Calcium Ion Accumulation and Volume Changes of Isolated Liver Mitochondria

REVERSAL OF CALCIUM ION-INDUCED SWELLING

BY A. R. CROFTS* AND J. B. CHAPPELL*
Department of Biochemistry, University of Cambridge

(Received 27 July 1964)

1. The excessive accumulation of Ca^{2+} by mitochondria suspended in an iso-osmotic buffered potassium chloride medium containing oxidizable substrate and phosphate led to extensive swelling and release of accumulated Ca^{2+} from the mitochondria. When the Ca^{2+} was removed from the medium by chelation with ethylene glycol bis(aminoethyl)tetra-acetate, the swelling was reversed in a respiration-dependent contraction. The contracted mitochondria were shown to have regained some degree of respiratory control. 2. The respiration-dependent contraction could be supported by electron transport through a restricted portion of the respiratory chain, and by substrates donating electrons at different levels in the respiratory chain. 3. Respiratory inhibitors appropriate to the substrate present completely inhibited the contraction. Uncoupling agents, and the inhibitors oligomycin and atractyloside, were without effect. 4. When the reversal of swelling had been prevented by respiratory inhibitors, the addition of ATP induced a contraction of the mitochondria. In the absence of added chelating agent the contraction was very slow. The ATP-induced contraction was completely inhibited by oligomycin and atractyloside, was incomplete in the presence of uncoupling agents and was unaffected by respiratory inhibitors. 5. The relationship between the energy requirements of respiration-dependent contraction and the requirements of ion transport and other contractile systems are discussed.

In the preceding paper (Chappell & Crofts, 1965a) it was shown that the process of Ca^{2+} and phosphate accumulation by isolated liver mitochondria leads to a swelling of large magnitude. Inhibitors that prevented the accumulation of Ca^{2+} , e.g. respiratory inhibitors and uncoupling agents, also prevented swelling. On the other hand, if Ca^{2+} accumulation had occurred already, then the addition of these inhibitors or anaerobic conditions caused extensive swelling. The addition of EGTA†, which avidly chelates Ca^{2+} (Schmid & Reilly, 1957), to suspensions of swollen mitochondria led, after a short lag period, to a respiration-dependent contraction of the mitochondria (Chappell, Cohn & Greville, 1963). The properties of this reversal system are discussed in the present paper. Reversal occurred on the institution of electron transport through even a restricted portion of the respiratory chain, in a process that was insensitive to uncoupling agents, e.g. 2,4-

dinitrophenol or carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine (Heytler & Prichard, 1962), or on the addition of ATP. When the mitochondria had contracted as a result of the addition of the chelating agent some measure of respiratory control was observed.

METHODS AND MATERIALS

The techniques used for measurement of oxygen consumption, changes in H^+ concentrations and light-scattering changes were as described by Chappell & Crofts (1965a).

RESULTS

Isolated liver mitochondria that had accumulated 100–300 $\mu\text{equiv.}$ of $\text{Ca}^{2+}/\text{g.}$ of protein underwent extensive swelling on: (a) sufficiently long incubation (3–5 min., depending on the age of the mitochondria) or the addition of excess of Ca^{2+} ; (b) on the addition of 2,4-dinitrophenol (100 μM) or carbonylcyanide-*p*-trifluoromethoxyphenylhydrazine (0.1 μM); (c) on the addition of 0.1 $\mu\text{g.}$ of antimycin/ml. or hydrogen cyanide (100 μM); or (d)

* Present address: Department of Biochemistry, Medical School, University of Bristol.

† Abbreviation: EGTA, ethylene glycol bis(aminoethyl)-tetra-acetate.

Fig. 1. Respiration-dependent reversal of Ca^{2+} -induced mitochondrial swelling. The broken trace is a recording of mitochondrial light-scattering at 180° , a downward deflection representing swelling. The upper continuous trace is a recording of the change in oxygen concentration in the suspending medium, a downward deflection representing oxygen uptake by the mitochondria. The lower continuous trace is a recording of changes in H^+ concentration in the suspending medium, an upward deflection representing H^+ production. 8 mM-Succinate, 2.1 mM-phosphate and $0.17 \mu\text{M}$ -rotenone were present initially. Additions were made where indicated as follows: A, mitochondria (approx. 10 mg. of protein); B, $1.0 \mu\text{mole}$ of CaCl_2 ; C, $4.0 \mu\text{moles}$ of EGTA; D, dinitrophenol ($100 \mu\text{M}$). For explanation of the Figure, see the text.

on the suspension becoming anaerobic (Chappell & Crofts, 1965a).

