Biochem. J. (1974) **140**, 143–155
Printed in Great Britain

The Inhibition of Mitochondrial Calcium Transport by Lanthanides and Ruthenium Red

By KEN C. REED and FYFE L. BYGRAVE
Department of Biochemistry, Faculty of Science, Australian National University, Canberra, A.C.T. 2600, Australia

(*Received* 15 *October* 1973)

An EGTA (ethanedioxybis(ethylamine)tetra-acetic acid)-quench technique was developed for measuring initial rates of $^{45}Ca^{2+}$ transport by rat liver mitochondria. This method was used in conjunction with studies of Ca^{2+}-stimulated respiration to examine the mechanisms of inhibition of Ca^{2+} transport by the lanthanides and Ruthenium Red. Ruthenium Red inhibits Ca^{2+} transport non-competitively with K_i 3×10^{-8} M; there are 0.08 nmol of carrier-specific binding sites/mg of protein. The inhibition by La^{3+} is competitive ($K_i = 2 \times 10^{-8}$ M); the concentration of lanthanide-sensitive sites is less than 0.001 nmol/mg of protein. A further difference between their modes of action is that lanthanide inhibition diminishes with time whereas that by Ruthenium Red does not. Binding studies showed that both classes of inhibitor bind to a relatively large number of external sites (probably identical with the 'low-affinity' Ca^{2+}-binding sites). La^{3+} competes with Ruthenium Red for most of these sites, but a small fraction of the bound Ruthenium Red (less than 2 nmol/mg of protein) is not displaced by La^{3+}. The results are discussed briefly in relation to possible models for a Ca^{2+} carrier.

The transport of Ca^{2+} by mitochondria has been studied in great detail with reference to the involvement of energy and of counterion movements (for review see Lehninger *et al.*, 1967). However, relatively little is known about the mechanism of the transport process itself. The first, and still the most convincing, evidence for the involvement of a specific carrier was the demonstration by Mela (1967, 1968*a*,*b*) of the marked inhibition by La^{3+} of Ca^{2+}-associated responses in mitochondria. This finding was later extended to show that Ca^{2+} transport itself is inhibited by all rare-earth cations at concentrations extrapolating to complete inhibition at less than 0.1 nmol of lanthanide/mg of protein (Mela, 1969*a*; Vainio *et al.*, 1970). This value has been used as a maximum estimate of the concentration of Ca^{2+}-specific carrier sites in the mitochondrion (Mela & Chance, 1969).

Moore (1971) has reported that Ruthenium Red also is a potent inhibitor of respiration-supported Ca^{2+} uptake, and detailed studies by Vasington *et al.* (1972*a*,*b*) have established that, at low concentrations, the inhibition by Ruthenium Red is specific for reactions associated with 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 Ca^{2+} transport, an approach essential to the development of a rational model of the mitochondrial Ca^{2+} carrier. In common with all membrane transport systems, it should be analysed initially by establishing its kinetic and binding properties, both in the presence and absence of specific inhibitors.

The purpose of this report is to define the interaction of Ruthenium Red and lanthanides with the mitochondrial Ca^{2+} carrier by examining their effects on both Ca^{2+}-stimulated respiration and the initial rate of Ca^{2+} transport. By treating the compounds as 'tight-binding' inhibitors (Henderson, 1972) we have shown that they have markedly different mechanisms of action. The kinetic studies have been complemented with measurements of the total binding of Ruthenium Red and La^{3+} to mitochondria which show that, although they share a relatively large number of 'low-affinity' sites in common, La^{3+} is unable to displace Ruthenium Red from a small number of high-affinity binding sites.

Experimental

Mitochondria

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

Cytochrome a measurement

Cytochrome *c* oxidase content was calculated from the $E_{605} - E_{630}$ of dithionite-reduced and ferricyanide-oxidized samples of mitochondria containing about 5 mg of mitochondrial protein/ml. Measurements were made in a Cary 14R split-beam

spectrophotometer with a scattered-transmittance accessory. Calculations were based on a value for $\Delta\varepsilon_{mM}^{605-630}$ of 19 for total haem *a*, i.e. cytochrome $a+a_3$ (Lemberg, 1969).

Oxygen uptake

Oxygen utilization by mitochondria was measured polarographically with a membrane-covered electrode (Reed, 1972).

Spectrophotometry

Absorbance changes of murexide present in mitochondrial suspensions were measured with dual-wavelength techniques (Chance, 1951) by using an Aminco–Chance spectrophotometer. All other measurements were made with a Varian-Techtron split-beam recording spectrophotometer with automatic wavelength programming.

Sonication

Suspensions of mitochondria were sonicated for 30–60s with a 3mm titanium probe operated at 8–10μm amplitude (MSE Ltd., London S.W.1, England).

Calcium transport

There are two basic problems associated with measurements of the initial rate of mitochondrial Ca^{2+} transport. First is the very high activity of the process, and secondly is the presence of a large number of external Ca^{2+}-binding sites (of the order of 30nmol/mg of protein; K. C. Reed & F. L. Bygrave, unpublished work; see also Reynafarje & Lehninger, 1969). The murexide technique (Mela & Chance, 1968) overcomes the first by continuous recording of the free Ca^{2+} concentration in a stopped-flow apparatus; the second problem can be ignored in this system, provided that the Ca^{2+} concentrations used are sufficiently high to saturate the external sites. However, the technical and theoretical disadvantages of this technique have led us to develop an alternative, which is both simpler and more generally applicable to kinetic studies.

The technique consists of incubating the mitochondria with $^{45}Ca^{2+}$ for a few seconds at low temperature and quenching with a tenfold excess of EGTA.* The combination of low temperature (to slow the reaction) and virtually instantaneous quenching with EGTA after a very short incubation period ensures that true initial rates are measured. This is verified by the similarity of data obtained when the incubation time is varied from 5 to 15s (K. C. Reed & F. L. Bygrave, unpublished work).

The stability constant of CaEGTA is sufficiently high (Sillén & Martell, 1964) to ensure that, under the quenching conditions, the concentration of free Ca^{2+} is lowered to about 10nM so that both transport (apparent $K_m \approx 2\times10^{-6}$M; Bygrave *et al.*, 1971*a*,*b*) and external binding ($K_d \approx 10^{-5}$–10^{-4}M; K. C. Reed & F. L. Bygrave, unpublished work; Reynafarje & Lehninger, 1969) are inhibited completely. The mitochondria are then separated by microfiltration (Millipore 13mm filters, 0.45μm pore size) or by centrifugation (Eppendorf microcentrifuge), and their radioactivity is assayed by scintillation counting in 10ml of a methylCellosolve 'cocktail' [6g of 2-(4′-t-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) dissolved in 400ml of methoxyethanol and 600ml of toluene].

