Uptake and Effects of Copper in Rat Liver Mitochondria

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The rate and extent of Cu²⁺ uptake by rat liver mitochondria was measured under various conditions. 1. The uptake is both greater and faster without an energy supply. 2. The uptake, when occurring in ionic media, has a biphasic character, that is it always slows down after an initial burst, and then re-accelerates. 3. Uptake of Cu²⁺ in the presence of energy initiates K⁺ uptake from K⁺-containing media with accompanying swelling and respiratory stimulation. Depending on the amount of Cu²⁺ added and the K⁺ concentration, an inhibition of respiration later ensues. 4. Chelation of the Cu²⁺ by substrates (notably glutamate) decreases the effects. 5. Prior exposure to Cu²⁺ decreases or prevents energy-dependent Ca²⁺ uptake.

When transition-metal ions are applied to cells, metabolic activity is modified, indicating that enzymes are influenced (Bremner, 1974). If an appreciable concentration of free metal arises in the cytosol, the well-known cation-accumulating power of the mitochondria (Lehninger et al., 1969) will come into play. The mechanism and consequences of metal uptake are important, because although many metals are essential, an excess of any one may become inhibitory and toxic. In two reports of the action of Cu²⁺ on ox heart mitochondria, the first (Cederbaum & Wainio, 1972b) noted only inhibitory effects of the metal, whereas the second (Hwang et al., 1972) describes a stimulation of mitochondrial respiration at low Cu²⁺ concentrations. This latter effect was ascribed to a presumed increase in permeability to K⁺ which, it was postulated, gives rise to an energy-dependent K⁺ uptake. Here we present unequivocal evidence that Cu²⁺ ions induce in liver mitochondria changes in alkali-cation permeability, energy-dependent K⁺ uptake, swelling and H⁺ loss. The energy-dependent K⁺ uptake and swelling leads to further accumulation of Cu²⁺, additional to the limited amount taken up in the absence of K⁺ from the medium.

The present work is a part of a programme of studies investigating the effects of heavy metals on mitochondrial metabolism. Because we consider aquatic organisms in polluted environments to be more susceptible to damage by dissolved toxic metals than are terrestrial mammals, we are studying the effects of metals on fish liver mitochondria. A preliminary report of this work has already appeared (Zaba & Harris, 1975). In the present work we have used rat liver mitochondria as a conveniently prepared test system.

Methods

Preparation of mitochondria

Mitochondria were prepared as described previously (Harris et al., 1971). Where uptake of Cu²⁺ or Ca²⁺ was to be measured, the mitochondria were washed for a third time in a medium free from EGTA⁺ and bovine serum albumin. Protein was measured by the biuret method.

Cu²⁺ uptake

The uptake of Cu²⁺ was measured kinetically by two techniques. The first of these was an EDTA 'quench' technique, a modified version of that described by Reed & Bygrave (1974). Liver mitochondria were incubated in an appropriate medium containing ²H₂O and [¹⁴C]dextran, and the desired amount of Cu²⁺ was added as CuSO₄. At suitable time-intervals, 0.5 ml samples were withdrawn and added to 20 µl of 0.1 M-EDTA in conical 1 ml tubes. Speed of addition and the shape of the tubes ensured rapid mixing of the mitochondria with the EDTA. Control experiments (with and without EDTA) showed that within the time required for sampling and centrifugation, no more than 10–15% of the mitochondrial copper was lost to the EDTA-containing supernatant fluid. The tubes were centrifuged in a Coleman Microfuge and the supernatant fluid was poured off into 1 ml tubes. The pellets were washed, the sides of the tubes dried with gauze and the pellet was extracted with 0.1 ml of 1.5 M-HClO₄. Samples of this extract and of the supernatant were suitably diluted and analysed for *Abbreviations: EGTA, ethanedioxybis(ethylamine)-tetra-acetate; Hepes, 2-(N-2-hydroxyethyl)piperazin-N'-yI)ethanesulphonic acid.
Cu by atomic-absorption spectrophotometry. Further samples were taken for liquid-scintillation counting by using Unisolve scintillation cocktail (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) and a Packard 3375 Tri-Carb counter, whence the mitochondrial water and external ‘carry down’ could be determined as described by Harris & Van Dam (1968).