Under appropriate conditions swelling induced in each of these ways was partially or wholly reversed on the addition of an excess of the Ca^{2+} -chelating agent. In the experiment shown in Fig. 1 swelling resulted from the addition of $1 \mu\text{mole}$ of Ca^{2+} . The pH trace indicates that Ca^{2+} was rapidly accumulated by the mitochondria (H^+ production), and associated with this process there was a rapid rate of respiration and a variable apparent contraction of the mitochondria (increased light-scattering). After 20 sec. the rate of respiration fell to a low value and the rate of H^+ production fell, both of which indicate that Ca^{2+} accumulation was completed. After a further 3–5 sec. the mitochondria began to swell, and after some measure of swelling had occurred there was a reversal of the H^+ change associated with the release of the accumulated Ca^{2+} and phosphate. The addition of EGTA at the completion of the swelling period led to a very rapid production of H^+ , as a result of the chelation of free Ca^{2+} released from the mitochondria, and an increase in the rate of respiration, and after a lag period of 7 sec. the mitochondria began to contract.

Fig. 2. Respiration-dependent reversal of Ca^{2+} -induced swelling in the presence of cytochrome c. 8 mM-Succinate, 2.1 mM-phosphate, $0.17 \mu\text{M}$ -rotenone and $2.5 \mu\text{M}$ -cytochrome c were present initially. The convention of the traces is the same as that given in Fig. 1. Additions were made where indicated as follows: A, mitochondria (approx. 10 mg. of protein); B, $1.0 \mu\text{mole}$ of CaCl_2 ; C, $4.0 \mu\text{moles}$ of EGTA; D, dinitrophenol ($100 \mu\text{M}$). For explanation of the Figure, see the text.

After 1 min. this contraction process was completed and the rate of respiration fell to a new steady value.

Swollen mitochondria that had not been exposed to EGTA did not show an increase of respiration on the addition of dinitrophenol ($100 \mu\text{M}$), but, if EGTA was added after the dinitrophenol, then after a period that coincided with that time necessary for reversal of swelling to commence there was an increase in the rate of respiration. The addition of dinitrophenol to mitochondria that had contracted in response to the addition of EGTA led to an immediate increase in the rate of respiration. These experiments (Figs. 3 and 4) demonstrate that under these conditions fully swollen mitochondria do not respond to the addition of dinitrophenol, whereas contracted mitochondria do.

Release of accumulated Ca^{2+} as a result of the process of swelling was a necessary prerequisite for the occurrence of EGTA-induced respiration-dependent contraction. If liver mitochondria were treated with quantities of Ca^{2+} ($1.25 \mu\text{moles}$ of calcium chloride/6 ml.) just sufficient to cause some measure of swelling, but without rapid release of Ca^{2+} (see Fig. 7 of Chappell & Crofts, 1965a), then there was no rapid production of H^+ on addition of EGTA, since little of the Ca^{2+} was available to the chelating agent. Also, there was no increase in the rate of respiration and no reversal of the intermediate degree of swelling that had occurred. If

Fig. 3. Respiration-dependent reversal of Ca^{2+} -induced mitochondrial swelling: the recoupling of respiration on contraction. The convention of the traces is the same as that given in Fig. 1. 8mm-Succinate, 2.1mm-phosphate, 0.17 μM -rotenone and 2.5 μM -cytochrome *c* were present initially. Additions were made where indicated as follows: A, mitochondria (approx. 5mg. of protein); B, 1.0 μmole of CaCl_2 ; C, 4.0 μmoles of EGTA; D, dinitrophenol (100 μM). For explanation of the Figure, see the text.

