Accumulation of Lanthanum by Rat Liver Mitochondria

By KEN C. REED and FYFE L. BYGRAVE

Department of Biochemistry, Faculty of Science, Australian National University,
Canberra, A.C.T. 2600, Australia

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The interaction of La^{3+} with rat liver mitochondria was examined with a wide variety of techniques permitting measurement of respiratory and structural responses. It is concluded that La^{3+} is accumulated by mitochondria in a process that is at least partially energy-dependent, and is bound with quite high affinity to membrane-associated sites both external and internal to the inner membrane. The relative insensitivity of the accumulation process to respiratory inhibitors and to the permeant anion acetate has interesting implications for the mechanism of active cation transport.

The trivalent rare-earth cations are extremely potent inhibitors of the energy-linked accumulation of Ca^{2+} by mitochondria (Mela, 1968*a*, 1969*a*; Mela & Chance, 1969; Vainio *et al.*, 1970; Scarpa & Azzone, 1970). Indeed, the most convincing evidence for the existence of a Ca^{2+} carrier in the mitochondrial membrane is obtained from the dose-response relationship between lanthanide concentration and the rate of Ca^{2+} accumulation (Mela & Chance, 1969). The inhibition by lanthanides is thought to differ from that by the bivalent metal ions (Mn^{2+} , Sr^{2+} , Ba^{2+}), not only with respect to their higher potency, but also because the lanthanides are thought not to be accumulated by mitochondria (cf. Mg^{2+} , K^{+} ; Vainio *et al.*, 1970). Lehninger & 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 La^{3+} with mitochondria that suggest that it may be transported by these organelles, the most obvious being the release of its inhibition of Ca^{2+} uptake with time (Mela, 1968*a,b*, 1969*b*; Lehninger & Carafoli, 1971). We felt that these indications were sufficiently strong to warrant a re-examination of the question of La^{3+} transport. The results of the present experiments show that La^{3+} is accumulated by mitochondria in a process similar in many respects to the uptake of Ca^{2+} . This report does not attempt to characterize optimal conditions for La^{3+} uptake, but concentrates instead on a thorough analysis of the numerous responses induced by La^{3+} in mitochondria.

Experimental

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–

* Abbreviations: Hepes, 2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid; EGTA, ethanedioxybis(ethylamine)tetra-acetic acid; ATPase, adenosine triphosphatase.

Tris buffer (pH 7.4 at 0°C) followed by differential centrifugation (Johnson & Lardy, 1967). The preparation was washed twice in 250 mM-sucrose–2 mM-Hepes–Tris buffer (pH 7.4) and finally resuspended in the same medium.

Incubation media

The standard incubation medium contained 250 mM-sucrose, 5 mM-Hepes–Tris, 10 mM-acetate–Tris and 5 mM substrate (succinate–Tris or sodium DL- β -hydroxybutyrate), final pH 7.4. Other additions were made as indicated in the legends to Figures.

Assays

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

ATPase activity was measured by following the release of P_i . Incubation samples were quenched with trichloroacetic acid and assayed by a micro-method with high chromophore stability (Baginski & Zak, 1960; Baginski *et al.*, 1967).

Mitochondrial swelling

Mitochondrial volume changes were monitored semi-quantitatively by measuring E_{520} in a Unicam SP.800 spectrophotometer with constant temperature cell housing.

Isopycnic centrifugation

Discontinuous sucrose density gradients were prepared in 5 ml tubes by using data compiled by Dawson *et al.* (1969) with the inclusion of 2 mM-Hepes–Tris buffer (pH 7.4 at 0°C) in all solutions. Samples were prepared by centrifuging a mitochondrial incubation mixture at 8000 g for

5 min and resuspending the pellets in 1 ml of 250 mM-sucrose–2 mM-Hepes–Tris buffer (pH 7.4). These were layered on top of the freshly prepared gradients and centrifuged at 2°C in a Beckman ultracentrifuge at 32 500 rev./min for 3 h in a SW50 head (Greenawalt *et al.*, 1964).

Electron microscopy

Mitochondria were treated with 1 mM-EGTA, then fixed for microscopy by the dropwise addition of 10% (v/v) glutaraldehyde (diluted with incubation medium) to the stirred incubation mixtures to give a final glutaraldehyde concn. of 2% (v/v). The solutions used in these experiments were neutralized with KOH rather than Tris to avoid the reaction of Tris with glutaraldehyde. After being fixed for 1 h at room temperature, the mitochondria were separated by centrifugation at 8000g for 5 min (2°C). The pellets were washed with cold 250 mM-sucrose–10 mM-collidine, pH 7.4, and post-fixed overnight with 1% (w/v) osmium tetroxide 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, 1968).

