Uptake and efflux of copper-64 in Menkes'-disease and normal continuous lymphoid cell lines

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The accumulation of copper over 2 h by normal lymphoid cells and those from Menkes'-disease patients (Menkes' cells) was found to be biphasic, with an initial phase of rapid uptake, an approach to steady state at around 40–60 min, followed by a further accumulation phase. The accumulation of copper was not diminished by the addition of a variety of metabolic inhibitors, suggesting that copper uptake is not an active process. The presence of carbonyl cyanide *m*-chlorophenylhydrazine in the culture medium stimulated the uptake and accumulation of copper in both normal and Menkes' cells to the same absolute level. This effect appeared to be specific for copper, since the accumulation of Zn and Cd was unaffected. Menkes' cells did not differ from normal in their initial rate of copper uptake. Analysis of the uptake curve suggested that the membrane transport of copper involves both passive and facilitated diffusion. Initial rate of efflux from the cells was approximated by two methods. Menkes' cells did not appear to be affected in this function. It seems likely that the basic defect in Menkes' disease involves a step in intracellular copper transport rather than the membrane transport of copper.

INTRODUCTION

Menkes' disease, first described in 1962 (Menkes *et al.*, 1962), is characterized by a disturbance of copper transport (Danks *et al.*, 1972). In cultured cells, the phenotype of elevated intracellular copper levels, increased accumulation of copper isotope and decreased percentage loss of isotope is well established (Goka *et al.*, 1976; Horn, 1976; Chan *et al.*, 1978; Beratis *et al.*, 1978; Lott *et al.*, 1979; Kirby & Wood, 1980; Camakaris *et al.*, 1980; Riordan & Jolicouer-Paquet, 1982).

The excess copper in Menkes' cells is bound to metallothionein (Riordan & Jolicouer-Paquet, 1982). The phenotype could result from an increased rate of uptake of Cu, a decreased rate of efflux or a defective step in intracellular transport. Cu would then accumulate and be sequestered by metallothionein. Alternatively there may be a primary defect which results in enhanced rates of synthesis of metallothionein (Leone *et al.*, 1985).

The aim of the present studies was to determine whether a defect exists in initial rates of copper uptake or efflux in cultured continuous lymphoid cells established from Menkes'-disease patients.

Studies on copper uptake and accumulation in normal cells have been performed by using a variety of different extracellular media (Weiner & Cousins, 1980; Schmitt *et al.*, 1983; Darwish *et al.*, 1984b). The distribution between, and binding of copper to, the various components of the media are important in determining the chemical activity of free copper with respect to uptake. Indeed, it is possible that the rate-determining step in uptake may be the dissociation of copper from copper-binding ligands in the medium.

Schmitt *et al.* (1983) used a balanced salt solution not containing any serum proteins for their measurements of copper uptake and accumulation in isolated rat hepatocytes. Uptake of copper was found to occur via a passive saturable process. Initial rapid uptake was followed by a slower increase before a steady state was reached at 30–40 min. In medium containing Cu at concentrations of more than 2 μ M, this was followed by a decrease in the level of accumulated copper isotope. The system described above was also able to function in the net efflux direction (Darwish *et al.*, 1984a).

Weiner and Cousins (1980) observed linear copper accumulation over 12 h in cultured rat hepatocytes incubated in serum-free tissue-culture medium with added albumin. A rapid uptake phase was observed in the first hour that was not observed in experiments *in vivo* and which was attributed to surface binding of the metal.

Darwish *et al.* (1984b) found that, when albumin was present in the uptake medium at a Cu²⁺/albumin ratio of 1:1, the accumulation of copper by isolated rat hepatocytes was decreased and did not approach steady state at 40 min. A rapid phase of uptake was observed in the first few minutes, followed by a near-linear accumulation that continued beyond 40 min, as described by Weiner & Cousins (1980). Darwish *et al.* (1984b) postulated that albumin binding decreases the availability of copper for uptake. Histidine (the active species being His₂-Cu) and, to a lesser extent cystine, abolished the effect of albumin.