Fig. 4. Effect of dinitrophenol on the respiration-dependent reversal of Ca^{2+} -induced mitochondrial swelling. Initial conditions and conventions were as given in Fig. 3. Additions were made where indicated as follows: A, mitochondria (approx. 5mg. of protein); B, 1.0 μmole of CaCl_2 ; C, dinitrophenol (100 μM); D, 4.0 μmoles of EGTA. For explanation of the Figure, see the text.

more extensive swelling was induced, before the addition of the EGTA, by adding either more Ca^{2+} or dinitrophenol (100 μM), then the subsequent addition of EGTA led to reversal of swelling, and the other effects described above.

Inhibitors of respiration-dependent contraction. When succinate was used as substrate, 0.1 $\mu\text{g.}$ of antimycin/ml. or hydrogen cyanide (100 μM) com-

pletely inhibited the contraction that occurred on the addition of EGTA. With the former inhibitor the addition of ascorbate (2mm) plus tetramethyl-*p*-phenylenediamine (0.2mm) restored both respiration and contraction. Again, when succinate was used as substrate but when respiration and contraction had been inhibited by addition of hydrogen cyanide, then the further addition of ferricyanide

(2mm), to act as electron acceptor in place of oxygen (the rate of ferricyanide reduction could be followed easily by recording the consequent production of H^+), restored both electron transport and contraction.

The use of those substrates with NAD-linked dehydrogenases, e.g. malate dehydrogenase, in the presence of a transaminating system (Chappell, 1961, 1964*a,b*) was complicated by severe inhibition caused by loss of nicotinamide nucleotide resulting from the swollen state of the mitochondria (see Lehninger, 1960*b*). The mitochondria also lost some of their cytochrome *c* when swollen, but retained sufficient to allow maximal rates of contraction, since the addition of cytochrome *c* (2.5 μM), although it enhanced the rate of respiration, had little effect on the contraction process (Fig. 2). However, with 5mm-glutamate plus 5mm-malate as substrate and with the further addition of NAD (1mm) reversal of Ca^{2+} -induced swelling occurred. In this case both respiration and contraction were inhibited by the presence of 0.17 μM -rotenone. With succinate as substrate, neither respiration nor contraction was affected by addition of this inhibitor of NAD-linked oxidation.

Mitochondria swell readily as a consequence of Ca^{2+} accumulation supported by endogenous substrates (Chappell & Crofts, 1965*a*). In this case the addition of EGTA did not produce contraction unless an oxidizable substrate, e.g. 5mm-succinate or 2mm-ascorbate together with 0.2mm-tetramethyl-*p*-phenylenediamine, was also added.

If care was taken to ensure that respiration itself was not inhibited by addition of uncoupling agents (see Chappell, 1964*b*), then these compounds were almost completely without effect on the contraction process. This result was obtained with 100 μM -dinitrophenol, 0.1 μM -carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone and 1 μM -pentachlorophenol. However, one difference in behaviour was apparent in the presence of uncoupling agents. When the suspension became anaerobic in the presence of an uncoupling agent, then swelling occurred (see Fig. 3), but in the absence of uncoupling agents there was no change in light-scattering (see Fig. 2). The same difference in behaviour was observed when antimycin was added rather than if the suspension was allowed to become anaerobic.

Concentrations of oligomycin well in excess of those required to block completely ADP-stimulated respiration (up to 2 $\mu g./ml.$) and atractyloside (up to 40 μM) were completely without effect on the respiration-dependent reversal of Ca^{2+} -induced swelling.

ATP-induced contraction. When contraction had been inhibited by addition of rotenone (with

glutamate plus malate as substrate), antimycin (with glutamate plus malate or succinate) or hydrogen cyanide (with all substrates tested), then the addition of ATP (2mm) induced a reversal of swelling consequent on the accumulation of Ca^{2+} and phosphate. Only a very slow and incomplete reversal was obtained on the addition of ATP, unless EGTA was also added. This appears to indicate that ATP is unable to bind Ca^{2+} sufficiently well to allow reversal to occur in the absence of added EGTA.

By making use of the fact that Ca^{2+} will induce swelling of mitochondria with only the endogenous substrates present (Chappell & Crofts, 1965*a*), it was possible to show that the ATP- and EGTA-induced contraction was not affected by the addition of respiratory inhibitors (rotenone, antimycin and hydrogen cyanide). It was, however, completely inhibited by oligomycin and atractyloside. Dinitrophenol and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone had a variable but small inhibitory effect on the initial phases of the ATP- and EGTA-induced contraction. In the later phases these uncoupling agents produced a more marked effect. It appears that this was possibly due to depletion of ATP by stimulation of adenosine-triphosphatase activity.