The validity of the basic technique has been established in a number of experiments. The data of

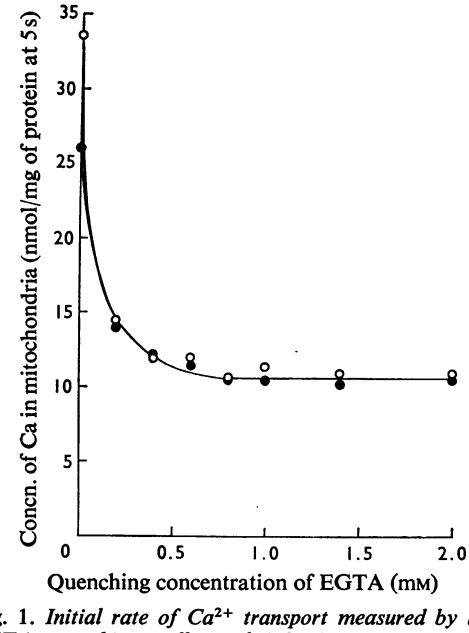

Fig. 1. *Initial rate of Ca^{2+} transport measured by using EGTA quenching: effect of EGTA concentration and quenching time*

Incubations contained 250mM-sucrose, 2mM-Hepes–Tris (pH7.4) and 2mM-succinate–Tris in a total volume of 0.5ml. Mitochondria (1mg of protein) were incubated for 1min before the addition of 200μM-$^{45}Ca^{2+}$ (0.05μCi) and then for 5s before being quenched by the addition of EGTA–Tris (pH7.4) at the concentrations shown. Samples (50μl) were filtered 10s (●) or 3min (○) after quenching, washed and assayed for radioactivity. Incubation temperature was 10°C.

* Abbreviations: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N*′-yl)ethanesulphonic acid; EGTA, ethanedioxybis-(ethylamine)tetra-acetic acid.

Fig. 1 show the effect of EGTA concentration on the amount of Ca^{2+} retained by mitochondria after a 5s incubation at 10°C. The non-removable fraction reaches a minimum when a fourfold excess of EGTA is used. The complete absence of further effects at higher concentrations indicates that EGTA is acting solely as an impermeant chelating agent. The second important feature seen in these 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. When EGTA is added to mitochondria before $^{45}Ca^{2+}$, the radioactivity associated with the mitochondria remains at the background value for at least 10min.

Fig. 2 shows that the pool of EGTA-inaccessible Ca^{2+} increases with time, as would be expected if it represents transported Ca^{2+}. It should be noted that the initial rate in this experiment is faster than in that of Fig. 1 owing to the presence of phosphate (Chance & Yoshioka, 1966). The reaction is first-order with a half-time of 17s, and after about 2min virtually no Ca^{2+} remains accessible to EGTA. We have found that the inclusion of respiratory inhibitors or uncouplers prevents the movement of Ca^{2+} into the EGTA-inaccessible pool.

On the basis of the above data we have equated this pool with the internal space of mitochondria to which Ca^{2+} is actively transported, i.e. the matrix and/or inner surface of the inner membrane.

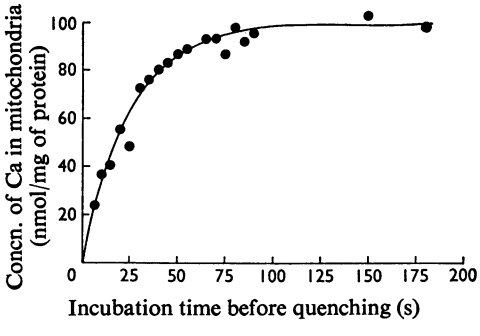

Fig. 2. *Time-course of Ca^{2+} transport measured by using EGTA quenching*

The incubation contained 250mM-sucrose, 2mM-Hepes–Tris (pH7.4), 1 mM-phosphate–Tris and 2mM-succinate–Tris in a total volume of 4.0ml. Mitochondria (13mg of protein) were preincubated for 1min before the addition of 325μM-$^{45}Ca^{2+}$. At the times shown samples (100μl) were removed into 160μl of ice-cold quench medium [250mM-sucrose, 2mM-Hepes–Tris (pH7.4), 2mM-EGTA–Tris] and mixed. At the end of the experiment, all tubes were centrifuged (4min at 12000*g*) and samples (100μl) of the supernatants were assayed for radioactivity. Incubation temperature was 10°C.

The experiments reported in this paper were done at low temperatures to ensure that incubation periods of no less than 5s would give an accurate assessment of initial rates. Shorter incubations involve impossibly high timing errors when manual starting and stopping techniques are used. In fact the major source of error in the present experiments is caused by variations in incubation time (an error of $\pm$0.25s in starting and quenching 5s incubations gives rise to an overall error of $\pm$10%). Better resolution and accuracy could be obtained with an automatic-injection apparatus or with continuous-flow systems.

Ruthenium Red

Fletcher *et al.* (1961) have shown that the structure of Ruthenium Red is $[(NH_3)_5Ru–O–Ru(NH_3)_4–O–Ru(NH_3)_5]^{6+}Cl_6^-,4H_2O$ with a corresponding mol.wt. of 858.3 and ε_{mM} of 61.5 at 532nm ($\lambda_{max.}$) in water. 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 ε_{mM} of 68 at 533nm ($\lambda_{max.}$) in 0.1M-ammonium acetate.

The pure Ruthenium Red used in the present experiments was recrystallized from a commercial sample (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as described by Fletcher *et al.* (1961). The yield was 11% and the product's extinction coefficient was identical with that found by Luft (1971).

Stock solutions were prepared at a concentration of about 1mM in water and stored in the dark at 4°C. These were diluted to the required working concentration on the day of use and analysed 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 1h in 1M-HNO_3. The brown suspension was centrifuged and the precipitate was dissolved in water. Spectroscopy showed that quantitative conversion into Ruthenium Brown had occurred ($\varepsilon_{460} = 42000$ in 0.01M-HNO_3; Fletcher *et al.*, 1961). In experiments with this solution, 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 micropipettes were used thereafter.

Solutions of 'crude Ruthenium Red' (see Fig. 4) were prepared by filtering aqueous 2mM (nominal) suspensions of the unpurified commercial product (Schmid and Co., Stuttgart, Germany) through a Millipore filter (0.22μm pore diameter). Analysis showed these to contain approx. 25–30% Ruthenium Red (uncorrected for absorbance of impurities at

533 nm). The unfiltered crude suspension contained less than 15% Ruthenium Red (again, uncorrected for absorbance of impurities; cf. similar purity of commercial products reported by Luft, 1971).

In the brief report of Moore (1971) a molecular weight of roughly one-third the correct value was used, based on the data of Morgan & Burstall (1936). Neither in that work nor in the subsequent studies of Vasington *et al.* (1972*a*,*b*) is there any indication that recrystallized Ruthenium Red was used. It must therefore be assumed that the data of Moore (1971) and Vasington *et al.* (1972*a*,*b*) apply to a crude mixture containing no more than 15% Ruthenium Red, and that the molar concentrations cited have little relevance to the actual amount of Ruthenium Red present.

