apparently depends on the respiratory state of the mitochondria (Mitchell \textit{et al}, 1968); it would be anticipated that, being an amphipathic weak acid, its distribution would be at least partially governed by \(\Delta p\text{H}\) across the inner membrane (cf. Palmieri \textit{et al}, 1970).

Thus, the time course of BTB response cannot be assumed to reflect the time course of proton removal from the inner phase. Until this can be measured with confidence the temporal relation between Ca\(^{2+}\) transport, respiratory responses and H\(^+\) movements must remain hypothetical.

Further refinement of the model (Fig. 3.22) can only be realized by a systematic kinetic analysis of Ca\(^{2+}\) transport along the lines developed in this chapter coupled with similar studies of cytochrome \(b\) responses. One of the major requirements is for an automated technique permitting more precise measurements of Ca\(^{2+}\) transport over a wider temperature range, but even then the quantitative evaluation of the few rate constants which can be assigned to specific steps must await measurements of the concentration of the carrier itself and of the number of Ca\(^{2+}\) binding sites associated with it. The final evaluation of all rate constants can only be achieved with a purified preparation of the carrier in a synthetic bilayer membrane (cf. Mueller and Rudin, 1969; Läuger, 1972).
Nevertheless, the data presented in this chapter provide the basis for a clearer understanding than was hitherto possible of the means by which mitochondria accumulate $\text{Ca}^{2+}$, and have led to the development of a model which is susceptible to experimental testing.

Theoretical considerations

The concept of a $\text{Ca}^{2+}$-specific carrier in the inner mitochondrial membrane has been treated in some detail in the preceding chapters. Since the binding of $\text{Ca}^{2+}$ to the carrier is an obligatory initial step in the transport process it might be possible to devise experimental conditions for measuring the concentration and dissociation constant of carrier-specific $\text{Ca}^{2+}$ binding sites. These are necessary for a more detailed analysis of the kinetic data, but in addition they furnish the criteria which must be met by any isolated protein purported to be the $\text{Ca}^{2+}$ carrier (cf. Bornkessel and Lehninger, 1969; Lehninger, 1971; Cozzi-Fusco et al., 1970).

Certain characteristics of carrier-specific $\text{Ca}^{2+}$ binding can be inferred from the kinetic data of the preceding chapter (see Table 3.2). The most relevant of these are the cooperative nature of $\text{Ca}^{2+}$ binding ($K_0$ coefficient = 1.7), the $K_m$ (4 pH at 0°C and pH 7.4) and its pH dependence ($K_m$ pH 7.4 active in unprotonated form). The $K_m$ has been concluded to represent the steady-state dissociation constant of the $\text{Ca}^{2+}$ carrier complex, and as such it is probably quite similar to the $K_0$ for $\text{Ca}^{2+}$ binding. However, the cooperativity suggests that the measured $K_m$ is a combined expression for the dissociation constants of two binding steps, i.e., for formation of $\text{RA}$ and $\text{RA}_2$:

\[
S + A \rightleftharpoons E_A
\]

\[
R + A \rightleftharpoons E_{RA}
\]

The overall dissociation constant, $K_{d1} = K_1 K_2$.

$E_A + A \rightleftharpoons E_{RA}$

$E_{RA} + A \rightleftharpoons E_{RA} A$

The overall dissociation constant, $K_{d1} = K_1 K_2$. 

$E_{RA} A + A \rightleftharpoons E_{RA} A_2$

$E_{RA} A_2 + A \rightleftharpoons E_{RA} A_3$
A RE-EVALUATION OF THE ENERGY-INDEPENDENT BINDING OF 

Ca\(^{2+}\) BY RAT LIVER MITOCHONDRIA

Theoretical considerations

The concept of a Ca\(^{2+}\)-specific carrier in the inner mitochondrial membrane has been treated in some detail in the preceding chapters. Since the binding of Ca\(^{2+}\) to the carrier is an obligatory initial step in the transport process it might be possible to devise experimental conditions for measuring the concentration and dissociation constant of carrier-specific Ca\(^{2+}\) binding sites. These are necessary for a more detailed analysis of the kinetic data, but in addition they furnish the criteria which must be met by any isolated protein purported to be the Ca\(^{2+}\) carrier (cf. Reynafarje and Lehninger, 1969; Lehninger, 1971a; Gomez-Puyou et al, 1972).

Certain characteristics of carrier-specific Ca\(^{2+}\) binding can be inferred from the kinetic data of the preceding chapter (see Table 3.2). The most relevant of these are the cooperative nature of Ca\(^{2+}\) binding (Hill coefficient = 1.7), the \(K_m\) (4 \(\mu\)M at 0\(^\circ\)C and pH 7.4) and its pH dependence (pKa \(\approx\) 7.5; active in unprotonated form). The \(K_m\) has been concluded to represent the steady-state dissociation constant of the Ca\(^{2+}\)-carrier complex, and as such it is probably quite similar to the \(K_d\) for Ca\(^{2+}\) binding. However, the cooperativity suggests that the measured \(K_m\) is a combined expression for the dissociation constants of two binding steps, i.e. for formation of EA and EA\(_2\):

\[
\begin{align*}
E + A &\rightleftharpoons EA \\
K'_{d} &= \frac{E.A}{EA} \\
\end{align*}
\]

\[
\begin{align*}
EA + A &\rightleftharpoons EA_2 \\
K''_{d} &= \frac{E.A}{EA_2} \\
\end{align*}
\]

The overall dissociation constant, \(K_{do}\) = \(K'_d.K''_d\)

\[
K_{do} = \frac{E.A^2}{EA_2}
\]
This treatment is an approximation in that it assumes two infinitely co-operative binding sites; the real number may be higher. The expected relationship between free and carrier-bound Ca\(^{2+}\) is, by rearrangement,

\[
EA_2 = \frac{E_T \cdot A^2}{K_{do} + A^2}
\]

where \(E_T\) = total concentration of carrier binding sites.

Since the approximate value of \(K_{do}\) is known, \(EA_2\) (bound Ca\(^{2+}\)) may be calculated for any value of \(A\) (free Ca\(^{2+}\)). Fig. 4.1a shows simulated Scatchard plots calculated from the above expression where \(K_{do} = 4 \times 10^{-6}\) M. Curves were constructed with the total concentration of binding sites \(E_T\) at 0.01, 0.1 and 1.0 nmoles/mg protein, with the resultant alteration in scales described in the legend.

The difficulties anticipated 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 1 n mole/mg protein, the concentration of Ca\(^{2+}\) bound is at best less than one-thousandth of its free concentration. This is in the region of the curve's maximum; to obtain a valid extrapolation to the number of binding sites requires accurate measurements under even less favourable conditions.

Fig. 4.1b shows the theoretical Scatchard plot calculated from identical data with the exception that binding occurs at non-interacting sites (Hill coefficient = 1). If this

---

1 The Scatchard plot (Scatchard et al, 1957) is based on a rearrangement of the expression

\[
K_d = \frac{E \cdot A}{EA}
\]

and substitution of \((E_T - EA)\) for \(E\) so that

\[
K_d \cdot \frac{EA}{A} = E_T - EA
\]

A plot of \(\frac{EA}{A}\) versus \(EA\) thus has a slope of \(-\frac{1}{K_d}\) and abscissa intercept of \(E_T\). This is generally the most satisfactory means of determining both the concentration of binding sites and their dissociation constant.
Fig. 4.1. Simulated Scatchard plots of carrier-specific mitochondrial Ca\(^{2+}\) binding

In all plots bound and free Ca\(^{2+}\) concentrations are expressed as moles/litre (M) in a solution containing 5 mg mitochondrial protein/ml. The dissociation constant for carrier-specific binding (K\(_d\)) is 4 \times 10^{-6} M.

(a) \[ EA_2 = \frac{E_T \cdot A^2}{K_d + A^2} \]

The concentration of bound Ca\(^{2+}\) (EA\(_2\)) was calculated over a wide range of free Ca\(^{2+}\) concentrations (A) for ET = 0.01, 0.1 and 1.0 nmoles/mg protein. The ordinate scale is multiplied by 10\(^5\), 10\(^4\) and 10\(^3\) respectively for the three values of ET, and the abscissa scale by 10\(^8\), 10\(^7\) and 10\(^6\).

(b) \[ EA = \frac{E_T \cdot A}{K_d + A} \]

The ordinate is multiplied by 10\(^2\), 10 and 1, the abscissa by 10\(^8\), 10\(^7\) and 10\(^6\) respectively for ET = 0.01, 0.1 and 1.0 nmoles/mg protein.

(c) \[ EA = \frac{E'_T \cdot A}{K'_d + A} + \frac{E''_T \cdot A}{K''_d + A} \]

where \( E'_T = 30 \) nmoles/mg protein
\( K'_d = 100 \times 10^{-6} M \)
\( E''_T = 1.0 \) (upper curve) nmoles/mg protein
\( K''_d = 4 \times 10^{-6} M \). \( E''_T = 0.1 \) (middle curve)
\( 0 \) (lower curve)

When ET is 0.01 nmoles/mg protein, or when the binding is expressed by the relation of (a), its total binding curve is not significantly different from that for ET = 0.
represents the true situation, analysis of carrier-specific binding is obviously more feasible, but the sigmoidal substrate-velocity relationship then requires an alternative explanation to that which has been proposed.

It might therefore be possible to measure carrier-specific binding of this nature, but there remain two further difficulties in the analysis of mitochondrial Ca\(^{2+}\) binding. The first of these 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 and Azzi, 1968; Scarpa and Azzzone, 1969; Lehninger, 1969; Lehninger et al., 1969; Reynafarje and Lehninger, 1969; Carafoli and Lehninger, 1971). There can be little doubt that these sites are preponderantly, if not entirely, phospholipids (Scarpa and Azzzone, 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. 4.1c shows simulated Scatchard plots of the simultaneous binding of Ca\(^{2+}\) to such low affinity sites (total concentration = 30 nmoles/mg protein; \(K_d = 100 \text{ \mu M}\): data from above references) and to the non-cooperative 'carrier' sites of Fig. 4.1b (the bound/free ratio for cooperative carrier sites (Fig. 4.1a) is so small that it does not 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 concentration of binding sites approaches 1 nmole/mg protein. Since the inhibition of Ca\(^{2+}\) transport by La\(^{3+}\) indicates <0.001 nmoles carrier/mg protein (based on the assumption that a single La\(^{3+}\) ion inhibits a single carrier molecule), this situation requires that each carrier molecule has in excess of 1,000 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\(^{2+}\) which has been transported by the carrier and released from it to the internal phase. The magnitude of this critical problem has not been recognised previously. Experimental data to be
presented in this chapter are concerned mainly with the assessment of such transport.

The basic conclusion to be drawn from these theoretical considerations is that it is virtually impossible to measure accurately carrier-specific Ca\textsuperscript{2+} binding by rat liver mitochondria if data derived from the kinetic studies of the previous chapter are indicative of the properties of carrier binding sites.

Experimental considerations

It seems futile to discuss optimal experimental conditions for the measurement of Ca\textsuperscript{2+} binding in view of the above comments, but only insofar as they relate to the Ca\textsuperscript{2+} carrier sites. The low affinity sites should be susceptible to analysis. In addition, a re-examination of the interaction of Ca\textsuperscript{2+} with respiration-inhibited mitochondria should be of some benefit in understanding the energy dependence of Ca\textsuperscript{2+} transport and may help to clarify the nature of the 'high-affinity energy-independent binding sites' first reported by Lehninger (1969) and subsequently studied in great detail (Reynafarje and Lehninger, 1969; Lehninger et al., 1969; Carafoli and Lehninger, 1971).

The most sensitive means of assaying Ca\textsuperscript{2+} binding is to use \textsuperscript{45}Ca\textsuperscript{2+} but, as with kinetic studies, one is faced immediately with the dual problems of dilution of endogenous Ca\textsuperscript{2+} (Mela and Chance, 1969) and chelation by incubation components, both of which would give values that overestimate the dissociation constant. There seems to be no acceptable means of completely overcoming the latter problem since the use of a strong buffer to generate known free Ca\textsuperscript{2+} concentrations makes the accurate measurement of bound Ca\textsuperscript{2+} very difficult (cf. previous chapter). Endogenous Ca\textsuperscript{2+} can be removed by pretreatment of all solutions with a chelating resin (Chelex-100).
The second requirement is to generate conditions where externally bound $\text{Ca}^{2+}$ is a large proportion of total mitochondrial $\text{Ca}^{2+}$. This requires an explicit definition of the mitochondrial pools available to $\text{Ca}^{2+}$, represented in the diagram below:

A gross distinction has been made between the pools separated by the selectively permeable inner membrane, denoted by E (external) and I (internal), and between bound (B) and free (F) $\text{Ca}^{2+}$. The need to distinguish between the internal and external pools is quite clear: it is only in this way that transported $\text{Ca}^{2+}$ can be distinguished from binding which occurs independently of transport. This topographical definition is more useful in quantifying $\text{Ca}^{2+}$ binding sites than is the 'energetic' criterion applied in previous studies (compare Reynafarje and Lehninger (1969) with Rossi et al. (1967) and Scarpa and Azzone (1970)).

The binding of $\text{Ca}^{2+}$, in every previous instance, has been determined as the total $\text{Ca}^{2+}$ associated with mitochondria whose respiration has been inhibited with rotenone and/or antimycin. The properties of the $\text{Ca}^{2+}$ carrier discussed in previous chapters suggest that under these conditions it will still transport external $\text{Ca}^{2+}$ to the internal pools, implying that 'metabolism-independent binding' is a nebulous combination of both externally-bound and internal $\text{Ca}^{2+}$. The total extent of movement of $\text{Ca}^{2+}$ to the internal pools would be a function
of the electrochemical gradient across the inner membrane. Since this is not able to be regenerated in respiration-inhibited mitochondria, the extent of net Ca\(^{2+}\) uptake should be small compared with that in actively-respiring mitochondria. It would further be expected that the distribution ratio of Ca\(^{2+}\) in the internal and external pools would be close to unity in the presence of uncouplers since these agents dissipate the pre-formed potential; net Ca\(^{2+}\) uptake should therefore be even less than in inhibited mitochondria, as has been observed (Reynafarje and Lehninger, 1969).

On the other hand, the inclusion of respiratory inhibitors and uncouplers to increase the ratio of externally-bound/total mitochondrial Ca\(^{2+}\) (and thus permit more accurate measurement of external binding) exaggerates the problem associated with dilution of \(45\text{Ca}^{2+}\) since both agents induce a release of endogenous mitochondrial Ca\(^{2+}\) (Carafoli, 1967; Reynafarje and Lehninger, 1969; Chance et al, 1969). The observation that respiratory inhibitors induce the release of a smaller amount of Ca\(^{2+}\) than do uncouplers (Reynafarje and Lehninger, 1969) is relevant to their anticipated effects on the distribution ratio of Ca\(^{2+}\).

It is concluded that neither with inhibited nor with uncoupled mitochondria could one expect to discriminate between external and internal pools of Ca\(^{2+}\). The lack of respiratory response (measured by oxygen polarography, double-beam spectrophotometry and fluorometry) and proton ejection following the addition of Ca\(^{2+}\) to inhibited mitochondria (Reynafarje and Lehninger, 1969) should be treated as experimental observations, not as evidence that Ca\(^{2+}\) is not transported into the internal pools under these conditions.

A final point relevant to the experimental criteria defining external binding is that it should be rapidly reversible. Analysis of binding data by the method of Scatchard (Scatchard et al, 1957) requires that binding be reversible, but this fundamental requirement was not considered by Reynafarje and 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 EGTA after incubation with \(^{45}\)Ca\(^{2+}\). Since EGTA cannot penetrate to the internal pools (see diagram above), the decrease in bound \(^{45}\)Ca\(^{2+}\) after such treatment equals that fraction which was reversibly bound at sites external to the permeability barrier, while the remaining fraction is equivalent to irreversibly-bound \(^{45}\)Ca\(^{2+}\), presumably identical with the internal pools.

Fig. 4.2 shows the results of such an experiment with 1 nmole \(^{45}\)Ca\(^{2+}\)/mg protein. The incubation conditions were similar to those of Reynafarje and Lehninger (1969) except that the temperature was 0°C and duplicate samples were analyzed for free Ca\(^{2+}\), one being centrifuged and the other treated with EGTA prior to centrifugation. While there appears to be little significant change in external free Ca\(^{2+}\) with time (Reynafarje and 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 later experiments showed that the EGTA-inaccessible pool decreases with time following EGTA addition (cf. EGTA-induced Ca\(^{2+}\) efflux from mitochondria in state 6). This means that the proportion of external (EGTA-removable)/total mitochondrial Ca\(^{2+}\) is overestimated in most of the data\(^1\). In all experiments the mitochondria were initially preincubated at the desired concentration in the presence of antimycin, rotenone (and CCCP when used), centrifuged, and resuspended in Chelex-treated sucrose/buffer.

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\(^1\)In the remainder of this chapter the terms 'EGTA-removable' and 'externally-bound' are used synonymously, as are 'EGTA-inaccessible' and 'internally-bound' or 'transported'.
Fig. 4.2. Reversible and irreversible components of 'high-affinity energy-independent Ca$^{2+}$ binding'

Mitochondria (30 mg protein) were preincubated for 2 min in 250 mM-sucrose and 2 mM-hepes-tris (pH 7.4) containing 1 µM-rotenone and 1 µM-antimycin in a total volume of 6 ml. 30 nmoles $^{45}$Ca$^{2+}$ (0.1 µCi) was added and 0.5 ml aliquots were then removed into micro-centrifuge tubes. At the times shown the contents of a tube were centrifuged (O) (Eppendorf: 12,000 x g for 2 min), or treated with 0.1 mM-EGTA-tris (●) and centrifuged. 0.1 ml aliquots of all supernatants were counted for radioactivity. The temperature was 0°C for all operations.