O₂ uptake

O₂ utilization by mitochondria was measured polarographically with a membrane-covered electrode (Reed, 1972).

Dual-wavelength spectrophotometry

Changes in absorbance of respiratory components were measured with an Aminco–Chance spectrophotometer by using the dual-wavelength technique (Chance, 1951). The same technique was used to measure the concentration of free La³⁺ by the absorbance of its murexide complex (cf. Mela & Chance, 1968).

Fluorescence measurements

The fluorescence of mitochondrial nicotinamide nucleotides and flavoproteins was monitored with a Zeiss spectrofluorimeter with dual monochromators.

Materials

Lanthanum solutions were prepared with LaCl₃·7H₂O and were standardized by titrating the acid equivalents displaced from a column of Dowex (H⁺ form) against a portion of the solution (K. J. Ellis, unpublished work). Antimycin solutions were standardized spectrophotometrically (Strong *et al.*, 1960). All reagents were of analytical grade.

Results

Passive swelling

When mitochondria with inhibited respiration 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 the cation (Chappell & Haarhoff, 1967).

A variation on this approach (suggested by Selwyn *et al.*, 1970) was used in preliminary experiments to investigate the permeability of the inner membrane towards La³⁺. The results of the control experiments with Mg²⁺ and Ca²⁺ additions to KCl, acetate and thiocyanate (Figs. 1a and 1b) are as expected from previous results (Selwyn *et al.*, 1970). Experiments with La³⁺ (Fig. 1c) indicate that, similarly to Ca²⁺ but in contrast with Mg²⁺, it enters the osmotically active space. The only noticeable difference between the effects of Ca²⁺ and La³⁺ is the considerable lag preceding the *E*₅₂₀ decrease of the latter, particularly in the presence of thiocyanate. It is probable that the *E*₅₂₀ decrease is a result of the penetration of La³⁺ into the osmotically active space, because it varies

Fig. 1. Passive swelling of mitochondria with Mg²⁺, Ca²⁺ and La³⁺

Mitochondria (4.25 mg of protein) were added to cuvettes containing 90 mM-KCl (curve 1), potassium acetate (curve 2) or potassium thiocyanate (curve 3), 5 mM-Hepes–Tris buffer (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 concn. of 7.3 mM: (a) MgCl₂; (b) CaCl₂; (c) LaCl₃. Total volume, 2.75 ml; temperature 30°C.

Fig. 2. Active swelling of mitochondria in response to La^{3+} addition

Mitochondria [2.5 mg of protein in (a) and (c), 2.3 mg of protein in (b)] were added to cuvettes containing the standard medium and succinate in a final volume of 3 ml (temperature 30°C). La^{3+} was added (arrows) at the concns. (μM) shown. (c) (i) Antimycin and rotenone ($0.12 \mu\text{M}$ and $0.17 \mu\text{M}$ respectively) added to mixture; (ii) carbonyl-cyanide *m*-chlorophenylhydrazone ($10 \mu\text{M}$) added to mixture; (iii) no additions.

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 La^{3+} may also be actively accumulated against a concentration gradient and thus induce swelling at low concentrations. This appears to be the case (Fig. 2). As seen in Fig. 2(a), 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 $100 \mu\text{M}$. The decrease 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 E_{520} decrease is inhibited (Fig. 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. 2c).

In both these experiments and others 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 8 min, treated with EGTA and then subjected to isopycnic centrifugation. Incubation with about 70 nmol of La^{3+}/mg of protein increased the density of most of the mitochondrial population by about 0.03 mg/ml (Figs. 3a and 3b). 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 & Chance (1968), to follow free La^{3+} concentrations.

Calibration curves for the $E_{510}-E_{540}$ difference as a function of La^{3+} concentration are shown in Fig. 4(a). The curve obtained in the presence of β -hydroxybutyrate is linear only to a concentration of La^{3+} about 60% that of murexide ($20 \mu\text{M}$), which is indicative of the high stability of the La-murexide complex (Sillén *et al.*, 1971). Absorbance traces were therefore converted into La^{3+} concentration to give more meaningful results. A separate calibration was required for experiments using succinate because within the concentration range used, it chelates roughly 75% of the available La^{3+} (see Sillén & Martell, 1964; Sillén *et al.*, 1971). Control experiments showed that La^{3+} causes negligible $E_{510}-E_{540}$ changes in the absence of murexide.