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, Teflon, 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% 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% 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, U.K.) 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; E. Merck A.-G., 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 μM), in either Pyrex or polyallomer at slightly acidic pH (K. J. Ellis, personal communication).

Other materials

Antimycin (Sigma Chemical Co.) solutions were standardized spectrophotometrically (Strong *et al.*, 1960). Soluene-100 was a product of Packard Instrument Co. (Ill., U.S.A.).

All reagents were of analytical grade.

Results

Ca^{2+}-stimulated oxygen uptake

The rate of Ca^{2+} transport can be estimated from measurements of the respiratory stimulation induced by saturating concentrations of Ca^{2+}, since the energy requirement in respiration-linked Ca^{2+} transport is reflected in an increased rate of oxygen consumption (Chance, 1965). This is seen in the control experiments of Fig. 3. The permeant anion 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+}, the response is somewhat different (Fig. 3*a*); respiration is again 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, 1969*a*). The potency of the inhibition is not obvious from these experiments, since 1.8 nmol of La^{3+}/mg of protein inhibited the maximally stimulated rate only by about 50% (cf. Mela, 1969a; Vainio *et al.*, 1970).

The final trace of Fig. 3*a* shows that both the initial lag period and the subsequent partial inhibition of respiratory stimulation are abolished by preincubating the mitochondria with La^{3+} for about 5 min before addition of Ca^{2+}. The recovery of the respiratory response with increased incubation time, in both the presence and the absence of Ca^{2+}, is due to the accumulation of La^{3+} by mitochondria (Reed & Bygrave, 1974) as had been suggested by Mela (1968*a*, 1969*b*).

Fig. 3(*b*) 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*b*, final trace) and is more complete even at very low concentrations. Ruthenium Red thus remains bound to a component of the Ca^{2+}-transport system for a considerable time, in contrast with La^{3+}.

When the rate of oxygen uptake immediately after Ca^{2+} addition is plotted as a function of Ruthenium Red concentration, a sigmoidal curve is obtained. The cause of the sigmoidal shape is not clear; it is

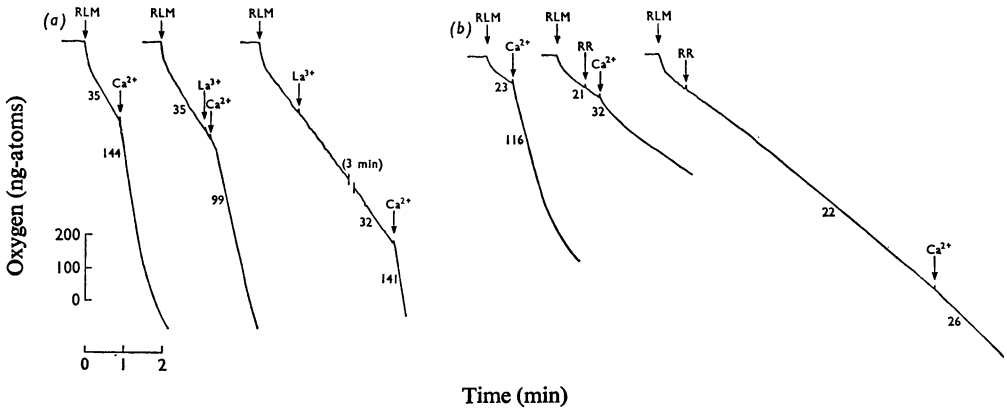

Fig. 3. *Effect of preincubation time on the inhibition of Ca^{2+}-stimulated respiration by La^{3+} and Ruthenium Red*

(*a*) The incubations contained 250mM-sucrose, 5mM-Hepes–Tris (pH7.4), 5mM-succinate–Tris, 10mM-acetate–Tris, 0.33μM-rotenone and 5.4mg of mitochondrial protein (RLM) in a final volume of 3.1ml; the temperature was 30°C. Additions of Ca^{2+} (323μM) and La^{3+} (3.1μM) were made at the times shown. (*b*) Incubation conditions were similar to those of (*a*) except that 4.3mg of mitochondrial protein was used. Ruthenium Red (RR) was added as shown at a concentration of 0.265μM. Numbers below the traces are rates of oxygen uptake in ng-atoms of oxygen/min per mg of protein.

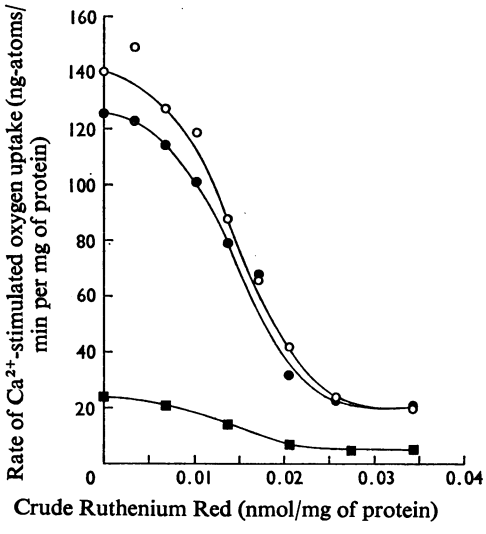

Fig. 4. *Inhibition of Ca^{2+}-stimulated respiration by crude Ruthenium Red*

Incubations contained 250mM-sucrose, 5mM-Hepes–Tris (pH7.4), 5mM-succinate–Tris (■) and either 2mM-phosphate–Tris (○) or 10mM-acetate–Tris (●), with 4.2mg of mitochondrial protein in a final volume of 2ml; the temperature was 25°C. Mitochondria were preincubated for 15s with the indicated concentration of crude Ruthenium Red (see the Experimental section) before addition of 500μM-Ca^{2+}.

not seen in the inhibition of Ca^{2+} uptake itself (see below). Virtually complete inhibition of the respiratory response is attained with 0.15nmol of Ruthenium Red/mg of protein.

Spectroscopic studies showed that the Ruthenium 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 filtered solution of unpurified commercial Ruthenium Red (see the Experimental section) had inhibitory properties qualitatively similar to those of the pure complex (Fig. 4). However, it was far more potent in terms of the amount of Ruthenium Red required for complete inhibition (about 0.02nmol/mg of protein). This value refers to the concentration of Ruthenium Red itself in the crude solution, calculated from E_{533}; obviously some low-molecular-weight component(s) of the unpurified solution possess inhibitory properties similar to those of Ruthenium Red.