Serum-free medium was chosen as a simple defined medium for the present experiments. The inclusion of foetal-calf serum in the uptake medium would raise the basal copper level in the medium 8–10-fold, thereby

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DCCD, *N,N*-dicyclohexylcarbodi-imide; 2,4-DNP, 2,4-dinitrophenol; PBS, phosphate-buffered saline [NaCl (8.0 g/l)/KCl (0.2 g/l)/Na₂HPO₄ (1.15 g/l)/KH₂PO₄ (0.2 g/l), pH 7.4]; RPMI 1640, Roswell Park Memorial Institute 1640 (culture medium); Menkes' cells, lymphoid cells of Menkes'-disease patients.

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restricting our ability to measure uptake and efflux at low extracellular copper concentrations. In addition, the studies would be complicated by the introduction of variable concentrations of defined and undefined copper-binding ligands present in serum.

MATERIALS AND METHODS

Human continuous lymphoid cells (Epstein-Barr-virus-transformed) were grown in Dulbecco-modified Eagle's medium (purchased from Flow Laboratories, Sydney, Australia) + 10% (v/v) foetal-calf serum ([Cu] 0.15 µg/ml). For each experiment cells were incubated in RPMI 1640 ([Cu] 0.002 µg/ml) + ^{64}Cu or ^{67}Cu ([Cu] 0.01–5.00 µg/ml) at a cell density of 1×10^6 cells/ml.

Cell viability, measured as the cells' ability to exclude Trypan Blue, was maintained at more than 90%. Incubation of cells in ice-cold 10 mM-EDTA (used for the cell washes) was not found to alter cell viability when it was judged by this criterion. Possible disruptive effects of 10 mM-EDTA on the cell membrane were further tested by using an assay for leakage of the cytoplasmic enzyme lactate dehydrogenase. Washing cell pellets in PBS or PBS/10 mM-EDTA caused ~0.8% leakage of lactate dehydrogenase (close to the limit of sensitivity of the assay). This may have resulted from manual disruption of membranes during cell resuspension. It was concluded that 10 mM-EDTA does not disrupt the cell membrane and is therefore suitable for the removal of non-specific copper binding on the cell surface.

$^{64}\text{CuCl}_2$ was purchased from the Australian Atomic Energy Commission, Lucas Heights, Australia. It was used because ^{67}Cu was not available during most of the period of this study. $^{67}\text{CuCl}_2$ was purchased from Atomic Energy of Canada Ltd., Ottawa, Canada. Levels of ^{64}Cu and ^{67}Cu in cells and in culture media were measured with an LKB Ultragamma counter. Non-radioactive copper was measured using a Perkin-Elmer model 5000 atomic-absorption spectrophotometer with an HGA 500 graphite furnace, and converted into µM units. The specific radioactivity of copper in the culture medium was determined for each experiment by using these measurements. Carrier-free $^{109}\text{CdCl}_2$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. (50–1000 µCi/µg Cd). $^{65}\text{ZnCl}_2$ was obtained from New England Nuclear Corp., Boston, MA, U.S.A. Proteins were measured after solubilization of the cell pellet in 0.1% (w/v) SDS by using the Bio-Rad reagent (purchased from Bio-Rad, Melbourne, Australia), with human γ-globulin (Commonwealth Serum Laboratories, Melbourne, Australia) as the standard (Bradford, 1976).

Ouabain, CCCP, DCCD and 2,4-DNP were purchased from Sigma, valinomycin from Boehringer-Mannheim, gramicidin D from Calbiochem-Behring and the phosphorylation uncoupler SF6847 from Wako Pure Chemical Industries, Osaka, Japan).

Analysis of kinetic data

Measurements of the initial rates of uptake (v) at different copper concentrations (s) were fitted to the equation describing a combination of facilitated and passive diffusion:

$$v = \frac{V \cdot s}{K + s} + k \cdot s \quad (1)$$

by non-linear regression, using the modification of the STEPT program described by Duggleby & Morrison (1979). The STEPT program was obtained from the Quantum Chemistry Program Exchange, Department of Chemistry, Indiana University, Bloomington, IN, U.S.A., as part of the STEPT package (QCPE 307).

V is the maximum velocity for carrier-mediated uptake of Cu (s), K is the apparent Michaelis constant for this saturable process and k is the first order rate constant for passive diffusion of Cu.