By making small additions of La^{3+} to mitochondria it was found that $25\text{--}30 \text{ nmol}$ of La^{3+}/mg of protein were bound within the mixing time (5 s). This amount is similar to the number of external low-affinity Ca^{2+} -binding sites of liver mitochondria (K. C. Reed & F. L. Bygrave, unpublished work), and probably reflects the binding of La^{3+} to these sites. Addition of La^{3+} to substrate-supplemented mitochondria at concentrations higher than about 12 nmol/mg of protein causes an immediate decrease in E_{540} relative to E_{510} ; the absorbance subsequently increases with time (Fig. 4b), but the rate of increase is considerably slower than that seen with Ca^{2+} or Mn^{2+} (Mela & Chance, 1968) suggesting that the rate of

Fig. 3. Isopycnic centrifugation of mitochondria loaded with La^{3+}

Mitochondria (3.6 mg of protein) were incubated with La^{3+} for 8 min under conditions identical with those of Fig. 2. EGTA was added to give a final concn. of 0.83 mM and the tubes were centrifuged at 8000g for 5 min (at 2°C). The pellets were then resuspended, layered on discontinuous sucrose density gradients and centrifuged as described in the Experimental section. 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. 4. Free La^{3+} concentrations measured by murexide absorbance changes

(a) Calibration curves for murexide absorbance changes at 510–540 nm as a function of La^{3+} concn. Cuvettes contained the standard medium, 20 μM -murexide and succinate (○) or β -hydroxybutyrate (●) in a final volume of 3 ml, at room temperature. (b) Uptake of La^{3+} by mitochondria. Cuvettes contained the standard medium, 20 μM -murexide, β -hydroxybutyrate and 7.2 mg of mitochondrial protein in a final volume of 3 ml at room temperature; antimycin and rotenone [included in incubation for curve (i)] and carbonyl cyanide *m*-chlorophenylhydrazone [added at arrow in curve (ii)] were used at concns. of 0.24 μM , 0.33 μM and 10 μM respectively. Murexide absorbance changes (510–540 nm) after the addition of 100 μM La^{3+} were converted into free La^{3+} concns. by using the appropriate calibration curve (a).

accumulation of La^{3+} is slower (cf. stimulation of oxygen uptake; see below).

The inclusion of antimycin and rotenone partially inhibits the decrease in free La^{3+} concentration

(Fig. 4b) as does the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (not shown), but the murexide absorbance decrease is less sensitive than the light-scattering response (Fig. 2). The addition of

EXPLANATION OF PLATE I

Effects of La^{3+} on mitochondrial ultrastructure

Rat liver mitochondria (4.1 mg of 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 before the addition of La^{3+} (at the concentrations shown) and then for a further 10 min before being prepared for microscopy as described in the Experimental section. (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.

EXPLANATION OF PLATE 2

Effects of La^{3+} on mitochondrial ultrastructure

Conditions were identical with those of Plate 1. (a) and (b) $100\mu\text{M-La}^{3+}$; (c) and (d) $333\mu\text{M-La}^{3+}$. 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 $1\mu\text{m}$.

inhibitors or uncouplers to mitochondria fully loaded with La^{3+} does not cause its release (Fig. 4b).

Electron microscopy

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

The control preparation, incubated for 10 min in the absence of La^{3+} (Plate 1a), exhibits the condensed conformation typical of freshly isolated liver mitochondria. The addition of $33\text{ }\mu\text{M}$ - La^{3+} (Plate 1b) 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 & Connelly, 1971). When the La^{3+} concentration is further increased to $67\text{ }\mu\text{M}$ (Plates 1c and 1d) a proportion of the population becomes swollen. Some of these swollen mitochondria show a broken outer membrane [Plate 1c(1)], which remains in contact with the inner membrane in regions that still exhibit remnants of cristal structure. The sequence of transitions induced by La^{3+} to this stage (condensed $\rightarrow$ twisted, orthodox $\rightarrow$ swollen, broken) is typical of the ultrastructural changes induced by swelling agents (Myron & Connelly, 1971) or by changes in osmotic strength of the suspending medium (Stoner & Sirak, 1969).

