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Studies of Copper Ion-Induced Mitochondrial Swelling *in vitro*

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1. A study of the mode and mechanism of Cu^{2+}-induced mitochondrial swelling was carried out. 2. Mitochondrial swelling curves (E_{520} turbidity changes) were obtained as a function of [Cu^{2+}], pH, temperature and mitochondrial protein concentration. ED_{50} was approx. 70 mμmoles of Cu^{2+}. Calculation of the activation energy from the Arrhenius equation gave a value of 22900 cal./mole per degree with Q_{10} 4·02. 3. No lipid peroxides were formed during swelling. 4. Changes in oxygen consumption (Clark-type electrode) were dependent on the substrate used, but revealed no increased uptake in presence of Cu^{2+}. 5. Cu^{2+}-induced swelling was inhibited by EDTA, 8-hydroxyquinoline, cyanide, citrate, bovine serum albumin, ATP, glutamate, GSH, dithiothreitol and sucrose. Azide, Amytal, antimycin A and oligomycin had no significant effect. Potentiation of swelling was seen with ascorbate, 2,4-dinitrophenol and succinate. 6. The occurrence of different types of mitochondrial swelling and the suggestion that Cu^{2+}-induced swelling is mediated through a stoicheiometric interaction with a thiol-containing membrane receptor are discussed.

The investigation of effects of heavy-metal ions on membrane functions in biological systems may help to explain the nature of the toxicity of heavy-metal ions as well as providing a tool for elucidating membrane properties and function. The interaction of heavy-metal ions with subcellular organelles *in vitro* has provided limited but valuable data in this respect. Changes in mitochondrial K^+ binding and oxidative phosphorylation occur after exposure to Hg^{2+} (Gamble, 1957; Scott & Gamble, 1961), mitochondrial swelling occurs in the presence of Ag^+, Hg^{2+}, Fe^{2+} and Cu^{2+} (Tapley, 1956; Lehninger, 1962; Riley & Lehninger, 1964; Hunter, Gebicki, Hoffsten, Weinstein & Scott, 1963) and Hg^{2+} induces an irreversible loss of structure-linked latency with enzyme activation in hepatic lysosomes (Verity & Reith, 1967).

Mitochondrial swelling induced by heavy-metal ions is thought to be associated with the formation of mercaptides with protein thiol groups in the membrane (Lehninger, 1962). This view is supported by observations in which mitochondrial swelling occurs with *p*-chloromercuribenzoate and *N*-ethylmaleimide, agents known to alkylate thiol groups (Dickens & Salmony, 1956; Lehninger & Ray, 1957). Moreover, it has been suggested that compounds containing thiol groups cause swelling by reducing specific membrane disulphide groups (Neubert & Lehninger, 1962). Mitochondrial thiols are associated with oxidative phosphorylation and electron transport, processes intimately related with some forms of large-amplitude swelling (Fluharty & Sanadi, 1962; Chappell & Greville, 1963). Other mitochondrial thiols are necessary for adenosine triphosphatase activity (Cooper, 1960), which also is intimately associated with mitochondrial swelling and contraction.

We now report studies on the nature and mechanism of Cu^{2+}-induced mitochondrial swelling and its relationship to electron transport and oxidative phosphorylation. The observations suggest a stoicheiometric interaction with a membrane receptor probably containing a thiol group. The kinetics of the swelling are dependent on dose and temperature with a high coefficient of activation.

