*Biochem. J.* (1965) **95**, 378

# Calcium Ion Accumulation and Volume Changes of Isolated Liver Mitochondria

## CALCIUM ION-INDUCED SWELLING

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

(*Received* 27 *July* 1964)

1. Liver mitochondria suspended in an iso-osmotic buffered potassium chloride medium containing an oxidizable substrate and phosphate accumulated added $Ca^{2+}$. During this process $H^+$ appeared in the medium and the mitochondrial suspension showed increased light-scattering. Respiration was markedly stimulated. 2. The addition of excess of $Ca^{2+}$, respiratory inhibitors or uncoupling agents caused extensive mitochondrial swelling associated with release of $Ca^{2+}$ into the suspending medium. When the suspension became anaerobic extensive swelling also occurred. Only under conditions when the addition of uncoupling agents would have produced high rates of electron transport, e.g. in the presence of succinate, was the structural integrity of the mitochondrion maintained after $Ca^{2+}$ accumulation. 3. Conditions that prevented respiration-dependent $Ca^{2+}$ accumulation also prevented $Ca^{2+}$-induced swelling. Bovine plasma albumin was without effect, indicating that U-factor was not involved. Oligomycin together with ADP or ATP partially stabilized the mitochondria against $Ca^{2+}$-induced swelling. 4. It is suggested that a 'high-energy' intermediate generated by coupled electron transport is required to prevent the mitochondrial swelling that results as a consequence of $Ca^{2+}$ accumulation.

It has been known for a number of years that $Ca^{2+}$ causes a swelling of large magnitude of isolated mitochondria (Raaflaub, 1953; Slater & Cleland, 1953; Hunter & Ford, 1955; Tapley, 1956). It has been claimed that this swelling action of $Ca^{2+}$ is more immediately due to the enzymically catalysed release of U-factor from an endogenous precursor, a process activated by $Ca^{2+}$. It is characteristic of swelling of this type, including that produced by $Ca^{2+}$, that it is prevented by low concentrations of serum albumin, probably because of the binding of U-factor (Lehninger & Remmert, 1959; Wojtczak & Lehninger, 1961). Like other forms of large-magnitude swelling, e.g. that produced by phosphate or thyroxine, $Ca^{2+}$-induced swelling is abolished by the addition of respiratory inhibitors and appears to be dependent on electron transport through even a restricted portion of the respiratory chain (see Chappell & Greville, 1963*a*). Use of selective inhibitors of phosphorylation (2,4-dinitrophenol, oligomycin) rather than of electron transport has indicated that this type of swelling is perhaps directly dependent on electron transport (Chappell & Greville, 1959, 1960, 1961, 1963*a*).

Recent work from a number of Laboratories has revealed that mitochondria isolated from a variety of tissues are able to accumulate $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Sr^{2+}$ with the simultaneous uptake of phosphate in either a respiration-dependent process or one that requires the presence of ATP (e.g. Brierley, Bachmann & Green, 1962; Brierley, Murer, Bachmann & Green, 1963; Chappell, Cohn & Greville, 1963; Chappell & Greville, 1963*b*; Chappell, Greville, & Bicknell, 1962; Lehninger, Rossi & Greenawalt, 1963*a*,*b*; Saris, 1959, 1963*a*,*b*). During the investigation of this process in this Laboratory it was discovered that $Ca^{2+}$ causes a swelling of mitochondria that appears to be intimately related to the accumulation process (Chappell *et al.* 1963). In the present paper the results of this investigation are presented. It has been shown that U-factor is probably not involved in this type of swelling, but rather that the accumulation of $Ca^{2+}$ by the mitochondria is the causal factor. Under conditions where accumulation is prevented swelling does not occur. In an accompany-

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

ing paper (Crofts & Chappell, 1965) the reversal of this type of swelling is described.

## METHODS AND MATERIALS

Rat-liver mitochondria were isolated in a freshly prepared medium consisting of 0·25 M-sucrose in 5 mM-tris–chloride buffer, pH 7·2, essentially by the method of Hogeboom, Schneider & Pallade (1948). The mitochondria were washed twice and stored for use at a protein concentration of 60–80 mg. of protein/ml. at 0° for not more than 4 hr.

Oxygen consumption, changes in $H^+$ concentration and light-scattering were measured simultaneously in the reaction vessel shown diagrammatically in Fig. 1. The total volume of the reaction vessel, which was thermostatically controlled at 30°, was 6·0 ml. The reaction medium consisted of 80 mM-KCl in 20 mM-tris–chloride buffer containing the various additions indicated in the text and legends to Figures. The initial pH was 7·20–7·25 in each case.

*Oxygen consumption.* This was followed by using the Clark oxygen electrode (Yellow Springs Instrument Co., Ohio, U.S.A.), as described by Chappell (1964*a*). In some experiments the rate of respiration was recorded directly by differentiating the amplified signal from the oxygen electrode (see Longmuir, 1957) by means of the circuit shown in Fig. 2. The 'noise' of the differentiated signal was 5–10% of the ADP-stimulated succinate rate (0·2 $\mu$g.atom of O/min.), the small fluctuations in the electrode current due to imperfect stirring of the medium contributing most of this 'noise'. In the Figures shown in the present paper the traces of the recorded rate of oxygen consumption have been smoothed; the other traces have been presented as they were recorded.

pH *changes.* Changes in $H^+$ concentration were followed by using a Radiometer (Copenhagen, Denmark) concentric glass electrode (model GK 2026C) and radiometer pH-meter (model PHM 22r) adapted for recording. The saturated KCl solution of the concentric glass electrode was replaced by 0·1 M-KCl. It was found that this diminished considerably the 'noise' due to stirring of the medium, presumably because of the decrease in the fluctuations of the junction potential at the porous plug separating the 0·1 M-KCl and the reaction medium. The use of 0·1 M-KCl instead of saturated KCl had no effect on the behaviour of the pH-meter. The output from the pH-meter was used to drive a Honeywell–Brown (Motherwell, Scotland) recorder (1 mv full-scale deflection, 1 sec. response time). A suitable resistance network was used to match the pH-meter and recorder, and a suitable backing-off circuit was incorporated to enable a pH change of 0·1–0·2 unit to give a full-scale deflection on the recorder, starting at any initial pH value.