There was no demonstrable requirement for Mg^{2+} in either the respiration- or ATP-dependent contraction processes. Indeed with ATP, 1–5mm-magnesium sulphate caused a marked decrease in the rate of contraction.

DISCUSSION

The ATP-dependent contraction of mitochondria, swollen as a consequence of a variety of treatments, has been widely studied (see e.g. Chappell & Greville, 1963). Isolated pigeon-breast-muscle mitochondria were first shown to have a Mg^{2+} -dependent contractile system (Chappell, 1954; Chappell & Perry, 1954). With the realization that sucrose markedly inhibits the contractile system of liver mitochondria it was possible to demonstrate a similar ATP-dependent reversal of swelling with those mitochondria (Chappell & Greville, 1958; Lehninger, Ray & Schneider, 1959). The isolation from liver mitochondria of a protein fraction with properties similar to those of actomyosin has been reported (Ohnishi & Ohnishi, 1962).

Lehninger and his colleagues have carried out an extensive investigation of the requirements for the demonstration of maximal rates of ATP-induced reversal of the various types of large-magnitude swelling of liver mitochondria (Lehninger *et al.* 1959; Lehninger, 1959*a,b*, 1960*a,b*, 1961*a,b*, 1962; Wojtczak & Lehninger, 1961; Neubert & Lehninger, 1962*a,b*; Neubert, Foster & Lehninger, 1962;

Neubert, Rose & Lehninger, 1962). Most germane to this present investigation is their demonstration that the requirements for the maximal rates of reversal depends on the swelling agent used. Thus, in the type of Ca^{2+} -induced swelling (due to U-factor) that they studied, either EDTA or serum albumin was required in addition to ATP and Mg^{2+} or Mn^{2+} . With the ATP-dependent reversal of the swelling that occurred as a result of the accumulation of Ca^{2+} and phosphate only EGTA was required and Mg^{2+} was inhibitory. However, Lehninger and his colleagues showed that the reversal of thyroxine-induced swelling did not require an added bivalent metal ion. With the exception of thyroxine-induced swelling, oligomycin proved to be a potent inhibitor of the reversal produced by ATP. Oligomycin was also an inhibitor of the type of ATP-induced contraction described in the present paper, as was atractyloside. The inhibition of the ATP-dependent reversal of swelling of liver mitochondria by atractyloside was observed by Bruni & Luciani (1962).

The respiration-dependent contraction of liver mitochondria described in the present paper does, however, show some unusual features. Reversal of swelling was observed when electron transport through even a restricted portion of the respiratory chain occurred (cytochrome *b* to cytochrome *c*, cytochrome *c* to oxygen). However, the insensitivity of the reversal process to the uncoupling agents, dinitrophenol, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone and pentachlorophenol,

and to the inhibitors of phosphorylation, oligomycin and atractyloside, indicates that a very early phase in the energy-conservation system is involved. During the contraction process, subsequent to the addition of EGTA, the rate of respiration was markedly increased (see Figs. 1 and 3). It seems possible that this increase in respiration reflects the energy requirement for the contraction process, just as ATP synthesis or ion accumulation in mitochondria causes increased rates of electron transport. In all three processes, when ATP synthesis is complete, or when nearly all the added bivalent metal ion is accumulated or when the contraction process is complete (Figs. 1 and 3), respiration returns to the resting level.

In Scheme 1 the possible relationships between the processes of electron transport, energy conservation, ion accumulation and the contractile system are shown diagrammatically. This Scheme accounts for many of the observations described in this and the accompanying paper (Chappell & Crofts, 1965a). The justification for the assumed sites of action of the inhibitors shown and the interaction of the ion accumulation process with the energy-conservation system are discussed by Chappell & Crofts (1965b). Scheme 1 accounts for the respiration-dependent contraction if it is assumed that the contractile system is related to some intermediate between the respiratory chain and 'X ~ I', the concentration and state of this intermediate determining the extent of contraction of the mitochondria. Such a hypothesis would

Scheme 1. Schematic representation of the sites of action of inhibitors (in **bold type**) and functions associated with the respiratory chain and the energy-coupling process.

also account for the small-magnitude contraction that occurs when ADP is added to respiring mitochondria (Chance & Packer, 1958; Packer, 1960), and perhaps also for the contraction observed on adding Ca^{2+} , Mn^{2+} or Sr^{2+} to mitochondria (Chappell *et al.* 1963; Chappell & Crofts, 1965a).