MATERIALS AND METHODS

All chemicals were reagent grade. ATP, ADP, GSH, L-cysteine hydrochloride, 2,4-dinitrophenol, *p*-chloromercuribenzoate, Cleland's reagent (dithiothreitol), human serum albumin (crystalline) and 2-thiobarbituric acid were obtained from Calbiochem, Los Angeles, Calif., U.S.A. Crystalline bovine serum albumin was purchased from Pentex Inc., Kankakee, Ill., U.S.A. Sodium Amytal was obtained from Smith, Kline and French, Philadelphia, Pa., U.S.A. Antimycin A was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and oligomycin from K & K Laboratories, Los Angeles, Calif., U.S.A. All chemicals used in swelling experiments were made up in 25 mM-tris adjusted to pH 7·4 with 0·1 N-HCl and made

iso-osmotic with respect to 0·15 M-KCl. Stock solutions of antimycin A and 2,4-dinitrophenol were dissolved in glass-redistilled ethanol and *p*-chloromercuribenzoate was dissolved in N-NaOH.

Adult male (70–90-day-old) Swiss albino mice weighing approx. 25 g. were used. Animals were killed by cervical compression and the livers were rapidly excised, placed in 10 ml. of ice-cold 0·3 M-sucrose and homogenized for 30 sec. at 350 rev./min. in a smooth-walled Potter–Elvehjem homogenizer fitted with a plastic pestle. Mitochondrial fractions were prepared at 2° in a Spinco model L ultracentrifuge (rotor 50). The homogenate was centrifuged at 550*g* for 10 min. ($R_{av.}$ 5·4 cm.). The supernatant was spun at 6300*g* for 10 min., yielding a mitochondrial pellet. This was washed twice in 0·3 M-sucrose.

Measurement of swelling. Swelling changes in a final volume of 3 ml. of 25 mM-tris–0·15 M-KCl, pH 7·4, were recorded as decrease in E_{520} in a Beckman model DK–2 ratio spectrophotometer fitted with a constant-time-drive attachment at 24°. In experiments at different temperatures, measurements were made at 30 sec. intervals in a Beckman model DU spectrophotometer fitted with double thermospacer plates attached to a controlled-temperature-water circulator.

Anaerobic experiments were performed in Thunberg cuvettes. The swelling reagent was placed in the side tube, other constitutents were in the main tube. Evacuation and flushing with nitrogen was carried out for 15 min. at 2° with intermittent shaking. The contents were warmed to room temperature before being mixed and readings were taken.

Measurement of oxygen uptake. Studies of mitochondrial oxygen consumption were performed with a YSI Oxygen Monitor with Clark-type polarographic electrode. The incubation medium (final volume 3 ml.) contained in 25 mM-tris–0·15 M-KCl: Na_2HPO_4, 13 mM; KH_2PO_4, 3 mM; KCl, 84 mM; $MgCl_2$, 6 mM; washed mitochondria; 16 μmoles of β-hydroxybutyrate or L-glutamate as exogenous substrate. The $CuSO_4$ solution (10–50 μl.) was added by microsyringe via the overflow groove. Bovine serum albumin (4·4 mg.) was present as indicated.

Determination of lipid peroxides. This was carried out by a slight modification of the thiobarbituric acid reaction (Hunter *et al.* 1963). The values were plotted directly as $E^{1\,cm.}_{532}$ without conversion into malonaldehyde equivalents.

Determination of protein. This was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline human serum albumin as standard.

Statistical and mathematical treatments. These were performed by the procedures of Bernstein & Weatherall (1952) and Patton (1965) respectively.

RESULTS

Characteristics of Cu^{2+}*-induced swelling* (E_{520} *turbidity changes*)