Appropriate corrections could then be applied to the Cu-concentration measurements to calculate the net mitochondrial Cu in ng-atoms/mg of mitochondrial protein. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. except for [14C]dextran, which was from the New England Nuclear Corp., Boston, MA, U.S.A.

Most of the Cu²⁺-uptake measurements were performed by using the cation-sensitive dye murexide, which exhibits a high sensitivity in its spectral properties towards the Cu²⁺ ion. Cu²⁺ uptake could therefore be monitored by the changes in \(E_{540}\) with respect to the \(E_{507}\) in an Amino dual-wavelength spectrophotometer (DW2). The extinction due to the Cu²⁺-murexide complex varied with the experimental conditions (pH, ionic strength) and a calibration curve was constructed for each particular set of conditions. A simple rapid-mixing device was constructed to enable us to add Cu to the mitochondrial suspension + murexide and observe the first phase of the reaction. A twin-syringe assembly with a Teflon mixing nozzle was connected via plastic tubing to a flow-through cuvette. The cuvette was first loaded with mitochondrial suspension + murexide (0.14 mm) from the larger syringe, and a baseline obtained for the absorbance differences (\(E_{540} - E_{507}\)). The smaller syringe contained CuSO₄ solution of the required strength, and the contents of the two syringes were next injected simultaneously so as to replace, within 1 s, the mixture in the cuvette with one identical except for the additional presence of the required amount of Cu²⁺. The pen recorder monitoring the absorbance difference gave a highly reproducible deflexion equal to that expected for the Cu²⁺ concentration being provided and followed, when flow ceased, by a return to the zero Cu²⁺ reading, thus showing that during flow there had not been time for appreciable Cu²⁺ uptake.

**Measurements of K⁺ and H⁺ movements, swelling and oxygen consumption**

These parameters were measured simultaneously in a multi-electrode device which consisted of a water-jacketed vessel, into the inner chamber of which were intruded H⁺- and K⁺-specific glass electrodes (respectively Jena combination and EIL type GKN 33C), an oxygen electrode and, by means of a length of fibre optic, a light source from a small (‘pea’) bulb. A photoresistor, receiving reflected light from the above source, was connected, also by a length of fibre optic, with the interior of the chamber. The walls of the vessel were painted black, and stray light was excluded from the assembly. The apparatus was housed in a metal screening box, which was earthed. The outputs from the glass electrodes were fed into standard pH-meters, outputs from which were displayed on a two-channel potentiometric pen recorder. A second two-channel recorder was used for recording the amplified outputs of the oxygen electrode and changes in the potential difference across the photoresistor. The inner chamber of the assembly had a volume of 7 ml and mitochondria were used at a concentration of about 2 mg/ml. The medium for the experiments described consisted of 250 mm sucrose, 10 mm-Tris/Hepes, 10 mm-Tris/HCl, 5 mm KCl and suitable substrates as described in the Figure legends. Additions were made to the reaction mixture during the run by means of syringes connected via plastic tubes to the vessel, to minimize electrical and mechanical disturbances during recording.

**Ca²⁺-uptake studies**

Ca²⁺ uptake was studied by the same two techniques as described above for Cu²⁺ uptake, except that EGTA (10 mm final concentration) was substituted for EDTA to stop the uptake and ⁴⁶Ca was used to obtain measurements of the Ca contents of pellets and supernatants; alternatively, a record was obtained by dual-wavelength spectrophotometry of the absorbance changes of murexide (at 25 μm) after initiation of the uptake by addition of succinate to a mixture of mitochondria (1 mg/ml) and medium supplemented with rotenone (2 μg/ml). The same wavelength pair (540, 507 nm) was used as for the Cu²⁺-uptake experiments. The sensitivity necessary to follow the Ca²⁺ movement was ten times that needed when Cu²⁺ was being observed.

