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Effect of medium copper concentration on the growth, uptake and intracellular balance of copper and zinc in Menkes' and normal control cells

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The precise nature of the variation in cellular copper load against medium copper concentration is defined using a comprehensive logarithmically incremented series of medium copper concentrations ranging from low levels (4.8 p.p.b.) through 'normal' to toxic levels (40 p.p.m.) in which fibroblasts were grown followed by determination of intracellular content. Menkes' fibroblasts showed an unexpected plateau region of stable intracellular copper content against a change in medium concentration of over 100-fold, albeit only when sufficient copper was present in the medium (0.08–8.0 p.p.m.). Thus, Menkes' cells are clearly capable of balancing uptake/efflux providing copper availability allows. Simultaneous analysis of cellular copper and zinc load at various medium copper concentrations shows an indistinguishable intracellular copper:zinc ratio between the two cell lines. The nature of non-labeled copper uptake by fibroblasts over a 40 min and 7 day period is reported. During the 40 min period copper uptake (20 p.p.m.) was essentially the same in both cell lines. However, copper absorbed was superimposed upon large pre-existing copper pools in the case of Menkes' cells only. Advantages of techniques determining non-labeled copper in copper uptake/efflux experiments are discussed in the light of these results. Fibroblast growth studies showed that, compared with normal cells, Menkes' cells are significantly ($P < 0.01$) more growth sensitive to extended exposure to low copper concentrations. Thus, Menkes' disease appears to be not only a result of copper maldistribution but also a direct result of an inability of Menkes' cells to function normally in low copper environments.

Keywords: copper balance, copper metabolism, copper uptake, fibroblasts, intracellular copper, intracellular zinc, Menkes' disease, zinc balance

Introduction

The diverse catalytic properties of copper ions, usually involving oxidation/reduction systems and a Cu^{+}/Cu^{2+} oxidation/reduction cycle, have made this metal an essential element in human nutrition (Underwood 1977). However, copper ions are also potentially lethal to biological systems and can generate highly toxic free radicals which subsequently lead to protein and membrane damage, impairment of cell function, and cell death (Miller *et al.* 1990, Stadtman 1990). Cellular response to copper must, therefore, be carefully regulated to minimize the side-effects of this metabolite whilst maintaining an efficient balance to satisfy cellular requirements. The ability of cells and organs to maintain such a homeostasis is a prerequisite for normal growth which is not achieved in the case of patients with Menkes' disease.

Menkes' disease is a recessive, X-linked disorder, almost invariably leading to death in early infancy, occurring with a frequency of ~1 in 100000 live births. A direct link between the disease and disturbed copper homeostasis, a universal biochemical signature of the disease, was first made by Danks *et al.* (1972). The tissues responsible for cell-mediated transposition of low molecular weight copper species into the blood, i.e. the intestine

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(absorption of dietary copper) and the kidney (resorption of copper from the glomerular filtrate), are accumulation sites of copper in Menkes' disease.

Copper maldistribution is now recognized at all levels of cellular organization studied in Menkes' disease; organs (Nooijen *et al.* 1981, Williams & Atkin 1981), individual cells (Allan *et al.* 1991) and subcellular organelles (Kodama *et al.* 1989). With the notable exception of hepatocytes (Darwish *et al.* 1983) elevation of intracellular copper content is a common denominator of diseased cells. Increased amounts of intracellular copper found in affected cell lines relative to control cells are identified as bound to metallothionein (Leone *et al.* 1985, Hamer 1987, Kodama *et al.* 1991).

Intracellular copper accumulation is considered to occur through the plasma membrane mediated by facilitated and passive diffusion (possibly via a zinc/copper port) although direct evidence is lacking (Herd *et al.* 1987). The existence of a corresponding energy dependent efflux port specific for copper only (as found in *Escherichia coli*; Silver *et al.* 1989) has been speculated for mammalian systems but not proven, partly because studies on the kinetics of copper balance (uptake verses efflux) have been severely restricted due to the lack of copper isotopes with long half lives (Danks 1990); ^{61}Cu, ^{64}Cu and ^{67}Cu have half lives of 3.4, 12.7 and 61.9 h, respectively. Recently, evidence for a candidate gene for Menkes' disease has been presented (Chelly *et al.* 1993, Mercer *et al.* 1993, Vulpe *et al.* 1993) which possibly codes for a copper-transporting ATPase.

We describe here the effects of the manipulation of medium copper concentration on the growth and net accumulation of medium copper by control and Menkes' fibroblasts after 7 days incubation and examine trends in intracellular copper/zinc balance. Further, the kinetics of non-radiocopper uptake over a 40 min and 7 day period are reported. The validity of determining kinetics of copper homeostasis at the cellular level using copper radioisotopes is discussed in the light of the results presented.