*Light-scattering.* This was measured by using a 2·2 v pre-focus torch bulb (Ever Ready 2225) supplied from a stabilized 2 v source (model 115D spectrophotometer power supply; Labgear Ltd., Cambridge) and measurement of the light-intensity at 180° to the incident beam by using a red-sensitive vacuum photocell (Cintell VS 50S) covered with a gelatin filter with maximum transmission at 750 m$\mu$. The current from the photocell was amplified with a Pye Instrument Co. (Cambridge) d.c. amplifier (model 11370) and recorded on a Control Instruments Ltd. (Birkenhead) potentiometric recorder (1 mv full-scale deflection, 0·3 sec. response time).

There was no detectable interaction between the oxygen and glass electrode systems if care was taken to see that there was no electrical leak between the Clark electrode and the reaction vessel.

*Reagents.* Most organic chemicals used in the present investigation were obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Sucrose used in the preparation of mitochondria and inorganic

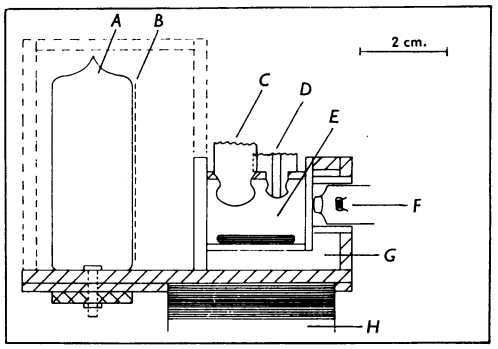


Fig. 1. Reaction vessel for the simultaneous recording of oxygen consumption, changes in $H^+$ concentration and changes in light-scattering at 180°. *A*, Photocell; *B*, filter; *C*, concentric glass and calomel electrode; *D*, oxygen electrode; *E*, reaction vessel; *F*, lens-ended bulb; *G*, water jacket; *H*, stirrer. The apparatus was constructed of Perspex, the cell from clear Perspex, and the base, water jacketing, electrode support and photocell hood from black Perspex. Details of components are given in the Methods and Materials section.

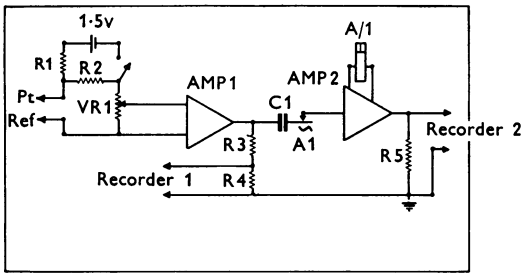


Fig. 2. Circuit for recording oxygen uptake together with the rate of oxygen uptake. Components were as follows: VR 1, 1 kΩ; R 1, 1·5 kΩ; R 2, 1·0 kΩ; R 3, 100 kΩ; R 4, 50Ω; R 5, 5Ω; C 1, 8 $\mu$F, 750 v d.c., paper; A/1, A 1, Associated Electrical Industries Ltd. synchronous chopper (CK 3); AMP 1, Pye d.c. amplifier (model 11370); AMP 2, Princetown Applied Research lock-in amplifier (model JB 4); Recorders 1 and 2, Control Instruments Ltd. recorders (0–5 mv full-scale deflection, 1·3 sec. response time). The frequency of chopping was determined by a tuning circuit incorporated into the lock-in amplifier, which then filtered out all signals except those at the set frequency, thus eliminating 'noise' from the signal.

chemicals were of A.R. grade. EGTA* was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Oligomycin was a gift from Charles Pfizer Inc., Groton, Conn., U.S.A., and antimycin was purchased from the Wisconsin Alumni Foundation, Madison, Wis., U.S.A. Atractyloside was a kind gift from Dr E. C. Slater and rotenone from Dr R. W. Estabrook. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone was provided generously by Dr P. G. Heytler of E. I. du Pont de Nemoirs and Co. Inc., Wilmington, Del., U.S.A.

## RESULTS

Some bivalent metal ions ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Sr^{2+}$) are accumulated by mitochondria suspended in the presence of phosphate and an oxidizable substrate by a process that has the following characteristics.

(1) There is an increased rate of respiration (Chance, 1959) that decreases when the added ion has been accumulated by the mitochondria (Chappell *et al.* 1962). The rates of electron transport produced by $Ca^{2+}$ are two- to four-fold greater than those that occur during ADP-stimulated respiration (Chance, 1959, 1963; Chappell *et al.* 1963; Gutfreund & Jones, 1964); in this respect $Ca^{2+}$ resembles 2,4-dinitrophenol, which can also induce rates of electron transport well in excess of those produced by ADP together with phosphate (Chappell, 1962, 1964*b*). It is emphasized that this high rate of electron transport is not necessarily associated with any increase in the permeability of the mitochondrial membrane.