**Results**

**Binding of copper by liver mitochondria**

Under the correct conditions, liver mitochondria accumulate large amounts of Cu from their suspending medium. Fig. 1 shows a typical uptake curve obtained by using the murexide technique with a 150 mm-KCl medium, and the mitochondria in an energized state. Provided that the added Cu²⁺ concentration exceeded 8 nmol/mg of protein, the uptake takes place in two distinct stages, an initial process (A), which may not be complete before 1 s, and a faster process (B), responsible for the binding of the greater proportion of the added Cu²⁺, ensues. In this example, 28.5 ng-ions of Cu²⁺ were added per mg of mitochondrial protein, and 88% of the added dose was bound within 4 min. The Cu²⁺ concentrations referred to are values for free Cu²⁺; an additional 5 ng-ions/mg were
present in a chelated form, which does not react with the murexide. In experiments made with additions of up to 33 ng-ions/mg of protein, about 90% of the added Cu²⁺ was eventually accumulated. The initial rate of uptake of Cu²⁺ by energized mitochondria is linearly related to the concentration of free Cu²⁺ added; this is illustrated in Fig. 2, which summarizes results of a series of experiments made by the stopped-flow method. The initial rates are of the same order as those found for Ca²⁺, Mn²⁺, Sr²⁺ and Ba²⁺ (Vainio et al., 1970).

The rate and extent of Cu²⁺-accumulation depend on the supply of energy. Mitochondria which have been treated with antimycin+oligomycin, to prevent use of respiratory substrates and of endogenous ATP, accumulate Cu²⁺ faster than do mitochondria which are respiring, and the uptake proceeds to close to 100% of the added Cu²⁺. The two phases of the uptake are present in each condition; it was technically more convenient to compare the rates of the second phase (Fig. 3), though the first phase was also seen to be faster in absence of energy.

The binding of Cu²⁺ to mitochondria depends on the composition of the incubation medium. In both the experiments illustrated in Fig. 4, a similar

![Cu²⁺ uptake by rat liver mitochondria](image)

Fig. 1. Cu²⁺ uptake by rat liver mitochondria

Mitochondria (3.75 mg of protein) were incubated in 3 ml of medium containing 150 mM-KCl, 10 mM-Tris/Hepes, 3 mM-succinate and rotenone (1.7 µg/ml). Murexide was present at 85 µM. The absorbance difference (E₅₄₀ - E₅₀₇) was monitored in the dual-wavelength spectrophotometer at room temperature (25°C). At the point indicated by the arrow, 12.5 µl of 10 mM-CuSO₄ was added. (A) and (B) denote the initial and later stages respectively.

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![Graph](image)

Fig. 2. Relationship of first-phase Cu²⁺ uptake to free Cu²⁺ concentration

Mitochondrial suspension (4.0 ml; 0.75 mg/ml) in a medium containing 150 mM-KCl, 10 mM-Tris/Hepes, 3 mM-succinate, rotenone (0.8 µg/ml) and 85 µM-murexide, at pH 7.2, were pumped into a flow-through cuvette in the dual-wavelength spectrophotometer at room temperature. The small syringe was loaded with 12 µl of CuSO₄ solution (1, 3.3, 5, 6.6 or 10 mM), and this was rapidly mixed together with a further 5.2 ml of the mitochondrial suspension, and the mixture pumped into the cuvette, replacing the original 4 ml within 1 s. This enabled the initial rate of accumulation to be measured.

![Graph](image)

Fig. 3. Relationship between second-phase Cu²⁺ uptake and free Cu²⁺ concentration for energized and non-energized mitochondria

Mitochondria (3.75 mg of protein) were incubated in 3 ml of medium containing 150 mM-KCl, 10 mM-Tris/Hepes, 85 µM-murexide, pH 6.85. For the energized state (○), the medium was supplemented with 3 mM-succinate+rotenone (1.7 µg/ml). For the de-energized state (●), antimycin (0.55 µg/ml) and oligomycin (1.1 µg/ml) were added. Reaction was initiated by addition of CuSO₄ solution (10 mM) and the rate of the second phase of the reaction was measured by monitoring the absorbance difference (E₅₄₀ - E₅₀₇).
amount of Cu\textsuperscript{2+} was added to mitochondria in a sucrose medium (a) and in a KCl medium (b). The uptake curve in the KCl medium shows the ion-dependent, second phase of accumulation; in the same time, only 20% of the Cu\textsuperscript{2+} is accumulated from the sucrose medium. Experiments in which KCl was added to the sucrose medium at 5, 10 and 20mM showed an increasing rate and extent of Cu\textsuperscript{2+} accumulation with the increasing proportion of KCl. For the appearance of the second phase, the presence of KCl was necessary, and the rate of the second phase increased as the KCl concentration in the medium was augmented. If NaCl was used in place of KCl as the main component of the incubation medium, a second phase of Cu\textsuperscript{2+} accumulation was again apparent, but this proceeded at approximately half the rate which was obtained with KCl medium.