Mitochondrial swelling and contraction are processes dependent on largely unknown chemical mechanisms. Criteria must still be defined to provide an adequate description of the event. Three criteria (Connelly & Lardy, 1964) are utilized to describe Cu^{2+}-induced swelling: (*a*) time of onset is defined as the time when the swelling process begins, as observed from a change in extinction, and is estimated by extrapolation from the rate-of-swelling slope; (*b*) rate of swelling is the maximal slope of the E_{520}–time curve, $\Delta E/\Delta t$; (*c*) extent of swelling is represented by the difference between the initial extinction, E_0, and the extinction of the final asymptote plateau, E_f. Fig. 1 demonstrates these criteria for mouse liver mitochondrial swelling induced by Cu^{2+}. Spontaneous swelling in tris–potassium chloride, pH 7·4, at room temperature was negligible over the time-period involved. A flat sigmoid swelling curve was obtained with Cu^{2+} concentrations greater than 5 mμmoles/3 ml. In some experiments with 30 mμmoles of Cu^{2+}/3 ml. a biphasic swelling curve was obtained (Fig. 1), suggesting the presence of two reactive sites, one of which becomes unmasked as swelling proceeds. The extent of swelling approximated $0{\cdot}35(E_0 - E_f)$, but in no instance did the E_f value go below 0·15, in contrast with low E_f values induced by Fe^{2+}, GSH + GSSG and ascorbate (McKnight, Hunter & Oehlert, 1965; Schneider, Smith & Hunter, 1964; Hunter *et al.* 1964*a*).

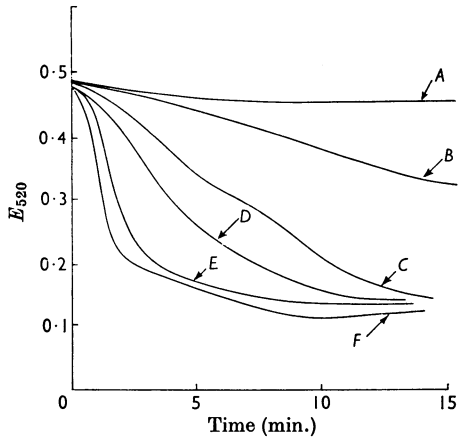

Fig. 1. Effect of Cu^{2+} concentration on the character of turbidity changes (E_{520}) as a function of time at 24°. Medium (final volume 3 ml.) contained 25 mM-tris–0·15 M-KCl, pH 7·4, and Cu^{2+} to give the following final concentrations: *A*, none (control); *B*, 5 mμmoles/3 ml.; *C*, 30 mμmoles/3 ml.; *D*, 50 mμmoles/3 ml.; *E*, 0·1 μmole/3 ml.; *F*, 0·2 μmole/3 ml. Mitochondria in 0·3 M-sucrose were added last.

The initial rate of swelling determined from the maximum slope and corrected for mitochondrial protein was linearly related to log[Cu^{2+}] over a tenfold range (Fig. 2). At 30 mμmoles of Cu^{2+}/3 ml. an abrupt change was noted in the slope, accompanied by swelling rates significantly lower than in the major portion of the relationship. The rate of swelling is dependent on the concentration of mitochondrial protein at constant Cu^{2+} concentration. A twofold increase in protein was accompanied

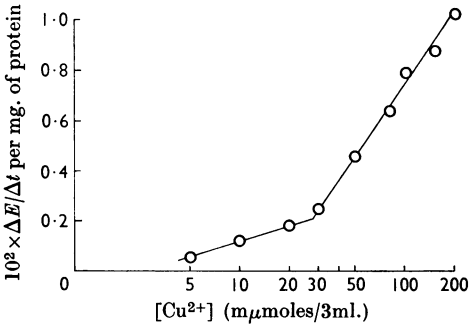

Fig. 2. Relation of absolute rate of swelling (expressed as a function of protein concentration) to Cu^{2+} concentration in the incubation system. Individual values represent means of three to eight experiments.