Fig. 4.3. Effect of CCCP on components of energy-independent Ca$^{2+}$ binding

Mitochondria (12 mg protein) were preincubated for 2 min in 250 mM-sucrose (Chelex-treated), 2 mM-hepes-tris (pH 7.4) containing 1 µM-rotenone, 1 µM-antimycin (circles) and where indicated, 1 µM-CCCP (triangles) in a total volume of 6 ml. The suspension was centrifuged (16,000 x g for 5 min) and the pellets were resuspended in 6 ml of the same medium. 12 nmoles $^{45}$Ca$^{2+}$ (0.05 µCi) was added and 0.25 ml aliquots were removed into micro-centrifuge tubes. At the times shown the contents of a tube were centrifuged (open symbols) (Eppendorf: 12,000 x g for 2 min) or treated with 0.4 mM-EGTA-tris and centrifuged (closed symbols). 0.1 ml aliquots of all supernatants were counted for radioactivity. The temperature was 0°C for all operations.
to minimize the dilution of isotopic label by endogenous Ca\(^{2+}\) released by the inhibitors.

Fig. 4.3 compares the time course of distribution of a low concentration of Ca\(^{2+}\) between the internal and external pools in the presence and absence of CCCP. Transfer between pools occurs in both cases but the internal pool reaches a lower steady-state level in the presence of CCCP. The addition of CCCP to respiration-inhibited mitochondria preincubated with \(^{45}\)Ca\(^{2+}\) induces an efflux from the internal pool as shown by Fig. 4.4 (the higher concentration of free Ca\(^{2+}\) in this experiment is due to the use of a relatively high concentration of antimycin: see later). The rate of efflux is considerably faster than the initial rate of entry of Ca\(^{2+}\) into the internal pool (cf. Figs. 4.2 and 4.3).

Both the total number of external binding sites (19 nmoles Ca\(^{2+}\)/mg protein) and their dissociation constant (25 µM) are identical with and without CCCP (Fig. 4.5). In this and in subsequent experiments involving the concentration dependence of binding, measurements were made after a 10 to 15 min incubation with \(^{45}\)Ca\(^{2+}\) at 0°C, i.e. at the completion of the time-dependent transfer of Ca\(^{2+}\) between mitochondrial pools.

The external binding sites are competitively inhibited by K\(^+\), Na\(^+\), and the local anaesthetic nupercaine (Fig. 4.6); the inhibition constants (K\(_i\)) are 17 mM, 6.6 mM and 54 µM respectively.

Additional experiments concentrated on measuring binding at 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. 4.7 shows data from an experiment in which the total Ca\(^{2+}\) concentration was varied from 0.025 to 2 nmoles/mg protein. The Scatchard plot (Fig. 4.7c) is horizontal and extrapolates to the ordinate at a value not significantly different from those of Figs. 4.5 and 4.6 where higher concentrations were used. Similar data are obtained when the mitochondria are pretreated with CCCP, although the internal pool is much smaller in this case. These experiments were repeated in a medium containing 125 mM-NaCl to see if inhibition by Na\(^+\) of the numerous
Fig. 4.4. **CCCP-induced efflux of Ca\(^{2+}\) from respiration-inhibited mitochondria**

The incubation procedure was similar to that of Fig. 4.3 except that the concentration of mitochondria was 5 mg protein/ml and of \(^{45}\text{Ca}^{2+}\), 5 µM. Antimycin and rotenone were each present at 0.25 µM and 5 µM-CCCP was added at the time shown by the arrow.

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**Fig. 4.5. Scatchard plot of external Ca\(^{2+}\) binding (+ CCCP)**

Mitochondria (2 mg protein/ml) were pre-treated as described in Fig. 4.3. 0.5 ml aliquots of the suspension were transferred to 9 microcentrifuge tubes containing from 4 to 100 nmoles \(^{45}\text{Ca}^{2+}\) (sp.act. 2 µCi/µmole) in 0.1 ml of incubation solution. The tubes were mixed and 250 µl of each was transferred to a second series of tubes. Exactly 10 min after the addition of mitochondria, the first set of tubes was centrifuged, and 0.8 mM-EGTA-tris was simultaneously added to the second set and these too were centrifuged. Radioactivity was measured in 0.1 ml aliquots of all supernatants. The concentration of free Ca\(^{2+}\) is calculated from the radioactivity of the untreated supernatant while the externally-bound Ca\(^{2+}\) is calculated from the difference between the EGTA-treated and untreated supernatants. All operations were performed at 0°C. ○, 1 µM-CCCP; ●, no CCCP.
Fig. 4.6. Inhibition of external Ca\(^{2+}\) binding by nupercaine, K\(^+\) and Na\(^+\)

The experimental procedure was similar to that of Fig. 4.5 except that CCCP was included in all incubations (similar data were obtained in all cases when CCCP was omitted). The pretreated mitochondria were resuspended, prior to incubation with \(^{45}\)Ca\(^{2+}\), in 250 mM-sucrose (O), 250 mM-sucrose containing 83 μM-nupercaine (●), 125 mM-KCl (△) or 125 mM-NaCl (▲). Other conditions were as described in Fig. 4.5.
Fig. 4.7. External binding at very low concentrations of Ca\textsuperscript{2+}

The experimental procedure was similar to that of Fig. 4.5 except that the concentration of mitochondria was 5 mg protein/ml and CCCP was omitted. The concentration of antimycin used was not sufficient to inhibit succinate oxidation completely; this was to ensure that no significant uncoupling occurred (see Fig. 4.8). The rotenone concentration was sufficient to block \( \beta \)-hydroxybutyrate oxidation. The closed and open symbols refer to two separate experiments, in each of which duplicate aliquots of supernatant were counted for radioactivity (sp.act. \( ^{45}\text{Ca}^{2+} = 0.3 \mu\text{Ci/nmole} \)).

(a) triangles: \( \text{Ca}^{2+} \) free in supernatant; circles: \( \text{Ca}^{2+} \) in supernatant after EGTA treatment. The dashed line shows the relation between '\( \mu\text{M} \)' and 'nmoles/mg protein'.

(b) External \( \text{Ca}^{2+} \) is that in the ECTA supernatants; externally-bound \( \text{Ca}^{2+} \) is the difference between that value and free \( \text{Ca}^{2+} \) in the untreated supernatants.

(c) Scatchard plot of external binding.
external $\text{Ca}^{2+}$ binding sites would permit identification of possible carrier sites above the lowered background. However, the ordinate intercept obtained was indistinguishable from that of the appropriate experiment in Fig. 4.6.

The concentration of antimycin used in experiments of this type has a marked effect on the equilibrium distribution of $\text{Ca}^{2+}$, although it does not affect the external binding parameters. Fig. 4.8 shows the results of experiments in which internal, externally-bound and free $\text{Ca}^{2+}$ were measured as a function of antimycin concentration in the presence and absence of rotenone. In both cases, approximately 88 percent of the $\text{Ca}^{2+}$ is inaccessible to EGTA at concentrations of antimycin up to 0.1 nmoles/mg protein, the approximate concentration required for complete inhibition of respiration (Estabrook, 1962; Ernster et al., 1963). At higher concentrations of antimycin, the internal pool decreases and both the external and free pools increase. Rotenone exaggerates these effects, presumably since the combination provides more complete respiratory inhibition than is afforded by antimycin alone. It is apparent that absolute measurements of each pool size will vary with the concentration of antimycin.

The effect of antimycin at higher concentrations is similar to that of the uncoupler CCCP (Fig. 4.3). Löw and Vallin (1963) and Haas (1964) have previously described an uncoupling effect of antimycin on beef heart mitochondria and particles at concentrations in excess of those required for respiratory inhibition.

Table 4.1 shows the effects of respiratory inhibitors, CCCP and oligomycin, on the distribution of 1 nmole $\text{Ca}^{2+}$/mg protein between the three pools. Oligomycin causes a measurable but relatively slight decrease in the internal fraction only when antimycin and rotenone also 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 $\text{Ca}^{2+}$ binding, but there remains the possibility of detecting it by using the specific inhibitors
Fig. 4.8. Effect of antimycin concentration on components of Ca\(^{2+}\) binding

Mitochondria (5 mg protein/ml) were pre-treated in the absence of respiratory inhibitors as described in Fig. 4.5. 0.5 ml aliquots were added to tubes containing 0.1 ml incubation solution plus antimycin at the final concentrations shown (identical volumes (6 µl) of ethanol, the antimycin solvent, were included in all tubes). The mitochondria were preincubated for 5 min before adding 1 n mole \(^{45}\text{Ca}^{2+}\)/mg protein (sp.act. 10 µCi/µmole) and then incubated for 10 min. After this time radioactivity was measured in duplicate 0.1 ml aliquots of supernatant (○) and supernatant after EGTA treatment (●). All operations were at 0°C.

(a) No rotenone; (b) plus rotenone (0.2 n moles/mg protein)
TABLE 4.1. Effect of respiratory and energy-transfer inhibitors on components of Ca\(^{2+}\) binding

The experimental procedure was similar to that described in Fig. 4.8 except that the inhibitors were varied as shown (total Ca\(^{2+}\) added = 1 n mole/mg protein, or 4.17 \(\mu\)M).

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration of Ca(^{2+}) in appropriate pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free ((\mu)M)</td>
</tr>
<tr>
<td>Nil</td>
<td>0.021</td>
</tr>
<tr>
<td>Oligomycin (1 (\mu)g/mg protein)</td>
<td>0.022</td>
</tr>
<tr>
<td>Antimycin (0.15 n moles/mg protein)</td>
<td>0.052</td>
</tr>
<tr>
<td>Antimycin + oligomycin</td>
<td>0.059</td>
</tr>
<tr>
<td>Antimycin + rotenone (0.2 n moles/mg protein)</td>
<td>0.382</td>
</tr>
<tr>
<td>Antimycin + rotenone + oligomycin</td>
<td>0.585</td>
</tr>
<tr>
<td>Antimycin + rotenone + oligomycin + CCCP (5 (\mu)M)</td>
<td>2.085</td>
</tr>
</tbody>
</table>
La$^{3+}$ and ruthenium red. In the experiments of Fig. 4.9, these inhibitors were added at various concentrations after 10 min incubation of the mitochondria with 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. Each time these experiments were carried out, ruthenium red had a more pronounced effect than did La$^{3+}$.

An interesting 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 so 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 more mundane interpretation proved to be correct. The lack of effect of the inhibitors on the concentration of free Ca$^{2+}$ (Fig. 4.9) is incompatible with the above interpretation. Furthermore, Fig. 4.10 shows that the increase in internal Ca$^{2+}$ due to ruthenium red addition exceeds 1 nmole/mg protein which is far too high to be due to carrier-specific binding (cf. earlier considerations). In fact, the apparent increase is due to inhibition of EGTA-induced efflux as seen in Fig. 4.11. Further experiments in which ruthenium red was found to inhibit the entry of Ca$^{2+}$ into the internal (EGTA-inaccessible) pool have confirmed this interpretation.

These effects of ruthenium red and La$^{3+}$ strongly support the assumption made earlier 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$^{3+}$ is probably due to a combination of the release of its inhibition with time (a consequence of its being transported; Reed and Bygrave, 1973a) and the difference in inhibitory mechanisms of La$^{3+}$ and ruthenium red seen in the kinetic data of the previous chapter.
Mitochondria (2 mg protein/ml) were pre-treated as described in Fig. 4.3 (minus CCCP). 2 µM-45Ca2+ (sp.act 5 µCi/µmole) was added and after 10 min incubation, 0.5 ml aliquots were transferred to tubes containing (in 50 µl) either La3+ (a) or ruthenium red (b) at the final concentrations shown. These were mixed immediately and 0.2 ml aliquots were treated with 0.25 mM-EGTA and immediately centrifuged; the remaining suspensions also were centrifuged. Radioactivity was measured in 0.1 ml aliquots of all supernatants (total concentration of 45Ca2+ = 1.82 µM). All operations were at 0°C. O: untreated supernatants; •: supernatants after EGTA treatment.

The ruthenium red solution used in this and subsequent experiments was a crude solution (see Section II). Its concentration is expressed as approximate inhibitory equivalents of ruthenium red, as determined from its inhibition of respiration-supported Ca2+ uptake.

Fig. 4.9. **Effect of La3+ and ruthenium red on components of "energy-independent" Ca2+ binding**
Mitochondria (2 mg protein/ml) were pre-treated as described in Fig. 4.3 (minus CCCP) and then 0.5 ml aliquots were transferred to microcentrifuge tubes containing 0.1 ml 45Ca2+ (sp.act. 0.25 µCi/nmole) as shown. After 10 min incubation, 0.016 nmoles ruthenium red (Δ), 0.02 nmoles La3+ (O) or nil (•) was added and the tubes incubated for a further 5 min. Aliquots were then centrifuged before and after treatment with 0.4 mM-EGTA and 0.1 ml of all supernatants was counted for radioactivity. The figure shows the amount of Ca2+ not removed by EGTA (i.e. internal Ca2+). All operations were at 0°C.

Fig. 4.11. Time-dependent removal of internal Ca2+ by EGTA

The preparation of mitochondria was identical to that in Fig. 4.4. In each experiment, 10 nmoles 45Ca2+ was added to 2 ml of the suspension. This was either immediately preceded by the addition of 1 mM-EGTA (●) or followed after 15 min by the addition of EGTA (O) or 1.5 µM-ruthenium red and EGTA (Δ).
The belated realization that EGTA treatment induces an efflux from the internal pool in addition to removing externally-bound Ca$^{2+}$ means that the preceding data are not quantitatively correct. The resultant overestimation of external binding would vary with both the concentration of mitochondria used and the lapsed time between EGTA addition and centrifugation. Obviously, ruthenium red should be included in EGTA quenching solutions to ensure valid measurement of external binding.

The final figure (Fig. 4.12) shows the results of an experiment in which this technique was used in conjunction with pretreatment of the mitochondria with both CCCP and EGTA to ensure maximum removal of endogenous Ca$^{2+}$. Again it can be seen that ruthenium red does not alter the total concentration of mitochondrial Ca$^{2+}$ (cf. Fig. 4.9) but it does decrease the measured values of external binding. The Scatchard plots of external binding (Fig. 4.12(c)) show that the correct number of external Ca$^{2+}$ binding sites (measured with ruthenium red/EGTA quenching) is approximately 15 nmole/mg protein, 25 per cent less than their number measured with EGTA alone (Figs. 4.5, 4.6 and 4.12). Since the slope of the plot is identical in both cases, the overestimation of external binding in the latter case is due to an overestimate of the total number of binding sites rather than an effect on the dissociation constant ($K_d = \frac{-1}{\text{slope}}$). The dissociation constant itself (~140 uM) was far higher in this experiment than in previous ones. The reason for this is not clear, but it may be due partly to the lower pH of the incubations (7.2 cf. 7.4); it might also be related to the extensive depletion of endogenous divalent cations during pre-treatment 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 to those of Fig. 4.12(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
Fig. 4.12. Energy-independent Ca\(^{2+}\) binding: titration of Ca\(^{2+}\)-depleted mitochondria in the presence and absence of ruthenium red

Mitochondria were suspended at 2.5 mg protein/ml in 250 mM-sucrose, 2 mM-hepes-tris (pH 7.2), 0.5 mM-EGTA-tris and 10 µM-CCCP at 0°C. After 15 min the suspension was centrifuged (16,000 x g for 5 min) and the pellet was resuspended and recentrifuged twice more in 250 mM-sucrose (Chelex-treated), 2 mM-hepes-tris (pH 7.2), then finally suspended in the same medium at 5 mg protein/ml. \(^{45}\)Ca\(^{2+}\) (sp.act. 0.3 µCi/µmole) was added to 1 ml of this suspension at the concentrations shown; 0.2 ml aliquots were then transferred to four separate microcentrifuge tubes. Exactly 15 min later 1 µM-ruthenium red was added to two of these tubes followed immediately by 1 mM-EGTA to one of these and to one of the remaining two tubes. All four tubes were immediately centrifuged (Eppendorf: 12,000 x g for 2 min) and the supernatants were removed completely. The pellets were dissolved in 0.1 ml Soluene and counted for radioactivity.

(a) Total Ca\(^{2+}\) in mitochondria before (O) and after (●) EGTA treatment.

(b) Total Ca\(^{2+}\) in mitochondria after the addition of ruthenium red before (O) and after (●) EGTA treatment.

(c) Scatchard plots of external binding with (△) and without (▲) ruthenium red.

\(^{1}\) The mitochondria remained completely uncoupled as assessed by the lack of effect of extra CCCP on succinate-supported respiration.
about 15 nmoles/mg protein. It might eventually prove possible to identify carrier-specific Ca\(^{2+}\) binding sites by pretreating mitochondria with sufficient ruthenium red to completely inhibit transport, provided that ruthenium red does not inhibit the binding of Ca\(^{2+}\) to the carrier as suggested by its non-competitive inhibition (see later).

**General Discussion**

The 'high-affinity' binding of Ca\(^{2+}\) by respiration-inhibited rat liver mitochondria, reported in detail by Reynafarje and Lehninger (1969), has frequently been equated with carrier-specific binding on the basis of its high affinity, low total number of sites, sensitivity to uncouplers and to ruthenium red (Vastington et al., 1972a,b) and La\(^{3+}\) (Lehninger et al., 1969), its specificity for Ca\(^{2+}\), Sr\(^{2+}\), Mn\(^{2+}\) (Reynafarje and Lehninger, 1969) and La\(^{3+}\) (Lehninger and Carafoli, 1971), and its species distribution (Carafoli and Lehninger, 1971). Such an interpretation presents a number of anomalies: not all preparations capable of oxidative phosphorylation (and, presumably, active Ca\(^{2+}\) transport) show 'high affinity' Ca\(^{2+}\) binding (Lehninger et al., 1969); of those that do, both the number and affinity of sites vary over a wide range. The number of binding sites in rat liver mitochondria has been variously reported as 0.6, 0.1-1.0, 1.2, 3.0 and 1.2-8 nmoles/mg protein while their dissociation constant, a reflection of their affinity for Ca\(^{2+}\), ranges from 0.01 µM to 1.6 µM (Lehninger, 1969; Lehninger et al., 1969; Reynafarje and Lehninger, 1969; Lehninger, 1971a; Lehninger and Carafoli, 1971; Carafoli and Lehninger, 1971) in spite of both the maximum velocity and K\(_m\) (for Ca\(^{2+}\)) of energy-linked Ca\(^{2+}\) transport being remarkably uniform between different preparations (Reed, unpublished observations).