Initial rates of uptake

Normal and Menkes' lymphoid cells were washed in RPMI 1640 medium then resuspended in RPMI 1640 medium at a density of 2×10^6 cells·ml⁻¹ for 15 min at 37 °C. At zero time the cell suspension was mixed 1:1 with RPMI 1640 medium (previously equilibrated at 37 °C) containing $^{64}\text{Cu} \pm 50$ µM-CCCP. After 30 s the cell suspension was diluted 2:3 with ice-cold 16.67 mM-EDTA in PBS, pH 7.4 (final concentration of EDTA, 10 mM). The cells were centrifuged, washed twice with cold 10 mM-EDTA in PBS, pH 7.4, and finally with PBS at 4 °C. The levels of radioisotope and protein in the cell pellets were determined, together with the levels of radioisotope and total copper in the culture medium. Rates of uptake were measured at 16 different copper concentrations (between 0.01 and 5.00 µg/ml) in each experiment.

Initial rate of efflux

(a) The first part of the experiment estimated the rate of copper efflux according to the assumption made by Darwish *et al.* (1984a). Cells were incubated for 40 min in RPMI 1640 + ^{67}Cu , at which time, according to the assumption, the rapidly equilibrating pool has reached steady state and is in equilibrium with the extracellular medium. Then the concentration of copper in this pool may be defined as being equal to that in the extracellular medium. After 40 min incubation the cells were washed in 10 mM-EDTA in PBS, pH 7.4, at 4 °C, and resuspended in RPMI 1640 containing no added copper, at 37 °C.

The loss of ^{67}Cu from the cells over 1 min was measured. The total amount of copper lost was estimated from the measured ratio of ^{67}Cu to unlabelled Cu in the medium at the end of the 40 min preincubation and the levels of intracellular ^{67}Cu before and after the efflux. Protein levels in the cell pellets were also determined.

(b) The second part of the experiment estimated the rate of copper efflux by means of the measurement of the intracellular ^{67}Cu and total copper before and after efflux. Cells were incubated in RPMI 1640 + ^{67}Cu for 40 min, then washed and resuspended in RPMI 1640 for 1 min (as for *a*). Samples were taken after the 40 min incubation and wash, and after the 1 min efflux. Levels of intracellular ^{67}Cu were determined, together with atomic-absorption-spectrophotometric measurements of total intracellular copper. These values give an alternative estimate of the ratio of ^{67}Cu to unlabelled Cu effluxed from the cell. Dissolution of the cell pellets in HNO_3 for atomic-absorption spectroscopy makes the sample unsuitable for protein estimation. The ratios of ^{67}Cu to unlabelled Cu effluxed at each copper concentration were substituted into part (*a*) in place of the media ^{67}Cu /unlabelled copper ratios.

The amount of copper effluxed from the cells/min

Fig. 1. Accumulation of ^{64}Cu by lymphoid cells

Normal (■) and Menkes' (●) lymphoid cells were incubated in $10.86\ \mu\text{M}$ -Cu (as $^{64}\text{CuCl}_2$) for 2 h at 37°C . Samples were removed at intervals for ^{64}Cu and protein determinations. After rapid dilution of the sample with ice-cold $16.67\ \text{mM}$ -EDTA (final concn. $10\ \text{mM}$) in PBS, pH 7.4, the cells were centrifuged and washed twice with $10\ \text{mM}$ -EDTA in PBS, pH 7.4, with a final wash in PBS, pH 7.4, all of these steps being performed at 4°C . Each value represents the average of duplicate determinations (which varied by less than 10%).

per mg of protein was calculated for each copper concentration by using the two methods.

RESULTS

Copper uptake and accumulation

In normal cells the uptake/accumulation curve for copper was found to be biphasic during the first 2 h. The results of a typical experiment are shown in Fig. 1. Uptake was rapid during the first few minutes, slowing to approach a steady state at 40–50 min. This was followed by a further phase of accumulation. The accumulation of radioisotope was more constant in Menkes' cells, with some decrease at 40–50 min (Fig. 1).

The energy dependence of copper uptake and accumulation over the first 30 min was tested by including a variety of metabolic inhibitors in the medium. Table 1 shows that none of the metabolic inhibitors tested caused a significant reduction in copper accumulation. The unusual finding was the significant enhancement of accumulation caused by CCCP. The effect was concentration dependent: maximal at $100\ \mu\text{M}$ (media [Cu] $5.2\ \mu\text{M}$) and undetectable at concentrations of less than $1\ \mu\text{M}$.