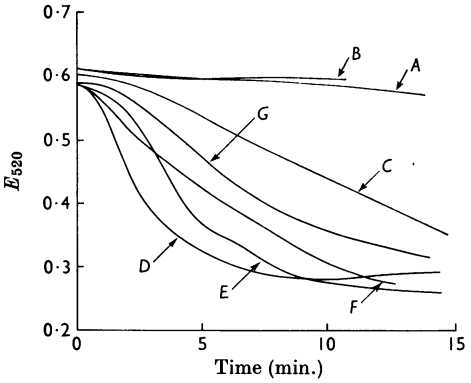

Fig. 3. Effect of pH on spontaneous and Cu^{2+}-induced mitochondrial swelling at 24°. Incubation medium contained 25mM-tris–0·15M-KCl. Spontaneous swelling: *A*, at pH 6·8; *B*, at pH 7·4; *C*, at pH 8·9. Swelling induced by 30mμmoles of Cu^{2+}/3ml.: *D*, at pH 6·85; *E*, at pH 7·0; *F*, at pH 7·4; *G*, at pH 8·3.

by an approximate doubling of the swelling rate, indicating a constant linear relationship.

Effect of pH *and temperature.* Experiments testing the effect of pH on the rate of swelling are shown in Fig. 3, and indicate that maximum rates of Cu^{2+}-induced swelling occur at pH 6·85 or lower. On the other hand, spontaneous swelling appears to be most rapid at pH 8·9.

The Arrhenius equation describes the relationship between temperature and the velocity of a process and states that:

$$k = se^{-A/-RT}$$

The plot of $\log k$ versus $1/T$ at [Cu^{2+}] 10 and 40mμmoles/3ml. provides two parallel slopes over the temperature range studied (16–37°). Calcula-

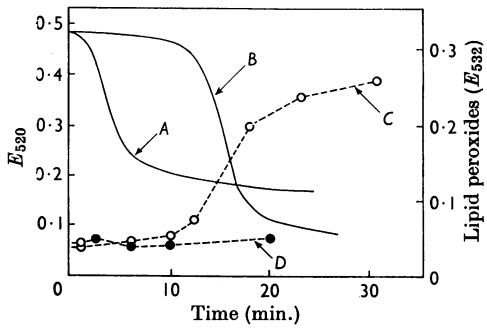

Fig. 4. Comparison of swelling curves (*A* and *B*) and rate of lipid peroxide formation (*C* and *D*) in the presence of 10μM-Cu^{2+} (*A* and *D*) and 30μM-Fe^{2+} (*B* and *C*). A 1ml. sample of the mitochondria-swelling system containing approx. 0·45mg. of mitochondrial protein was added to 0·3ml. of 35% (w/v) trichloroacetic acid in 3N-HCl. The thiobarbituric acid reaction was performed as described in the Materials and Methods section.

tion of the apparent activation energy for Cu^{2+}-induced swelling gives a value of 22900cal./mole per degree. The temperature coefficient, Q_{10}, at 20° was calculated to be 4·02 by substituting in the equation:

$$\log\left(\frac{k_2}{k_1}\right) = 0{\cdot}219A \cdot \left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

Effect of aging and formation of lipid peroxides. Mitochondria were maintained at room temperature for 1·5 and 3hr. in 0·3M-sucrose. After 1·5hr. the initial E_{520} had fallen to 70% of the original value. Though an increase in the rate of spontaneous swelling was noted, especially after aging *in vitro* for 3hr., no change in Cu^{2+}-induced swelling rate was apparent. The time of onset, however, was decreased.

Lipid peroxides, as measured by the thiobarbituric acid colour reaction, accumulated during swelling lysis of liver mitochondria in the presence of GSH + GSSG, Fe^{2+} and ascorbate. No formation of lipid peroxides occurred during Cu^{2+}-induced mitochondrial swelling (Fig. 4), whereas a control system in the presence of 0·1μmole of Fe^{2+}/3ml. developed a rapid swelling phase and accumulation of lipid peroxides after a lag period of 10min.

Mitochondrial oxygen consumption during Cu^{2+}*-induced swelling.* Oxygen uptake was measured in the presence of β-hydroxybutyrate and glutamate. In the absence of bovine serum albumin no significant difference was observed between the two substrates, but in the presence of albumin respiration supported by β-hydroxybutyrate was inhibited by approx. 50%. Added Cu^{2+} (0·1μmole/3ml.) partially inhibited oxygen uptake, but no further

inhibition of β-hydroxybutyrate-supported respiration in the presence of albumin was noted. No rapid burst or slow increase in oxygen consumption was demonstrated during Cu^{2+}-induced swelling, in contrast with the swelling lysis occurring with ascorbate, Fe^{2+}, GSH + GSSG and phosphate (Schneider *et al.* 1964).