(2) Phosphate is accumulated at the same time as the bivalent metal ion. Arsenate is able to replace phosphate (Chappell *et al.* 1963). In the presence of phosphate, analysis shows that it is probable that the product accumulated has the composition $M_3(PO_4)_2$ for $Mg^{2+}$ (Brierley *et al.* 1962) and $Mn^{2+}$ (Chappell *et al.* 1963) and something very close to this for $Ca^{2+}$ (Rossi & Lehninger, 1963). This accumulation process accounts adequately for the appearance of $H^+$ in the medium:

$$3M^{2+} + 2HPO_4^{2-} \rightarrow M_3(PO_4)_2 + 2H^+ \quad (1)$$

$$3M^{2+} + 2H_2PO_4^{-} \rightarrow M_3(PO_4)_2 + 4H^+ \quad (2)$$

At pH 7·2, which is close to the second acid dissociation constant of phosphate, it would be expected that approx. $1H^+$ would appear in the medium for each metal ion accumulated by the mitochondria. This has been observed for $Mn^{2+}$ (Chappell & Greville, 1963*b*; Chappell *et al.* 1963).

(3) The accumulation of bivalent metal ions and phosphate is prevented by respiratory inhibitors (hydrogen cyanide, Amytal, rotenone, antimycin) appropriate to the oxidizable substrates present and by a variety of uncoupling agents. It is not affected by oligomycin (Chappell & Greville, 1963*b*; Chappell *et al.* 1963; Rossi & Lehninger, 1963) or by concentrations of atractyloside that completely block ADP-stimulated respiration and ATP synthesis (J. B. Chappell, unpublished work). The ATP-dependent accumulation of bivalent metal ions is inhibited by oligomycin, but is unaffected by respiratory inhibitors (Rossi & Lehninger, 1963).

Light-scattering measurements have revealed that during the accumulation of $Mn^{2+}$ and $Ca^{2+}$ mitochondria undergo an apparent contraction (Chappell *et al.* 1963; and Fig. 3). This increased light-scattering by the mitochondrial suspension may well have been due to an increased opacity of the mitochondria as a result of the accumulated metal phosphate, rather than the type of effect that is observed on addition of ADP (see Packer, 1960), although this latter alternative cannot be ruled out.

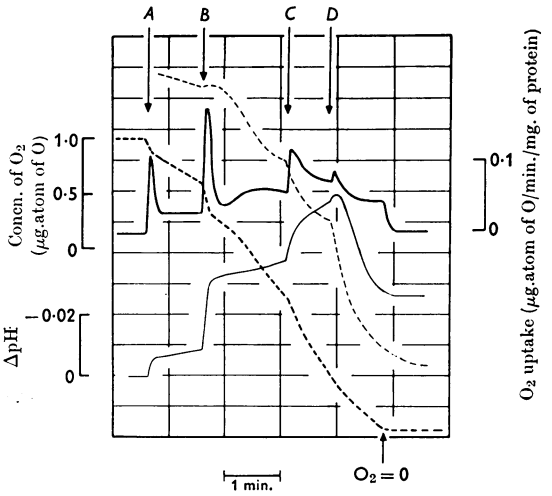


Fig. 3. $Ca^{2+}$ accumulation and $Ca^{2+}$-induced swelling of mitochondria. The upper broken trace is a recording of the light-scattering at 180°, a downward deflection representing mitochondrial swelling. The lower broken trace is a recording of the change in oxygen tension in the suspending medium, a downward deflection representing oxygen uptake by the mitochondria. The upper continuous trace is a recording of the differential of the change in oxygen concentration, an upward deflection representing an increase in the rate of oxygen uptake, and the height of the trace giving the rate of oxygen uptake, which can be read off on the scale. The lower continuous line is a recording of the change in $H^+$ concentration, an upward deflection representing a production of $H^+$. 8 mM-Succinate, 2·1 mM-phosphate and 0·17 $\mu$M-rotenone (to prevent oxaloacetate accumulation; see the text), were present initially, and additions were made where indicated as follows: *A*, mitochondria (9·7 mg. of protein); *B*, 0·75 $\mu$mole of $CaCl_2$; *C*, 0·5 $\mu$mole of $CaCl_2$ *D*, 0·5 $\mu$mole of $CaCl_2$.

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

The ADP-induced contraction is smaller than that produced by $Ca^{2+}$ (Fig. 3). Successive additions of $Mn^{2+}$ or $Sr^{2+}$ led to further increases in light-scattering synchronous with the accumulation process, but with $Ca^{2+}$ at some stage extensive mitochondrial swelling occurred and $Ca^{2+}$ was released again (Fig. 3).

*Inhibition of* $Ca^{2+}$*-induced swelling.* Inhibitors that prevented the accumulation of $Ca^{2+}$ by mitochondria, as revealed by $H^+$ production and increased respiration, also prevented the swelling action. Thus if 100 $\mu$M-hydrogen cyanide or 0·1 $\mu$g. of antimycin/ml. was present when succinate, or glutamate together with malate, served as substrate then there was no pH change on adding $Ca^{2+}$, i.e. there was no accumulation, and the mitochondrial volume remained unchanged, i.e. neither swelling nor contraction occurred. However, if ascorbate (2mM) plus tetramethyl-*p*-phenylenediamine (0·2mM) were added when swelling had been inhibited by antimycin, then $Ca^{2+}$ was accumulated and extensive swelling occurred (Fig. 4). Ascorbate plus tetramethyl-*p*-phenylenediamine had no action when respiration had been blocked by hydrogen cyanide. With this latter inhibitor and with succinate as substrate, neither accumulation of $Ca^{2+}$ nor mitochondrial swelling occurred, but the further addition of ferricyanide (2mM) as electron acceptor allowed both $Ca^{2+}$ accumulation and extensive swelling. These and other similar results indicate that $Ca^{2+}$ accumulation and $Ca^{2+}$-induced swelling can both be supported by electron transport through even a restricted portion of the respiratory chain, e.g. from succinate to ferricyanide (cytochrome *b* to cytochrome *c*) and from tetramethyl-*p*-phenylenediamine to oxygen (cytochrome *c* to oxygen).