The outstanding feature of Plate 1(d) 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 1(c) and 1(d). 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 polyvesicular structure becomes even more pronounced after treatment with $100\text{ }\mu\text{M}$ - La^{3+} (Plates 2a and 2b) and reaches its maximal frequency in samples treated with about $100\text{--}200\text{ }\mu\text{M}$ - 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 $100\text{ }\mu\text{M}$ is mitochondrial aggregation. This phenomenon is dependent on both La^{3+} concentration and time and is optimally enhanced by P_i at a concentration about half that of La^{3+} . The junction between the membranes of aggregated mitochondria is generally very electron-dense [Plates 2a(1) and 2b(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 $333\text{ }\mu\text{M}$ (Plates 2c and 2d), aggregation occurs to such an extent that mitochondria are rarely seen in isolation. There are virtually no polyvesicular structures. A proportion of the mitochondria are swollen (e.g. Plate 2d), but a large number retain relatively 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 more than $100\text{ }\mu\text{M}$ - 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 O_2 uptake

Lehninger & 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(a) 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 β -hydroxybutyrate as substrate (Fig. 5b), but no stimulation is observed with α -oxoglutarate plus malonate (Fig. 5c) 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 α -oxoglutarate (plus malonate) and pyruvate plus malate probably has a similar explanation (see Sillén & Martell, 1964; Sillén *et al.*, 1971).

Fig. 5. Stimulation of mitochondrial O_2 uptake by La^{3+}

Mitochondria (3.8 mg of protein) were incubated under conditions similar to those of Fig. 2 except that the substrate was varied as follows: (a) succinate; (b) β -hydroxybutyrate; (c) α -oxoglutarate-Tris (5mM) plus malonate-Tris (5mM). Additions were mitochondria (RLM) and La^{3+} (167 μ M).

Fig. 6. 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 of mitochondrial protein (RLM), 20°C; (b) β -hydroxybutyrate, 3.8 mg of mitochondrial protein (RLM), 30°C; (c) β -hydroxybutyrate, 3.6 mg of mitochondrial protein, (RLM), 30°C. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (10 μ M) was added where indicated.

Both the rate and extent of the stimulation shown in Fig. 5 are dependent on La^{3+} concentration. With β -hydroxybutyrate, both parameters are maximal at about 100 μ M, but at concentrations less than

30 μ M little or no stimulation occurs. With succinate, higher concentrations of La^{3+} are required.

Within quite narrow concentration limits, the stimulation of succinate oxidation by La^{3+} is cyclic,

Fig. 7. Response of mitochondrial respiratory components to $100\text{ }\mu\text{M-La}^{3+}$

Dual-wavelength spectrophotometry was used to record the changes in redox state of respiratory components in response to the addition of $100\text{ }\mu\text{M-La}^{3+}$ (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 unit for nicotinamide nucleotides and cytochrome *b* [in (a) and (b) only], and 0.005 unit for all other traces. Experiment; were done with the standard incubation medium containing (a) β -hydroxybutyrate; (b) succinate plus rotenone ($0.33\text{ }\mu\text{M}$)s (c) succinate. Mitochondrial protein was 7.2 mg in (a) and (b), 6.8 mg in (c). The final volume was 3 ml at room temperature.

as illustrated by the experiment of Fig. 6(a). The difficulties involved in obtaining this type of response are the lack of stimulation by low concentrations ($<75\text{ }\mu\text{M}$) on the one hand, and the apparent irreversibility of the response induced by high concentrations ($>150\text{ }\mu\text{M}$) on the other. It is thus difficult to assess accurately the La^{3+}/O activation ratio (cf. Ca^{2+}/O ; Rossi & Lehninger, 1964). Those values that 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/energy-conserving site (Chance, 1965).

In contrast, no cyclic response could be obtained in the presence of β -hydroxybutyrate. At concentrations exceeding $50\text{ }\mu\text{M}$ the stimulated rate of respiration is followed by an inhibited state, but this is not the normal state 4 $\uparrow$, as it is not relieved by the addition of an uncoupling agent (Fig. 6b). The inhibition does not extend to succinate oxidation, as shown in Fig. 6(c).

† The numerical respiratory states of mitochondria used in the text are those defined by Chance & Williams (1956) and Chance & Schoener (1966).