A further factor which greatly influenced the extent of copper uptake was chelation by certain substrates, most notably by glutamate. The uptake kinetics from solutions in which Cu\textsuperscript{2+} was present as a chelate were measured by the EDTA quench technique. The extent of uptake under these conditions (mitochondria energized with glutamate+succinate as substrates) was rather small, only 7ng-atoms taken up per mg of protein in a 10min period after the addition of 110ng-atoms of Cu\textsuperscript{2+}/mg of protein. When these experiments were repeated by using the murexide technique, the reason for the very small uptake became clear, namely that in the presence of 3mm-glutamate the Cu\textsuperscript{2+} concentration is vastly decreased. This observation was confirmed in a system free from mitochondria, by using murexide absorbance at a single wavelength (540 nm) to measure the free Cu\textsuperscript{2+} concentration. Several substrate anions were found to have considerable chelating properties for Cu\textsuperscript{2+}, the order of efficacy in decreasing the Cu\textsuperscript{2+} concentration being glutamate > oxoglutarate > citrate > isocitrate > pyruvate. Malate and succinate had little effect on the Cu\textsuperscript{2+} concentration.

Although it was shown that mitochondria deprived of an energy source accumulated Cu\textsuperscript{2+} more rapidly and to a greater extent than did those that were energized, it was also noted that aged mitochondria (48h old) lost the property of Cu\textsuperscript{2+} accumulation completely, indicating that some structural integrity must be required for the reaction to occur.

**Effect of Cu\textsuperscript{2+} on alkali-ion permeability**

Fig. 5 shows the effect of adding 94ng-ions of Cu\textsuperscript{2+}/mg of protein to mitochondria respiring in a medium containing 5mm-KCl. The substrates were 3mm-glutamate+3mm-succinate. The conditions were such as to minimize Cu\textsuperscript{2+} uptake, as will be seen from a consideration of the results presented in the preceding section, but the effects of such an addition are nevertheless dramatic. The

![Fig. 4. Cu\textsuperscript{2+} accumulation from sucrose and KCl media](image)

Mitochondria (3.75 mg of protein) were incubated in 3.0 ml of medium. For (a), the medium consisted of 300 mm-sucrose, 10mm-Tris/Hepes, 85 mM-murexide, 3mm-succinate and rotenone (1.7μg/ml), pH 6.85. For (b) the medium was identical, except for the presence of 150mm-KCl instead of 300mm-sucrose. In (a), 10mm-CuSO\textsubscript{4} was added to give a free Cu\textsuperscript{2+} concentration of 21ng-ions/mg of protein; in (b) the free Cu\textsuperscript{2+} added was 17.5ng-ions/mg.

![Fig. 5. Cu\textsuperscript{2+}-induced K\textsuperscript{+} accumulation](image)

Mitochondria (16 mg of protein) were incubated in the multi-electrode chamber in 7ml of medium containing 250mm-sucrose, 5mm-KCl, 10mm-Tris/Hepes, 10mm-Tris/HCl and 3mm-succinate+glutamate, pH 7.2. At the indicated point, 15μl of 0.1m-CuSO\textsubscript{4} (equivalent to 94ng-ions/mg of protein) was injected into the incubation mixture. The calibrations shown refer to the 7ml volume and are drawn opposite the appropriate traces, that is, from top to bottom at the origin: H\textsuperscript{+} concentration, K\textsuperscript{+} concentration, light-scattering (LS) and oxygen concentration. At O\textsubscript{2}=0 the suspension became anaerobic.
respiration rate increases from 12.4 to a maximum of 32 ng-atoms of O/min per mg. The K+ concentration falls in the medium as the ion moves into the mitochondria, which accumulate 148 ng-atoms of K+/mg. Also, the rate of acidification of the medium increases more than in proportion to the respiration, and so is indicative of a K+-for-H+ exchange. The light scattered by the suspension decreases, which indicates that the particles are swelling. When the mitochondria have consumed all the available oxygen, the changes are reversed, either wholly or partially. The particles shrink to their original volume and the K+ concentration in the medium returns to its original value; these changes indicate that the reaction had been energy-dependent. This feature was confirmed by addition of ATP to the anaerobic suspension, which caused a re-accumulation of K+ with concomitant swelling.