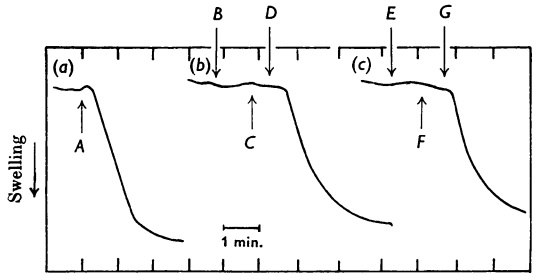


Fig. 4. Swelling supported by electron transport through a restricted portion of the respiratory chain. In each of these experiments, 8mM-succinate, 2·1mM-phosphate and 0·17 $\mu$M-rotenone, together with mitochondria (9·3mg. of protein), were present before the recording was started. The traces are of light-scattering at 180°, downward deflection representing swelling. Oxygen uptake and ferricyanide reduction were followed by recording changes in oxygen tension and $H^+$ concentration respectively. These traces have been omitted for clarity. Additions were made where indicated as follows: (*a*) *A*, 1·5 $\mu$moles of $CaCl_2$. (*b*) *B*, 0·4 $\mu$g. of antimycin; *C*, 1·5 $\mu$moles of $CaCl_2$; *D*, ascorbate (2·0mM) with tetramethyl-*p*-phenylenediamine (0·2mM). (*c*) *E*, HCN (100 $\mu$M); *F*, 1·5 $\mu$moles of $CaCl_2$; *G*, 5·0 $\mu$moles of $K_3Fe(CN)_6$.

The addition of dinitrophenol (100 $\mu$M) or carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (0·1 $\mu$M), a potent uncoupler of respiratory-chain phosphorylation (Heytler & Prichard, 1962), before the addition of $Ca^{2+}$ prevented the pH change associated with the accumulation process, any further increase in respiration beyond that due to the uncoupling agent alone, and swelling.

EDTA (5mM) or EGTA (0·5mM), a potent chelating agent for $Ca^{2+}$, $Sr^{2+}$ and $Mn^{2+}$ (Schmid & Reilly, 1957), completely inhibited the accumulation process and swelling. However, when $Ca^{2+}$ was added in excess over the EGTA present then both accumulation and swelling occurred.

The ease with which $Ca^{2+}$ produced swelling of mitochondria was found to depend on a variety of factors, many of which are discussed below. Freshly prepared mitochondria could accumulate much larger quantities of $Ca^{2+}$ (up to 300 $\mu$equiv. of $Ca^{2+}$/g. of protein) without swelling occurring than mitochondria that had been stored for 3–6hr. at 0°. The presence of 1mg. of defatted bovine plasma albumin/ml. had no effect on the rate or extent of $Ca^{2+}$-induced swelling.

*Relationship between rate of respiration and* $Ca^{2+}$*-induced swelling.* Inhibition of respiration (anaerobic conditions, respiratory inhibitors) prevented both $Ca^{2+}$ accumulation and $Ca^{2+}$-induced swelling. In contrast, in the absence of respiratory inhibitors substrates capable of producing potentially low rates of electron transport, e.g. endogenous substrates or DL-$\beta$-hydroxybutyrate, much more readily supported $Ca^{2+}$-induced swelling than those substrates, e.g. succinate or glutamate together with malate, that were capable of supporting relatively high rates of electron transport (Chappell, 1962, 1964*b*). Some explanation of these results is provided by the following experiments.

The addition of either respiratory inhibitors or uncoupling agents to mitochondria that had already accumulated $Ca^{2+}$ resulted in a rapid swelling and a release of the accumulated $Ca^{2+}$, as revealed by a reversal of the pH change that occurred during the accumulation process. In Fig. 5 the results of an experiment of this type are shown. In this case oligomycin was present before the mitochondria were added. The addition of ADP led to no increase in respiration and only a small pH change, very much smaller than that which would have occurred as a result of the

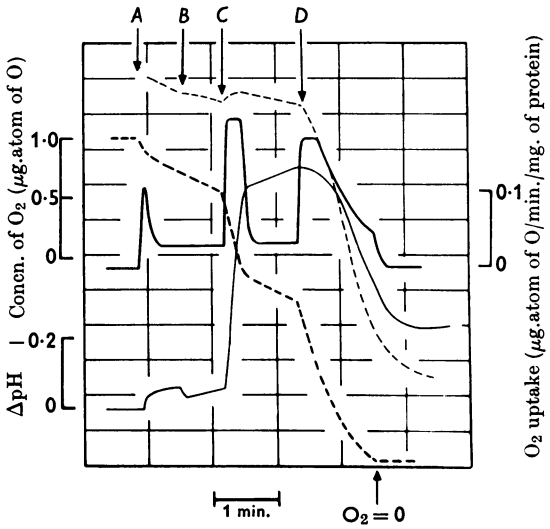


Fig. 5. Swelling of mitochondria that had accumulated $Ca^{2+}$, initiated by uncoupling agents. The convention of the traces is the same as that given in Fig. 3. 8mM-Succinate, 2·1mM-phosphate, 0·17$\mu$M-rotenone and 0·33$\mu$g. of oligomycin/ml. were present initially. Additions were made where indicated as follows: *A*, mitochondria (9·7mg. of protein); *B*, 0·5$\mu$mole of ADP; *C*, 2·0$\mu$moles of $CaCl_2$; *D*, dinitrophenol (133$\mu$M).