When phosphate (1 mM) is substituted for acetate, the respiratory stimulation induced by La^{3+} is barely noticeable, probably owing 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. 7). With both β -hydroxybutyrate (Fig. 7a) and succinate (Figs. 7b and 7c) as substrates, $100\text{ }\mu\text{M-La}^{3+}$ elicits a change in the redox state of all respiratory carriers.

With β -hydroxybutyrate (Fig. 7a), nicotinamide nucleotides are extensively oxidized. Simultaneously, flavoprotein components (and/or non-haem iron

protein), cytochrome *b* and cytochrome *c* (+*c*₁) become more reduced, indicating a cross-over (Chance & 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. 8). After about 1 min the rate of nicotinamide nucleotide oxidation decreases, flavoproteins become more oxidized and cytochrome *b* undergoes a second phase of reduction.

The response of cytochrome *a* (+*a*₃) is anomalous in that it initially tends towards a more oxidized state. We have been unable to measure an initial 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. 7*b*). The two bottom traces in this Figure compare absorbance changes of the α - and β -bands respectively of cytochrome oxidase. The contribution of cytochrome *a* to the former is about 80%, whereas it contributes only about 35% to the Soret band (Lemberg, 1969). From a comparison of the responses measured near the maxima of these two bands, it seems that cytochrome *a*₃ does not undergo the second-phase reduction and that this response is due to cytochrome

a alone. Experiments with β -hydroxybutyrate (results not shown) give similar results.

When succinate alone is used as the substrate (Fig. 7*c*) the effects observed are similar to those seen in the presence of rotenone, with the notable exception that cytochrome *a* (+*a*₃) shows an initial reduction.

The initial responses of the respiratory components occurring within the first minute after La^{3+} addition apparently correlate with the lag period noted in the measurements of light-scattering and O_2 uptake. It is therefore the second-phase responses which correlate with respiratory stimulation and mitochondrial swelling.

The responses to $100\text{ }\mu\text{M-La}^{3+}$ described above are irreversible, reflecting the irreversibility of the stimulation of O_2 uptake at higher concentrations. At low concentrations of La^{3+} , the oxidation of nicotinamide nucleotides (Fig. 8*a*) and cytochrome *a*₃ (+*a*) is again irreversible, but in contrast, cytochrome *b* undergoes a cycle of rapid reduction followed by slow reoxidation (Fig. 8*b*). A number of such additions can be made until a point is reached at which cytochrome *b* becomes irreversibly reduced (Figs. 8*b* and 8*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

Fig. 8. Effects of low La^{3+} concentrations on nicotinamide nucleotides and cytochrome *b*

Conditions were identical with those of Fig. 7 except that the substrate was β -hydroxybutyrate in each case. The absorbance calibration represents 0.01 unit for nicotinamide nucleotides (340–374 nm) and 0.005 unit 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 of mitochondrial protein.

Fig. 9. Effects of La^{3+} and Ca^{2+} on nicotinamide nucleotide fluorescence

Mitochondria (3.1 mg of protein) were added to cuvettes containing the standard medium and β -hydroxybutyrate [(a) and (d)], succinate [(b) and (e)] or succinate plus $0.13 \mu\text{M}$ -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. Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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. Since it is unable to penetrate the osmotically active inner compartment of mitochondria (K. C. Reed & F. L. Bygrave, unpublished work; see also Chappell *et al.*, 1963; Chappell & Crofts, 1965), it does not remove La^{3+} that 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 $33 \mu\text{M}$ - La^{3+} (Fig. 8c), it stimulates the rate of cyclic reoxidation but has no instantaneous effect (cf. Fig. 8d). Similarly, EGTA has no effect on the irreversible oxidation of cytochrome *a*₃ (+a) induced by $33 \mu\text{M}$ - 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 $67 \mu\text{M}$ - La^{3+} is immediately reversed by EGTA (Fig. 8d); this phenomenon must be associated with La^{3+} that 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 $67 \mu\text{M}$ - La^{3+} is not affected by a similar concentration of EGTA.