Effect of inhibitors on Cu^{2+}*-induced swelling.* (*a*) Metal ions and agents forming complexes with metal ions. Mitochondria were pre-exposed to the complex-forming agent or metal ions for 3 min. at 24° in tris–potassium chloride medium before the addition of 10 μM-Cu^{2+}. Mg^{2+} (0·1–0·5 mM) had no effect on spontaneous or Cu^{2+}-induced swelling. Mn^{2+} (0·5 mM) was also without significant inhibitory effect. It is noteworthy that 10–100 μM-Mn^{2+} blocks GSH + GSSG- and ascorbate-induced swelling through an antioxidant action (Hunter *et al.* 1964*b*). EDTA (0·1 mM) and 8-hydroxyquinoline (0·1 mM) completely inhibited Cu^{2+}-induced swelling. Citrate (0·1 mM) produced partial inhibition, but more significantly clearly revealed the biphasic nature of the swelling process (Fig. 5), which proceeded to the same limiting E_t produced by Cu^{2+} alone. Nitrate, sulphate and acetate were without significant effect on spontaneous or Cu^{2+}-induced swelling.

(*b*) Inhibitors of electron transport, phosphorylation and uncoupling agents. Some of the established respiratory inhibitors and uncoupling agents were used and a summary of their effects is presented in Table 1. Azide, Amytal and antimycin A failed to inhibit Cu^{2+}-induced swelling. There was slight potentiation of the swelling rate in the presence of 2,4-dinitrophenol and antimycin A. Inhibition by sodium cyanide and fluoride may be due to chelation of Cu^{2+}, and not through their selective action on the respiratory chain.

Effect of substrates and ATP. α-Oxoglutarate (5 mM) had no significant effect on the kinetics of Cu^{2+}-induced mitochondrial swelling. The presence of succinate (5 mM) or glutamate (5 mM) as respiratory substrate in the tris–potassium chloride control medium induced slight swelling (Fig. 6). However, with Cu^{2+} plus succinate there was a shortened time of onset and increase in initial swelling rate with appearance of a biphasic curve. Glutamate inhibited Cu^{2+}-induced swelling.

Table 1. *Effect of uncoupling agents and respiratory-chain inhibitors on the rate of* Cu^{2+}*-induced swelling of mitochondria*

Reagents were dissolved as indicated in the Materials and Methods section and buffered to pH 7·4 before addition to 25 mM-tris–0·15 M-KCl medium. Cu^{2+} (30 mμmoles) was added last.

Reagent	Final concn.	Effect
Sodium azide	3·3 mM	No effect
Sodium fluoride	20 mM	Inhibition (25%)
NaCN	1 mM	Inhibition (90–100%)
Antimycin A	4·5 μg./3 ml.	Increased swelling (10%)
Sodium Amytal	1–5 mM	No effect
2,4-Dinitrophenol	0·33 mM	Increased swelling (20%)
Oligomycin	1·0 μg./3 ml.	No effect