In fact, these 'high-affinity' sites show very few of the binding properties expected of the mitochondrial Ca\(^{2+}\) carrier. In particular, their dramatic sensitivity to uncouplers and their relative insensitivity to La\(^{3+}\) and ruthenium red, together with the variable estimates of their concentration and affinity, are incompatible with kinetic data of active Ca\(^{2+}\) transport; furthermore, even the lowest measured
concentration of these sites is far too high to be consistent with the concentration of inhibitor binding sites determined from kinetic measurements (cf. Mela and Chance, 1969; see also the previous chapter). The reason for the widespread misinterpretation of the nature of 'high-affinity' binding is that the binding studies have been carried out without any prior attempt to establish the properties of the Ca\(^{2+}\) carrier by kinetic studies, a situation which surely must be unprecedented in enzymology.

The data of this chapter show 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 powerful 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 CCCP.

From the considerations of the Ca\(^{2+}\) carrier in previous chapters, there is every reason to believe that the carrier should continue net transport of Ca\(^{2+}\) in either the forward or reverse direction until the distribution ratio appropriate to the energy status of the mitochondria has been achieved (it would also catalyse isotope exchange between inner and outer pools, as distinct from net transport). There is no reason to suppose that CCCP will inhibit the carrier except insofar as it alters the pH in the region of the binding sites. The data obtained with both respiration-inhibited and uncoupled mitochondria are thus quite consistent with the model of the Ca\(^{2+}\) carrier outlined earlier; so too is the lack of identification of carrier-specific binding sites.

It is clear that the 'high affinity' energy-independent binding represents net Ca\(^{2+}\) transport to the internal pool.
It is therefore a measure of the distribution ratio of Ca\(^{2+}\) across the inner membrane in respiration-inhibited mitochondria. This cannot be assessed in terms of Ca\(^{2+}\) concentrations since the internal free concentration is not known, but the total capacity for net uptake is presumably within the range of 0.6 to 8 nmol Ca\(^{2+}\)/mg protein. The variation in capacity is probably due to relatively minor differences in energy status of different preparations, as can be inferred from the data of Fig. 4.8. It is unfortunate that 'high-affinity' binding has been used so frequently as a diagnostic tool for carrier-specific Ca\(^{2+}\) binding.

The second pool of mitochondrial Ca\(^{2+}\) is one which can be removed by EGTA and is therefore reversibly-bound Ca\(^{2+}\) located on surfaces external to the inner phase. The present data indicate that these external sites can bind approximately 15 nmol Ca\(^{2+}\)/mg protein and that their dissociation constant is 20-25 \(\mu\)M. Their sensitivity to Na\(^{+}\) and K\(^{+}\) and more particularly to nupercaine suggests them to be phospholipids (Dawson and Hauser, 1970; Papahadjopoulos, 1972; Seeman, 1972). Previous studies of 'low-affinity' energy-independent binding have suffered from the lack of recognition of carrier-mediated transport and so are not strictly comparable (but see Scarpa and Azzi, 1968 and Scarpa and Azzone, 1969).

The major conclusion of this chapter 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 independently measured with the techniques described in this and the previous chapter.

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, viz Mn\(^{2+}\) (Chappell et al, 1963; Puskin and Gunter, 1973) and some lanthanides (cf. Williams, 1970).
SECTION II

THE INTERACTION OF SPECIFIC Ca\(^{2+}\) TRANSPORT INHIBITORS WITH RAT LIVER MITOCHONDRIA

The trivalent rare-earth metal ions are extremely potent inhibitors of the energy-linked accumulation of Ca\(^{2+}\) by mitochondria (Hala, 1968a, b; Hala, 1969a; Hala and Chang, 1969; Vainio et al., 1970; Scarpe and Arnone, 1970). The inhibition by lanthanides is thought to differ from that by the divalent metal ions (Mn\(^{2+}\), Sr\(^{2+}\), Ba\(^{2+}\), not only with respect to their higher potency, but also by virtue of the supposition that the lanthanides are not accumulated by mitochondria (cf. Mg\(^{2+}\), Sr\(^{2+}\); Vainio et al., 1970). Lemberg and Czarnecki (1972) have concluded that this is the case from studies with the radioactive lanthanides. However, there are certain features of the interaction of La\(^{3+}\) with mitochondria which suggest that it may be transported by the mitochondrial ATPase. The evidence for the presence of La\(^{3+}\) in the mitochondria is provided by the data of Lemberg and Czarnecki (1971) in which: (a) there is no obvious increase in La\(^{3+}\) binding with time (Figs. 2, 4, 5); (b) the amount of La\(^{3+}\) bound in the presence of succinate at 25\(^\circ\) is greater than 50 nanomoles/mg protein (than the amount added is 80 nanomoles/mg protein), i.e. in excess of the number of energy-independent La\(^{3+}\) binding sites at 25\(^\circ\), which total about 30 nanomoles/mg protein; (c) the number of 'high affinity' energy-independent La\(^{3+}\) binding sites is 8 nanomoles/mg protein; as has been reported in the previous chapter, such 'high affinity binding' of Ca\(^{2+}\) is in fact due to carrier-mediated transport; (d) the inhibition of 'high affinity' Ca\(^{2+}\) binding (energy-independent transport) by La\(^{3+}\) is also overcome with time (Lemberg and Czarnecki, 1971, Fig. 6, cf. Scarpe and Arnone, 1970, Fig. 10).

We felt that these indications were sufficiently strong to warrant a re-examining of the question of La\(^{3+}\) transport, since a clear understanding of its interaction with mitochondria is essential to the development of a rational model for Ca\(^{2+}\) transport. Using a wide variety of experimental techniques, we have been able to show that La\(^{3+}\) is accumulated by mitochondria in a process similar to many respects to that of Ca\(^{2+}\), although its rate of transport is much slower. In addition, we have found that La\(^{3+}\) has unique effects on
THE INTERACTION OF $\text{La}^{3+}$ WITH RAT LIVER MITOCHONDRIA

The trivalent rare-earth metal ions are extremely potent inhibitors of the energy-linked accumulation of $\text{Ca}^{2+}$ by mitochondria (Mela, 1968a; Mela, 1969a; Mela and Chance, 1969; Vainio et al., 1970; Scarpa and Azzone, 1970). The inhibition by lanthanides is thought to differ from that by the divalent metal ions ($\text{Mn}^{2+}$, $\text{Sr}^{2+}$, $\text{Ba}^{2+}$), not only with respect to their higher potency, but also by virtue of the supposition that the lanthanides are not accumulated by mitochondria (cf. $\text{Mg}^{2+}$, $\text{K}^+$: Vainio et al., 1970). Lehninger and Carafoli (1971) have concluded that this is the case from studies with the radioisotope $^{140}\text{La}^{3+}$.

However, there are certain features of the interaction of $\text{La}^{3+}$ with mitochondria which suggest that it may be transported by these organelles. The most obvious of these is the release of its inhibition of $\text{Ca}^{2+}$ uptake with time (Mela, 1968a; Lehninger and Carafoli, 1971). Further indications are provided by the data of Lehninger and Carafoli (1971) in which: (a) there is an obvious increase in $\text{La}^{3+}$ binding with time (Figs. 2, 4, 5); (b) the amount of $\text{La}^{3+}$ bound in the presence of succinate at $25^\circ$ is greater than 60 nmoles/mg protein (when the amount added is 80 nmoles/mg protein), far in excess of the number of energy-independent $\text{La}^{3+}$ binding sites at $0^\circ$, which total about 30 nmoles/mg protein; (c) the number of 'high affinity' energy-independent $\text{La}^{3+}$ binding sites is 3 nmoles/mg protein; as has been reported in the previous chapter, such 'high affinity binding' of $\text{Ca}^{2+}$ is in fact due to carrier-mediated transport; (d) the inhibition of 'high affinity' $\text{Ca}^{2+}$ binding (energy-independent transport) by $\text{La}^{3+}$ is also overcome with time (Lehninger and Carafoli, 1971, Fig. 6, cf. Scarpa and Azzone, 1970, Fig. 10).

We felt that these indications were sufficiently strong to warrant a re-examination of the question of $\text{La}^{3+}$ transport, since a clear understanding of its interaction with mitochondria is essential to the development of a rational model for $\text{Ca}^{2+}$ transport. Using a wide variety of experimental techniques, we have been able to show that $\text{La}^{3+}$ is accumulated by mitochondria in a process similar in many respects to that of $\text{Ca}^{2+}$, although its rate of transport is much slower. In addition, we have found that $\text{La}^{3+}$ has unique effects on
mitochondrial structure and function, including aggregation, gross changes in morphology and changes in respiratory activity. No attempt has been made to characterize optimal conditions for La\(^{3+}\) uptake, but rather we have concentrated on a thorough analysis of the numerous responses induced by La\(^{3+}\) in mitochondria.

**Passive swelling**

When mitochondria whose respiration is inhibited are suspended in iso-osmotic salt solutions, the osmotically active compartment (matrix space) swells if the inner membrane is permeable to both the anion and cation (Chappell and Haarhoff, 1967).

A variation of this approach (suggested by Selwyn et al, 1970) was used in preliminary experiments to investigate the permeability of the inner membrane towards La\(^{3+}\). The results of the control experiments with Mg\(^{2+}\) and Ca\(^{2+}\) additions to potassium chloride, acetate and thiocyanate (Fig. 5.1a and b) are as expected from previous data (Selwyn et al, 1970). Experiments with La\(^{3+}\) (Fig. 5.1c) indicate that, similarly to Ca\(^{2+}\) but in contrast to Mg\(^{2+}\), it enters the osmotically active space. The only noticeable difference between the effects of Ca\(^{2+}\) and La\(^{3+}\) is the considerable lag preceding the absorbance decrease at 520 nm with the latter, particularly in the presence of thiocyanate. It is probable that the absorbance decrease is a result of the penetration of La\(^{3+}\) to the osmotically active space since it varies with the permeability properties of the anion. However, the high concentrations of La\(^{3+}\) used in these experiments are undesirable because they also cause aggregation of mitochondria (see below).

**Active swelling**

The apparent passive permeability of mitochondria to La\(^{3+}\) raised the possibility that it may also be actively accumulated against a concentration gradient, and thus induce swelling at low concentrations. This appears to be the case (Fig. 5.2). As seen in Fig. 5.2a, the extent of swelling induced by La\(^{3+}\) in the presence of succinate and acetate is dependent on the concentration of La\(^{3+}\) added, up to about 100 \(\mu\)M. The decrease
Mitochondria (4.25 mg protein) were added to cuvettes containing 90 mM-potassium chloride (curves 1), potassium acetate (2) or potassium thiocyanate (3), 5 mM-hepes-tris (pH 7.4), 0.18 µM-rotenone and 0.14 µM-antimycin. At the times indicated (arrows) the following solutions were added at a final concentration of 7.3 mM: (a) MgCl₂; (b) CaCl₂; (c) LaCl₃. Total volume, 2.75 ml; temperature 30°C.
Fig. 5.2. Active swelling of mitochondria in response to La\(^{3+}\) addition.

Mitochondria (2.5 mg protein in (a) and (c), 2.3 mg protein in (b)) were added to cuvettes containing the standard medium (250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 10 mM-acetate-tris) and 5 mM-succinate-tris in a final volume of 3 ml. Temperature 30°C. La\(^{3+}\) was added (arrows) at the concentrations shown. The concentrations of antimycin, rotenone and CCCP used in (c) were 0.12 µM, 0.17 µM and 10 µM respectively.
is preceded in each case by a lag period similar to that observed in the passive swelling experiments. At higher concentrations, the lag period is lengthened and the subsequent absorbance decrease is inhibited (Fig. 5.2b). That the observed responses are dependent on active transport of the La$^{3+}$ is further indicated by the inhibition produced by respiratory inhibitors and uncouplers (Fig. 5.2c).

In both these experiments and others to be described below, it became apparent that acetate is not essential for the responses induced by La$^{3+}$. However, it does accentuate them and was therefore included in all experiments.

**Isopycnic centrifugation**

The La$^{3+}$ associated with respiring mitochondria is in a compartment inaccessible to EGTA, as shown by experiments in which mitochondria were incubated with La$^{3+}$ for eight min, treated with EGTA and then subjected to isopycnic centrifugation. Incubation with about 70 nmoles La$^{3+}$/mg protein increased the density of the bulk of the mitochondrial population by about 0.03 g/ml (Fig. 5.3a and b). Doubling the concentration of La$^{3+}$ had little further effect on the density of the mitochondria. This apparent limit to the accumulation of La$^{3+}$ may be a function of the incubation time, but it was also evident when light scattering and respiratory activities were measured (see below).

**Murexide absorbance changes**

If the above results are due to La$^{3+}$ transport, they should correlate with a decrease in concentration of La$^{3+}$ external to the mitochondria. We have therefore used the murexide technique introduced by Mela and Chance (1968) to follow free La$^{3+}$ concentrations.

Calibration curves for the 510-540 nm absorbance difference as a function of La$^{3+}$ concentration are shown in Fig. 5.4a. The curve obtained in the presence of $\beta$-hydroxybutyrate is linear only to a concentration of La$^{3+}$ about 60 per cent that of murexide (20 $\mu$M), indicative of the high stability of the La-murexide complex (Sillén et al, 1971). Absorbance
Fig. 5.3. Isopycnic centrifugation of mitochondria loaded with La$^{3+}$

Mitochondria (3.6 mg protein) were incubated with La$^{3+}$ for 8 min under conditions identical to those of Fig. 5.2; EGTA was added to give a final concentration of 0.83 mM and the tubes were centrifuged at 8,000 x g for 5 min (at 20°C). The pellets were then resuspended, layered on discontinuous sucrose density gradients and centrifuged as described in the Appendix.

The position and density (g/ml) of the gradient bands before centrifuging is shown on the left. The final position of the mitochondria is represented by the shaded areas in (a), (b) and (c); hatched and dark areas reflect the relative distribution of mitochondria.

Fig. 5.4. Free La$^{3+}$ concentration measured by murexide absorbance changes

(a) Calibration curves for murexide absorbance changes at 510-540 nm as a function of La$^{3+}$ concentration. Cuvettes contained the standard medium, 20 µM-murexide and succinate (○) or β-hydroxybutyrate (●) in a final volume of 3 ml. Room temperature

(b) Uptake of La$^{3+}$ by mitochondria. Cuvettes contained the standard medium, 20 µM-murexide, β-hydroxybutyrate and 7.2 mg mitochondrial protein in a final volume of 3 ml; antimycin, rotenone and CCCP were used at concentrations of 0.24 µM, 0.33 µM and 10 µM respectively. Room temperature. Murexide absorbance changes (510-540 nm) following the addition of 100 µM-La$^{3+}$ were converted to free La$^{3+}$ concentrations using the appropriate calibration curve (a).
traces were therefore converted to $\text{La}^{3+}$ concentration to give more meaningful data. Separate calibrations were required for experiments using succinate, since within the concentration range used it chelates roughly 75 per cent of the available $\text{La}^{3+}$ (see Sillén and Martell, 1964; Sillén et al, 1971) and for those from which substrate anions were omitted. Control experiments showed that $\text{La}^{3+}$ causes negligible changes at 510-540 in the absence of murexide.

Addition of $\text{La}^{3+}$ to substrate-supplemented mitochondria at concentrations higher than about 12 nmoles/mg protein (see below) causes an immediate decrease in absorbance at 540 nm relative to 510 nm; the absorbance subsequently increases with time (Fig. 5.4b), but the rate of increase is considerably slower than that seen with $\text{Ca}^{2+}$ or $\text{Mn}^{2+}$ (Mela and Chance, 1968) suggesting that the rate of accumulation of $\text{La}^{3+}$ is slower (cf. stimulation of oxygen uptake; see below).

The inclusion of antimycin and rotenone partially inhibits the decrease in free $\text{La}^{3+}$ concentration (Fig. 5.4b) as does the uncoupler CCCP (not shown), but the murexide absorbance decrease is less sensitive than the light scattering response (Fig. 5.2). The addition of inhibitors or uncouplers to mitochondria fully loaded with $\text{La}^{3+}$ does not cause its release (Fig. 5.4b).

The binding of $\text{La}^{3+}$ to external sites of respiration-inhibited mitochondria (defined as that occurring within five sec) was measured with murexide using two different approaches: the first involved its use as an internal indicator in much the same way that it was used in the experiments described above. Since the absorbance difference at 540-510 nm increased with time, the data on the binding of $\text{La}^{3+}$ to external sites were obtained from individual experiments in which only the immediate decrease at 540-510 nm following $\text{La}^{3+}$ addition was measured.

The second approach was to use murexide as an external indicator (Fig. 5.5). Similar incubation conditions were used except that the temperature was decreased to $0^\circ\text{C}$ to minimize $\text{La}^{3+}$ transport in the short interval between its addition and the subsequent separation of mitochondria by centrifugation. Murexide was added to the supernatants and the concentration of $\text{La}^{3+}$ in them was again estimated by a dual-wavelength technique, but in this case the sensitivity was increased by
Incubations contained 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 0.56 µM-antimycin, 0.77 µM-rotenone and where indicated, 4.1 mg mitochondrial protein, in a total volume of 1.3 ml. Following 1 min preincubation, La$^{3+}$ was added at the appropriate concentration and the tube immediately centrifuged (Eppendorf microcentrifuge: 2 min at 12,000 x g). At the completion of the experiment, all tubes were recentrifuged to pack the pellets more firmly and 1.0 ml aliquots of supernatant were diluted with 2.0 ml of 5 mM-hepes-tris (pH 7.4) containing 150 µM-murexide. The absorbance of all samples was read at 470 and 540 nm and the difference ($\Delta E_{470-540}$) was corrected for the difference of control incubations from which Ca$^{3+}$ had been omitted. The incubations and centrifugation were done at 0°C.

(a) Absorbance increase at 470-540 nm as a function of final La$^{3+}$ concentration in the presence (•) or absence (○) of mitochondria (the concentration of La$^{3+}$ in the incubations was three times that shown).

(b) The above data replotted to show La$^{3+}$ bound by mitochondria as a function of total La$^{3+}$ added. The absorbance differences measured in the presence of mitochondria were converted to La$^{3+}$ concentrations by using the calibration curve obtained in the absence of mitochondria.