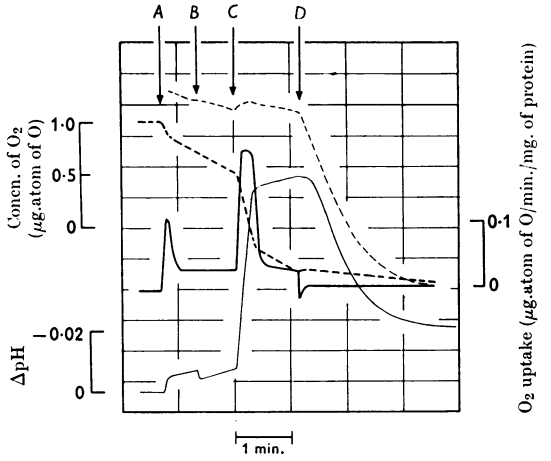


Fig. 6. Swelling of mitochondria that had accumulated $Ca^{2+}$, initiated by respiratory inhibitors. The convention of the traces is the same as that given in Fig. 3. 8mM-Succinate, 2·1mM-phosphate, 0·17$\mu$M-rotenone and 0·33$\mu$g. of oligomycin/ml. were present initially. Additions were made where indicated as follows: *A*, mitochondria (9·7mg. of protein); *B*, 0·5$\mu$mole of ADP; *C*, 2·0$\mu$moles of $CaCl_2$; *D*, 0·4$\mu$g. of antimycin.

phosphorylation of ADP. The addition of $Ca^{2+}$ led to a marked increase in the rates of $H^+$ production and respiration. After a new steady state had been reached the addition of dinitrophenol led to increased respiration, swelling of the mitochondria and $Ca^{2+}$ release. In Fig. 6 a similar experiment is shown; in this case antimycin was added instead of dinitrophenol.

In brief, it appears that if the potential rate of accumulation of $Ca^{2+}$ exceeds the rate at which electron transport is able to supply the energy necessary for this accumulation then swelling results. This action of respiratory inhibitors and uncoupling agents in inducing mitochondrial swelling is to be contrasted with their inhibitory effect when they are added before the $Ca^{2+}$, i.e. at a time when they prevent the accumulation of the bivalent metal ion.

The hypothesis that an inadequate rate of electron transport is responsible for $Ca^{2+}$-induced swelling once some measure of accumulation has occurred is supported by results obtained when succinate served as substrate. As mentioned above $Ca^{2+}$, like dinitrophenol, is able to induce rates of electron transport in excess of those produced by ADP together with phosphate, and it may be predicted therefore that $Ca^{2+}$ ought to lead to non-linear rates of succinate oxidation as a result of the accumulation of oxaloacetate and the consequent inhibition of succinate dehydrogenase (Pardee & Potter, 1948; Chappell, 1961, 1963, 1964*b*; Gutfreund & Jones, 1964) and that this would in turn lead to $Ca^{2+}$-induced swelling as a result of failure of respiration. If this were so, then rotenone or Amytal, both of which inhibit the oxidation of those substrates with NAD-linked dehydrogenases, including malate dehydrogenase (Ernster, Jalling, Löw & Lindberg, 1955; Fukami & Tomizawa, 1956; Lindahl & Oberg, 1961), should diminish to some extent the onset of $Ca^{2+}$-induced swelling in the presence of succinate. This in fact proved to be the case. Glutamate (5mM), which also prevents the inhibition of succinate oxidation by high concentrations of dinitrophenol (greater than 20$\mu$M) (Chappell, 1964*b*), also stabilized the mitochondria against $Ca^{2+}$-induced swelling when succinate served as substrate.

However, even in the presence of rotenone, the addition of malonate (0·5–1·0mM) to mitochondria that had accumulated $Ca^{2+}$ in the presence of succinate (4mM) led to both inhibition of respiration and to swelling. This action of malonate resembled that of antimycin and uncoupling agents. It appears therefore that once accumulation of $Ca^{2+}$ has occurred a high concentration of some 'energy-rich' intermediate is required by the mitochondria for the maintenance of their morphological integrity. Anaerobic conditions, or

the addition of a respiratory inhibitor or uncoupling agent, leads to immediate and extensive swelling.

*Effects of oligomycin, adenine nucleotides and arsenate.* Oligomycin (up to 0·8 μg./ml.) was without effect on the respiration-dependent accumulation of $Ca^{2+}$ by liver mitochondria and had only a small and variable inhibitory effect on $Ca^{2+}$-induced swelling. However, in the presence of 200 μM-ADP and oligomycin the mitochondria were able to accumulate very much greater quantities of $Ca^{2+}$ than in the absence of these compounds. ATP was as effective as ADP in the presence of oligomycin. This stabilizing action of adenine nucleotides provides an explanation of the observation that ATP is required for extensive accumulation of $Ca^{2+}$ and phosphate by mitochondria (Brierley *et al.* 1962; Lehninger *et al.* 1963*a*).

Arsenate could replace phosphate at the same concentration in both the accumulation and swelling processes. Oligomycin completely inhibits arsenate-stimulated respiration (Estabrook, 1961), but had no effect on the rate of accumulation of $Ca^{2+}$, $Mn^{2+}$ or $Sr^{2+}$ in the presence of arsenate. However, under these circumstances it was found that mitochondria were more prone to the swelling action of $Ca^{2+}$ in the presence of arsenate as compared with phosphate. With $Mn^{2+}$ or $Sr^{2+}$ no difference in behaviour with regard to $H^+$ production or oxygen consumption was observed. Even in the presence of arsenate neither $Mn^{2+}$ nor $Sr^{2+}$ caused mitochondrial swelling.