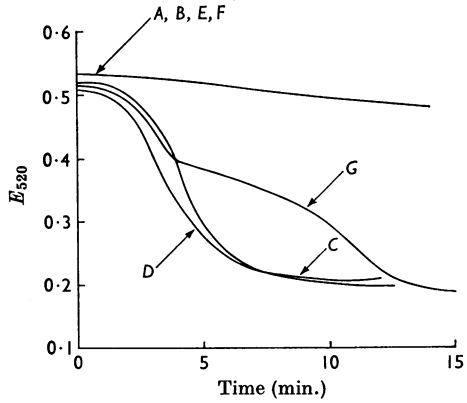

Fig. 5. Effect of Mn^{2+} and reagents forming complexes with metal ions on Cu^{2+}-induced mitochondrial swelling. Mitochondria were preincubated in 25 mM-tris–0·15 M-KCl containing reagents for 3 min. before the addition of Cu^{2+}. Additions were as follows: *A*, none (control); *B*, Mn^{2+} (0·5 mM); *C*, Cu^{2+} (10 μM); *D*, Cu^{2+} (10 μM) + Mn^{2+} (0·5 mM); *E*, Cu^{2+} (10 μM) + 8-hydroxyquinoline (0·1 mM); *F*, Cu^{2+} (10 μM) + EDTA (0·1 mM); *G*, Cu^{2+} (10 μM) + citrate (0·1 mM).

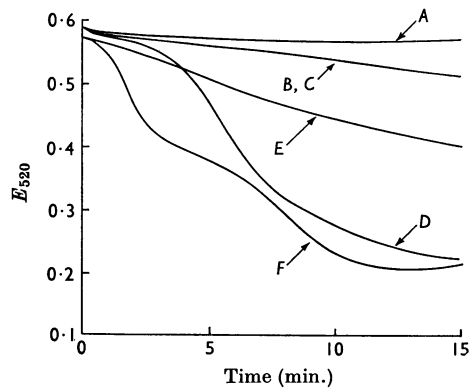

Fig. 6. Effect of glutamate and succinate on spontaneous and Cu^{2+}-induced mitochondrial swelling. Additions were as follows: *A*, none (spontaneous swelling); *B*, glutamate (5 mM); *C*, succinate (5 mM); *D*, Cu^{2+} (9 μM); *E*, Cu^{2+} (10 μM) + glutamate (5 mM); *F*, Cu^{2+} (10 μM) + succinate (5 mM).

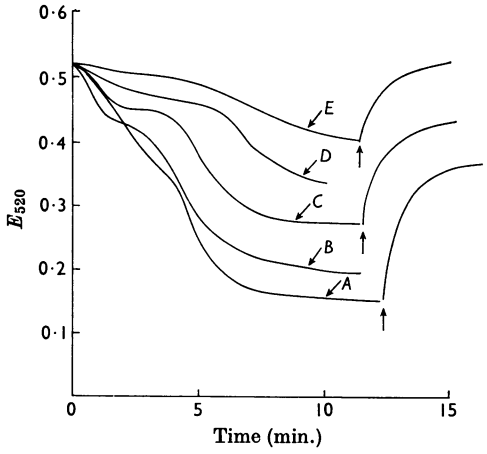

Fig. 7. Inhibition of Cu^{2+}-induced mitochondrial swelling after the addition of various sucrose concentrations to the basal 25 mM-tris–0·15 M-KCl swelling medium. Swelling was induced by 10 μM-Cu^{2+} in the presence of the following concentrations of sucrose: *A*, none; *B*, 0·03 M; *C*, 0·1 M; *D*, 0·2 M; *E*, 0·3 M.