Nicotinamide nucleotide fluorescence

In mitochondria supplemented with β -hydroxybutyrate, La^{3+} causes an initial rapid increase in nicotinamide nucleotide fluorescence, followed by a slower decrease (Fig. 9a) which reflects the oxidation seen in absorbance measurements (Fig. 7a). Both the extent of the rapid increase and the rate of the subsequent decrease are dependent on concentration, but at all concentrations tested (25–150 μM) the response is irreversible and qualitatively similar. When succinate is substituted for β -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. 9b). On the further inclusion of rotenone, La^{3+} causes a slow increase in fluorescence (Fig. 9c). The subsequent addition of carbonyl cyanide *m*-chlorophenylhydrazone decreases fluorescence only to the initial value, in contrast with its effect in the absence of rotenone (Figs. 9b and 9c).

These effects of La^{3+} can be compared with those of Ca^{2+} under similar conditions (Figs. 9d–9f). A

series of rapid oxidation-reduction cycles are obtained with β -hydroxybutyrate (Fig. 9d), but with succinate the nicotinamide nucleotides become irreversibly oxidized when the total Ca^{2+} concentration exceeds $250\text{ }\mu\text{M}$ (Fig. 9e). In contrast, the addition of Ca^{2+} to a medium further supplemented with rotenone causes a large increase in nicotinamide nucleotide fluorescence, an effect similar to but more pronounced than that of La^{3+} .

The increase in fluorescence intensity induced by Ca^{2+} in the presence of succinate and rotenone

(Chance & Azzi, 1968) is probably due to fluorescence enhancement of reduced nicotinamide nucleotides resulting from their binding Ca^{2+} in a non-aqueous environment, presumably a membrane lipid phase. Vinogradov *et al.* (1972) have reported that La^{3+} causes a much greater enhancement of NADH fluorescence in a model system [aq. 90% (v/v) methanol] than does Ca^{2+} , suggesting that our results with La^{3+} (Figs. 9a and 9c) reflect a similar association of this cation with reduced nicotinamide nucleotides in the inner membrane.

Fig. 10. Inhibition of succinate oxidation by Ca^{2+} and its reversal by La^{3+} and rotenone

Mitochondria (3.5 mg of 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 $0.13\text{ }\mu\text{M}$ -rotenone. The final concn. of Ca^{2+} added was in each case $500\text{ }\mu\text{M}$ and that of La^{3+} , $3\text{ }\mu\text{M}$. The final concn. of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in (a) was $10\text{ }\mu\text{M}$.

The surprising sensitivity of nicotinamide nucleotides to Ca^{2+} in succinate-supplemented mitochondria prompted its further investigation. As seen in Fig. 10(a), the oxidation of nicotinamide nucleotides correlates with a dramatic inhibition of O_2 uptake to a rate less than that of state 4. The Ca^{2+} -inhibited state is relatively insensitive to carbonyl cyanide *m*-chlorophenylhydrazone, in marked contrast with state 6 (Chance & Schoener, 1966).

Complete cycles of Ca^{2+} -induced respiration can be restored in two ways. The first is by the inclusion of rotenone (Fig. 10c); the second is by the prior addition of a low concentration of La^{3+} . It should be noted that $3\text{ }\mu\text{M}$ - La^{3+} causes only partial inhibition of Ca^{2+} -stimulated respiration (Figs. 10b and 10d; cf. Figs. 10a and 10c), reflecting the accumulation of La^{3+} shown by the preceding results.

Flavoprotein fluorescence

The addition of $150\text{ }\mu\text{M}$ - La^{3+} to mitochondria oxidizing either β -hydroxybutyrate or succinate causes a relatively slow but extensive oxidation of fluorescing flavoprotein(s) (Figs. 11a and 11b);

the further inclusion of rotenone in the succinate-supplemented system decreases both the rate and extent of oxidation (Fig. 11c). In contrast, La^{3+} causes a reduction of components absorbing at 475 nm (see Fig. 7). The La^{3+} -induced cross-over shown by Fig. 7 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 O_2 side (Chance *et al.*, 1967, 1969; Hatefi, 1968).