*Changes in rate of respiration during* $Ca^{2+}$*-induced swelling.* The addition of $Ca^{2+}$ to mitochondria suspended in the presence of phosphate led to an increased rate of respiration, $H^+$ production and an increased light-scattering. With low concentrations of $Ca^{2+}$ (approx. 1 μmole of calcium chloride/6 ml.), once $Ca^{2+}$ accumulation was completed then the rate of respiration fell to a low value, characteristic of the resting state, $H^+$ production ceased and the mitochondrial volume, as reflected by light-scattering, remained unaltered. With higher concentrations of $Ca^{2+}$, after a period of accumulation, characterized by a rapid rate of $H^+$ production and respiration, extensive swelling occurred. This led to an inhibition of respiration and a reversal of the pH change, owing to release of $Ca^{2+}$. When succinate was the substrate it could be shown that this inhibition of respiration probably derived from two causes, loss of cytochrome *c* and accumulation of oxaloacetate, since the addition of cytochrome *c* (2·5 μM) and rotenone very largely prevented the decrease in rate of respiration (cf. Gutfreund & Jones, 1964).

More informative results were obtained when intermediate concentrations of $Ca^{2+}$, which caused some measure of, but incomplete, swelling of the mitochondria, were used (Fig. 7). Here four phases of behaviour in response to the addition of $Ca^{2+}$ could be characterized.

Phase 1 occurred immediately after the addition of $Ca^{2+}$. This phase was characterized by high rates of respiration and $H^+$ production and an increased light-scattering of the mitochondria;

Phase 2 started when $Ca^{2+}$ accumulation was complete. It was possible to show this by making use of the fact that, where EGTA is added to a solution containing free $Ca^{2+}$, 2 $H^+$/metal ion are produced as a result of chelation. As mentioned above, when $Ca^{2+}$ is accumulated by mitochondria only 1 $H^+$/metal ion is produced (Chappell *et al.* 1963). It is possible to assess therefore the extent to which $Ca^{2+}$ is free at any time and it is also possible to determine the rate at which bound $Ca^{2+}$ leaves the mitochondria, once accumulation has occurred. Thus the addition of EGTA in this phase led to no immediate $H^+$ production (Fig. 7*b*), which it would have done had any significant quantity of $Ca^{2+}$ been free (see Fig. 7*a*).

Phase 3 occurred some 10–20 sec. after phase 2 and was characterized by a relatively slow swelling of the mitochondria to a new steady state and an increased rate of respiration (Fig. 7*c*). During this phase relatively little $Ca^{2+}$ was available to added EGTA, either at the start of this phase (Fig. 7*b*) or later (Fig. 7*a*), since no rapid change of pH occurred on the addition of EGTA. It seems that under these conditions $Ca^{2+}$ was being lost and taken up again by the mitochondria, which would account for the increased rate of respiration. However, EGTA did not prevent the swelling. The addition of cytochrome *c* (2·5 μM) during this phase considerably increased the rate of respiration, indicating that the mitochondria had lost much of their cytochrome *c*, yet were able to retain the $Ca^{2+}$ that they had accumulated.

Phase 4 occurred when the suspension became anaerobic. In this phase, all the accumulated $Ca^{2+}$ was rapidly lost (since added Triton X-100 caused no further $Ca^{2+}$ release; Fig. 7*a*) and extensive mitochondrial swelling occurred, even in the presence of EGTA (Figs. 7*b* and 7*c*).

The most reasonable interpretation of these results may be made in terms of the hypothesis developed above. The behaviour in phase 3 is perhaps the most interesting. Here it is apparent that the mitochondria, probably as a result of their partially swollen condition, are losing $Ca^{2+}$ and accumulating it at the same time. This process requires energy and this must be provided by an increased rate of respiration. In this respect phase 3 resembles the state that occurs when liver mitochondria have been treated with $Mn^{2+}$ in the absence of added phosphate (see Fig. 2 of Chappell