Fig. 5.5. Binding of La$^{3+}$ to external sites of mitochondria measured with murexide as an external indicator.
using wavelengths corresponding to the peak (470 nm) and the trough (540 nm) in the difference spectrum. The need for a close wavelength pair is not critical since the absorbance of the supernatant itself is similar in all samples (cf. Mela and Chance, 1968).

Identical data were obtained with both techniques. Scatchard plots show that the total number of external La$^{3+}$ binding sites is 27-30 nmoles/mg protein and the apparent Kd is about 10 µM, in reasonable agreement with the number and affinity of 'low affinity' binding sites measured by Lehninger and Carafoli (1971) with $^{140}$La$^{3+}$ under conditions similar to those of Fig. 5.5.

**Electron microscopy**

Mitochondria which had been treated with La$^{3+}$ were examined by electron microscopy, firstly to verify that the absorbance decreases measured at 520 nm do in fact reflect swelling of the matrix space, and secondly in the hope that intramitochondrial deposits of La$^{3+}$ could be visualized (cf. Sr$^{2+}$; Greenawalt and Carafoli, 1966).

The control preparation, incubated for 10 min in the absence of La$^{3+}$ (Plate la), exhibits the condensed conformation typical of freshly isolated liver mitochondria. The addition of 33 µM-La$^{3+}$ (Plate lb) results in a fairly general expansion of the matrix space to give a mixed population of twisted, intermediate and orthodox conformations (for nomenclature, see Myron and Connelly, 1971). When the La$^{3+}$ concentration is further increased to 67 µM (Plates lc and d) a proportion of the population becomes swollen. Some of these swollen mitochondria show a broken outer membrane (Plate lc(l)) which remains in contact with the inner membrane in regions which still exhibit remnants of cristal structure. The sequence of transitions induced by La$^{3+}$ to this stage (condensed → twisted, orthodox → swollen, broken) is typical of the ultrastructural changes induced by swelling agents (Myron and Connelly, 1971) or by changes in osmotic strength of the suspending medium (Stoner and Sirak, 1969).
Plate 1. Effects of La$^{3+}$ on mitochondrial ultrastructure

Rat liver mitochondria (4.1 mg protein) were added to a medium containing 250 mM-sucrose, 5 mM-succinate, 10 mM-acetate and 20 mM-hepes neutralized to pH 7.4 with KOH. The final volume was 3 ml and the temperature was 30°C. Mitochondria were incubated for 2 min prior to the addition of La$^{3+}$ (at the concentration shown) and then for a further 10 min before being prepared for microscopy as described in the Appendix. (a) Control: no La$^{3+}$; (b) 33 µM-La$^{3+}$; (c) and (d) 67 µM-La$^{3+}$.

The numbered lines indicate the following features:
1. broken outer membrane of swollen mitochondria; 2. discontinuity of inner membrane; 3. 'swollen' intramitochondrial vesicle; 4. 'orthodox' intramitochondrial vesicle; 5. 'condensed' intramitochondrial vesicle; 6. invagination of outer membrane.

The horizontal bar represents 1 µm.
The outstanding feature of Plate 1d is the presence of mitochondria containing discrete pockets of condensed matrix material situated within the outer membrane and adjacent to it, a completely novel conformation. Examination of a large number of representative fields has revealed possible intermediate steps in the adoption of this stage, each of which is illustrated in Plates 1c and 1d. The first step is proposed to be breakage of the inner membrane (Plate 1c(2)) followed by resealing to form small internal vesicles with a structure similar to that of swollen mitochondria (Plate 1d(3)). The vesicles then condense to states characteristic of the orthodox and condensed conformations (Plate 1d(4) and (5) respectively).

The poly-vesicular structure becomes even more pronounced after treatment with 100 µM-La$^{3+}$ (Plate 2a and b) and reaches its maximal frequency in samples treated with about 100-200 µM-La$^{3+}$. Two other features predominate both in these samples and in those observed at higher La$^{3+}$ concentrations: the first is the surprisingly large proportion of mitochondria in the orthodox or intermediate conformations, all of which appear to have double membranes. This reflects what seems to be the final stage in the transformations induced by La$^{3+}$, in which the condensed pockets of matrix material are pinched off by invaginations of the outer membrane (Plate 1d(6)).

The second feature which becomes obvious at La$^{3+}$ concentrations exceeding 100 µM is mitochondrial aggregation. This phenomenon is dependent on both La$^{3+}$ concentration and time, and is optimally enhanced by inorganic phosphate at a concentration about half that of La$^{3+}$. The junction between the membrane of aggregated mitochondria is generally very electron dense (Plate 2a and b(1)). It seems likely that aggregation is due to ion-bridging between La$^{3+}$ binding sites on the external surface of the mitochondrial outer membrane.

When the La$^{3+}$ concentration is increased to 333 µM (Plate 2c,d), aggregation occurs to such an extent that mitochondria are rarely seen in isolation. There are virtually no poly-vesicular structures. A proportion of the mitochondria are swollen (e.g. Plate 2d), but a large number retain relatively
Plate 2. Effects of La$^{3+}$ on mitochondrial ultrastructure

Conditions were identical to those of Plate 1. (a) and (b) 100 µM-La$^{3+}$; (c) and (d) 333 µM-La$^{3+}$.

The numbered lines indicate the following features:
1. dense deposits in the junction between aggregated mitochondria;
2. convex profile of the inner membrane;
3. densely stained boundary and cristal membranes;
4. dense deposits at the point of contact between inner and outer membranes.

The horizontal bar represents 1 µm.
normal orthodox and condensed conformations, except that the inner membrane surrounding the dense matrix material appears to be tightly stretched between points of attachment to the outer membrane (e.g. Plate 2c(2)). These latter contracted forms contain numerous electron-dense deposits in the matrix (Plate 2c). It is highly probable that these are intramitochondrial deposits of La\(^{3+}\), although we lack supporting evidence.

The boundary and cristal membranes of mitochondria treated with 100 µM-La\(^{3+}\) are generally densely stained (Plate 2d(3)). As noted above, staining is also intensified in the regions of contact between adjacent mitochondria and at points of attachment of the inner to the outer membrane (Plate 2a(4)).

**Stimulation of oxygen uptake**

Lehninger and Carafoli (1971) have reported that La\(^{3+}\) does not stimulate mitochondrial respiration in a medium of 80 mM-NaCl, supplemented with 10 mM-succinate. The data of Fig. 5.6a show, on the other hand, that in 250 mM-sucrose supplemented with 5 mM-succinate, a marked stimulation is obtained. As with the swelling experiments, this response is preceded by a short lag time. The stimulation is even more dramatic with \(\beta\)-hydroxybutyrate as substrate (Fig. 5.6b), but no stimulation is observed with \(\alpha\)-oxoglutarate plus malonate (Fig. 5.6c) or with pyruvate and malate (not shown).

Further experiments revealed that both 80 mM-NaCl and high succinate concentrations inhibit the response of mitochondria to La\(^{3+}\), the latter by virtue of chelation. The lack of response with \(\alpha\)-oxoglutarate (plus malonate) and pyruvate plus malate probably has a similar explanation (see Sillén and Martell, 1964; Sillén et al., 1971).

Both the rate and extent of the stimulation shown in Fig. 5.6 are dependent on La\(^{3+}\) concentration. With \(\beta\)-hydroxybutyrate, both parameters are maximal at about 100 µM, but at concentrations less than 30 µM little or no stimulation occurs. With succinate, higher concentrations of La\(^{3+}\) are required.
Stimulation of mitochondrial oxygen uptake by La\(^{3+}\)

Mitochondria (3.8 mg protein) were incubated under conditions similar to those of Fig. 5.2 except that the substrate was varied as follows: (a) succinate; (b) \(\beta\)-hydroxybutyrate; (c) \(\alpha\)-oxoglutarate-tris (5 mM) plus malonate-tris (5 mM). Additions were mitochondria (RLM) and La\(^{3+}\) (167 \(\mu\)M).

Effects of La\(^{3+}\) on mitochondrial respiration

Mitochondria were incubated in 3 ml of the standard medium. The conditions for each experiment varied as follows: (a) succinate, 7.2 mg mitochondrial protein (RLM), 20°C; (b) \(\beta\)-hydroxybutyrate, 3.8 mg mitochondrial protein (RLM), 30°C; (c) \(\beta\)-hydroxybutyrate, 3.6 mg mitochondrial protein (RLM), 30°C.
Within quite narrow concentration limits, the stimulation of succinate oxidation by La$^{3+}$ is cyclic, illustrated by the experiment of Fig. 5.7a. The difficulties involved in obtaining this type of response are the lack of stimulation by low concentrations (<75 µM) on the one hand, and the apparent irreversibility of the response induced by high concentrations (>150 µM) on the other. It is thus difficult to assess accurately the La$^{3+}$/0 activation ratio (cf. Ca$^{2+}$/0; Rossi and Lehninger, 1964). Those values which have been obtained with succinate are of the order of 2.6 and may be related to a stoichiometry of four cation-charge-equivalents transported per energy-conserving site under these experimental conditions (Chance, 1965).

In contrast, no cyclic response could be obtained in the presence of β-hydroxybutyrate. At concentrations exceeding 50 µM the stimulated rate of respiration is followed by an inhibited state, but this is not the normal state 4 since it is not relieved by the addition of an uncoupling agent (Fig. 5.7b). The inhibition does not extend to succinate oxidation as shown in Fig. 5.7c.

When phosphate (1 mM) is substituted for acetate, the respiratory stimulation induced by La$^{3+}$ is barely noticeable, probably due to the formation of a stable lanthanum phosphate complex. No visible precipitation of lanthanum phosphate occurs at these concentrations but mitochondrial aggregation is quite pronounced.

Additional experiments have shown that oligomycin has no noticeable effect on the responses described above.

**Redox changes in respiratory components**

To obtain more direct evidence for energy-dependent transport of La$^{3+}$, the individual components of the respiratory chain were studied by dual-wavelength spectrophotometry. (Fig. 5.8). With both β-hydroxybutyrate (Fig. 5.8a) and succinate (Fig. 5.8b and c) as substrates, 100 µM-La$^{3+}$ elicits a change in the redox state of all respiratory carriers.

In the case of β-hydroxybutyrate (Fig. 5.8a), pyridine nucleotides are extensively oxidized. Simultaneously, flavoprotein components (and/or non-haem iron protein), cytochrome b
Fig. 5.8. Response of mitochondrial respiratory components to 100 µM-La$^{3+}$

Dual-wavelength spectrophotometry was used to record the changes in redox state of respiratory components in response to the addition of 100 µM-La$^{3+}$ (arrows). The tracings in each horizontal row were obtained with the wavelength pair indicated on the left (measuring wavelength - reference wavelength (nm)). The vertical absorbance calibration represents 0.02 units for pyridine nucleotides and cytochrome b (in (a) and (b) only), and 0.005 units for all other traces. Experiments were done with the standard incubation medium containing (a) β-hydroxybutyrate; (b) succinate plus rotenone (0.33 µM); (c) succinate. Mitochondrial protein was 7.2 mg in (a) and (b), 6.8 mg in (c). The final volume was 3 ml. Room temperature.
and cytochrome c \(+\text{c}_1\) become more reduced, indicating a cross-over (Chance and Williams, 1956) in the flavoprotein chain. The fast primary response is succeeded by a slow phase of reduction of flavoproteins and cytochrome c, while cytochrome b is slightly more oxidized (cf. Fig. 5.9). After about 1 min the rate of pyridine nucleotide oxidation decreases, flavoproteins become more oxidized and cytochrome b undergoes a second phase of reduction. The response of cytochrome a (\(\text{a}_3\)) is anomalous in that it initially tends towards a more oxidized state. We have been unable to measure an initial rapid response so it is possible that the primary response is a rapid transitory reduction. A phase of reduction occurs synchronously with the second phase responses of the other respiratory components.

A qualitatively similar pattern is evident with succinate plus rotenone (Fig. 5.8b). The two bottom traces in this Figure compare absorbance changes of the \(\alpha\) and \(\beta\) bands respectively of cytochrome oxidase. The contribution of cytochrome a to the former is about 80 per cent while it contributes only about 35 per cent to the Soret band (Lemberg, 1969). From a comparison of the response measured near the maxima of these two bands, it seems that cytochrome \(\text{a}_3\) does not undergo the second phase reduction and that this response is due to cytochrome a alone. Experiments with \(\beta\)-hydroxybutyrate (data not shown) give similar results.

When succinate alone is used as the substrate (Fig. 5.8c) the effects observed are similar to those seen in the presence of rotenone, with the notable exception that cytochrome a (\(\text{a}_3\)) shows an initial reduction.

The initial responses of the respiratory components occurring within the first minute after \(\text{La}^{3+}\) addition apparently correlate with the lag period noted in the measurements of light scattering and oxygen uptake. It is therefore the second phase responses which correlate with respiratory stimulation and mitochondrial swelling.

The responses to 100 \(\mu\text{M}\)-\(\text{La}^{3+}\) described above are irreversible, reflecting the irreversibility of the stimulation of oxygen uptake at higher concentrations. At low concentrations of \(\text{La}^{3+}\), the oxidation of pyridine nucleotides
Fig. 5.9. Effects of low La$^{3+}$ concentrations on pyridine nucleotides and cytochrome b

Conditions were identical to those of Fig. 5.8 except that the substrate was 8-hydroxybutyrate in each case. The absorbance calibration represents 0.01 units for pyridine nucleotides (340-374 nm) and 0.005 units for cytochrome b (430-410 nm). Additions were made as shown to cuvettes containing 7.2 ((a) and (b)) or 4.8 ((c) and (d)) mg mitochondrial protein.
(Fig. 5.9a) and cytochrome $a_3$ (+a) is again irreversible, but in contrast, cytochrome $b$ undergoes a cycle of rapid reduction followed by slow reoxidation (Fig. 5.9b). A number of such additions can be made until a point is reached at which cytochrome $b$ becomes irreversibly reduced (Fig. 5.9b,d).

In a further series of experiments, EGTA was used to clarify the site of interaction of La$^{3+}$. The rationale underlying its use in this context is that EGTA avidly chelates La$^{3+}$ (see Sillén et al., 1971). Thus, any free La$^{3+}$, as well as that bound to the outer membrane and to the external surface of the inner membrane, will be chelated by EGTA (cf. Section I). Since it is unable to penetrate the osmotically active inner compartment of mitochondria, it does not remove La$^{3+}$ which has been transported to this space (similar use of EGTA was made in the isopycnic centrifugation studies described above).

When 0.67 mM-EGTA is added immediately after the reduction of cytochrome $b$ induced by 33 µM-La$^{3+}$ (Fig. 5.9c), it stimulates the rate of cyclic reoxidation but has no instantaneous effect (cf. Fig. 5.9d). Similarly, EGTA has no effect on the irreversible oxidation of cytochrome $a_3$ (+a) induced by 33 µM-La$^{3+}$. It can thus be concluded that the initial reduction of cytochrome $b$ is due to La$^{3+}$ situated within the EGTA-permeability barrier; i.e. the inner membrane. In contrast, the irreversible reduction of cytochrome $b$ induced by 67 µM-La$^{3+}$ is immediately reversed by EGTA (Fig. 5.9d); this phenomenon must be associated with La$^{3+}$ which is accessible to EGTA, by virtue either of external binding or of increased membrane permeability. However, the extensive oxidation of cytochrome $a_3$ (+a) induced by 67 µM-La$^{3+}$ is not affected by a similar concentration of EGTA.

Pyridine nucleotide fluorescence

In mitochondria supplemented with β-hydroxybutyrate, La$^{3+}$ causes an initial rapid increase in pyridine nucleotide fluorescence followed by a slower decrease (Fig. 5.10a) which reflects the oxidation seen in absorbance measurements (Fig. 5.8a). Both the extent of the rapid increase and the rate of subsequent decrease are dependent on concentration, but at all concentrations tested (25-150 µM), the response is irreversible.
Fig. 5.10. Effects of La$^{3+}$ and Ca$^{2+}$ on pyridine nucleotide fluorescence

Mitochondria (3.1 mg protein) were added to cuvettes containing the standard medium and β-hydroxybutyrate ((a) and (d)), succinate ((b) and (e)) or succinate plus 0.13 µM-rotenone ((c) and (f)) in a total volume of 2 ml at room temperature. Further additions were made as indicated. Fluorescence emission was recorded at 460 nm with excitation at 355 nm.
and qualitatively similar. When succinate is substituted for 3-hydroxybutyrate, the same features are observed but in this case the rate of oxidation is considerably faster and the total extent of oxidation is less (Fig. 5.10b). On the further inclusion of rotenone, La\textsuperscript{3+} causes a slow increase in fluorescence (Fig. 5.10c). The subsequent addition of CCCP decreases fluorescence only to the initial level, in contrast to its effect in the absence of rotenone (Fig. 5.10b,c).

These effects of La\textsuperscript{3+} can be compared with those of Ca\textsuperscript{2+} under similar conditions (Fig. 5.10d-f). A series of rapid oxidation-reduction cycles are obtained with 3-hydroxybutyrate (Fig. 5.10d), but with succinate the pyridine nucleotides become irreversibly oxidized when the total Ca\textsuperscript{2+} concentration exceeds 250 \( \mu \text{M} \) (Fig. 5.10e). In contrast, the addition of Ca\textsuperscript{2+} to a medium further supplemented with rotenone causes a large increase in pyridine nucleotide fluorescence, an effect similar to, but more pronounced than that of La\textsuperscript{3+}.

The increase in fluorescence intensity induced by Ca\textsuperscript{2+} in the presence of succinate and rotenone (Chance and Azzi, 1968) is probably due to fluorescence enhancement of reduced pyridine nucleotides resulting from their binding Ca\textsuperscript{2+} in a non-aqueous environment, presumably a membrane lipid phase. Vinogradov \textit{et al} (1972) have reported that La\textsuperscript{3+} causes a much greater enhancement of NADH fluorescence in a model system (90 per cent methanol) than does Ca\textsuperscript{2+}, suggesting that our results with La\textsuperscript{3+} (Fig. 5.10a,c) reflect a similar association of this cation with reduced pyridine nucleotides in the inner membrane.

The surprising sensitivity of pyridine nucleotides to Ca\textsuperscript{2+} in succinate-supplemented mitochondria prompted its further investigation. As seen in Fig. 5.11a, the oxidation of pyridine nucleotides correlates with a dramatic inhibition of oxygen uptake to a rate less than that of state 4. The Ca\textsuperscript{2+}-inhibited state is relatively insensitive to CCCP, in marked contrast to state 6 (Chance and Schoener, 1966).