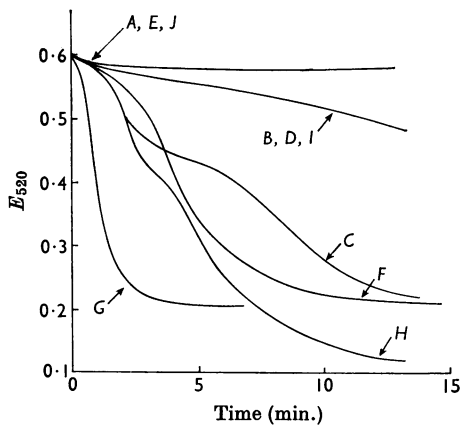

Fig. 8. Effect of ascorbate, cysteine, GSH and dithiothreitol on spontaneous and Cu^{2+}-induced mitochondrial swelling. Additions were as follows: *A*, none (spontaneous swelling); *B*, ascorbate (3 mM); *C*, cysteine (3·3 mM); *D*, GSH (2 mM); *E*, dithiothreitol (3·3 mM); *F*, Cu^{2+} (10 μM); *G*, Cu^{2+} (10 μM) + ascorbate (3 mM); *H*, Cu^{2+} (10 μM) + cysteine (3·3 mM); *I*, Cu^{2+} (10 μM) + GSH (2 mM); *J*, Cu^{2+} (10 μM) + dithiothreitol (3·3 mM).

Disodium ATP added initially at 1–5 μmoles/3 ml. had no inhibitory effect on Cu^{2+}-induced swelling, but at 20 μmoles/3 ml. there was complete inhibition of swelling. Reversal of Cu^{2+}-induced swelling ('contraction') occurred rapidly after the addition of ATP (10 mM). At this concentration of ATP, Mg^{2+}, EDTA or bovine serum albumin did not potentiate, though at ATP concentrations less than 3·3 mM Mg^{2+} or serum albumin + EDTA slightly enhanced the ATP-induced contraction.

Lehninger (1959), in a detailed study of the action of ATP in causing the reversal of thyroxine-induced swelling of rat liver mitochondria, observed that sucrose added to the basal tris–potassium chloride medium inhibited the swelling and ATP reversal, suggesting a common denominator in these two events. Fig. 7 reveals that sucrose inhibited Cu^{2+}-induced swelling in a complex manner, as indicated by the changing biphasic nature of the swelling curve as a function of sucrose concentration. Contrary to the observations with thyroxine, sucrose in the medium did not inhibit ATP-induced contraction.

Effect of anaerobiosis, ascorbate and thiols on Cu^{2+}-induced swelling. Anaerobiosis had no significant effect on Cu^{2+}-induced swelling. In a control of the anaerobic conditions, cysteine failed to induce swelling, which did occur once air was readmitted to the system in the presence of cysteine. Ascorbate (3·3 mM) initiated slight swelling in an aerobic environment. Preincubation of mitochondria with ascorbate (for 3 min. at 2°) before addition to Cu^{2+}-containing swelling medium induced a significant potentiation of swelling rate to an E_f value similar to that obtained with Cu^{2+} alone (Fig. 8). Addition of ascorbate during Cu^{2+}-induced swelling prevented E_f from approaching the limiting value given by Cu^{2+}. L-Cysteine produced a typical biphasic swelling curve, whose E_f approached values less than 0·1 with variable displacement of the Cu^{2+}-swelling curve in the presence of cysteine, which progressed to similar low E_{520} values (Fig. 8). There was inhibition of Cu^{2+}-induced swelling by 2 mM-GSH and 3·3 mM-dithiothreitol. Once swelling had been initiated, the addition of GSH or thiols to the incubation system failed to stop or reverse the rate of swelling.

DISCUSSION

Active large-amplitude swelling of mitochondria is a manifestation of water uptake and can be produced through three fundamentally different mechanisms. One mechanism is linked to the oxidation of substrates through the electron-transport chain and can be induced by phosphate, thyroxine (Lehninger, Ray & Schneider, 1959) or addition of substrate to aged mitochondria (Chappell & Greville, 1963). A further mode of swelling is initiated after peroxidation of mitochondrial lipids by ascorbate, Fe^{2+} and GSH + GSSG. A third mechanism, less understood, relates to the swelling caused by thiols and disulphides including numerous peptide hormones and cyclic

polypeptide antibiotics (Neubert & Lehninger, 1962; Lehninger & Neubert, 1961; Riley & Lehninger, 1964). Support for such a role of thiols is found also in the swelling resulting from alkylation of thiol groups by *N*-ethylmaleimide and *p*-chloromercuribenzoate or mercaptide formation with Ag^+.