Initial rate of Ca^{2+} transport

In the experiment of Fig. 5, 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. 6 below, and Mela, 1969*a*). This experiment was done at 10°C with a 60s preincubation of the mitochondria with

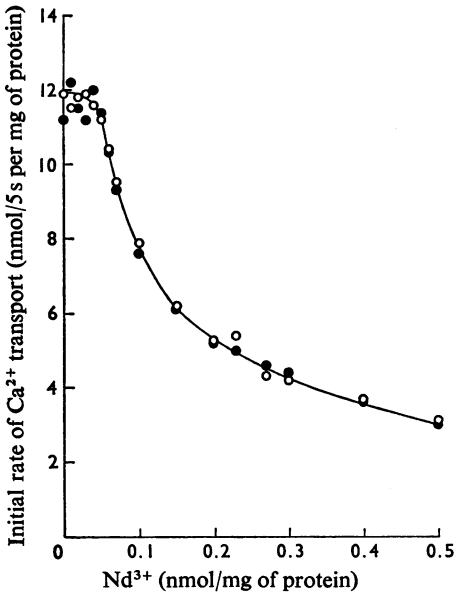

Fig. 5. *Inhibition of the initial rate of Ca^{2+} transport by Nd^{3+} (1 min preincubation)*

Incubations contained 250mM-sucrose, 2mM-Hepes–Tris (pH7.4) and 2mM-succinate–Tris in a total volume of 0.5ml. Mitochondria (1mg of protein) were preincubated with Nd^{3+} for 1min before the addition of 200μM-$^{45}Ca^{2+}$ (0.05μCi); incubation was continued for 5s and quenched by the addition of 1mM-EGTA–Tris. Samples (50μl) were filtered 10s (●) or 3min (○) after quenching, washed and assayed for radioactivity. Incubation temperature was 10°C.

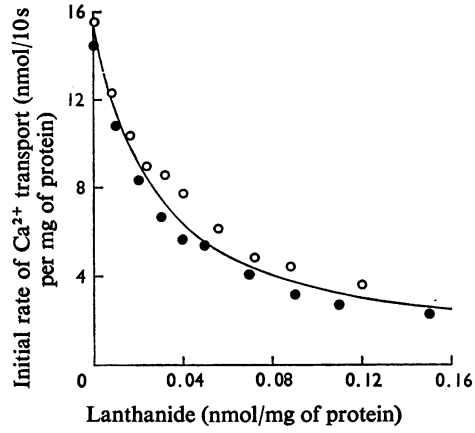

Fig. 6. *Inhibition of the initial rate of Ca^{2+} transport by lanthanides (5s preincubation)*

Incubations contained 250mM-sucrose, 2mM-Hepes–Tris (pH7.4) and 2mM-succinate–Tris in a total volume of 0.54ml in Eppendorf microcentrifuge tubes. Mitochondria (1.0mg of protein) were preincubated for 1min without inhibitors, then for 5s with Nd^{3+} (●) or La^{3+} (○). Each incubation was started by the addition of 100nmol of $^{45}Ca^{2+}$ (0.01μCi) and was continued for 10s before being quenched with 1μmol of EGTA–Tris. The tube was immediately centrifuged (2min at 12000*g*), the supernatant was removed and the pellet was prepared for scintillation counting by dissolution in 0.1ml of Soluene. Incubation temperature was 0°C.

Nd^{3+}, the one set of data being obtained with immediate filtration and the other with a 3min delay before filtration. 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+} transport is not hyperbolic. It seems that the lanthanides should be treated as 'alternate substrate and/or product' inhibitors, since inhibition of the 'dead-end' type is accompanied by hyperbolic kinetics. This is borne out by an earlier report (Reed & Bygrave, 1974) which shows that La^{3+} is accumulated by rat liver mitochondria, presumably on the 'Ca^{2+} carrier'. By using a shorter preincubation time and lower temperature to minimize lanthanide transport, inhibition would be expected to more nearly approximate a 'dead-end' pattern.

The results of such an experiment, with La^{3+} and Nd^{3+} as the inhibitors, are shown in Fig. 6. Inhibition occurs at lower concentrations under these conditions and the curve is hyperbolic. Analysis of the effects of lanthanides on Ca^{2+} transport is thus critically dependent on the assumption of 'dead-end' inhibition in short-term measurements, which is supported by the linearity of the derived kinetic plots (see below).

The initial slope of inhibition curves similar to that shown in Fig. 6 has, in previous reports (e.g. Mela & 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, 1969*a*; Vainio *et al.*, 1970). 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 & Goldstein, 1943; Morrison, 1969; Henderson, 1972).

The most informative derived plot for such data is that of $i_t/(1-\alpha)$ against $1/\alpha$ where $\alpha = v_i/v_0$, 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 the value of the ratio E_t/K_i is within the limits 0.01–100 (Henderson, 1972).

The data from a large number of experiments with La^{3+}, Nd^{3+}, Eu^{3+} and Tb^{3+} have been replotted in the derived form outlined above. In all cases the plots have a positive slope that can be extrapolated to the origin. It is concluded that under the particular conditions used in these experiments, E_t/K_i is less than or equal to 0.01 (Henderson, 1972). Most of the inhibitor is therefore 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. 6) is invalid (cf. Mela & 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 further substantiated by the linearity of the Dixon plots (Dixon, 1953) obtained with various La^{3+} concentrations at two different concentrations of Ca^{2+} (Fig. 7). The extrapolated lines intersect above the abscissa, showing competitive inhibition (verified by double-reciprocal plots) with a K_i of 2×10^{-8} M. Scarpa & Azzone (1970) similarly have found competitive inhibition of mitochondrial Ca^{2+} uptake by La^{3+} by using the murexide technique, but in earlier experiments with the same technique Mela (1969*a*) found non-competitive inhibition by Pr^{3+}; both groups obtained linear Dixon plots and a value for K_i of 5×10^{-8} M.

The concentration of La^{3+}-binding sites cannot be determined from the present data, but it has an approximate upper limit of 2×10^{-9} M, or 0.001 nmol/mg of protein, based on the upper limit of the ratio 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°C is shown in Fig. 8(*a*). The derived plots again have a positive slope, but in this case they can be extrapolated to intersect the ordinate above the origin (Fig. 8*b*). The degree of scatter in

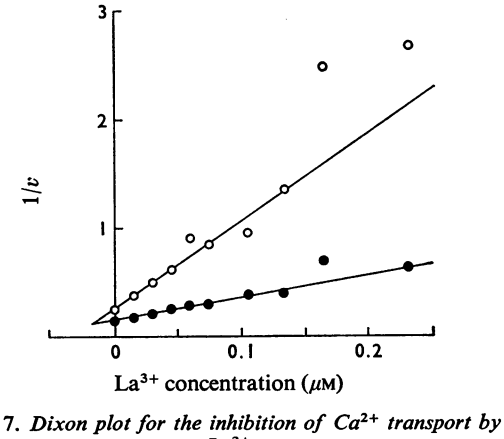

Fig. 7. *Dixon plot for the inhibition of Ca^{2+} transport by La^{3+}*

Experimental conditions were similar to those of Fig. 6 except that 0.93 mg of protein was present and the incubation time was 5 s. 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 $1/v$, where v is the initial rate of Ca^{2+} transport expressed as nmol of Ca^{2+} in mitochondria/5 s per mg of protein.