Complete cycles of Ca\textsuperscript{2+}-induced respiration can be restored in two ways. The first is by the inclusion of rotenone (Fig. 5.11c); the second is by the prior addition of a low concentration of La\textsuperscript{3+}. It should be noted that 3 \( \mu \text{M}-\text{La}^{3+} \)
Fig. 5.11. **Inhibition of succinate oxidation by Ca$^{2+}$ and its reversal by La$^{3+}$ and rotenone**

Mitochondria (3.5 mg protein) were added as shown (RLM) to the standard medium containing succinate in a total volume of 2 ml at 25°C. Incubations (c) and (d) were further supplemented with 0.13 µM-rotenone. The final concentration of Ca$^{2+}$ added was in each case 500 µM and that of La$^{3+}$, 3 µM. The final concentration of CCCP in (a) was 10 µM.

Fig. 5.12. **Effects of La$^{3+}$ and Ca$^{2+}$ on flavoprotein fluorescence**

Mitochondria (3.5 mg protein) were incubated exactly as described for Fig. 5.10, the substrates being β-hydroxybutyrate ((a) and (d)), succinate ((b) and (e)), or succinate plus 0.14 µM-rotenone ((c) and (f)).

The final concentration of La$^{3+}$ was in each case 150 µM and that of Ca$^{2+}$, 250 µM. Fluorescence emission was recorded at 570 nm with excitation at 436 nm.
causes only partial inhibition of Ca\(^{2+}\)-stimulated respiration (Fig. 5.11b,d; cf. Fig. 5.11a,c), reflecting the accumulation of La\(^{3+}\) shown by the preceding data.

**Flavoprotein fluorescence**

The addition of 150 µM-La\(^{3+}\) to mitochondria oxidizing either β-hydroxybutyrate or succinate causes a relatively slow but extensive oxidation of fluorescent flavoproteins (Fig. 5.12a,b); the further inclusion of rotenone in the succinate-supplemented system decreases both the rate and extent of oxidation (Fig. 5.12c). In contrast, La\(^{3+}\) causes a reduction of components absorbing at 475 nm (see Fig. 5.8). The La\(^{3+}\)-induced cross-over shown by Fig. 5.8 is thus very close to, or identical with, the rotenone-sensitive site and the first energy-coupling site, located between fluorescing flavoprotein(s) on the substrate side and non-fluorescing flavoproteins and/or non-haem iron protein on the oxygen side (Chance *et al.*, 1967 and 1969; Hatefi, 1968).

Again, no cyclic response is obtained, in contrast to the effects of Ca\(^{2+}\) (Fig. 5.12d-f). It should be noted that the specific inhibition of succinate oxidation by Ca\(^{2+}\) is accompanied by increased oxidation of the fluorescing flavoproteins.

**ATP-supported La\(^{3+}\) accumulation**

It was anticipated that ATP could support La\(^{3+}\) uptake by analogy with energy-linked Ca\(^{2+}\) transport (Bielawski and Lehninger, 1966). Control experiments showed no interference of La\(^{3+}\) with the phosphate assay and no precipitation of lanthanum phosphate at concentrations up to 0.5 mM; when a precipitate did form at higher concentrations, it was completely soluble in the 5 per cent (w/v) trichloroacetic acid used to quench reactions.

The appropriate experiments were carried out but the results were equivocated by pronounced aggregation of the mitochondria, an effect associated with the release of inorganic phosphate from ATP. When incubation conditions were adjusted to minimize this effect, a slight stimulation of ATP hydrolysis was evident accompanied by a marginal increase in mitochondrial density as estimated by isopycnic centrifugation, but the effects of La\(^{3+}\) were in no case sufficiently marked to warrant
definite conclusions. The recurring problem of La$^{3+}$ chelation, in this case by ATP, is probably responsible for the lack of effect in this system. No data are available for the stability constant of LaATP, but it probably exceeds $10^6$ M$^{-1}$.

Discussion

The primary aim of this work was to determine unequivocally whether La$^{3+}$ is transported across the inner membrane of rat liver mitochondria. The foregoing data leave little doubt that this is indeed the case, although the La$^{3+}$ accumulation process shows some rather unusual features. Thus, the contrary conclusions of Lehninger and Carafoli (1971) are due in part to the incomplete inhibition of La$^{3+}$ transport by respiratory inhibitors and uncouplers, partly to an unfortunate choice of incubation conditions, and partly to the restricted experimental approaches used. The time-dependent relief of the inhibition of Ca$^{2+}$ transport by La$^{3+}$ is seen to be a consequence of its accumulation. Clearly, data relating to its use as a specific inhibitor must be interpreted with caution.

Functional aspects of La$^{3+}$ accumulation

The interaction of La$^{3+}$ with mitochondria is markedly dependent on its concentration and is probably a function of the ratio of La$^{3+}$ to 'mitochondrial protein'. At very low concentrations a number of similarities are apparent between the effects of La$^{3+}$ and Ca$^{2+}$.

Thus, all respiratory components show an initial rapid response, but the direction of this response to La$^{3+}$ is open to question because of the poor time resolution in these experiments. It is essential that more sensitive kinetic studies be undertaken, not only to determine the correct position of cross-over points, but more significantly to enable a comparison to be made between the initial rates of interaction of La$^{3+}$ and Ca$^{2+}$. Such a comparison would be extremely useful in terms of understanding the mechanism of active cation transport. La$^{3+}$ has a far greater affinity for the Ca$^{2+}$ carrier than does Ca$^{2+}$ itself (illustrated by its remarkably strong competitive inhibition of Ca$^{2+}$
transport) but its rate of accumulation is much slower (compare respiratory stimulation shown in Figs. 5.6 and 5.11). On the other hand, Mn$^{2+}$ similarly causes less stimulation of oxygen uptake than does Ca$^{2+}$ (Chappell et al., 1963; Chance and Schoener, 1966) and is accumulated more slowly (Mela and Chance, 1968), but the initial response of the respiratory components is quite slow (Chance and Schoener, 1966; Vainio et al., 1970). Since the affinity of Mn$^{2+}$ for the Ca$^{2+}$ carrier appears to be relatively low (Chappell et al., 1963; Vinogradov and Scarpa, 1973), it is probable that the initial rate of response of the respiratory components, particularly those associated with energy-coupling sites, to cations is directly related to the affinity of the cations for the carrier.

Implicit in this suggestion is the assumption that Ca$^{2+}$, Mn$^{2+}$ and La$^{3+}$ are transported on the same carrier. This remains to be definitely proven, but the known interactions between these cations (Mela and Chance, 1968; Reynafarje and Lehninger, 1969; Vainio et al., 1970; Vinogradov and Scarpa, 1973) make it a strong possibility.

The reoxidation of cytochrome b reduced by the addition of La$^{3+}$ may provide further insight into the mechanism of cation transport. This recovery phase is much slower than the corresponding phase of reduction associated with the response to Ca$^{2+}$. It suggests that release of La$^{3+}$ from internal binding sites of the carrier may be the rate-limiting step in the accumulation of La$^{3+}$, a conclusion completely in accord with the high affinity of La$^{3+}$ for the carrier. This is strengthened by the ineffectiveness of EGTA in evoking an immediate response when added at the point of maximum reduction (Fig. 5.9c). The stimulated rate of recovery under these latter conditions is taken to be a result of the decreased availability of La$^{3+}$ for external binding to the carrier prior to transport, so that it gives an estimate of the rate of release of La$^{3+}$ to the matrix from the loaded carrier. Unfortunately, the release of cations to the internal space is a feature of the carrier mechanism which has drawn little previous attention. It is anticipated that comparative
studies of both the initial and recovery phases of the cytochrome responses to Mn\(^{2+}\), Ca\(^{2+}\) and La\(^{3+}\) will be extremely valuable.

The effects of La\(^{3+}\) on the flavoprotein chain seem to imply a site(s) of interaction common with Ca\(^{2+}\) with one notable exception. The cross-over immediately following the addition of La\(^{3+}\) appears to be in this region, in contrast to the case with Ca\(^{2+}\) where it has been localized between cytochromes \(b\) and \(c_1\) (Chance, 1965). The shift towards the substrate end of the chain may be due to the lower respiratory activity induced by La\(^{3+}\).

The most interesting feature of the La\(^{3+}\) accumulation process described in this paper is its relative insensitivity to inhibitors of energy production and to the permeant anion, acetate. This can be rationalized by proposing that La\(^{3+}\) is bound and transported by a specific cation carrier in an energy-independent process and is released (more slowly than Ca\(^{2+}\)) to a large number of internal binding sites. It is assumed that the affinity of these sites for La\(^{3+}\) is lower than that of the carrier, but that they are present at high concentration (probably in the membrane). The differing sensitivities of La\(^{3+}\) and Ca\(^{2+}\) accumulation to uncouplers and respiratory inhibitors reflect the difference in affinity of these cations for internal sites.

Such an interpretation is supported by differences between the binding of La\(^{3+}\) and Ca\(^{2+}\) to external sites of mitochondria. The work described in the previous chapter shows that a total of about 15 nmoles Ca\(^{2+}\)/mg protein is bound externally (Kd \(\approx\) 25 \(\mu\)M), whereas Fig. 5.5. shows that approximately 30 nmoles La\(^{3+}\)/mg protein can be bound within five sec at 0°C (Kd \(\approx\) 10 \(\mu\)M). This is assumed to be a good estimate of external binding since the rate of La\(^{3+}\) transport is too low to permit significant net uptake within this time. The close similarity in binding properties of Ca\(^{2+}\) and La\(^{3+}\) (Williams, 1970) and the demonstrated inhibition of low affinity Ca\(^{2+}\) binding by La\(^{3+}\) (Lehninger and Carafoli, 1971) suggest that La\(^{3+}\) binds to the same external sites as does Ca\(^{2+}\), but also binds to an approximately equal number of
additional sites.

Data of Lehninger and Carafoli (1971) are relevant in this regard since they show that the external binding of La$^{3+}$ is accompanied by the release of protons in approximate 1:1 stoichiometry, whereas Reynafarje and Lehninger (1969) and Wenner and Hackney (1967) have found that 'energy-independent' Ca$^{2+}$ binding is not accompanied by proton release. The additional La$^{3+}$ binding may thus be to sites which are normally protonated at pH 7.4, but for which La$^{3+}$ has a sufficiently high affinity to cause deprotonation (in contrast to Ca$^{2+}$). It is possible that both the external Ca$^{2+}$ binding sites and the external La$^{3+}$ binding sites might involve the same molecular species, one with a pKa close to 7.4. A likely candidate would be phosphatidylserine (Dawson and Hauser, 1970; Rojas and Tobias, 1965; Rojas et al., 1966).

Studies of the effect of pH on external Ca$^{2+}$ and La$^{3+}$ binding would be of benefit in resolving this question.

Structural aspects of La$^{3+}$ accumulation

The effects of La$^{3+}$ on respiratory activity become irreversible at concentrations exceeding about 50 µM with β-hydroxybutyrate, or about 100 µM with succinate as substrate. The mitochondria similarly become maximally swollen at this concentration of La$^{3+}$ (Fig. 5.2 and Plate 1). It thus seems possible that the non-cyclic stimulation of oxygen uptake, and irreversible changes in steady state of the respiratory components, could be a direct result of the swelling process. It has been assumed throughout this chapter that the swelling is a simple osmotic response to the accumulation of soluble lanthanum acetate in the matrix space. However, this is probably not so. In the first place, acetate has only a marginal stimulatory effect on the absorbance decreases at 520 nm, indicating it is not required for La$^{3+}$-induced swelling. Secondly, the magnitude of the swelling induced by the accumulation of La$^{3+}$ is far greater than that of the purely osmotic swelling induced by the accumulation of a similar concentration of Ca$^{2+}$ in the presence of acetate (data not shown). Thirdly, the non-cyclic response of pyridine
nucleotides and flavoproteins at very low concentrations of \( \text{La}^{3+} \) argues for the direct interaction of \( \text{La}^{3+} \) with a component(s) in this region, which necessitates the association of \( \text{La}^{3+} \) with sites in the membrane, even at low concentrations. Finally, the arguments developed in the preceding discussion to rationalize the insensitivity of the \( \text{La}^{3+} \) accumulation process to respiratory inhibitors and uncouplers might be construed as further evidence that the accumulated \( \text{La}^{3+} \) is membrane-bound rather than soluble.

It has been well-established that the accumulation of \( \text{Ca}^{2+} \) by liver mitochondria similarly results in 'non-osmotic' swelling (Lehninger, 1962; Chappell and Crofts, 1965). This is an effect quite distinct from an osmotic response to the accumulation of a soluble \( \text{Ca}^{2+} \) salt, since it occurs some time after the accumulation process itself, the lag period being dependent on \( \text{Ca}^{2+} \) concentration (see Introduction). Furthermore, it is inhibited by the presence of acetate. Under these latter conditions, immediate osmotic swelling accompanies the accumulation of \( \text{Ca}^{2+} \) and is followed only after a prolonged lag period by the second swelling response (data not shown).

The mechanism of this effect of \( \text{Ca}^{2+} \) remains unclear in spite of the considerable attention focused on various aspects of it. A more detailed study of the swelling effect of \( \text{La}^{3+} \) could be useful in view of the possible similarity between it and that of \( \text{Ca}^{2+} \). At this preliminary stage, it appears that the only difference between their effects is that \( \text{La}^{3+} \) has a higher affinity for membrane sites involved in the swelling response.

The ultrastructural features seen at higher \( \text{La}^{3+} \) concentrations may again be a direct consequence of its binding with relatively high affinity to membrane components. Thus, aggregation is proposed to be due to ion-bridging between the external surfaces of membranes. The condensation of inner membrane fragments to form discrete vesicles within the outer membrane occurs at the same concentrations, suggesting that the same phenomenon may be responsible for both effects. In the case of the inner membrane, ion-bridging between
opposing faces of the membrane would induce a tightly-folded, dense configuration. The subsequent 'budding' of the vesicles formed in this way and the final tightly-stretched appearance of the inner membrane may be further consequences of ion-bridging but, in these later stages, between sites on the outer and inner membranes.

Prospects

The data reported in this chapter establish the basic features of La$^{3+}$ accumulation by mitochondria, but also raise many interesting questions. The answers to these are seen as being valuable, not so much in terms of studying the effects of La$^{3+}$ itself, as in the insight they can provide into the interaction of physiological cations with mitochondria. In most physiological systems La$^{3+}$ and other rare earth cations mimic the effects of Ca$^{2+}$, resulting in either a strong enhancement or inhibition of the normal Ca$^{2+}$ response, due to the similarity in bonding behaviour of the lanthanides and the alkaline earths. In both cases, bonding is primarily electrostatic, but the higher charge density of the lanthanides provides far greater bond stability. In addition, some of the lanthanides have analytically useful spectral and magnetic properties, suggesting their use as probes for the alkaline earth cations (Williams, 1970; Birnbaum et al., 1970); they have already been used as such in a number of studies (e.g., Darnall and Birnbaum, 1970; Luk, 1971; Dwek et al., 1971; Sperow and Butler, 1972; see also Barry et al., 1971).

In the case of mitochondrial cation accumulation, La$^{3+}$ provides an analogue whose utility extends beyond its use as an inhibitor of the Ca$^{2+}$ carrier. Indeed, its main value may lie in defining the mechanism for active cation transport. At this stage, the most obvious need in this regard is for more sensitive kinetic studies, together with multiparameter analyses of respiration, light scattering and proton ejection. In addition, the accumulation and binding of $^{140}$La$^{3+}$ (or a more stable lanthanide radioisotope; e.g., $^{147}$Nd, $^{143}$Pr,
$^{154}$Eu should be re-assessed using EGTA to discriminate between La$^{3+}$ bound to internal and external sites. All of these studies must be made with low La$^{3+}$ concentrations to prevent interference from non-specific effects associated with large amplitude swelling.

The effects of lanthanides on mitochondrial ultrastructure should also be examined in more detail with a view to clarifying the role of Ca$^{2+}$ in particular (and metal ions in general) in maintaining and modifying the structure of biological membranes. Electron microscopy provides a means of studying this at the level of supramolecular organization, while the spectroscopic and magnetic properties of the rare earth cations should reveal details at the molecular level.
STUDIES OF RUTHENIUM RED IN RELATION TO ITS INTERACTION WITH MITOCHONDRIA

The inhibition of mitochondrial Ca\textsuperscript{2+} transport by ruthenium red was examined in an earlier section. It was shown that the number of carrier-specific ruthenium red binding sites is 0.08 nmoles/mg protein and their $K_d$ is $0.03 \times 10^{-6}$ M. That work raised two additional questions which are discussed here in some detail.

The first is the need for direct measurements of the binding of ruthenium red to mitochondria. It is not known if ruthenium red binds solely to the Ca\textsuperscript{2+} carrier. Clearly, the interpretation of its effects on Ca\textsuperscript{2+} binding and transport depends on this knowledge.

The second point of interest in the kinetic studies was the demonstration of colourless inhibitory impurities in commercial preparations of ruthenium red. Experiments were undertaken to identify these in the hope that more information would thereby be gained of the binding sites involved in non-competitive inhibition of the Ca\textsuperscript{2+} carrier. A second advantage, inherent in a colourless inhibitor, is that it would not interfere with optical measurements of mitochondrial redox components, and would therefore be more useful than ruthenium red itself in determining the relation between non-competitive carrier inhibition and energy transduction.