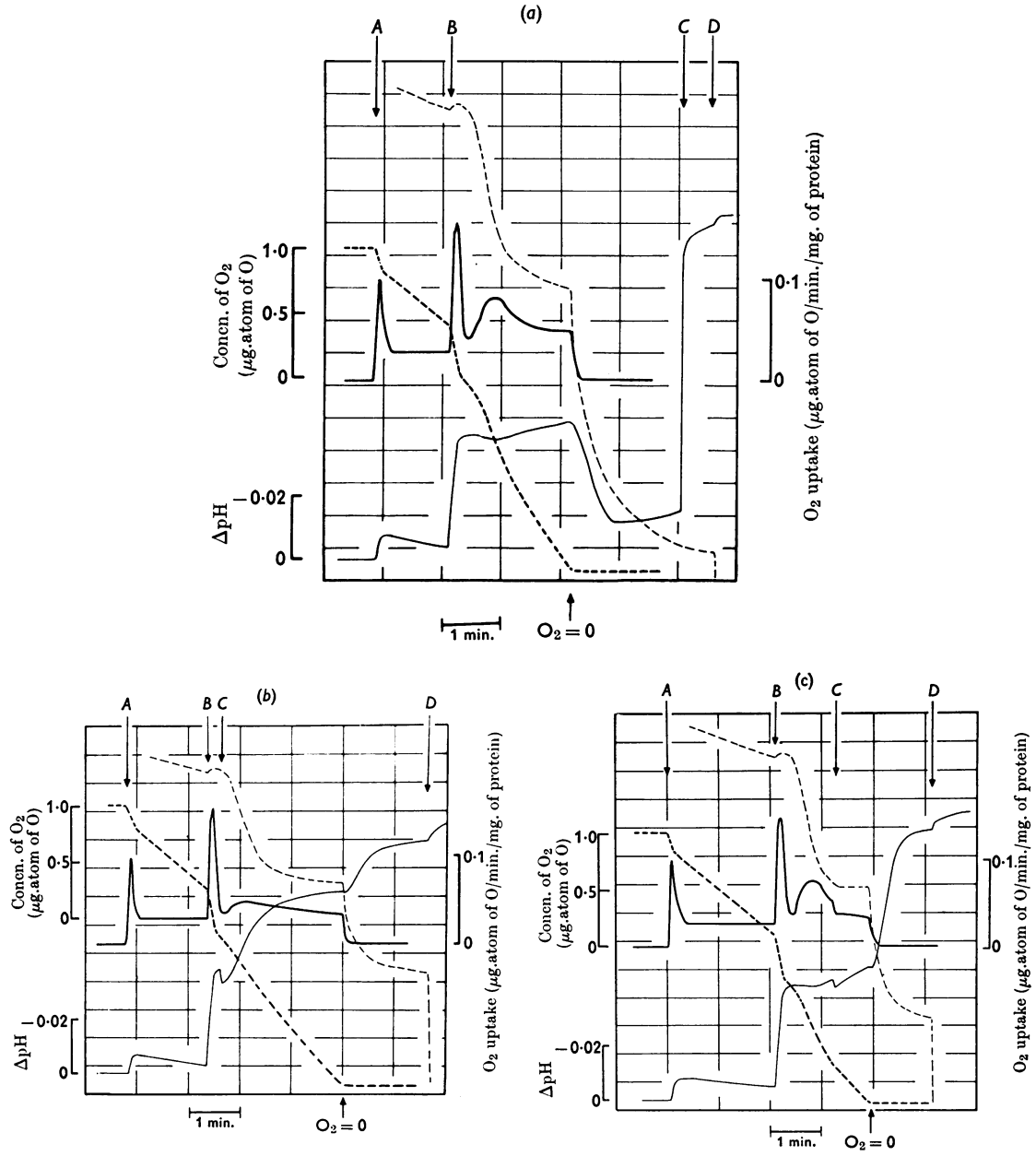


Fig. 7. Experiments showing the sequence of events leading to complete mitochondrial swelling induced by accumulation of $Ca^{2+}$. The convention of the traces is the same as that given in Fig. 3. In each experiment 8 mM-succinate, 2·1 mM-phosphate and 0·17 $\mu$M-rotenone were present initially. Additions were made where indicated as follows: (*a*) *A*, mitochondria (14·7 mg. of protein); *B*, 1·25 $\mu$moles of $CaCl_2$; *C*, 4·0 $\mu$moles of EGTA; *D*, 50 $\mu$l. of 5% Triton X-100. (*b*) and (*c*) Additions were the same as for (*a*), though the EGTA was added before the suspending medium had become anaerobic, in order that the release of bound $Ca^{2+}$ from the mitochondria could be followed.

*et al.* 1963, and the Discussion section of the present paper).

## DISCUSSION

The respiration-dependent accumulation of $Ca^{2+}$ together with phosphate or arsenate led to an extensive mitochondrial swelling. If the accumulation was prevented by inhibition of respiration or by uncoupling agents, then swelling did not occur. Chelating agents also prevented accumulation and the associated swelling. However, the addition of EGTA to mitochondria that had accumulated $Ca^{2+}$ did not prevent the swelling that occurred on the addition of a respiratory inhibitor or uncoupling agent.

Once accumulation had occurred, a variety of factors either induced swelling or increased the tendency of mitochondria to swell. These factors included excess of $Ca^{2+}$, uncoupling agents, respiratory inhibitors and anaerobic conditions. Under each of these circumstances it would be reasonable to assume that the concentration of 'high-energy' intermediates, which are perhaps necessary for the maintenance of the structural integrity of the mitochondrial membrane, would be relatively low.

Evidence has been presented by Chappell (1963) that the alkylguanidines interact with a non-phosphorylated 'high-energy' intermediate in mitochondria, causing inhibition of those oxidations involving the use of NAD. The alkylguanidines fail to act in the presence of uncoupling agents, under anaerobic conditions, or in the presence of $Ca^{2+}$. The conditions for the inhibitory action of alkylguanidines and for the prevention of $Ca^{2+}$-induced swelling after accumulation are therefore similar. These conditions are also those under which $Mn^{2+}$ accumulated by liver mitchondria in the absence of added phosphate is released. Considerable quantities of $Mn^{2+}$ can be accumulated by liver mitochondria in the absence of added phosphate in a respiration-dependent process that has the same inhibitor sensitivity as the uptake that occurs in the presence of phosphate. However, when these inhibitors are added to mitochondria that have accumulated $Mn^{2+}$ in the absence of phosphate, the $Mn^{2+}$ is released into the solution again, but without swelling of the mitochondria (Chappell *et al.* 1963).

The effects of dinitrophenol on $Ca^{2+}$-induced swelling of the type discussed in the present paper are similar, at least superficially, to the effects of dinitrophenol on the phosphate-induced swelling of mitochondria (Chappell & Greville, 1959). The addition of dinitrophenol at zero time to mitochondrial suspensions in buffered 0·3 M-sucrose prevented phosphate-induced swelling, but the delayed addition of dinitrophenol accelerated the process. However, although antimycin or Amytal inhibited phosphate-induced swelling, they did not cause swelling when added subsequently, in contrast with the results obtained with $Ca^{2+}$ reported in the present paper.