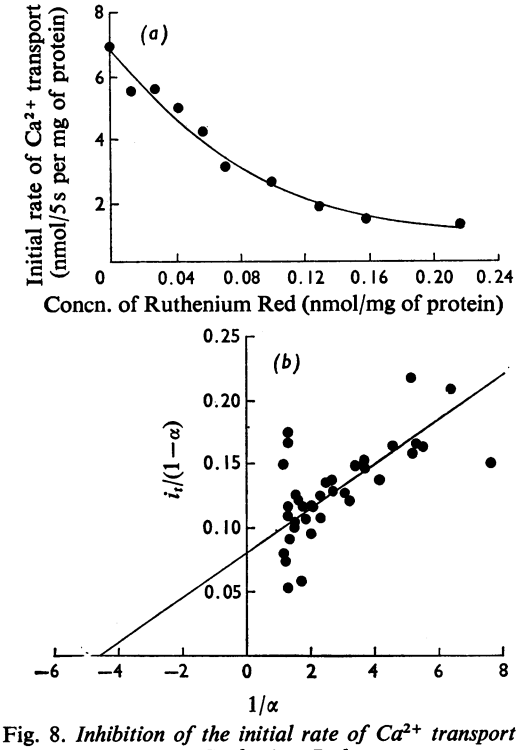

Fig. 8. *Inhibition of the initial rate of Ca^{2+} transport by Ruthenium Red*

(*a*) Experimental conditions were similar to those of Fig. 6 except that 0.95 mg of protein was present and the incubation time was 5 s. Each point is the mean of duplicate incubations. (*b*) Data of five separate experiments similar to (*a*) replotted in the form discussed in the text. The abscissa is $1/\alpha$ where $\alpha = v_i/v_0$ (the ratio of the inhibited rate to the uninhibited rate at the same substrate concentration) and the ordinate is $i_t/(1-\alpha)$ where i_t is the total concentration of Ruthenium Red (nmol/mg of protein). The intercept on the ordinate gives the concentration of inhibitor binding sites (E_T) and the intercept on the abscissa is $-E_T/K_i$.

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. 8*b*).

The concentration of Ruthenium-Red-binding sites is 0.08 ± 0.01 nmol/mg of protein. However, in contrast with the binding of 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 3×10^{-8} M, a value similar to that for La^{3+}.

Henderson (1973) has described an iterative statistical treatment for the analysis of experimental results obtained with tight-binding inhibitors that has the advantage of conferring the same weight to measured values of v_0 and v_i. Scatter of data points in the derived linear plot is thereby decreased. The programme developed by Henderson (1973) yields the statistically best values of v_0, E_t and K_i, and their standard deviations. However, the magnitude of experimental errors is too high in the present work (see the Experimental section) for this treatment to be of great value.

External binding

The above data show that both lanthanides and Ruthenium Red bind to the Ca^{2+} carrier with very high affinity, but do not show whether they bind to the carrier in amounts exceeding those required for inhibition, or to non-carrier sites. To determine this, binding was measured at external sites only (defined as that occurring within 5 s), since with the lanthanides total binding includes a large component of transport in experiments conducted over longer time-periods (Reed & Bygrave, 1974).

The binding of La^{3+} to mitochondria was measured with murexide by using two different approaches: the first involved its use as an indicator (see the Experimental section) in much the same way that it has previously been used to measure the kinetics of cation transport (Mela & Chance, 1968; Vainio *et al.*, 1970). Although the incubations included antimycin and rotenone and lacked a respiratory substrate and permeant anion, the $E_{540-510}$ increased with time, reflecting the transport of La^{3+}. The data on the binding of La^{3+} to external sites were thus obtained from individual experiments in which only the immediate decrease in $E_{540-510}$ after La^{3+} addition was measured.

The second approach was to use murexide as an external indicator (Fig. 9). Similar incubation conditions were used except that the temperature was decreased to 0°C to minimize La^{3+} transport in the short interval between its addition and the subsequent separation of mitochondria by centrifuga-

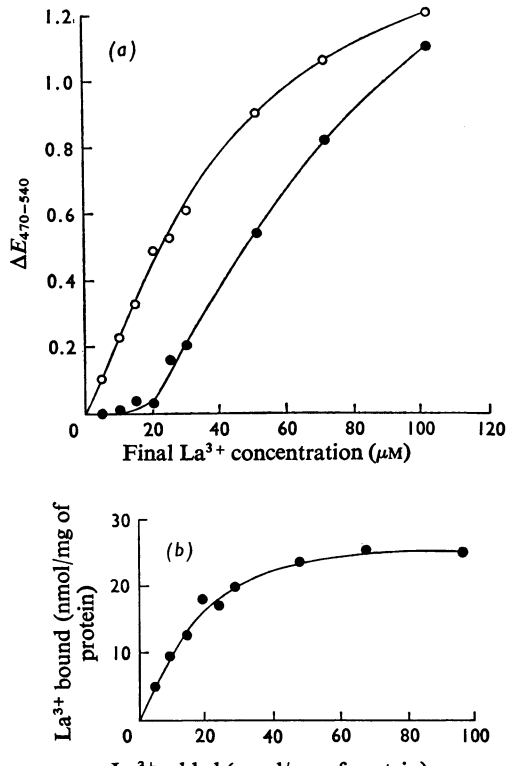

Fig. 9. *Binding of La^{3+} to external sites of mitochondria measured with murexide as an external indicator*

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 of mitochondrial protein, in a total volume of 1.3 ml. After 1 min preincubation, La^{3+} was added at the appropriate concentration and the tube immediately centrifuged (Eppendorf microcentrifuge: 2 min at 12000*g*). At the completion of the experiment, all tubes were re-centrifuged to pack the pellets more firmly and samples (1 ml) 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 La^{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 into La^{3+} concentrations by using the calibration curve obtained in the absence of mitochondria.

tion. Murexide was added to the supernatants and the concentration of La^{3+} in them was again measured by a dual-wavelength technique, but in this case the sensitivity was increased by using wavelengths corresponding to the peak (470nm) and the trough (540nm) 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 & Chance, 1968).

Identical data were obtained with both techniques. Scatchard plots show that the total number of external La^{3+}-binding sites is 27–30nmol/mg of protein and the apparent K_d is about 10μM, in reasonable agreement with the number and affinity of 'low-affinity' binding sites measured by Lehninger & Carafoli (1971) with $^{140}La^{3+}$ under conditions similar to those of Fig. 9 ($n = 29$nmol of La^{3+}/mg of protein; $K_d = 42\mu$M).