(a) **External binding of ruthenium red to mitochondria**

The ruthenium red used in these studies was recrystallized to spectroscopic purity (refer to Appendix). It was found that its binding to mitochondria can be measured by direct titration with dual-wavelength spectrophotometry (Fig. 6.1). The absorbance maximum for ruthenium red in water is at 533 nm, in the sucrose-hepes-tris incubation medium at 535 nm, and in a suspension of mitochondria in the incubation medium at 540 nm. The 5 nm shift in the presence of mitochondria, a
Fig. 6.1. Binding of ruthenium red to intact and sonicated mitochondria

Reference and sample cuvettes contained mitochondria (1.28 mg protein) in 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 0.15 µM-rotenone and 0.15 µM-antimycin in a total volume of 3 ml (●), or mitochondria (1.28 mg protein) sonicated for 30 sec in 3 ml water (○). Ruthenium red was added to the sample cuvettes at the concentrations shown and the absorbance was recorded at the appropriate wavelength pairs. The difference $\Delta E_{540-535}$ (●) or $\Delta E_{539-533}$ (○) is plotted as a function of ruthenium red concentration. The experiments were carried out at room temperature.
consequence of its binding, enables the number of binding sites to be estimated by extrapolating the ascending and descending portions of a plot of $\Delta E_{540-535}$ against ruthenium red concentration (Fig. 6.1) since these portions represent bound and free ruthenium red, respectively. The accuracy of this procedure obviously increases with the affinity of the binding sites. The data of Fig. 6.1 show that intact mitochondria bind approximately 15 nmoles ruthenium red/mg protein. This is assumed to be at external sites since there is no increase in binding with time (cf. stability of inhibition of Ca$^{2+}$ transport: Section I).

In these experiments it was noticed that the mitochondria aggregate at concentrations of ruthenium red sufficient to saturate the binding sites (similar effects are associated with La$^{3+}$ binding; Reed & Bygrave, 1973a). The above experiment was therefore repeated using mitochondria which had been sonicated in water. Aggregation still occurred but it caused less 'noise' because of the smaller particle size. The amount of ruthenium red bound to these particles is again 15 nmoles/mg protein but the binding affinity is less (Fig. 6.1).

It is clear that, similarly to Ca$^{2+}$ and La$^{3+}$, ruthenium red does not bind solely to the Ca$^{2+}$ carrier. The finding that mitochondria bind (externally) twice the amount of La$^{3+}$ as they do ruthenium red raised the question of whether their binding sites are identical. This was examined by measuring the effect of La$^{3+}$ on the binding of ruthenium red. In these experiments, mitochondria were incubated briefly with ruthenium red at 0°C, La$^{3+}$ was added at the desired concentration, and the tubes were centrifuged immediately. The absorbance of the supernatants was read at 535 nm and corrected for the slight loss of ruthenium red due to its adsorption to the incubation tubes (see Appendix) by reference to a standard curve prepared simultaneously. A Scatchard plot of the data of such an experiment is shown.
Fig. 6.2. Inhibition of ruthenium red binding by La$^{3+}$

(a) Incubations contained mitochondria (1.43 mg protein) in 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 0.15 µM-rotenone and 0.15 µM-antimycin in a total volume of 3.15 ml. Ruthenium red was added and followed immediately by nil (○), 127 µM-La$^{3+}$ (●) or 254 µM-La$^{3+}$ (△). The tubes were then centrifuged at 16,000 x g for 5 min and the absorbance of the supernatants was read at 535 nm. The concentration of ruthenium red remaining in the supernatants was calculated by referring to a standard curve constructed from identical, simultaneous incubations lacking mitochondria. These values were used to calculate the amount of ruthenium red bound (nmoles/mg protein) and the ratio bound:free (nmoles/mg protein:µM). Data plotted in the absence of La$^{3+}$ (●) are the mean values from experiments with two separate batches of mitochondria. The experiments were done at 0°C.

(b) The washed pellet from an incubation of (a) (shown by the arrow) was sonicated for 1 min in 3 ml water and its absorbance was recorded at 539 and 533 nm relative to a reference containing mitochondria similarly treated in the absence of ruthenium red. Both cuvettes were titrated with La$^{3+}$ as shown. The difference $\Delta E_{539-533}$ is plotted as a function of La$^{3+}$ concentration.
in Fig. 6.2(a). In the absence of $\text{La}^{3+}$, the number of binding sites is again found to be 14-15 nmoles/mg protein and their apparent $K_d$ is approximately 0.7 $\mu$M, confirming the high affinity shown qualitatively in Fig. 6.1.

In the presence of $\text{La}^{3+}$, two classes of ruthenium red binding sites can be distinguished (Fig. 6.2(a)). The first, numbering about 15 nmoles/mg protein, is inhibited competitively by $\text{La}^{3+}$ with a $K_i$ of approximately 10 $\mu$M (calculated from the 'apparent $K_d$' values). The second class is not inhibited by $\text{La}^{3+}$ and binds less than 2 nmoles ruthenium red/mg protein. The limited sensitivity of the spectrophotometric measurements prevented a detailed analysis at lower concentrations.

The inhibition of 'low-affinity' ruthenium red binding by $\text{La}^{3+}$ is also evident in the experiment of Fig. 6.2(b). In this case, the sonicated pellet from an incubation containing a saturating concentration of ruthenium red was titrated with $\text{La}^{3+}$ and the absorbance at 539 and 533 nm (the maxima for bound and free ruthenium red respectively under these conditions) was recorded. The decrease in $\Delta E_{539-533}$ shows the release of bound ruthenium red; the $K_i$ for $\text{La}^{3+}$ is approximately 50 $\mu$M (the concentration required for half-maximal inhibition). It therefore appears that the water-treated sonic particles bind both $\text{La}^{3+}$ and ruthenium red with lower affinity than do intact mitochondria (Figs. 6.1 and 6.2).

Attempts to measure carrier-specific ruthenium red binding

The competitive-binding experiments with $\text{La}^{3+}$ reveal the existence of a small number of high-affinity sites for ruthenium red which are not (or only weakly) inhibited by $\text{La}^{3+}$. Since $\text{La}^{3+}$ was added after ruthenium red in these experiments (to minimize $\text{La}^{3+}$ uptake), these could be sites to which ruthenium red is bound irreversibly. But they could be sites
at which La$^{3+}$ does not bind, a particularly interesting possibility in view of the non-competitive inhibition of Ca$^{2+}$ transport by ruthenium red. Their concentration is too high for them to be solely associated with inhibition of the Ca$^{2+}$ carrier, but nonetheless it may be possible to identify a class of sites within this group which have the properties of the carrier-specific binding sites revealed by the kinetic data.

The sensitivity demanded in such experiments is too high for spectrophotometric assays of binding. The alternative approach is to use radioactive ruthenium red. To this end, the synthesis described by Fletcher et al. (1961) was adopted, scaled down for 0.4 g of carrier RuCl$_3$$\cdot$3H$_2$O and 0.7 mCi $^{103}$Ru (final sp.act. ~ 0.5 mCi/m mole Ru). The yield of ruthenium red was too small to allow purification of the product by the crystallization procedure of Fletcher et al. (1961). The most successful method (of many attempted) involved its resolution on a column of silicic acid eluted with 0.1 M-ammonium acetate at pH 5.0 (Fig. 6.3).

Each time this purification procedure was used, two pink-red components were resolved. The fractions containing the second of these were combined and adjusted to pH 8.5 with NH$_3$ to minimise the oxidation of ruthenium red to the brown complex (Fletcher et al., 1961). The extremely dilute solution was concentrated by adding a few milligrams of silicic acid (previous tests had shown that ruthenium red adsorbs strongly to silicic acid at neutral or alkaline pH, but not in acid). When the solution had been decolourized, it was decanted and the red silicic acid was packed in the stem of a pasteur pipette, washed with water, and the red compound eluted with a small volume of 10 mM-HCl. The pH of the eluate was immediately adjusted to about 9 with dilute NH$_3$.

The absorbance spectrum of the product indicated the presence of ruthenium red heavily contaminated with ruthenium brown, but the composition and purity of the product were cast in doubt by the observation that a flocculent red precipitate developed within a short time; this behaviour is not typical of authentic ruthenium red. Binding
A portion of the products from the small-scale synthesis (see text) were adsorbed to a small amount of silicic acid at pH 8.5 (0.1 M-ammonium acetate), and this suspension was applied to a pre-washed column (1.5 x 35 cm) of silicic acid equilibrated with 0.1 M-ammonium acetate (pH 5.0). The column was eluted with the pH 5.0 buffer under nitrogen pressure at a flow rate of 100 ml/hr for the first 14 fractions, and 400 ml/hr thereafter. The first 20 fractions were colourless; these were followed by bands of pink and red material as indicated on the figure. Fractions 40-68, containing the bulk of the red material, were pooled and treated as described in the text. Fraction volume was 20 ml. Room temperature.
experiments were nevertheless conducted with the red complex which remained in solution, but these gave variable and inconsistent results.

Thus, 'high-affinity' binding of ruthenium red has not been analyzed successfully. The experimental approach of using the radioactively-labelled compound is undoubtedly the most promising, but it must await the development of an unequivocal purification procedure.

The effects of UO$_2^{2+}$

The competitive-binding experiments described above were supplemented by similar studies with the uranyl ion (UO$_2^{2+}$). Williams (1970) has suggested using UO$_2^{2+}$ as a probe for Ca$^{2+}$ in biological systems. Barton (1968) found that it binds to phosphatidylserine micelles with extremely high affinity; the data of McLaughlin et al (1971) similarly demonstrate a very strong interaction of UO$_2^{2+}$ with bilayers of both phosphatidylserine (acidic) and phosphatidylethanolamine (amphoteric). Shah (1970) extended these observations by showing that zwitterionic lecithin monolayers also bind UO$_2^{2+}$. These experimental measurements were anticipated by the widespread use of UO$_2^{2+}$ as an electron-dense stain for cellular membranes in general and mitochondrial membranes in particular.

We have found that UO$_2^{2+}$ does not inhibit the initial rate of energy-linked Ca$^{2+}$ transport at low concentrations (up to 0.6 nmoles/mg protein), although substantial inhibition of Ca$^{2+}$-induced respiration and active osmotic swelling is evident at 100 µM-UO$_2^{2+}$ (Fig. 6.4). This does not involve a specific inhibitory mechanism, since valinomycin-mediated K$^+$ transport is also inhibited (Fig. 6.4), as is CCCP-stimulated respiration (Fig. 6.4). The basis of these effects may lie in the increased cation charge density of the external surfaces of the mitochondria, which would cause
Fig. 6.4. Inhibition of mitochondrial ion transport by $\text{UO}_2^{2+}$

(a) Respiration: Mitochondria (2.8 mg protein) were added to the vessel of an oxygen polarograph containing 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 5 mM-succinate-tris, and 0.83 µM-rotenone in a final volume of 3.0 ml. Experiments (ii) and (iii) also contained 10 mM-acetate-tris and (iii) contained 5 mM-KCl. Further additions were 10 µM-CCCP, 333 µM-Ca$^{2+}$, 0.5 µg valinomycin, and 100 µM-UO$_2^{2+}$. Temperature was 30°C.

(b) Swelling: Mitochondria (3.6 mg protein) were added to a cuvette containing the medium described above and 10 mM-acetate-tris in a final volume of 3.0 ml; experiments (iii) and (iv) also contained 5 mM-KCl. Further additions were 333 µM-Ca$^{2+}$, 0.5 µg valinomycin, and 100 µM-UO$_2^{2+}$. Mitochondrial swelling was measured at room temperature as the decrease in absorbance at 520 nm.
electrostatic repulsion of Ca$^{2+}$ and K$^+$ (cf. Barton (1968) and Shah (1970)). No valid assessment could be made of the effects of UO$_2^{2+}$ on energy-linked parameters in a medium containing phosphate because of the extremely high stability (and insolubility) of uranyl phosphate.

The inhibitory effects of UO$_2^{2+}$ seen in Fig. 6.4 could also be due in part to its decreasing the fluidity of the inner membrane, and hence restricting the permeability of lipid-soluble species (CCCP, valinomycin, acetic acid). Shah (1970) noted that UO$_2^{2+}$ tends to solidify lecithin monolayers; Barton (1968) ascribes the particularly high affinity of UO$_2^{2+}$ for phosphatidylserine aggregates to the formation of uranyl-phosphate-type lattices, a view supported by the relative weak binding of UO$_2^{2+}$ to a dispersion of phosphatidylserine in chloroform/methanol (Blaustein, 1967).

That UO$_2^{2+}$ tends to stabilize ('solidify') mitochondrial membranes was borne out by preliminary experiments, which showed that mitochondria which had been incubated briefly with 96 µM-UO$_2^{2+}$ appeared to be totally resistant to prolonged sonication in water, since there was no obvious decrease in turbidity of the suspension. This effect was examined more precisely by measuring the effect of UO$_2^{2+}$ on the decreased light-scattering associated with osmotic shock of mitochondria (Fig. 6.5). Maximal protection against osmotic swelling (about 75 per cent) was afforded by concentrations in excess of 80 µM (approx. 80 nmoles UO$_2^{2+}$/mg protein). This is largely a protective action, rather than a contraction of pre-swollen mitochondria, since the addition of UO$_2^{2+}$ has little effect after swelling has occurred (Fig. 6.5), although it does induce a slight contraction of both swollen (Fig. 6.5) and condensed (Figs. 6.4 and 6.5) mitochondria. In contrast to both La$^{3+}$ and ruthenium red, UO$_2^{2+}$ does not cause aggregation.

Thus, UO$_2^{2+}$ is known to bind with high affinity and specificity to membranous phospholipids, and the above results suggest that this is the basis of its effects on mitochondria. Its use as a probe for the ruthenium red
Mitochondria (3.2 mg protein) were added to a cuvette containing 3.0 ml of an aqueous solution of UO$_2^{2+}$ at the concentrations shown. The absorbance of the resulting suspension was read at 520 nm 2 min later, when the absorbance was stable (●). The dashed line is the absorbance of the same concentration of mitochondria in 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4); the addition of 100 µM-UO$_2^{2+}$ to this suspension caused a slight increase (○). The effect of addition of UO$_2^{2+}$ after the suspension of mitochondria in water is also shown (□). Room temperature.
binding sites was predicated on this assumption. Experiments similar to those of Fig. 6.2(a) showed that, similarly to La$^{3+}$, UO$_2^{2+}$ is a competitive inhibitor of ruthenium red binding ($K_d \approx 12 \mu M$) but only at concentrations of ruthenium red less than about 15 nmoles/mg protein (Fig. 6.6). When the concentration of ruthenium red exceeds this value, UO$_2^{2+}$ induces virtually complete binding (Fig. 6.6). This was confirmed in experiments similar to that of Fig. 6.2(b), in which the addition of 33 µM-UO$_2^{2+}$ doubled $\Delta F_{539-533}$.

Direct attempts to measure the binding of UO$_2^{2+}$ to mitochondria by fluorimetry were unsuccessful, owing in part to strong quenching of its fluorescence, and partly to the proximity of the UO$_2^{2+}$ excitation and emission peaks to those of the reduced pyridine nucleotides.

Discussion

The total number of ruthenium red binding sites is similar to the number of external Ca$^{2+}$-binding sites, about 15 nmoles/mg protein. This might suggest that the sites for both are identical, particularly since Vasington et al (1972a, b) and Rossi et al (1973) have reported that crude ruthenium red inhibits 'low-affinity' Ca$^{2+}$ binding (but see Section 1 for a discussion of low-affinity Ca$^{2+}$ binding). On the other hand, the very high affinity of ruthenium red for the external sites ($K_d \approx 0.7 \mu M$) suggests that it may bind to the same total number of sites as does La$^{3+}$ by displacing protons from acidic residues. The competitive inhibition of ruthenium red binding by La$^{3+}$, in which the $K_i$ for La$^{3+}$ is similar to the $K_d$ for La$^{3+}$ binding, supports this interpretation.

Vasington et al (1972a) have used the sensitivity of the low-affinity Ca$^{2+}$ binding sites to (crude) ruthenium red to implicate glycoproteins in these sites, on the basis of a
Fig. 6.6. Effect of UO$_2^{2+}$ on the binding of ruthenium red to mitochondria

Experimental conditions were similar to those described for Fig. 6.2 (a) except that 96 µM-UO$_2^{2+}$ was added instead of La$^{3+}$. The results show the binding of ruthenium red before (●) and after treatment with UO$_2^{2+}$ (O).
supposed specificity of ruthenium red for glycoproteins. However, this assumption is based solely on its gross histological staining properties. Luft (1971) has shown in an exhaustive series of semi-quantitative tests that ruthenium red binds to a very broad spectrum of organic materials, among which some of the most reactive are the phospholipids that previously have been implicated in mitochondrial Ca\(^{2+}\) binding (phosphatidylethanolamine, phosphatidylserine, cardiolipin; Scarpa and Azzi, 1968; Scarpa and Azzone, 1969).

The probability of the ruthenium red binding sites being phospholipids is borne out by their strong inhibition by UO\(_2\)\(^{2+}\) at less-than-saturating concentrations of ruthenium red. The reason for the enhancement by UO\(_2\)\(^{2+}\) at high concentrations of ruthenium red is not clear, but it probably involves direct interaction between the two ion-complexes.

It is concluded that ruthenium red binds to membrane phospholipids at the same sites to which La\(^{3+}\) binds, but in half the total amount. The explanation for this may lie in the relative sizes of the La\(^{3+}\) and ruthenium red cations. Luft (1971) has calculated a mean molecular diameter for ruthenium red of 11.3 Å; X-ray diffraction data of synthetic and biological phospholipid membranes show that the hydrocarbon interchain-distance is 4.2 to 4.6 Å, with ordered hexagonal packing (Keith and Melhorn, 1972), so that the maximum separation between adjacent headgroups is 8.4 to 9.2 Å. A single ruthenium red molecule could therefore bridge the distance between the polar headgroups of two adjacent phospholipid molecules in a membrane with extensive bilayer domains. The polyvalency of the complex (and its ability to participate in ionic, hydrogen and van der Waals bonding) would allow strong bonding to two sites.

The remarkable feature revealed in the present work and in that of Luft (1971), is that ruthenium red is such a specific inhibitor of Ca\(^{2+}\) transport. Its interaction with
the carrier has not been clarified by these binding studies, but the insensitivity to La$^{3+}$ of a small number of ruthenium red binding sites suggests that it has a particularly high affinity for specific groups in the mitochondrial membrane. The nature of these is not known.