The effect of CCCP was also influenced by the concentration of copper in the culture medium (Fig. 2). The addition of CCCP to normal cell cultures caused the pattern of copper accumulation to resemble that seen in Menkes' cells. CCCP did not affect the accumulation of zinc or cadmium (Table 2).

The initial rate (30 s) of copper uptake by normal cells at various copper concentrations is shown in Fig. 3. The

Table 1. Effect of metabolic inhibitors on copper accumulation

The cells were incubated at 37°C for 30 min in RPMI 1640 + ^{64}Cu . The total copper in the medium was $2.4\ \mu\text{M}$ (2,4-DNP, valinomycin, gramicidin), $5.2\ \mu\text{M}$ (ouabain, DCCD, CCCP) or $10.9\ \mu\text{M}$ (SF6847). The inhibitors were first dissolved in ethanol, with the exception of ouabain, which was dissolved in water. ^{64}Cu accumulation was halted by dilution of the sample in ice-cold $16.7\ \text{mM}$ -EDTA in PBS, pH 7.4 (final [EDTA] $10\ \text{mM}$). The cell pellet was washed once in chilled $10\ \text{mM}$ -EDTA/PBS and once in chilled PBS. ^{64}Cu accumulation and protein were measured in the cell pellets. The accumulation of ^{64}Cu after 30 min is expressed as a percentage of control-cell values without inhibitors. Each value represents the average of duplicate determinations.

Metabolic inhibitor	Concn.	Accumulation (%)	
		Normal	Mutant
Ouabain	1 mM	111	106
DCCD	100 μM	114	128
SF6847	25 μM	95	107
2,4-DNP	6 mM	264	222
Valinomycin	10 μM	135	101
Gramicidin	4 $\mu\text{g/ml}$	126	111
CCCP	100 μM	744	694
	10 μM	275	364
	1 μM	109	113
	0.1 μM	94	95
	0.01 μM	102	94

Fig. 2. Effect of media copper concentration on ^{64}Cu accumulation in the presence of CCCP

Normal (■, □) and Menkes' (●, ○) lymphoid cells were incubated in RPMI 1640 + ^{64}Cu ($0.16, 1.57, 4.73, 9.44, 15.7\ \mu\text{M}$ -Cu) $\pm 20\ \mu\text{M}$ -CCCP for 2 h at 37°C . The cells were harvested and washed twice with chilled $10\ \text{mM}$ -EDTA in PBS, pH 7.4. The levels of ^{64}Cu and protein in each pellet were determined.

graph of velocity versus substrate concentration suggested a saturable uptake process at low copper concentrations, but the results deviated from the theoretical rectangular hyperbola expected for a single

Table 2. Effect of CCCP on the accumulation of Zn and Cd

Cells were incubated at 37 °C for 2 h in RPMI 1640 $\pm$ ^{65}Zn , $\pm$ 30 μM -Zn (basal level of Zn in the medium was 2 μM), $\pm$ ^{109}Cd , $\pm$ CCCP. The cell pellets were washed twice with chilled 10 mM-EDTA in PBS, pH 7.4, and once with chilled PBS. The levels of accumulated ^{65}Zn and ^{109}Cd were related to cell number. Each value is the mean result for duplicate determinations.

Incubation medium: RPMI plus:			Accumulated radioactivity (c.p.m./ 10^7 cells)	
Radioisotope*	[Zinc] (μM)†	[CCCP] (μM)	Normal	Mutant
^{65}Zn	—	—	9403	8856
	—	50	9018	8434
	30	—	2342	2205
	30	50	2227	2301
^{109}Cd	—	—	61 677	62 102
	—	50	57 705	62 662

* Carrier-free isotope.

† ZnSO_4 supplementation.

saturable process at high concentrations. A simple model was proposed where two processes, one saturable and one non-saturable, were contributing to the observed uptake as described in eqn. (1).

Fig. 3 shows the data as individual points together with the computer-generated curve of best fit. Since no systematic deviations were noted, it was concluded that the two-process model was a plausible explanation for the observed effects.