Of the three mechanisms proposed, the available evidence does not support mediation of electron transport as the underlying mechanism of Cu^{2+}-induced swelling. Though swelling is blocked by cyanide and possesses a higher temperature coefficient of activation, oligomycin is non-inhibitory. In addition, a decrease in oxygen consumption is detected during the swelling phase in the presence of β-hydroxybutyrate and DL-glutamate. Swelling is not substrate-supported, is independent of aging and is not inhibited by the electron-transport inhibitors antimycin A, Amytal or anaerobiosis. Inhibition by glutamate is probably due to the formation of a bidentate α-amino acid chelate (Freeman, 1966) and is unrelated to its electron-donating properties.

No significant accumulation of lipid peroxides occurred during or immediately after the completion of Cu^{2+}-induced swelling. This was in contrast with Fe^{2+}-induced swelling, which is accompanied by a rapid accumulation of lipid peroxides in the pre-swelling phase by bursts of increased oxygen uptake, and is inhibited by Mn^{2+}, antioxidants, anaerobiosis and chelating agents that bind Fe^{2+} (Schneider *et al.* 1964; McKnight *et al.* 1965). With Fe^{2+}, structural rupture with lysis by peroxidation rather than release of free fatty acids is the primary causal event, but these effects are believed not to contribute to the pathogenesis of Cu^{2+}-induced swelling, where E_t values do not approach the lytic level.

Hypo-osmotic, spontaneous, Fe^{2+}-induced and oleate-induced swelling proceeds equally rapidly at 0° and 24° (Lehninger *et al.* 1959; McKnight *et al.* 1965). The high temperature coefficient (Q_{10} 4·02) of Cu^{2+}-induced swelling in non-sucrose media suggests the presence of a rate-limiting chemical or enzymic step preceding the swelling phenomenon. Riley & Lehninger (1964) found an average of 95 mμmoles of total thiol groups/mg. of mitochondrial protein, 73% being associated with the membrane. Swelling induced by GSSG was accompanied by a decrease in total thiol groups, which were restored on the addition of albumin. Such restoration was a prerequisite for ATP-induced contraction. It is of note that albumin is not required for ATP-induced contraction after Cu^{2+}-induced swelling.

Assuming unit stoicheiometry for Cu^{2+}–thiol interaction, we have demonstrated (Fig. 2) that swelling is initiated by 10–30 mμmoles of Cu^{2+}/mg. of mitochondrial protein. This would represent only 10–30% of the available reactive sites, but agrees well with the proportion of total mitochondrial thiol groups considered to represent rapidly reacting groups (12% of the total), primarily located in the membrane (Riley & Lehninger, 1964). The immediate potentiation of Cu^{2+}-induced swelling in the presence of buffered ascorbate suggests that an increased number of thiol sites are available for combination resulting from an equilibrium shift to the left of the thiol $\rightleftharpoons$ disulphide equilibrium.

The complex membrane structure of the mitochondrion could permit different types and stages of mitochondrial swelling to occur (Lehninger, 1962), especially if the permeability of the inner or outer membrane systems differed. Amoore & Bartley (1958) have defined sucrose-accessible and -non-accessible spaces. The existence of such spaces allows for a partial interpretation of the biphasic swelling curves seen in some experiments, especially as a function of sucrose or citrate concentration. Under these circumstances, the rate of entry of water and solute is the sum of the relative permeability of the two spaces.

Mn^{2+} failed to protect against Cu^{2+}-induced swelling. Mn^{2+} is a powerful 'stabilizer' of membrane permeability and inhibits lipid peroxide formation (Thiele & Huff, 1960). Binding of Mn^{2+} to membrane carboxyl or phosphoryl groups (Passow, Rothstein & Clarkson, 1961) evokes no permeability response or protection of membrane thiol groups, strengthening the suggestion that Cu^{2+}-induced swelling is mediated through thiol group interaction.

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