The present data on the interaction of Ca$^{2+}$, La$^{3+}$, ruthenium red, and UO$_2$$^{2+}$ with mitochondria can be understood when considered in relation to previous findings of the interaction of heavy metal ions and ionic complexes with phospholipids. The major site of external binding of all these ions is probably the polar headgroups of membrane phospholipids. The relatively low affinity of Ca$^{2+}$ restricts its binding to those groups which are dissociated at neutral pH (e.g. phosphatidylserine; apparent pKa $\approx$ 7.5; Rojas and Tobias, 1965; Papahadjopoulos, 1968). It is suggested that both La$^{3+}$ and ruthenium red bind to these same sites, but in addition their high affinity (particularly in the case of ruthenium red) ensures that they can bind also to undissociated residues (e.g. phosphatidylserine and phosphatidylethanolamine) by displacing protons. The mitochondrial aggregation caused by these ions at concentrations in excess of those required for saturation of the binding sites is probably due to ion-bridging between mitochondria, made possible by their high catonic charge.

The effects of UO$_2$$^{2+}$ can be related to its ability to bind strongly to acidic, amphoteric, and zwitterionic phospholipids. Its binding sites thus include those for the ions discussed above, but in addition may encompass virtually all membrane phospholipids. The total number of UO$_2$$^{2+}$ binding sites may approach 80 nmoles/mg protein (Fig. 6.5). The specific steric requirements for UO$_2$$^{2+}$ binding prevent it from promoting mitochondrial aggregation by ion-bridging.

The high affinity binding of the heavy-metal cations must obviously have a profound influence on the surface charge characteristics of the mitochondrial membrane. This limits their utility as probes into the structural and functional
consequences of phospholipid involvement in membrane structure. The inhibition of mitochondrial energy-linked functions by high concentrations of ruthenium red studied in great detail by Vasington et al. (1972a,b) is, in all probability, a result of such non-specific effects.

(b) Attempts to identify the colourless inhibitory contaminants in commercial preparations of ruthenium red

The approach adopted in the first phase of this work was to synthesize ruthenium red according to Fletcher et al. (1961) with the inclusion of a trace of $^{103}$Ru to enable detection of all ruthenium complexes. Since commercial ruthenium red is probably synthesized under similar conditions, the inhibitory impurities of commercial preparations (see Section I) should be present and labelled in the resulting mixture of products.

Preparation of radioactive ruthenium complexes

Two gm of RuCl$_3$ and 34 µCi $^{103}$Ru were used as the starting materials in the procedure for the synthesis of ruthenium red described by Fletcher et al. (1961). The supernatants from successive ruthenium red crystallizations were combined, and this solution was used as the source of material in subsequent analyses.

Thin-layer chromatography

Partial separation of the components of this mixture was achieved by using sheets of cellulose, DEAE-cellulose and various thin-layer silica and alumina gel media as the supporting adsorbent phase, with combinations of methanol and ammonium acetate (Luft, 1971) as the solvent. The best resolution of visible components was attained on cellulose impregnated with silica gel (Gelman ITLC: Type SG) with a solvent of 95 per cent methanol: 25 per cent ammonium acetate (0.1 M final concentration). The nine visible bands from such a chromatogram were eluted immediately with
0.1 M-ammonium acetate (pH 5) and the eluates were centrifuged to remove suspended material. Each of these nine fractions was then analysed for visible and ultraviolet absorbing material, for radioactivity, and for its ability to inhibit mitochondrial Ca\textsuperscript{2+} transport.

Fig. 6.7 shows the absorbance spectra of the nine fractions. Six distinct peaks are apparent, three in the visible region and three in the ultraviolet. The peak at 536 and the shoulder at 477 nm are ruthenium red and ruthenium brown respectively (Luft, 1971); the peak at 355 nm is partially attributable to these complexes (Fletcher et al., 1961; Luft, 1971), but is due also to an additional component, since its height is not related to the height of the visible peaks for ruthenium red and brown.

The radioactivity due to \textsuperscript{103}Ru, and hence the proportion of total ruthenium, is greatest in fractions seven and eight (Fig. 6.8); so too is the potency of inhibition of Ca\textsuperscript{2+} transport (Fig. 6.8). Since ruthenium red is most concentrated in fraction six (Fig. 6.7), the inhibitory activity of the latter fractions is due to some other component.

It seemed possible that this material might correspond to one of the ultraviolet-absorbing species, so attempts were made to purify these. The compound absorbing at 290 nm was purified by passing fraction seven through a micro-column of silicic acid equilibrated with 0.1 M-ammonium acetate at pH 8.5. It alone was not adsorbed at this pH. However, the purified material did not inhibit Ca\textsuperscript{2+} transport. The column was then eluted with acid and the eluate chromatographed on a thin-layer of silica gel (as above) with a solvent of 90 per cent methanol; 10 per cent ammonium acetate (0.1 M final concentration). The solvent front was eluted as described above. The absorbance spectrum showed a single peak at 255 nm, but this material also caused no inhibition of Ca\textsuperscript{2+} transport.
A sample of the crude mixture resulting from the synthesis of ruthenium red from $^{103}$Ru-RuCl$_3$ as described by Fletcher et al. (1961) (see text) was chromatographed on Gelman ITLC (Type SG) with 75 per cent methanol, 25 per cent ammonium acetate (0.1 M final concentration). The nine visible bands were cut into thin strips and eluted in small volumes of 0.1 M-ammonium acetate (pH 5.0). The eluates were centrifuged (16,000 x g for 5 min) and their spectra recorded against a solvent blank.
Fig. 6.8. Radioactivity and inhibitory activity of fractions resolved by thin-layer chromatography

Samples of the nine fractions obtained from thin-layer chromatography (Fig. 6.7) were counted for radioactivity (○) and for their ability to inhibit the initial rate of respiration-supported Ca$^{2+}$ transport (●). The latter effect was assessed by measuring the initial rate of mitochondrial swelling (E520) when 333 µM-Ca$^{2+}$ was added to 3 ml of medium containing 4.2 mg of mitochondrial protein in 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 5 mM-succinate-tris, 1 µM-rotenone, and 10 mM-acetate-tris.
Silic acid column chromatography

The properties of the ruthenium complexes revealed by the foregoing separation and elution procedures, together with a series of qualitative adsorption tests, suggested that chromatography on a column of silica under mild acid conditions would resolve the components of the mixture more completely than the thin-layer procedures. The column absorbent was prepared by extensive washing and filtration of 70-325 mesh silicic acid in 0.1 M-ammonium acetate (pH 5.0 with acetic acid) which was used to pack a 1.5 x 50 cm column. The sample was prepared by adsorbing the crude mixture of $^{103}\text{Ru}$ complexes to a small amount of silicic acid at pH 8.5 in 0.1 M-ammonium acetate (adjusted with ammonia) and this was carefully layered on top of the column. This application procedure gave much better resolution than was achieved by applying the liquid directly to the column. The column was eluted with 0.1 M-ammonium acetate (pH 5.0) under nitrogen pressure and 10 ml fractions were collected until the first coloured band reached the bottom of the column (six distinct coloured components were separated).

Fig. 6.9 shows the distribution of radioactivity ($^{103}\text{Ru}$) in the eluate, and the activity of each fraction in causing inhibition of $\text{Ca}^{2+}$-stimulated respiration. Two peaks of inhibitory activity were obtained: the first corresponded to the first peak of radioactivity, but this was less potent than the material concentrated in the second peak (Fig. 6.9b). This second region of inhibitory material was associated with only a small shoulder in the radioactivity profile (Fig. 6.9a).

Ultraviolet spectra of the column fractions (Fig. 6.10) revealed the presence of four separate absorbing species, none of which had been completely resolved. There is no obvious correlation between any one of these species and the inhibition profile, with the possible exception of the small peak at 355 nm (Fig. 6.10). The material absorbing at 255 nm may show a reasonable correlation, but its absolute absorbance is difficult to establish due to the proximity of the spectral components at 235 and 275 nm (Fig. 6.10). If this is
Fig. 6.9. Radioactivity and inhibitory activity of fractions resolved by silicic acid chromatography

A sample of the crude mixture was chromatographed on a silicic acid column as described in the text. The fraction volume was 10 ml and the flow rate was 85 ml/hr. Room temperature.

(a) radioactivity in 300 μl aliquots.

(b) per cent inhibition of the initial rate of respiration-supported Ca\(^{2+}\) transport caused by 30 μl (O) or 5 μl (●) samples of each fraction. The initial rate of Ca\(^{2+}\) transport was measured as the respiratory stimulation induced by the addition of 500 μM-Ca\(^{2+}\) to 5.6 (O) or 2.8 (●) mg of mitochondrial protein in 2 ml of 250 mM-sucrose, 5 mM-hepes-tris (pH 7.4), 5 mM-succinate-tris and 10 mM-acetate-tris at 30°C.
Fig. 6.10. Ultraviolet absorbance spectra of fractions resolved by silicic acid chromatography

The spectra of the inhibitory fractions shown in Fig. 6.9 were recorded at room temperature against a solvent blank.
the same component seen in the thin-layer chromatograms (Fig. 6.8), it is not responsible for the inhibition (refer above). On the other hand, the quantitative differences in the relation of spectral peak height at 355 nm to inhibitory activity for the active fractions obtained from the thin-layer and column chromatography (Figs. 6.7 and 6.8 cf. Figs. 6.9 and 6.10) suggest that neither is this material the inhibitory factor.

Thus, the analytical approach to determining the identity of the colourless ruthenium compound inhibitory to Ca\(^{2+}\) transport was not completely successful, although it established beyond doubt the existence of such a compound. Refinement of the column chromatography procedure (e.g. by slightly raising the eluant pH) could give better resolution of both the active compound and the ultraviolet-absorbing species.

**Ruthenium ammine complexes**

The problem was then approached by testing ruthenium ammine complexes for inhibitory activity. The rationale behind this search was provided by three separate lines of evidence: firstly, since the unknown compound probably inhibits at the same site as does ruthenium red, it would be expected to be structurally related. The mononuclear analogues are \([\text{Ru(NH}_3)_6\text{]}^{3+}\), \([\text{RuOH(NH}_3)_5\text{]}^{2+}\), and \([\text{Ru(OH)}_2(\text{NH}_3)_4\text{]}^{+}\) (see Appendix for structure of ruthenium red). Secondly, Tashmukhamedov *et al* (1972) have reported that 'hexamine cobaltichloride' \([\text{Co(NH}_3)_6\text{]}^{3+}\) inhibits mitochondrial Ca\(^{2+}\) transport. The ammine complexes of trivalent cobalt are similar in many respects to those of Ru\(^{III}\) (*e.g.* Broomhead *et al*, 1964), suggesting that inhibition by \([\text{Co(NH}_3)_6\text{]}^{3+}\) and the unknown ruthenium complex may be due to structurally-similar compounds. Finally, the ammoniacal reaction mixture from which ruthenium red is synthesized contains a large proportion of ruthenium ammine complexes (*J. Broomhead, personal communication*).
\([ \text{Ru(NH}_3\text{)}_6\text{]}^{3+} \) was tested by dissolving \( \text{Ru(NH}_3\text{)}_6\text{Cl}_3 \) in water (\( \lambda_{\text{max}} = 275 \text{ nm} \); Hartmann and Buschbeck, 1957) and using the solution immediately. It caused no inhibition of \( \text{Ca}^{2+} \)-stimulated respiration at concentrations up to 0.5 mM. Neither did \( \left[ \text{RuOH(NH}_3\text{)}_5\text{]}^{2+} \) (\( \lambda_{\text{max}} = 295 \text{ nm} \)), which was prepared as a fresh solution of \( \text{RuCl(NH}_3\text{)}_5\text{Cl}_2 \) in 0.1 M-NH\(_3\) (the chloro-complex (\( \lambda_{\text{max}} = 327 \text{ nm} \)) undergoes very rapid base-catalysed hydrolysis; see Broomhead et al., 1964). However, when a 10 mM solution of ruthenium pentammine chloride in 0.1 M-NH\(_3\) was allowed to stand overnight at room temperature, it developed inhibitory activity. The resulting solution was dark brown, and in addition to the hydroxypentammine absorption at 295 nm, its ultraviolet spectrum showed a shoulder at 355 nm. The ratio of the absorbance at 355 nm to the inhibitory activity of the solution was again different from the similar relations for the active fractions from the chromatography procedures described above.

A sample of the aged solution was applied to a micro-column of silicic acid and eluted with 0.1 M-ammonium acetate (pH 5.0). The only spectral component in the inhibitory fractions of eluate was a strong symmetrical peak at 295 nm due to \( \left[ \text{RuOH(NH}_3\text{)}_5\text{]}^{2+} \). The shoulder at 355 nm in the original spectrum had disappeared, and was apparently due to ruthenium red and/or a brown complex, both of which adsorbed more strongly to the column than did the major inhibitory compound. There was no evidence of components absorbing at 255 nm, but relatively weak absorbance in this region would be difficult to detect against the strong peak at 295 nm.

Unfortunately, time has not permitted further pursuit of these studies. The results discussed above confirm the existence of a colourless ruthenium complex inhibitory to mitochondrial \( \text{Ca}^{2+} \) transport, but little is known about the properties of this complex. It is of lower molecular weight than ruthenium red (see Section I) and is less strongly adsorbed to silicic acid at pH 5.0, implying that it has a lower cation charge. It may have an absorbance maximum at 255 nm.
There is insufficient information available in the literature to assign this peak to a specific complex or to establish the products arising from treatment of $[\text{RuOH(NH}_3)_5]^{2+}$ with ammonia. Work is continuing on both these problems.
ABBREVIATIONS

ADP  adenose-5’-diphosphate
ATP  adenose-5’-triphosphate
CCCP  carbonyl cyanide m-chlorophenylhydrazone
cpm  counts (radioactivity) per min
DTNB  5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA  ethylene diaminetetraacetic acid
EGTA  ethylene glycol-bis-(β-aminopropyl ether)-N,N,N',N'-tetraacetic acid
enase  2-(N,N-dimethylaminopropyl)ethanesulphonic acid
mes  1-(N-morpholinoo)ethanesulphonic acid
BADH  microsomal adenine dinucleotide (reduced)
NDA  nitrosothiatriacetic acid; trimethylamine-3,3',3''-tris(hydroxyethyl)aminomethane
SKP  submitochondrial particles prepared by ultra-sonication
State 3,4,5  respiratory states of mitochondria defined by Chance and Williams (1956) and Chance and Sとなって
TCA  trichloroacetic acid
trox  trimethylamine-3,3'(hydroxymethyl)-aminomethane
### Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>ADP</td>
<td>adenosine-5'-diphosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonylcyanide-m-chlorophenylhydrazone</td>
</tr>
<tr>
<td>cpm</td>
<td>counts (radioactivity) per min</td>
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<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis-(2-nitrobenzoic acid)</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine N,N'-tetraacetic acid</td>
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<td>EGTA</td>
<td>ethylene glycol-bis-(β-ethylene diamine ether)-N,N'-tetraacetic acid; ethanedioxy-bis-(ethylamine)-tetraacetic acid</td>
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<tr>
<td>hepes</td>
<td>2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonic acid</td>
</tr>
<tr>
<td>mes</td>
<td>2-(N-morpholino)-ethanesulphonic acid</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide (reduced)</td>
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<td>NTA</td>
<td>nitrilotriacetic acid; trimethylamine-α,α',α''-tricarboxylic acid</td>
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<td>State 3,4,6</td>
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<td>trichloroacetic acid</td>
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MATERIALS AND METHODS

(I) PRELIMINARY EXPERIMENTS

Mitochondria

Mitochondria were isolated from homogenates of rat liver in 0.25 M-sucrose by differential centrifugation (Myers and Slater, 1957). The protein concentration of mitochondrial suspensions was measured by the biuret method (Layne, 1957) following solubilization with deoxycholate (Jacobs et al., 1956).

ADP-ATP exchange activity

Incubation conditions were similar to those of Bygrave and Lehninger (1966). The nucleotides were separated by chromatography on DEAE-cellulose paper (Morrison, 1968) and the regions containing ATP were cut out and counted by liquid scintillation.

ATPase activity

(i) Total inorganic phosphate was measured by the method of King (1932).

(ii) The release of protons was continuously monitored with a combination glass electrode (Titron Instruments, Melbourne, Australia) and a Townson Expansion pH meter (Townson & Mercer, Sydney, Australia). The meter output was recorded on a 10 in. recorder (Rikadenki Kogyo Co. Ltd., Tokyo, Japan) with a full-scale deflection of 0.14 pH units. The apparatus was calibrated by the addition of standard HCl; under the conditions of the experiments, changes in pH were a linear function of changes in proton concentration.

(iii) The release of $^{32}$P$_i$ from (γ-32P)-ATP was measured as the radioactivity remaining in the supernatant after treatment of the incubation solution with trichloroacetic acid (final concentration 5 per cent (w/v)) and activated
charcoal (Norit A treated with hot conc. HNO₃, then washed and dried) (Crane and Lipmann, 1953). Tests over wide concentration ranges showed that < 0.5 per cent of the radioactivity of (γ-³²P)-ATP remained in the supernatant after such treatment whereas no significant inorganic phosphate or ³²Pᵢ was adsorbed to the charcoal.

Respiration

Mitochondrial oxygen uptake was measured polarographically with a Gilson Oxygraph (Gilson Medical Electronics, Wisconsin, U.S.A.) using a Clark-type electrode (Clark, 1956) obtained from the Yellow Springs Instrument Co. (Ohio, U.S.A.).

Phosphorylation

The incorporation of ³²Pᵢ into ATP (Fig. 1.2) was measured by the isobutanol/benzene extraction method of Nielsen and Lehninger (1955).

Swelling

(i) Light-scattering of mitochondrial suspensions was measured by continuously recording the absorbance at 520 nm with a Unicam SP 800 spectrophotometer.

(ii) The total water space and the sucrose-accessible space of mitochondria were calculated from the radioactivity in centrifuged mitochondrial pellets following incubation with (³H)-H₂O and (¹⁴C)-sucrose. The tubes containing the pellets were swabbed dry and the pellets were suspended in water and treated with 5 per cent (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation and samples of the supernatant were counted for radioactivity with the dual-channel technique. The pellet volume occupied by the appropriate isotope was calculated from its concentration in the original incubation.

(iii) Samples for electron microscopy were prepared by centrifugation of a mitochondrial suspension (containing approximately 0.1 mg protein) in a Beckman micro-centrifuge
for 45 sec. The supernatant was removed and the pellet fixed on ice for 90 min. with 2 per cent (w/v) \( \text{OsO}_4 \) in 40 mM-sucrose and 10 mM-phosphate (pH 7.4). The fixed pellet was rinsed, then dehydrated, embedded and stained according to Hackenbrock (1966). Sections were examined and photographed with an Hitachi HU-11E electron microscope.