Again, no cyclic response is obtained, in contrast with the effects of Ca^{2+} (Figs. 11d–11f). 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 expected that ATP could support La^{3+} uptake, by analogy with energy-linked Ca^{2+} transport (Bielawski & Lehninger, 1966). Control experiments showed no interference of La^{3+} with the phosphate assay and no precipitation of lanthanum

Fig. 11. Effects of La^{3+} and Ca^{2+} on flavoprotein fluorescence

Mitochondria (3.5 mg of protein) were incubated exactly as described for Fig. 9, the substrates being β -hydroxybutyrate [(a) and (d)], succinate [(b) and (e)], or succinate plus $0.13\text{ }\mu\text{M}$ -rotenone [(c) and (f)]. The final concn. of La^{3+} was in each case $150\text{ }\mu\text{M}$ and that of Ca^{2+} , $250\text{ }\mu\text{M}$. Fluorescence emission was recorded at 570 nm with excitation at 436 nm (Chance *et al.*, 1967).

phosphate at concentrations up to 0.5 mM; when a precipitate did form at higher concentrations, it was completely soluble in the 5% (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 P_i 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 La -ATP, 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 above 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 & 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 (as illustrated by its remarkably strong inhibition of Ca^{2+} uptake at concentrations 1000-fold lower; Mela, 1969a), but its rate of accumulation is much slower (compare respiratory stimulation shown in Figs. 5 and 10). On the other hand, Mn^{2+} similarly causes less stimulation of O_2 uptake than does Ca^{2+} (Chappell *et al.*, 1963; Chance & Schoener, 1966) and is accumulated more slowly (Mela & Chance, 1968), but the initial response of the respiratory components is quite slow (Chance & Schoener, 1966; Vainio *et al.*, 1970). Since the affinity of Mn^{2+} for the Ca^{2+} carrier appears to be relatively low (Reynafarje & Lehninger, 1969), 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 & Chance, 1968; Vainio *et al.*, 1970; Reynafarje & Lehninger, 1969) 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. 8c). 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 before transport, so that it gives an estimate of the rate of release of La^{3+} to the matrix from the carrier. Unfortunately, the release of cations to the internal space is a feature of the carrier mechanism which has drawn little previous attention. 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 after the addition of La^{3+} appears to be in this region, in contrast with 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 the internal sites. Thus the major proposal of this scheme is that the requirement for energy in cation accumulation is largely for increasing the affinity and/or number of internal binding sites. Their assumed higher affinity for La^{3+} in the 'non-energized' state (partially) relaxes this requirement in the case of La^{3+} accumulation.

This hypothesis for cation transport will be developed further in subsequent communications. It is not inconsistent with much previous data (in particular, see Gear *et al.*, 1967). The nature of the proposed energy-dependent increase in binding affinity (or amount) is not clear, but it is most readily conceived of in terms of either small-scale conformational changes or an increase in internal alkalinity such that dissociable groups with pK_a near neutrality become ionized (e.g. phosphatidylethanolamine and cardiolipin).

Structural aspects of La^{3+} accumulation

The effects of La^{3+} on respiratory activity become irreversible at concentrations exceeding about $50\mu\text{M}$ with β -hydroxybutyrate, or about $100\mu\text{M}$ with succinate as substrate. The mitochondria similarly become maximally swollen at this concentration of La^{3+} (Fig. 2 and Plate 1). It thus seems possible that the non-cyclic stimulation of O_2 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 the Results section 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 E_{520} decreases, indicating that 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 (K. C. Reed & F. L. Bygrave, unpublished work). Thirdly, the non-cyclic response of nicotinamide nucleotides and flavoproteins at very low concentrations of La^{3+} argues for the direct

interaction of La^{3+} with a component(s) in this region, which necessitates the association of 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 La^{3+} -accumulation process to respiratory inhibitors and uncouplers might be construed as further evidence that the accumulated La^{3+} is membrane-bound rather than soluble.

It has been well established that the accumulation of Ca^{2+} by liver mitochondria similarly results in non-osmotic swelling (Lehninger, 1962; Chappell & Crofts, 1965). This is an effect quite distinct from an osmotic response to the accumulation of a soluble Ca^{2+} salt, since it occurs some time after the accumulation process itself, the lag-period being dependent on Ca^{2+} concentration (K. C. Reed & F. L. Bygrave, unpublished work). Further, it is inhibited by the presence of acetate. Under these latter conditions, immediate osmotic swelling accompanies the accumulation of Ca^{2+} and is followed only after a prolonged lag period by the second swelling response (K. C. Reed & F. L. Bygrave, unpublished work).

The mechanism of this effect of 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 La^{3+} could be useful in view of the possible similarity between it and that of Ca^{2+} . At this preliminary stage, it appears that the only difference between their effects is that La^{3+} has a higher affinity for membrane sites involved in the swelling response.

The ultrastructural features seen at higher 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.

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