Table 1 shows the rate of K+ accumulation and respiratory stimulation as a function of the dose of Cu++. In all three experiments, the additional K+ accumulated remained at about 100 ng-atoms/mg of protein, but the rate of K+ uptake and of respiration was increased as the Cu++ addition was increased. For comparison, valinomycin at 3 ng/mg of protein induced an uptake of 225 ng-atoms/mg of protein, at an initial rate of 286 ng-atoms/min per mg. In other measurements of the effect of Cu++, especially when higher doses of Cu++ were used, the K+ uptake gave place to an efflux before anaerobiosis, though the high rate of respiration and swollen state persisted, despite the K+ loss. The rates of K+ accumulation, as well as swelling and respiratory stimulation, were increased if phosphate was present in the medium, so that the K+ uptake is otherwise limited by the anionic equivalents available in the mitochondrial structure. This feature parallels similar observations made with valinomycin-induced uptakes.

The K+/O ratio was rather variable, with an average of 3.0, over a range of 1.0-7.2 in eight experiments. This would appear to be a less efficient accumulation process than that initiated by valinomycin, which can give ratios near the theoretical maximum of 8 for succinate-supported respiration (Harris et al., 1966). The largest K+ uptake observed was 150 ng-atoms/mg of protein, in the presence of phosphate, which is also not as great as that reported for valinomycin-induced accumulation (400 ng-atoms/mg).

The effect of Cu++ on permeability to K+ is abolished if 0.33 mM-EDTA is added to the medium before Cu++ addition. If, however, EDTA is added during the course of a Cu++-induced K+ accumulation, the effects are not thereby reversed. If ADP is added to the suspension during the course of K+ accumulation, the rate of oxygen consumption is further stimulated and the rate of K+ accumulation remains the same. This indicates that respiratory energy can be used at the same time for K+ accumulation and for the phosphorylation of ADP.

If Rb+ or Cs+ are used in place of K+ in these experiments, uptake of these cations can be similarly measured. The amounts of these alkali ions that were accumulated were in the series Rb+ > K+ > Cs+, and the rates of accumulation, though similar for the three species, were in the same order. This selectivity is similar to that obtained with valinomycin, but the differences are even more marked for the Cu++-induced movement than for that caused by valinomycin.

**Effect of Cu++ on respiration**

In a 150 mM-KCl medium, Cu++-induced respiratory stimulation is rapid, in contrast with the slower effect in a 250 mM-sucrose + 5 mM-KCl medium (Fig. 5). A maximum rate is obtained within 1 min of the Cu++ addition; the relationship between the stimulated rate and the amount of Cu++ added is shown in Fig. 6. This shows that 25 ng-atoms of Cu++/mg of protein stimulate the oxidation of succinate (+rotenone) maximally, whereas 100 ng-atoms/mg have to be added to give the same effect if succinate + glutamate are used, or malate + glutamate, doubtless because of chelation by glutamate. The respiration becomes inhibited after the initial stimulation, if the free Cu++ exceeds 28 ng-atoms/mg.

In a sucrose medium, containing 5 mM-KCl, no inhibition is observed at Cu++ doses as high as 42 ng-atoms/mg of protein. The extent of the respiratory inhibition obtained in a high-KCl medium is difficult to assess, since after the initial stimulation the inhibition is progressive, until the available oxygen is used up. When pyruvate + malate are used as substrates, an addition of 118 ng-atoms/mg of protein causes a short-lived threefold stimulation of respiration, followed by complete inhibition.

**Effect of Cu++ on Ca++ accumulation**

The addition of Cu++ to an unenergized suspension before Ca++ (42.5 nmol/mg, equivalent to 33 μM...
initial concentration in the medium) decreased the succinate-induced Ca\(^{2+}\) uptake in proportion to the amount added. For example, with 17 nmol of Cu\(^{2+}\)/mg, the Ca\(^{2+}\) uptake was only 11 nmol/mg out of the possible 50 nmol/mg, which includes 7.5 nmol of endogenous Ca\(^{2+}\) shed during preincubation. The relation between Cu\(^{2+}\) addition and subsequent rate of succinate-induced Ca\(^{2+}\) uptake is shown in Fig. 7.