Table 3 lists the constants describing the two-component curves, showing that the normal and mutant cells did not differ significantly in their initial rates of copper uptake. Each experiment represents the average of duplicate determinations. Other normal and mutant cell lines were tested and were not found to differ significantly in their initial rates of uptake from those shown. These results are consistent with copper being taken up from the medium by a combination of passive and facilitated diffusion.

When the metabolic inhibitor CCCP was present in the medium, the uptake of ^{64}Cu over 30 s was greater in both normal and Menkes' cells. Fitting the curve to eqn. (1) showed that the apparent V_{max} and the apparent K_m were 50% lower (which makes the pseudo-first-order rate constant V_{max}/K_m the same) while the rate constant ' k ' (passive diffusion) was higher (Table 3).

Copper efflux

The efflux of copper from continuous lymphoid cells was measured over 2 h. Rapid efflux occurred over the first 15 min, followed by a slower efflux phase. This pattern was observed in both normal and Menkes' cells (Fig. 4).

The initial rate of efflux was estimated by two different methods, described above in the Materials and methods section. The rate of efflux was first calculated by the method of Darwish *et al.* (1984a) (method a). Normal and mutant cells showed the same direct relationship

Fig. 3. Dependence of uptake velocity on copper concentration

(a) and (b) show the results of the initial-rate measurements on normal lymphoid cells. (a) The proposed saturable and non-saturable components of copper uptake. (b) The theoretical line which was obtained by fitting the data to eqn. (1) and which represents the sum of the saturable and non-saturable components. The experimental points are shown in relation to the line. The experimental points for the five lowest media copper levels tested are not represented on the graph, but were included in the data on which the computer curves were based.

between the level of newly accumulated copper and the subsequent rate of efflux (Fig. 5).

The second part of the experiment attempted to measure copper efflux more directly (method b). The ratio of the radioactivity effluxed to the total copper effluxed was determined. The initial rate of copper efflux as estimated by the two methods, is shown in Table 4. The values for normal cells were similar for both methods. However, those for Menkes' cells differed dramatically. Direct measurement of copper efflux showed that much more copper effluxed from Menkes' cells than was estimated by the method of Darwish *et al.* (1984a).

Table 3. Kinetic effects of CCCP on copper uptake

Initial rates of copper uptake were determined as described in the Materials and methods section at 16 different copper concentrations, and the resultant data were fitted to eqn. (1), which describes passive plus facilitated diffusion. Results shown are fitted parameter values $\pm$ S.E.M. (see the Materials and methods section and Duggleby & Morrison, 1979).

Cells	K (μM)	$V_{\text{max.}}$ (pmol/min per mg)	$10^3 \times k$ (min^{-1})
Normal	6.48 ± 0.91	11.9 ± 1.7	0.47 ± 0.03
Mutant	4.75 ± 0.81	18.2 ± 2.8	0.66 ± 0.04
Normal + 50 μM -CCCP	2.31 ± 0.39	4.77 ± 0.78	1.62 ± 0.07
Mutant + 50 μM -CCCP	2.88 ± 0.64	9.57 ± 1.8	1.73 ± 0.08

Fig. 4. Efflux of ^{67}Cu from preloaded cells

Normal (■) and Menkes' (●) lymphoid cells were washed in RPMI 1640 then pre-incubated in RPMI 1640 + ^{67}Cu ($[\text{Cu}] = 4.25 \mu\text{M}$) for 1 h at 37°C . The cells were washed and resuspended in RPMI 1640 ($[\text{Cu}] = 0.03 \mu\text{M}$) at 37°C and samples were taken at 15 min intervals. The samples were spun and washed twice with 10 mM-EDTA in PBS, pH 7.4, and once in PBS, pH 7.4, at 4°C . The radioactivity and protein in the cell pellets were determined.