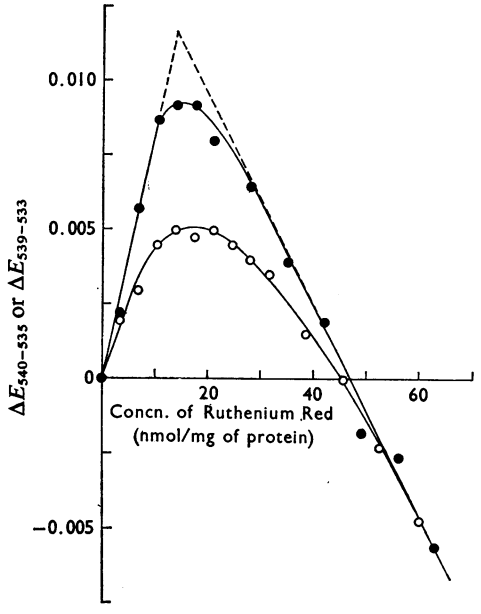

Fig. 10. *Binding of Ruthenium Red to intact and sonicated mitochondria*

Reference and sample cuvettes contained mitochondria (1.28mg of protein) in 250mM-sucrose, 5mM-Hepes–Tris (pH7.4), 0.15μM-rotenone and 0.15μM-antimycin in a total volume of 3ml (●), or mitochondria (1.28mg of protein) sonicated for 30s in 3ml of 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.

The binding of Ruthenium Red can be measured by direct titration with dual-wavelength spectrophotometry (Fig. 10). The absorbance maximum for Ruthenium Red in water is at 533nm, in the sucrose–Hepes–Tris incubation medium at 535nm, and in a suspension of mitochondria in the incubation medium at 540nm. The 5nm shift in the presence of mitochondria, a consequence of binding of Ruthenium Red, 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. 10) 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. 10 show that intact mitochondria bind approx. 15nmol of Ruthenium Red/mg of protein.

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, 1974). The above experiment was therefore repeated with mitochondria that had been sonicated in water. Aggregation still occurred, but it caused less optical interference because of the smaller particle size. The amount of Ruthenium Red bound to these particles is again 15nmol/mg of protein, but the binding affinity is less (Fig. 10).

The finding that mitochondria bind twice the amount of lanthanides 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 535nm and corrected for the slight loss of Ruthenium Red owing to its adsorption to the incubation tubes by reference to a standard curve prepared simultaneously. A Scatchard plot of the data of such an experiment is shown in Fig. 11(*a*). In the absence of La^{3+}, the number of binding sites was again found to be 14–15nmol/mg of protein and their apparent K_d is approx. 0.7μM, confirming the high affinity shown qualitatively in Fig. 10.

In the presence of La^{3+}, two classes of binding sites for Ruthenium Red can be distinguished (Fig. 11*a*). The first, numbering about 15nmol/mg of protein, is inhibited competitively by La^{3+} with a K_i of approx. 10μM (calculated from the 'apparent K_d' values). The second class is not inhibited by La^{3+} and binds less than 2nmol of Ruthenium Red/mg of protein. The limited sensitivity of the spectrophotometric measurements prevented a detailed analysis at lower concentrations.

The inhibition of 'low-affinity' binding of Ruthenium Red by La^{3+} is also evident in the experiment of

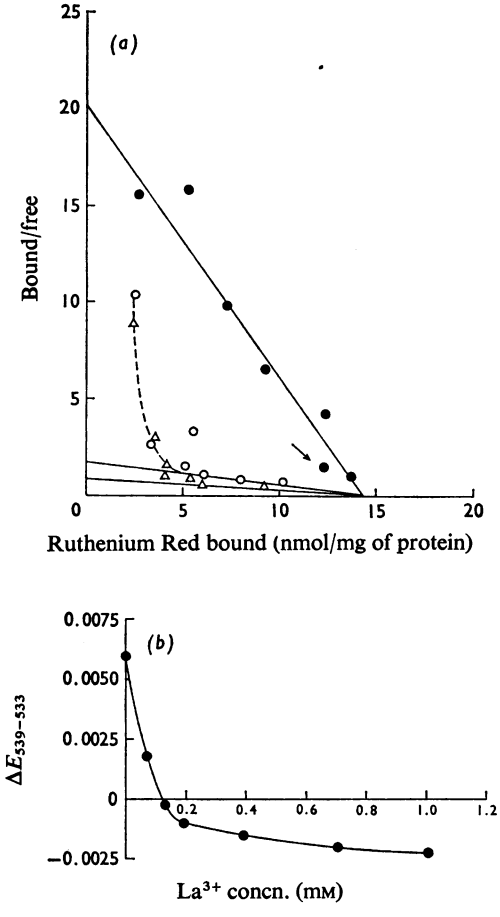

Fig. 11. *Inhibition of Ruthenium Red binding by* La^{3+}

(*a*) Incubations contained mitochondria (1.43mg of protein) in 250mM-sucrose, 5mM-Hepes–Tris (pH7.4), 0.15μM-rotenone and 0.15μM-antimycin in a total volume of 3.15ml. Ruthenium Red was added and followed immediately by 0 (●), 127μM-La^{3+} (○) or 254μM-La^{3+} (△). The tubes were then centrifuged at 16000*g* for 5min and the absorbance of the supernatants was read at 535nm. 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 (nmol/mg of protein) and the ratio bound:free (nmol/mg of 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 1min in 3ml of water and its absorbance was recorded at 539 and 533nm 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.

Fig. 11(*b*). In this case, the sonicated pellet from an incubation mixture containing a saturating concentration of Ruthenium Red was titrated with La^{3+} and the absorbance at 539 and 533nm (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 La^{3+} is approx. 50μM (the concentration required for half-maximal inhibition). It therefore appears that the water-treated sonic particles bind both La^{3+} and Ruthenium Red with lower affinity than do intact mitochondria (Figs. 10 and 11).

Discussion

The present paper establishes the inhibitory properties of the lanthanides and Ruthenium Red in sufficient detail for them to provide some insight into the mechanism of the Ca^{2+} carrier. However, the reiteration of the structure and properties of Ruthenium Red and the development of a generally applicable method for kinetic studies of Ca^{2+} transport described herein are valuable in themselves.

Ca^{2+} carrier

Previous workers have argued convincingly for the involvement of a specific carrier in mitochondrial Ca^{2+} transport (Lehninger & Carafoli, 1970; Vainio *et al.*, 1970; Selwyn *et al.*, 1970). The low number of transport-specific inhibitor binding sites and their extremely high affinity, seen in the results of the present work, support this proposal. On the basis of the combined evidence, the assumption has been made throughout this paper that the effects described pertain to such a carrier.