**Discussion**

In the available reports on the effect of Cu\(^{2+}\) on isolated mitochondria, a common technique has been to measure the swelling caused by the addition of Cu\(^{2+}\) to a mitochondrial suspension, as indicated by the changes in $E_{420}$ (Hwang et al., 1972; Verity & Gambell, 1968). The present results suggest that this parameter should be measured together with others, notably with the K\(^{+}\) movement. Although we see that energy-dependent K\(^{+}\) movements are closely correlated with the swelling and shrinkage of mitochondria, our results are in conflict with those of Verity & Gambell (1968), who describe a swelling that is not inhibited by lack of substrate or by respiratory inhibitors. It is not clear whether or not the swelling reported by these workers was associated with energy-dependent K\(^{+}\) uptake.

Hwang et al. (1972) inferred that the swelling that they found was correlated with K\(^{+}\) movements, because of differences in the rate of swelling when KCl was replaced by NaCl, and when the energy supply of the mitochondria was inhibited. However, Hwang et al. (1972) reported that the swelling was not reversed when anaerobic conditions ensued, a finding in conflict with our own, illustrated in Fig. 5. This discrepancy could be due to the fact that we have used rat liver mitochondria, whereas Hwang et al. (1972) used ox heart mitochondria. To avoid confusion, it appears to be useful to measure swelling, K\(^{+}\) movement and oxygen consumption simultaneously. Verity et al. (1967) have produced evidence for an increased liver mitochondrial permeability in mice after Cu\(^{2+}\) administration in vivo.

We have noted the poor efficiency of K\(^{+}\) accumulation as measured by the K\(^{+}\)/O ratio. The reason for this could be a stimulation of mitochondrial adenosine triphosphatase activity, as reported by both Hwang et al. (1972) and Cederbaum & Wainio (1972b) for ox heart mitochondria. Stimulation of adenosine triphosphatase by Cu\(^{2+}\) would lead to a diversion of metabolic energy from K\(^{+}\) accumulation to rephosphorylation of ADP. The possibility of this occurring was demonstrated in experiments where ADP caused a further increase in the rate of Cu\(^{2+}\)-stimulated respiration.

The second phase of Cu\(^{2+}\) uptake (illustrated in Fig. 1 and in Fig. 4b) is interesting in that it
seems to be the result of a reaction of Cu$^{2+}$ with the membrane in the first phase, which is dependent on the presence of K$^+$. The course of events that might be envisaged could be as follows: (a) an initial rapid binding of the Cu$^{2+}$ to the mitochondrion, resulting in increased permeability to K$^+$; (b) in the energized situation this is followed by K$^+$ uptake, giving rise to extensive swelling and (where the external KCl concentration is high), structural damage; (c) K$^+$ loss might then be expected to occur, and the second phase of Cu$^{2+}$ accumulation would ensue, Cu$^{2+}$ replacing K$^+$ on fixed sites, and this would lead to respiratory inhibition. This view of events is supported by experiments of the type illustrated in Fig. 5, where K$^+$ loss was found to occur before anaerobiosis. In such cases, the swelling was not reversed when K$^+$ influx occurred, which indicates that some irreversible structural change had taken place. In the de-energized state the Cu$^{2+}$ would similarly bind to the membrane in the first phase of the reaction, causing an increase in permeability to K$^+$; this allows K$^+$ loss to take place, and this is followed, as before, by the second phase of Cu$^{2+}$ uptake.

An interesting finding was that the mitochondria in the de-energized state took up more Cu$^{2+}$ than those that were energized. This is, of course, the opposite effect to that found with cations such as Ca$^{2+}$, and would appear to cast some doubt on the view that cation uptake in necessarily electrophoretic and obligatorily linked to proton ejection [the 'chemiosmotic' view as interpreted by Azzone & Massari (1973)]. It would appear that energization can lead to proton extrusion and cation uptake for certain cations (Ca$^{2+}$, K$^+$ + valinomycin), but for Cu$^{2+}$ the process leads to a decrease in the number of potential binding sites. Such a decreased binding capacity under conditions of energization may reflect the degree of ionization of membrane and/or matrix protein groups (particularly amino groups). Further interpretation of the phenomenon must, however, await the elucidation of the fate of the Cu$^{2+}$ that is taken up by the mitochondria; it is of some importance to the discussion how much of it is membrane-bound, and how much is transported into the matrix. Our experiments do not distinguish between these two types of uptake.