DISCUSSION

The well-known tendency of Menkes' cells to accumulate excessive amounts of copper isotope over a 20 h period (Goka *et al.*, 1976; Horn, 1976; Lott *et al.*, 1979; Kirby & Wood, 1980; Camakaris *et al.*, 1980) is most marked in medium containing low levels of copper

Fig. 5. Effect of intracellular ^{67}Cu on the rate of ^{67}Cu efflux into non-radioactive medium

Cells were preloaded for 40 min in RPMI 1640 + ^{67}Cu (0.16, 3.15, 6.30, 9.44, 12.6, $15.7 \mu\text{M}$). Intracellular ^{67}Cu was measured in the cells after the preloading period. The rate of ^{67}Cu efflux into non-radioactive medium was measured over 1 min and was converted into nmol of Cu/min per mg of protein by using the method of Darwish *et al.* (1984a). ■, Normal cells; ●, Menkes' cells.

(Riordan & Jolicœur-Paquet, 1982; S. M. Herd, unpublished work). Results obtained for continuous lymphoid cells showed that the ratio of copper in the two cell lines reaches a constant minimum (2:1, Menkes'/Normal) for copper at concentrations greater than $0.2 \mu\text{g/ml}$ (S. M. Herd, unpublished work).

An analysis of uptake and efflux rates and energy dependence, if any, was undertaken to elucidate the reasons for this difference.

The initial rate of copper uptake was measured in normal and Menkes' cells. The resulting velocity-versus-substrate-concentration curves fitted well to eqn. (1), which describes a two-process model (a saturable plus a non-saturable process) (Fig. 3).

The constants (Table 3) describing the component curves were not significantly different between normal and Menkes' cells. The 2-fold increase in $V_{\text{max.}}$ observed in Menkes' compared with normal cells is not considered substantial enough to account for the 9-fold increase observed in accumulated copper in Menkes' cells at low extracellular copper concentrations (S. M. Herd, unpublished work). This kinetic analysis, however, revealed two possible routes for uptake. One is a saturable process and hence probably a process of facilitated diffusion (or active transport) and the other non-saturable and most likely passive diffusion.

To determine whether saturable uptake is due to active transport rather than facilitated diffusion it is necessary to demonstrate transport against a concentration gradient of 'free' copper. Since intracellular copper may be bound to a variety of ligands and may not be considered as participating in the concentration gradient,

Table 4. Comparison of the rate of copper efflux estimated by two methods

Cells were preloaded with ^{67}Cu for 40 min at 37 °C before rates of efflux were determined. Samples were taken for measurement of ^{67}Cu , total intracellular Cu and protein before and after a 1 min efflux in non-radioactive medium as described in the Materials and methods section.

Media [copper] (μM)	Estimated from . . .	Copper effluxed (pmol/min per mg of protein)			
		Normal		Mutant	
		Media copper*	Cell copper†	Media copper*	Cell copper†
0.16		30.5	15.3	47.1	98.8
3.15		115	82.6	149	260
6.30		178	151	286	1023
9.44		220	206	411	1999
12.6		288	275	589	1763
15.7		269	288	505	1410

* Effluxed copper measured by the method of Darwish *et al.* (1984a) (see the Materials and methods section).

† Effluxed copper determined by direct measurement of intracellular copper before and after efflux, as described in the Materials and methods section.

knowledge of the total intracellular copper concentration is of little use in determining the direction and magnitude of the gradient across the membrane. For this purpose, metabolic inhibitors were used to determine whether or not metabolic energy was required for copper uptake. The lack of effect of valinomycin, gramicidin, ouabain, SF6847 and DCCD suggested that copper uptake was not linked to Na^+ , K^+ or proton gradients, nor was it dependent on the electrical potential of the membrane. Thus there was no evidence that copper was taken up by an active process.

The kinetic constants of copper uptake have been described for rat hepatocytes (Schmitt *et al.*, 1983) and suggest a saturable process of facilitated diffusion with a similar K_m but with a $V_{\max}$ some 200-fold higher than for continuous lymphoid cells. These results are consistent with the conclusion that a component of copper transport is facilitated in continuous lymphoid cells. The 200-fold higher value of $V_{\max}$ in hepatocytes is not surprising, considering the major role the liver plays in copper homeostasis, i.e. the incorporation of copper into, and the secretion of, caeruloplasmin, and the excretion of copper through the bile.

The finding of an additional non-saturable uptake process in continuous lymphoid cells constitutes a second difference between the two cell types.