An extension of this hypothesis also enables an explanation of the observations on the ability of dinitrophenol to stimulate or not the respiration of swollen and contracted mitochondria. The fact that Ca^{2+} -swollen mitochondria did not show dinitrophenol-stimulated respiration may be accounted for if it is assumed that in swollen mitochondria 'X~I' is physically dissociated from the respiratory chain, thus preventing dinitrophenol from having any action on the rate of electron transport. When contraction occurs the respiratory chain is brought into proximity with 'X~I', respiratory control is observed and dinitrophenol can stimulate respiration.

We thank Miss Jenny Flemans for her expert assistance, and the Medical Research Council for financial support.

REFERENCES

- Bruni, A. & Luciani, S. (1962). *Nature, Lond.*, **196**, 578.
 Chance, B. & Packer, L. (1958). *Biochem. J.* **68**, 295.
 Chappell, J. B. (1954). Ph.D. Dissertation: University of Cambridge.
 Chappell, J. B. (1961). In *Biological Structure and Function*, vol. 2, p. 71. Ed. by Goodwin, T. W. & Lindberg, O. London: Academic Press (Inc.) Ltd.
 Chappell, J. B. (1964a). *Biochem. J.* **90**, 225.
 Chappell, J. B. (1964b). *Biochem. J.* **90**, 237.
 Chappell, J. B., Cohn, M. & Greville, G. D. (1963). In *Energy-Linked Functions of Mitochondria*, p. 219. Ed. by Chance, B. New York: Academic Press Inc.
 Chappell, J. B. & Crofts, A. R. (1965a). *Biochem. J.* **95**, 378.
 Chappell, J. B. & Crofts, A. R. (1965b). *Biochem. J.* **95**, 393.
 Chappell, J. B. & Greville, G. D. (1958). *Abstr. 4th int. Congr. Biochem., Vienna*, p. 71.
 Chappell, J. B. & Greville, G. D. (1963). *Symp. biochem. Soc.* **23**, 39.
 Chappell, J. B. & Perry, S. V. (1954). *Nature, Lond.*, **173**, 1094.
 Heytler, P. G. & Pritchard, W. W. (1962). *Biochem. biophys. Res. Commun.* **7**, 272.
 Lehninger, A. L. (1959a). *J. biol. Chem.* **234**, 2187.
 Lehninger, A. L. (1959b). *J. biol. Chem.* **234**, 2465.
 Lehninger, A. L. (1960a). *Ann. N.Y. Acad. Sci.* **86**, 484.
 Lehninger, A. L. (1960b). *Biochim. biophys. Acta*, **37**, 387.
 Lehninger, A. L. (1961a). *J. Biochem., Tokyo*, **49**, 553.
 Lehninger, A. L. (1961b). *Biochim. biophys. Acta*, **48**, 324.
 Lehninger, A. L. (1962). *J. biol. Chem.* **237**, 946.
 Lehninger, A. L., Ray, B. L. & Schneider, M. (1959). *J. biophys. biochem. Cytol.* **5**, 97.
 Neubert, D., Foster, G. V. & Lehninger, A. L. (1962). *Biochim. biophys. Acta*, **60**, 492.
 Neubert, D. & Lehninger, A. L. (1962a). *J. biol. Chem.* **237**, 952.
 Neubert, D. & Lehninger, A. L. (1962b). *Biochim. biophys. Acta*, **62**, 556.
 Neubert, D., Rose, T. H. & Lehninger, A. L. (1962). *J. biol. Chem.* **237**, 2025.
 Ohnishi, T. & Ohnishi, T. (1962). *J. Biochem., Tokyo*, **51**, 380.
 Packer, L. (1960). *J. biol. Chem.* **235**, 242.
 Schmid, R. W. & Reilly, C. N. (1957). *Analyt. Chem.* **29**, 264.
 Wojtezak, L. & Lehninger, A. L. (1961). *Biochim. biophys. Acta*, **51**, 442.