Two further assumptions are implicit in the 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 & Bygrave, 1974), but has not been demonstrated for Ruthenium Red. 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. Detailed kinetic studies in our laboratory have verified this (K. C. Reed & F. L. Bygrave, unpublished work).

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). The Ca^{2+}-binding sites of the mitochondrial carrier are thus almost certainly acidic

Table 1. *Concentration of high-affinity inhibitor-binding sites and membrane-bound cytochromes in rat liver mitochondria*

Membrane component	Ligand	Number of binding sites (nmol/mg of protein) (±S.D.)	Method used for determination	Source of data
Ca^{2+} carrier	Lanthanides	≤0.001	Inhibition of Ca^{2+} transport	Present work
Ca^{2+} carrier	Ruthenium Red	0.08 (±0.01)	Inhibition of Ca^{2+} transport	Present work
Adenine nucleotide translocase	ADP	0.14	^{14}C-ADP binding (atractyloside-sensitive)	Weidemann *et al.* (1970)
Adenine nucleotide translocase	Atractyloside	0.12 to 0.16	^{35}S-atractyloside binding	Klingenberg *et al.* (1971)
Adenine nucleotide translocase	Bongkrekic acid	0.41 to 0.50	Inhibition of ATPase	Henderson (1972)
ATPase	Rutamycin	0.11 to 0.17	Inhibition of ATPase	Henderson (1972)
Cytochrome *b*	Antimycin	0.04	Inhibition of succinate oxidation	K. C. Reed, unpublished work
Cytochrome *b* (total)	—	0.28 (±0.005)	Spectroscopy	Williams (1968)
Cytochrome $a+a_3$ (total)	—	0.28 (±0.005)	Spectroscopy	Williams (1968)
Cytochrome $a+a_3$ (total)	—	0.26 (±0.007)	Spectroscopy	Present work

oxygen atoms, but no distinction is possible at this stage between phosphate (either protein- or lipid-bound) and carboxylate (protein or polysaccharide) groups, the two most obvious candidates.

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 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^{2+} carrier. The extremely low number of La^{3+}-binding sites is so small as to suggest that some unknown factor(s) may be involved, which invalidate the kinetic analysis. If the concentration of this carrier is of the order of 0.001 nmol/mg of protein, its molecular activity (single-site carrier) would be $1400\,s^{-1}$ at 0°C, an extraordinarily high value. However, previous evidence suggests that 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.*, 1971*a*,*b*), and the stimulation of Mn^{2+} transport by low concentrations of Ca^{2+} (Chance & Mela, 1966; Mela & Chance, 1968) or lanthanides (Vainio *et al.*, 1970).

We therefore suggest that the Ca^{2+} carrier has a number of Ca^{2+}-binding sites, but the (competitive) binding of La^{3+} to only one of these is sufficient to cause inhibition of Ca^{2+} transport by virtue of its very high affinity for the carrier and its consequent slow rate of release to the internal phase (Reed & Bygrave, 1974). The lanthanides may be able to bind to all the Ca^{2+} sites, but in so doing they would not further enhance their inhibition of the initial rate of Ca^{2+} transport.

The mechanism of the non-competitive inhibition by Ruthenium Red is not clear. One possibility is that it prevents the transport of Ca^{2+} (by inhibiting either the uptake or recovery phase of the carrier) without affecting Ca^{2+} binding. Regardless of its precise mechanism, it is clear that inhibition by Ruthenium Red requires its binding at sites 10–100-fold more numerous than those required for La^{3+} inhibition.

It is conceivable that the Ca^{2+} carrier is not a 'classical' mobile carrier as implied in the foregoing discussion, but a specific arrangement of high-affinity Ca^{2+}-binding sites in a membrane pore; doubtless there are many feasible models for Ca^{2+} transport compatible with existing data. It is expected that more detailed kinetic and binding studies of the available substrates and inhibitors will at least minimize the number of viable models.

External binding

The external La^{3+}-binding sites measured in these and previous experiments (Lehninger & Carafoli, 1971) are probably identical with the 'low-affinity' Ca^{2+}-binding sites studied by Reynafarje & Lehninger (1969). This is to be expected from the close similarity in binding properties of these two cations and is demonstrably so both from the identical concentration of binding sites for each (Reynafarje & Lehninger, 1969; Carafoli & Lehninger, 1971; Rossi *et al.*, 1967; Scarpa & Azzi, 1968; Lehninger & Carafoli, 1971; and Fig. 9 of the present paper) and by the inhibition of low-affinity Ca^{2+} binding by La^{3+} (Lehninger &

Carafoli, 1971). There can be little doubt that these sites are preponderantly, if not entirely, phospholipids (Scarpa & Azzi, 1968; Scarpa & Azzone, 1969).

The present work shows that Ruthenium Red binds to these same sites with an affinity higher than that of La^{3+} (K_d of 0.7μM, cf. 10μM for La^{3+}). The identity of the sites is shown by the competitive inhibition of low-affinity Ruthenium Red binding by La^{3+} in which the K_i for La^{3+} is identical with the K_d for its own binding. It has further been shown that crude Ruthenium Red inhibits the low-affinity binding of Ca^{2+} by mitochondria (Vasington *et al.*, 1972*a*,*b*; Rossi *et al.*, 1973). The conclusion can thus be drawn that the binding of Ruthenium Red measured in the present experiments is to the non-specific low-affinity Ca^{2+}-binding sites of mitochondria, which are probably phospholipids.

Vasington *et al.* (1972*a*) 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 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 which previously have been implicated in mitochondrial Ca^{2+} binding (phosphatidylethanolamine, cardiolipin; Scarpa & Azzi, 1968; Scarpa & Azzone, 1969).

The number of binding sites for Ruthenium Red requires further comment. Although they appear to be identical in nature with those binding La^{3+} (and Ca^{2+}), they bind only half the amount of Ruthenium Red as they do La^{3+}. It therefore seems probable that 1mol of Ruthenium Red binds simultaneously to 2mol of phospholipid. Luft (1971) has calculated a mean molecular diameter for Ruthenium Red of 1.13nm; X-ray-diffraction data of synthetic and biological phospholipid membranes show that the hydrocarbon interchain distance is 0.42–0.46nm with ordered hexagonal packing (Keith & Melhorn, 1972), so that the maximum separation between adjacent head groups is 0.84–0.92nm. A single Ruthenium Red molecule could bridge the distance between the polar head groups of two adjacent phospholipid molecules in a membrane with extensive bilayer domains. The polyvalency of the complex would allow simultaneous strong ionic bonding to two sites.