A significant response was observed when CCCP was added to the culture medium. CCCP stimulated copper accumulation (30 min) as well as uptake (30 s). This effect was apparently specific for copper, since the accumulation of zinc and cadmium was unaffected (Table 2). Gel filtration of the soluble proteins from cells incubated for 2 h ± CCCP showed that the increased amount of accumulated ^{64}Cu in cells + CCCP was not localized to a particular peak(s) in the profile. Although the soluble proteins bound more ^{64}Cu , the proportional distribution of radioisotope between the peaks in the profile was the same as for cells without CCCP (S. M. Herd, unpublished work). Analyses of the data fit to the two-process model (eqn. 1) showed that, whereas the apparent $V_{\max}$ and apparent K_m were lowered, 'k', the constant for the diffusion process, was increased. One

possible explanation is that CCCP may complex copper, causing enhanced passive diffusion. E.p.r. studies have not provided evidence for a primary copper complex either associated with cells or in the culture medium used, and the only significant copper complex in the medium detected by e.p.r. was the Cu-histidine complex (G. Hanson & J. Camakaris, unpublished work). It is also possible that eqn. (1) is inappropriate for analysis of the CCCP/Cu uptake data. The overall increase in 30 s uptake is in keeping with the longer-term observation of increased accumulation. Other models would need to be tested against the data before conclusions are made regarding the precise effect of CCCP on copper uptake and accumulation.

The unexpected effect of CCCP may depend on its ability to act as a thiol reagent (Kaback *et al.*, 1974). If CCCP stimulates uptake by activating cryptic binding sites for copper, it may be significant that CCCP elevates the accumulation of ^{64}Cu by normal and mutant cells to the same absolute level (see Fig. 2). This would suggest that the difference between Menkes' and normal continuous lymphoid cells may lie in the degree of activation of cryptic sites. Further experiments should examine this possibility more precisely.

A typical efflux curve is shown in Fig. 4. As with the uptake curve (Fig. 1), the rate of efflux is essentially zero after 40–60 min. It is interesting that ^{64}Cu effluxed from Menkes' cells at a rate similar to that found for normal cells, despite the difference in the starting level of accumulated ^{64}Cu .

Comparison of the initial rates of efflux from normal and Menkes' continuous lymphoid cells proved difficult. The assumption of Darwish *et al.* (1984a), as described in the Materials and methods section, was supported by the close agreement of the results obtained in normal continuous lymphoid cells with those obtained by using the more direct method of measuring whole-cell copper levels. The agreement between the results using the assumption of Darwish *et al.* (1984a) and those obtained by direct measurement was poor in Menkes' cells (Table 4).

It appears that the intracellular pool of 'free' copper

in the Menkes' cells had not reached equilibrium at the time of the efflux study, as suggested by the curve of copper-radioisotope accumulation for the Menkes' cells, which showed some decrease in the rate of accumulation at 40–60 min, but not a steady state. Delayed equilibration of the intracellular pool of 'free' copper in Menkes' cells suggests that this pool is enlarged, since the initial rate of copper uptake into the cell is not defective. Initial rates of efflux cannot therefore be determined for Menkes' cells, since the assumption of Darwish *et al.* (1984a) is not valid, and the concentration of copper in the intracellular 'free' pool is not known.

It was not possible, even for normal cells, to derive the kinetic parameters for efflux, since the rate of efflux was found to be directly proportional to the 'free' intracellular concentration of copper [using the assumption of Darwish *et al.* (1984a), which suggests non-saturation of the efflux mechanism (Fig. 5)]. However, the kinetic parameters of efflux have been derived for isolated rat hepatocytes, and they were found to correspond well with the parameters of uptake (Schmitt *et al.*, 1983). This suggested that, in these cells, uptake and efflux are mediated by the same system and fit the model of symmetrical facilitated diffusion (Christensen, 1975).

Although saturation of the efflux mechanism was not reached within the concentration range tested, the apparent K_m value for efflux is likely to be higher than that measured for uptake. Directional asymmetry of facilitated diffusion is not an uncommon phenomenon (Christensen, 1975).

The experiments presented here indicate that the defect in Menkes' disease is unlikely to involve the transport of copper across the plasma membrane in either direction. Disturbance of intracellular transport mechanisms seems a more likely explanation for the basic defect in Menkes' cells.

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