**Calcium uptake**

(i) In most experiments, the uptake of \( \text{Ca}^{2+} \) by mitochondria was calculated from the radioactivity remaining on the filter after a small sample (50-100 µl) of a mitochondrial suspension incubated with \(^{45}\text{Ca}^{2+}\) had been filtered on a cellulose membrane of 0.45 µm pore size (Millipore Corp., Massachusetts, U.S.A. (HAWP 025 00) or Gelman Instrument Corp., Michigan, U.S.A. (Metricel GN-6, 1 in.)). The filters were washed with 3 ml of incubation solution, dried at 90°C and counted by liquid scintillation.

(ii) In the experiment of Fig. 1.14, 2 ml of a mitochondrial incubation was filtered. A 50 µl sample of the filtrate was counted for radioactivity, and to the remainder was added \( \text{SrCl}_2 \) (1500 ppm) and HCl (0.2 M).

Total \( \text{Ca}^{2+} \) concentration was measured by atomic absorption spectrophotometry (by courtesy of Dr. J. David, CSIRO Soil Research, Canberra).

**Scintillation counting**

Aqueous samples were added to a glass vial containing 10 ml of Bray's cocktail (Bray, 1960); dried filters were counted in 10 ml of a solution containing 4 gm of 2,5-diphenyloxazole and 100 mg of 1,4-di-[2-(4-methyl-5-phenyloxazolyl)]-benzene (from Koch-Light, Bucks., England) in 1 litre of toluene. Counting was generally done with a Packard Tri-Carb scintillation counter, but in some cases a Beckman LS-100 was used.

**(\text{\( ^{32}\text{P} \)}\text{-ATP})**

ATP labelled in the terminal phosphate was prepared by the enzymatic isotope-exchange procedure of Post and Sen (1967). The once-purified product was re-chromatographed
as described by these authors to ensure complete removal of inorganic phosphate. More than 99.5% of the radioactivity in the product adsorbed to activated charcoal; after brief treatment with glucose, $\text{Mg}^{2+}$ and hexokinase, less than 5 per cent of the radioactivity was adsorbed (cf. Crane and Lipmann, 1953). The product was therefore assumed to be pure ($^{32}$P)-ATP labelled almost entirely in the $\gamma$-position.

Radioisotopes

$^{45}\text{Ca}^{2+}$, ($^{14}$C)-ADP, ($^{14}$C)-sucrose, ($^{14}$C)-inulin and ($^{3}$H)-H$_2$O were purchased from The Radiochemical Centre (Amersham, England); $^{32}$P$_i$ was a product of the Australian Atomic Energy Comission (Lucas Heights, Sydney, Australia).

Chemicals

Nucleotides and enzymes were obtained from The Boehringer Corp. (London, England); Ca$^{2+}$ solutions were purchased as 0.1000 M-CaCl$_2$ standards from Orion Research Incorp. (Massachusetts, U.S.A.) or were prepared from analytical grade CaCO$_3$ by treatment with HCl. All chemicals were of analytical grade.

(II) SECTION I

Mitochondria

Mitochondria were isolated from the livers of 200 g male rats (Wistar - albino) by homogenization in 250 mM-sucrose, 2 mM-hepes-tris, 0.5 mM-EGTA-tris (pH 7.4 at 0°) followed by differential centrifugation (Johnson and Lardy, 1967). The preparation was washed twice in 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4) and finally resuspended in the same medium.

Mitochondrial protein concentration was assayed by the biuret method (Layne, 1957) following solubilization with deoxycholate (Jacobs et al., 1956). Corrections were made for non-biuret colour and turbidity by subsequent cyanide treatment (Szarkowska and Klingenberg, 1963).
Oxygen uptake

Oxygen utilization by mitochondria was measured polarographically with the membrane-covered electrode assembly described by Reed (1972).

Spectrophotometry

All optical measurements were made with a Varian-Techtron split-beam recording spectrophotometer with automatic wavelength programming.

Cytochrome a measurement

Cytochrome oxidase content was calculated from the 605-630 nm absorbance difference of dithionite-reduced and ferricyanide-oxidized samples of mitochondria containing about 5 mg of mitochondrial protein per ml. Measurements were made in a Cary 14R split-beam spectrophotometer with a scattered-transmittance accessory. Calculations were based on a value for $\Delta \varepsilon_{605-630}$ of 19 for total haem a, i.e. cytochrome a + a₃ (Lemberg, 1969).

Scintillation counting

All radioactive samples were placed in glass vials with 10 ml of scintillation fluid (6 gm 2-(4'-n-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole (Koch-Light) dissolved in 400 ml methoxyethanol and 600 ml toluene) and counted in a Beckman LS-100 scintillation counter.

Miscellaneous techniques

A Townson expanded-scale pH meter was used in adjusting the pH of all solutions. The combination glass electrode (Titron) was equilibrated at the required temperature for 10 min before readings were taken.

All additions to incubation solutions were made with glass/stainless-steel microsyringes (from SGE Instruments, Victoria, Australia) or Hamilton dispensing syringes (from Hamilton Co., California, U.S.A.).

Ruthenium red

Fletcher et al (1961) have shown that the structure of ruthenium red is $[(\text{NH}_3)_5\text{Ru}-0-\text{Ru(NH}_3)_4-0-\text{Ru(NH}_3)_5]^6+ \cdot \text{Cl}^- \cdot 6\cdot 4\text{H}_2\text{O}$
with a corresponding molecular weight of 858.3 and a millimolar extinction coefficient in water of 61.5 at 532 nm, its absorption maximum. In a thorough analysis of the properties of ruthenium red relevant to biological studies in general, and to electron microscopy in particular, Luft (1971) has reported $\varepsilon_{\text{mM}} = 68$ at 533 nm ($\lambda_{\text{max}}$) in 0.1 M-ammonium acetate.

The pure ruthenium red used in the present experiments was recrystallized from a commercial sample (Sigma Chemical Co., Minnesota, U.S.A.) as described by Fletcher et al. (1961). The yield was 11 per cent and the product's extinction coefficient was identical with that found by Luft (1971).

Stock solutions were prepared at a concentration of about 1 mM in water and stored in the dark at 4°C. These were diluted to the required working concentration on the day of use and analyzed spectrophotometrically. The stock solution was stable for several weeks.

A sample of ruthenium red was oxidized to ruthenium brown (which has a similar structure to the red complex but a charge of +7; Fletcher et al., 1961) by treatment at 70°C for 1 h in 1 M-HNO$_3$. The brown suspension was centrifuged and the precipitate was dissolved in water. Spectroscopy showed that quantitative conversion to ruthenium brown had occurred ($\varepsilon_{460} = 42,000$ in 0.01 M-HNO$_3$; Fletcher et al., 1961).

In experiments with this solution (Fig. 3.17 and text) it was observed that on contact with the stainless-steel plunger of the microsyringe normally used for making additions, the brown solution quite rapidly turned red, presumably reflecting its reduction by a component of the steel. Disposable plastic micro-pipettes were used thereafter.

Solutions of 'crude ruthenium red' (see Figs. 3.17 and 4.9-4.12) were prepared by filtering aqueous 2 mM (nominal) suspensions of the unpurified commercial product (Schmid and Co., Stuttgart, Germany) through a Millipore filter (0.22 µm pore diameter). Analysis showed these to contain approx. 25-30 per cent ruthenium red (uncorrected for absorbance of impurities at 533 nm). The unfiltered crude suspension contained less than 15 per cent ruthenium red (again,
uncorrected for absorbance of impurities; cf. similar purity of commercial products reported by Luft, 1971).

In addition to the care needed to ensure that pure ruthenium red is used, attention must be given to two of its physical properties which provide potential sources of error. First, it is readily oxidized in mild acid conditions (see above); secondly, we have found that it adsorbs strongly to glass (soda and pyrex), quartz, Perspex, polythene and stainless-steel. The problems associated with adsorption are those of 'carry-over' in successive incubations (which can be prevented by treatment of the vessels with conc. HNO_3) and net loss from solutions. This latter problem was minimized by always adding ruthenium red after mitochondria.

**Lanthanides**

Stock solutions of 20 mM were prepared as follows: La_2O_3 and europium oxide (Eu_2O_3) (99.99 and 99.8 per cent pure respectively; K & K Laboratories, New York, U.S.A.) were dissolved in a slight molar excess of dilute HNO_3 and diluted with water; terbium oxide (a dark brown solid sometimes referred to as terbium peroxide, with the empirical formula Tb_4O_7; 99.9 per cent pure; K & K Laboratories) was dissolved in a small volume of hot conc. HNO_3 and diluted; neodymium nitrate (Nd(NO_3)_3·6H_2O) (Hopkin and Williams, Essex, England) was dissolved in dilute HNO_3. These stock solutions were diluted to 8 µM for use. The solution of Nd^{3+} was standardized by titration with EDTA ('Titration Grade'; Merck, Darmstadt, Germany) to the murexide end-point as determined spectrophotometrically from E_{540-470}.

As with ruthenium red, the lanthanides adsorb to various types of glass and synthetic polymer, although the problem is not so acute. It arises mainly with regard to the storage of dilute solutions. A detailed study of this property has shown that stock solutions are best stored at high concentrations in pyrex containers, or when lower concentrations are required (down to 1 µM), in either pyrex or
polyallomer at slightly acidic pH (K.J. Ellis, personal communication).

Other materials

Chelex-100 was a product of Bio-Rad Laboratories (California, U.S.A.); columns for the removal of Ca\(^{2+}\) from sucrose solutions were prepared according to Bio-Rad Technical Bulletin #114.

Ethanolic solutions of antimycin (Sigma) were standardized spectrophotometrically (Strong et al., 1960). All fine chemicals were obtained from Sigma, Calbiochem (California, U.S.A.), or Boehringer, with the following exceptions: nupercaine hydrochloride was a generous gift of CIBA Pharmaceuticals (Crows Nest, N.S.W., Australia); NTA was supplied by Fluka Chem. Fab. (Buchs, Switzerland), and Soluene-100 by the Packard Instrument Co. (Illinois, U.S.A.). The \(^{45}\)Ca\(^{2+}\) was from The Radiochemical Centre; solutions of CaCl\(_2\) were prepared as previously described.

All reagents used were of the highest purity available.

(III) SECTION II

Mitochondria

Mitochondria were isolated from the livers of 200 g male rats (Wistar - albino) by homogenization in 250 mM-sucrose, 2 mM-hepes-tris, 0.5 mM-EGTA-tris (pH 7.4 at 0°) followed by differential centrifugation (Johnson and Lardy, 1967). The preparation was washed twice in 250 mM-sucrose 2 mM-hepes-tris (pH 7.4) and finally resuspended in the same medium.

Mitochondrial protein concentration was assayed by the biuret method (Layne, 1957) following solubilization with deoxycholate (Jacobs et al., 1956). Corrections were made for non-biuret colour and turbidity by subsequent cyanide treatment (Szarkowska and Klingenberg, 1963).
Mitochondrial swelling

Mitochondrial volume changes were monitored semi-quantitatively by measuring absorbance at 520 nm in a Unicam SP 800 with constant temperature cell housing or a Varian-Techtron.

Isopycnic centrifugation

Discontinuous sucrose density gradients were prepared in 5 ml tubes using data compiled by Dawson et al (1969) with the inclusion of 2 mM-hepes-tris (pH 7.4 at 0°C) in all solutions. Samples were prepared by centrifuging a mitochondrial incubation mixture at 8,000 x g for 5 min and resuspending the pellets in 1 ml of 250 mM-sucrose, 2 mM-hepes-tris (pH 7.4). These were layered on top of the freshly-prepared gradients and centrifuged at 2°C in a Beckman ultracentrifuge at 32,5000 RPM for 3 h using the SW50 head (Greenawalt et al, 1964).

Electron microscopy

Mitochondria were fixed for microscopy by the drop-wise addition of 10 per cent glutaraldehyde (v/v) (diluted with incubation medium) to the stirred incubation mixtures to give a final glutaraldehyde concentration of 2 per cent (v/v). The solutions used in these experiments were neutralized with potassium hydroxide rather than tris to avoid the reaction of tris with glutaraldehyde. Following fixation for 1 h at room temperature, the mitochondria were separated by centrifugation at 8,000 x g for 5 min (20°C), the pellets washed with cold sucrose-collidine (250 mM-10 mM), pH 7.4 and post-fixed overnight with 1 per cent osmium tetroxide (w/v) in the same buffer. The pellets were rinsed, dehydrated with an ethanol series and embedded in Spurr's hard medium (Spurr, 1969). Sections were examined in a Hitachi HU 12 electron microscope after lead staining (Sato, 1967).

Spectrophotometry

Changes in absorbance of respiratory components were measured with an Aminco-Chance spectrophotometer using the dual-wavelength technique (Chance, 1951). The same technique
was used to measure the concentration of free \( \text{La}^{3+} \) by the absorbance of its murexide complex (cf. Mela and Chance, 1968). All other measurements were made with a Varian-Techtron split-beam recording spectrophotometer with automatic wavelength programming.

**Fluorescence measurements**

The fluorescence of mitochondrial pyridine nucleotides and flavoproteins was monitored with a Zeiss spectrofluorometer with dual monochromators; the output was recorded continuously on a Beckman potentiometric recorder.

**ATPase**

ATPase activity was measured by following the release of inorganic phosphate. Incubation samples were quenched with 5 per cent trichloroacetic acid (w/v) and assayed by a micro-method with high chromophore stability (Baginski and Zak, 1960; Baginski et al, 1967).

**Sonication**

Mitochondrial suspensions were sonicated with a 3 mm titanium probe operating at 8-10 \( \mu \text{m} \) amplitude (sonicator and probe from MSE Ltd., London, England).

**Materials**

Lanthanum solutions were prepared with \( \text{LaCl}_3, 7\text{H}_2\text{O} \) (Ajax Chemicals, Sydney, Australia) and were standardized by titrating the acid equivalents displaced from a column of Dowex (\( \text{H}^+ \) form) by an aliquot of the solution (K.J. Ellis, personal communication).

Solutions of uranyl nitrate (\( \text{UO}_2(\text{NO}_3)_2, 6\text{H}_2\text{O} > 99 \) per cent pure, from May and Baker, Dagenham, England) were prepared fresh immediately before use.

\( ^{103}\text{Ru} \) was purchased from The Radiochemical Centre, \( \text{RuCl}_3 \) from Hopkin and Williams, silicic acid from Merck, and thin-layer chromatography media from Gelman Instrument Corp. \( \text{RuCl(NH}_3)_5\text{Cl}_2 \) and \( \text{Ru(NH}_3)_6\text{Cl}_3 \) were kindly provided by Dr. J. Broomhead (Chemistry, S.G.S., A.N.U.).
RELATION BETWEEN FREE AND TOTAL Ca\(^{2+}\) CONCENTRATIONS IN Ca\(^{2+}\)-BUFFERED SYSTEMS

Fig. A.1. Chelation of Ca\(^{2+}\) by ATP and NTA

Free Ca\(^{2+}\) concentrations (M) were computed at selected total Ca\(^{2+}\) concentrations (M) by using a miniaturized version of the iterative COMICS programme (Concentration of Metal Ions and Complexed Species) developed by I.G. Sayce and published elsewhere (Perrin and Sayce, 1967). The computer used for these calculations was a PDP-8/i of Digital Equipment Corp. (Massachusetts, U.S.A.).

The solid lines are for 10 mM-NTA at pH 7.4 or pH 8.5 at 0.5°C (the curves for chelation at 25.3°C are virtually identical); the dashed line is for 6 mM-ATP at pH 7.4 and 25°C.

The following values for stability constants (Sillén and Martel, 1964) were used in the programme.

**NTA**
- \(\log K_{H^+}^+ : K_1 \approx -9.91 \text{ (0.5°C)}\)
- \(K_{12} \approx -2.49\)
- \(K_{13} \approx -1.89\)
- \(K_{12} \approx -9.63 \text{ (25.3°C)}\)
- \(K_{13} \approx -4.06\)

**ATP**
- \(\log K_{H^+}^+ : K_1 \approx -6.50\)
- \(K_{12} \approx 4.34\)
- \(K_{HL} \approx 2.13\)
log(free Ca concentration) vs log(total Ca concentration)
Fig. A.2. Chelation of Ca\(^{2+}\) by EGTA and EDTA

Free Ca\(^{2+}\) concentrations (M) were computed at selected total Ca\(^{2+}\) concentrations (M) in the presence of EGTA (a) or EDTA (b) at pH 7.4 and 20°C. The equation used for these calculations (below) was derived by substituting concentrations of metal species (in terms of equilibrium expressions) into mass-conservation equations for Ca\(^{2+}\) and ligand:

\[
M_F = \frac{-C + \sqrt{C^2 - 4BD}}{2B}
\]

where

\[
A = 1 + K_1[H^+] + K_1K_2[H^+]^2 + K_1K_2K_3[H^+]^3 + K_1K_2K_3K_4[H^+]^4
\]

\[
B = K_L + K_1K_{HL}[H^+] \\
C = A + B(L_T - M_T) \\
D = -AM_T
\]

\[
M_F = \text{free Ca}^{2+} \text{ concentration} \\
M_T = \text{total Ca}^{2+} \text{ concentration} \\
L_T = \text{total ligand concentration}
\]

\[
= 0.05 \ (1), \ 0.1 \ (2), \ 0.2 \ (3), \ 0.5 \ (4), \ 1 \ (5), \ 2 \ (6) \ \text{and} \ 5 \ (7) \ \text{mM.}
\]

The following values for stability constants (Sillén and Martell, 1964) were used:

(a) **EGTA** \[\log K_H^+ : K_1 -9.46 \ K_2 -8.85 \ K_3 -2.68 \ K_4 -2.00\]

\[\log K_{Ca}^{2+} : K_L 11.00 \ K_{HL} 5.33\]

(b) **EDTA** \[\log K_H^+ : K_1 -10.26 \ K_2 -6.16 \ K_3 -2.67 \ K_4 -1.99\]

\[\log K_{Ca}^{2+} : K_L 10.59 \ K_{HL} 3.51\]

The calculations were done with an IBM 360 computer using FORTRAN IV.