From previous work it has been concluded that, in order that mitochondria should swell, not only the presence of a swelling agent (e.g. $Ca^{2+}$, phosphate, thyroxine), but also some measure of electron transport through the respiratory chain, is necessary (see Chappell & Greville, 1963*a*). However, in many instances in the present investigation the most rapid swelling was observed on inhibition of respiration, e.g. on the suspension becoming anaerobic or on the addition of respiratory inhibitors. It is emphasized, however, that coupled respiration was necessary at a previous stage in order that $Ca^{2+}$ accumulation should occur.

The physiological significance of $Ca^{2+}$-induced swelling is not immediately apparent. However, preliminary experiments in collaboration with D. Tillotson (Dunn Nutritional Laboratories, Cambridge) have indicated that mitochondria from the livers and kidneys of vitamin D-deficient rats are much less prone to $Ca^{2+}$-induced swelling than are the mitochondria from vitamin D-dosed rats. There was no difference in rates of $Ca^{2+}$ uptake (as judged by $H^+$ production) or of respiration between mitochondria from vitamin D-deficient and vitamin D-dosed rats. These observations may account for the lowered rates of $Ca^{2+}$ release from mitochondria from vitamin D-deficient rats observed by DeLuca, Engstrom & Rasmussen (1962).

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

## REFERENCES

Brierley, G. P., Bachmann, E. & Green, D. E. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1928.

Brierley, G. P., Murer, E., Bachmann, E. & Green, D. E. (1963). *J. biol. Chem.* **238**, 3482.

Chance, B. (1959). In *Ciba Found. Symp.: Regulation of Cell Metabolism*, p. 91. Ed. by Wolstenholme, G. E. W. & O'Connor, G. M. London: J. and A. Churchill Ltd.

Chance, B. (1963). In *Energy-Linked Functions of Mitochondria*, p. 253. Ed. by Chance, B. New York: Academic Press Inc.

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. (1962). *Biochem. J.* **84**, 62P.

Chappell, J. B. (1963). *J. biol. Chem.* **238**, 410.

Chappell, J. B. (1964*a*). *Biochem. J.* **90**, 225.

Chappell, J. B. (1964*b*). *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. (1965). *Biochem. J.* **95**, 393.

Chappell, J. B. & Greville, G. D. (1959). *Nature, Lond.*, **183**, 1525.
Chappell, J. B. & Greville, G. D. (1960). *Biochim. biophys. Acta*, **38**, 483.
Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 502.
Chappell, J. B. & Greville, G. D. (1963*a*). *Symp. biochem. Soc.* **23**, 39.
Chappell, J. B. & Greville, G. D. (1963*b*). *Fed. Proc.* **22**, 526.
Chappell, J. B., Greville, G. D. & Bicknell, K. E. (1962). *Biochem. J.* **84**, 61P.
Crofts, A. R. & Chappell, J. B. (1965). *Biochem. J.* **95**, 387.
DeLuca, H. F., Engstrom, G. W. & Rasmussen, H. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1904.
Ernster, L., Jalling, O., Löw, H. & Lindberg, O. (1955). *Exp. Cell Res.* Suppl. 3, 124.
Estabrook, R. W. (1961). *Biochim. biophys. Res. Commun.* **4**, 89.
Fukami, J. & Tomizawa, C. (1956). *Botyu-Kagaku*, **21**, 122.
Gutfreund, H. & Jones, E. A. (1964). *Biochem. J.* **90**, 208.
Heytler, P. G. & Prichard, W. W. (1962). *Biochem. biophys. Res. Commun.* **7**, 272.
Hogeboon, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
Hunter, F. E. & Ford, L. (1955). *J. biol. Chem.* **216**, 357.
Lehninger, A. L. & Remmert, L. F. (1959). *J. biol. Chem.* **234**, 2459.
Lehninger, A. L., Rossi, C. S. & Greenawalt, J. W. (1963*a*). *Biochem. biophys. Res. Commun.* **6**, 444.
Lehninger, A. L., Rossi, C. S. & Greenawalt, J. W. (1963*b*). *Fed. Proc.* **22**, 526.
Lindahl, P. E. & Oberg, K. E. (1961). *Exp. Cell Res.* **23**, 228.
Longmuir, I. S. (1957). *Biochem. J.* **65**, 378.
Packer, L. (1960). *J. biol. Chem.* **235**, 242.
Pardee, A. B. & Potter, V. R. (1948). *J. biol. Chem.* **176**, 1085.
Raaflaub, J. (1953). *Helv. physiol. acta*, **11**, 142.
Rossi, C. S. & Lehninger, A. L. (1963). *Biochem. Z.* **338**, 698.
Saris, N. E. (1959). *Medd. finska Kemistamf.* **68**, 98.
Saris, N. E. (1963*a*). *Acta chem. scand.* **17**, 882.
Saris, N. E. (1963*b*). *Comment. phys. Math., Helsingf.*, **28**, 11.
Schmid, R. W. & Reilly, C. N. (1957). *Analyt. Chem.* **29**, 264.
Slater, E. C. & Cleland, K. W. (1953). *Biochem. J.* **53**, 557.
Tapley, D. F. (1956). *J. biol. Chem.* **222**, 325.
Wojtczak, L. & Lehninger, A. L. (1961). *Biochim. biophys. Acta*, **51**, 442.