The discovery that the second phase of Cu$^{2+}$ accumulation ensues in a NaCl medium suggests that permeability of the structure to either Na$^+$ or Cl$^-$ has been induced by the Cu$^{2+}$. An induction of permeability to Cl$^-$ by mercurials has been described by Brierley (1974). We have not measured Na$^+$ or Cl$^-$ movements in response to the addition of Cu$^{2+}$. Brierley (1974) has suggested that heavy metals and mercurials cause permeability changes by reacting at protein sites in the membrane, whereas molecules with ionophoretic activity, such as valinomycin, increase permeability at the phospholipid. Cederbaum & Wainio (1972c) produced evidence that the reaction of Cu$^{2+}$ is indeed at protein sites of the mitochondrial membrane, since removal of a large proportion of phospholipid from the mitochondria did not influence the degree of Cu$^{2+}$ binding. Southard et al. (1974) have, however, argued for a different mechanism of metal-ion-induced permeability changes. They envisage the addition of the metal ion causing the release or activation of an endogenous ionophore, which then acts in a manner similar to that shown by valinomycin. After considerable effort expended on the search for such a molecule, however, they were able to produce only scant evidence for its existence (Blondin, 1974). Our own findings suggest that, whatever the mechanism of the induction of the permeability change, there is a preference for accumulation of Rb$^+$ over K$^+$, and for both of these over Cs$^+$, a characteristic which argues for a selectivity residing either in the transporting system or at the site of the final binding of the alkali cation.

The effect of Cu$^{2+}$ on respiration in a KCl medium is greater than in a medium containing 250 mM-sucrose and 5 mM-KCl. It seems reasonable to suppose that this effect is also connected with K$^+$ movements. In particular, the inhibition noted in experiments where the free Cu$^{2+}$ concentration exceeded 30 ng-ions/mg of protein occurs at the same time after Cu$^{2+}$ addition as the second phase of Cu$^{2+}$ accumulation, which is likely to be associated with K$^+$ influx. The inhibition is presumably due to increased accessibility of the respiratory enzymes to the Cu$^{2+}$ ion. If the external K$^+$ concentration is kept low (5 mM), the inhibition does not take place until higher doses of Cu$^{2+}$ are used. This suggests that under the latter conditions the Cu$^{2+}$ is not reaching the inhibitory sites, since there is a diminished second-phase Cu$^{2+}$ uptake compared with that occurring in 150 mM-KCl. The effect of Cu$^{2+}$ on Ca$^{2+}$ accumulation is similar to that reported by this laboratory, of Pb$^{2+}$ on heart-mitochondrial Ca$^{2+}$ uptake (Parr & Harris, 1976). Pb$^{2+}$ has a similar inhibitory effect in liver mitochondria, and is more efficient than Cu$^{2+}$; 4 ng-ions of Pb$^{2+}$/mg of protein are required for almost total inhibition, whereas 18 ng-ions of Cu$^{2+}$/mg are responsible for a similar effect.

The massive amounts of Cu$^{2+}$ that can be accumulated by heart mitochondria in the absence of energy (Cederbaum & Wainio, 1972a), up to 4 μg-ion/mg of protein when no penetrating anion has been added, indicate the existence of a large population of potential cation-binding sites on the mitochondrial protein. Uptake of cations such as Zn$^{2+}$, Cu$^{2+}$, Pb$^{2+}$ and mercurials (Scott et al., 1970), Fe$^{3+}$ (Romslo & Flatmark, 1973), Ca$^{2+}$, Sr$^{2+}$ and Mn$^{2+}$ (Wainio et al., 1970), or K$^+$ in the presence of
valinomycin (Harris et al., 1966), supports the suggestions which have been made that the proteins' mutually interacting positive and negative groups (Azzone & Massari, 1973) can be dissociated by either energy+Ca2+, or K+ with an ionophore, or by sufficiently strongly multiply charged cations such as Fe3+, Cu2+ or Pb2+. In the absence of a supply of penetrant anions, the protonated amino groups could lose protons as their previously associated acid groups acquire metal ions. An opening-up of the structure by a strongly interacting metal attaching to both thiol and carboxyl groups may reasonably expose more singly charged anionic sites to which K+ ions could attach, giving a basis for Cu2+-induced swelling and K+ uptake. The stronger interaction with the sites shown by Cu2+ also would remove potential sites for binding of Ca2+, so preventing uptake of the latter.

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