In addition to identifying the nature of the relatively 'low-affinity' Ruthenium-Red-binding sites discussed above, the dual-binding experiments with La^{3+} reveal the existence of a smaller number of high-affinity sites which are not (or only weakly) inhibited by La^{3+}. Since La^{3+} was added after Ruthenium Red in these experiments (to minimize La^{3+} uptake), these could be sites to which Ruthenium Red is irreversibly bound, but they could equally be sites at which La^{3+} does not bind. This possibility is particularly interesting in view of the non-competitive inhibition of Ca^{2+} transport by Ruthenium Red. However, the concentration of the La^{3+}-insensitive sites is too high for them to be solely associated with inhibition of the Ca^{2+} carrier. Sensitive measurements of the high-affinity binding of the lanthanides and Ruthenium Red are essential to the further development of the concepts revealed by the data of the present paper.

The binding experiments discussed above show that neither the lanthanides nor Ruthenium Red bind to the Ca^{2+} carrier alone, although their affinity for the carrier is one to two orders of magnitude higher than for the non-specific phospholipid sites. Both cations provide powerful tools for analysis of the carrier mechanism. The main advantage offered by the lanthanides is that they bind to the Ca^{2+}-binding sites, which can thus be studied (necessarily at low temperatures to minimize the uptake of lanthanides) by every available spectroscopic method (Williams, 1970). On the other hand, the non-competitive 'irreversible' (with respect to time) inhibition by Ruthenium Red has obvious applications in quenching Ca^{2+} transport in kinetic studies of uptake and efflux, and in preventing transport in specific binding studies.

We thank Mrs. M. Gibberd and Mrs. A. Daday for their excellent technical assistance in many of the experiments, and Professor F. Gibson of the John Curtin School of Medical Research for the use of the Aminco–Chance spectrophotometer. We are also indebted to Dr. G. Barritt for helpful discussions.

References

Bygrave, F. L., Reed, K. C. & Spencer, T. (1971*a*) *Nature (London) New Biol.* **230**, 89

Bygrave, F. L., Reed, K. C. & Spencer, T. (1971*b*) in *Energy Transduction in Respiration and Photosynthesis* (Quagliariello, E., Papa, S. & Rossi, C. S., eds.), pp. 981–988, Adriatica Editrice, Bari

Carafoli, E. & Lehninger, A. L. (1971) *Biochem. J.* **122**, 681–690

Chance, B. (1951) *Rev. Sci. Instrum.* **22**, 634–638

Chance, B. (1965) *J. Biol. Chem.* **240**, 2729–2748

Chance, B. & Mela, L. (1966) *Biochemistry* **5**, 3220–3223

Chance, B. & Yoshioka, T. (1966) *Biochemistry* **5**, 3224–3229

Dixon, M. (1953) *Biochem. J.* **55**, 170–171

Fletcher, J. M., Greenfield, B. F., Hardy, C. J., Scargill, D. & Woodhead, J. L. (1961) *J. Chem. Soc. London* 2000–2006

Henderson, P. J. F. (1972) *Biochem. J.* **127**, 321–333

Henderson, P. J. F. (1973) *Biochem. J.* **135**, 101–107

Keith, A. D. & Melhorn, R. J. (1972) in *Membrane Molecular Biology* (Fox, C. F. & Keith, A. D., eds.), pp. 117–122, Sinauer Associates Inc., Stamford, Conn.

Klingenberg, M., Falkner, G., Erdelt, H. & Grebe, K. (1971) *FEBS Lett.* **16**, 296–300
Lehninger, A. L. & Carafoli, E. (1970) in *Biochemistry of the Phagocytic Process* (Schultz, J., ed.), pp. 9–22, North-Holland Publishing Co., Amsterdam
Lehninger, A. L. & Carafoli, E. (1971) *Arch. Biochem. Biophys.* **143**, 506–515
Lehninger, A. L., Carafoli, E. & Rossi, C. S. (1967) *Advan. Enzymol.* **29**, 259–320
Lemberg, M. R. (1969) *Physiol. Rev.* **49**, 48–121
Luft, J. H. (1971) *Anat. Rec.* **171**, 347–368
Mela, L. (1967) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **26**, 456
Mela, L. (1968*a*) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **27**, 828
Mela, L. (1968*b*) *Arch. Biochem. Biophys.* **123**, 286–293
Mela, L. (1969*a*) *Biochemistry* **8**, 2481–2486
Mela, L. (1969*b*) *Ann. N.Y. Acad. Sci.* **147**, 824–828
Mela, L. & Chance, B. (1968) *Biochemistry* **7**, 4059–4063
Mela, L. & Chance, B. (1969) *Biochem. Biophys. Res. Commun.* **35**, 556–559
Morgan, G. T. & Burstall, F. H. (1936) *J. Chem. Soc. (London)* 41–45
Moore, C. L. (1971) *Biochem. Biophys. Res. Commun.* **42**, 298–305
Morrison, J. F. (1969) *Biochim. Biophys. Acta* **185**, 269–286
Reed, K. C. (1972) *Anal. Biochem.* **50**, 206–212
Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **138**, 239–252
Reynafarje, B. & Lehninger, A. L. (1969) *J. Biol. Chem.* **244**, 584–593
Rossi, C., Azzi, A. & Azzone, G. F. (1967) *J. Biol. Chem.* **242**, 951–957
Rossi, C. S., Vasington, F. D. & Carafoli, E. (1973) *Biochem. Biophys. Res. Commun.* **50**, 846–852
Scarpa, A. & Azzi, A. (1968) *Biochim. Biophys. Acta* **150**, 473–481
Scarpa, A. & Azzone, G. F. (1969) *Biochim. Biophys. Acta* **173**, 78–85
Scarpa, A. & Azzone, G. F. (1970) *Eur. J. Biochem.* **12**, 328–335
Selwyn, M. J., Dawson, A. P. & Dunnett, S. J. (1970) *FEBS Lett.* **10**, 1–5
Sillén, L. G. & Martell, A. E. (1964) *Chem. Soc. Spec. Publ.* **17**
Straus, O. H. & Goldstein, A. (1943) *J. Gen. Physiol.* **26**, 559–585
Strong, F. M., Dickie, J. P., Loomans, M. E., van Tamelen, E. E. & Dewey, R. S. (1960) *J. Amer. Chem. Soc.* **82**, 1513–1514
Vainio, H., Mela, L. & Chance, B. (1970) *Eur. J. Biochem.* **12**, 387–391
Vasington, F. D., Gazzotti, P., Tiozzo, R. & Carafoli, E. (1972*a*) *Biochim. Biophys. Acta* **256**, 43–54
Vasington, F. D., Gazzotti, P., Tiozzo, R. & Carafoli, E. (1972*b*) in *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E. & Siliprandi, N., eds.), pp. 215–228, Academic Press, New York and London.
Weidemann, M. J., Erdelt, M. & Klingenberg, H. (1970) *Eur. J. Biochem.* **16**, 313–335
Williams, J. N. (1968) *Biochim. Biophys. Acta* **162**, 175–181
Williams, R. J. P. (1970) *Q. Rev. Chem. Soc. (London)* **24**, 331–365