Materials and methods

Reagents

Dulbecco's modified Eagles medium (DMEM) and trypsin were purchased from Flow Laboratories (McClean, VA). Fetal calf serum (FCS) was obtained from B.A. Bioproducts (Walkersville, MD). Penicillin and streptomycin were purchased from Meiji Seika (Tokyo, Japan). Fibroblast cultures purchased from the American Type Culture Collection (Rockville, MD) were normal control (CRL 1489) and Menkes' (CRL 1230) cell lines (both male donors of similar age). Falcon Labware Tissue culture flasks and 24-well plates were obtained from Becton-Dickinson (Tokyo, Japan) and Nunc 96-well MicroWellModule Plates (with individually removable strips $6 \times (2 \times 8)$) from Nihon Intermed (Tokyo, Japan). The following chemicals, EDTA, NH_4NO_3, HNO_3, $NaHCO_3$ and $CuCl_2$, were purchased from Wako Pure Chemical (Osaka, Japan). Chelex was purchased from BioRad (Richmond, CA) and Hanks' solution, bovine serum albumin and Triton X-100 from Sigma (St Louis, MO). Barnstead quality water was used throughout.

Cell culture

Fibroblast cells were grown in filter-sterilized DMEM supplemented with 10% (v/v) FCS, 3.7 mg ml^{-1} $NaHCO_3$, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 5% CO_2 (complete medium copper concentration 21 p.p.b.). Culture medium deficient in copper (4.8 p.p.b.) was prepared by extensive ion exchange treatment of complete medium. Briefly, ion exchange resin (Chelex) was sterilized with 100% alcohol (1 ml to 1 g Chelex) followed by evaporation of the alcohol overnight in a sterile air stream. The sterile resin was then added directly to freshly prepared sterile medium (4% w/v) and the suspension was stirred for 10 days at 4 °C. The medium was then decanted, and all major and trace metals removed (except copper) were resupplemented using a sterile stock metal solution. Cells of passage number 8–20 were used. For cell propagation confluent cultures were harvested with trypsin (0.25% w/v) in Hanks' solution (pH 7.4) and resuspended in complete medium as a 1:2 or 1:3 split.

Growth at low medium Cu concentrations

Both cell lines were cultured in flasks (25 cm^2) for 7 days with copper-deficient medium. Cells were then re-subcultured (96-well plates, 1:3 split) either into fresh copper-deficient medium or into fresh copper-deficient medium to which the copper concentration had been restored to be the same as that of the complete medium (21.3 p.p.b.) by addition of $CuCl_2$. Cells were then cultured for a further 7 days. Total cellular protein was determined after three washes with Hanks' solution (200 μl volumes at 4 °C).

Fibroblast copper uptake

Studies on copper uptake over a 40 min period were performed on confluent cultures (day 6–8) *in situ* after replacement of old with fresh medium. For longer uptake periods copper was added 2 h after plating. Stock solutions of $CuCl_2$ were filter-sterilized before use. For medium copper concentrations below complete medium levels appropriate volumes of complete medium were removed and replaced by copper-depleted medium. At the times specified after copper addition/depletion strips (2×8 wells) were taken from the 96-well plates and placed on ice. Medium was removed by aspiration and cells were

washed three times with Hanks' solution (200 μl each at 4 °C, pH 7.4). Intracellular copper content of fibroblasts grown for 7 days (24-well plates) at different medium copper concentrations were determined after washing cells with 300 μl aliquots as above.

Intracellular copper determination

Cellular copper was solubilized *in situ* by addition of 0.5 M HNO_3 (20 and 100 μl per well for 96- and 24-well plates, respectively). After 15 min incubation at 4 °C the acid was removed and pipetted into 1 volume of modifier (0.5 M HNO_3, 0.5% Triton X-100 and 10% NH_4NO_3) prior to metal analysis. An atomic absorption spectrophotometer model Z 9000 (Hitachi, Tokyo, Japan) fitted with a graphite furnace utilizing Zeeman background correction was employed for copper measurement on sample aliquots of 10 μl volume. Triplicate measurements were made and the results averaged. Standards and blanks were included at regular intervals to determine baseline drift and enable compensation for minor fluctuations in sensitivity.

Cell protein determination

Acid-fixed cellular protein, measured to be 94% of total cellular protein, was estimated by a modification of the Bradford procedure (Vincent and Nadeau 1983). Briefly, protein was solubilized by addition of aliquots (20 and 100 μl per well for 96- and 24-well plates, respectively) of 0.125 M NaOH in 0.0125% v/v Triton X 100 followed by incubation at 70 °C for 5 min. After cooling Bradford reagent was added and absorbances measured at 600 nm with a microplate spectrophotometer (EIA Reader Model 2550, BioRad). Absorbance values were converted to concentration units using bovine serum albumin as standard.

Results

As measured by protein yield after 7 days culture (Figure 1a) and as confirmed using phase contrast microscopic examination of the two morphologically indistinguishable fibroblast culture types (results not shown) both control and Menkes' cultures showed similar growth characteristics over a medium copper concentration of more than three orders of magnitude. Nevertheless, differences were apparent at the extremes of copper availability, above 10 p.p.m. and below 20 p.p.b. copper. At toxic medium copper levels control cells appeared more tolerant to copper than Menkes' cells (Figure 1b). The medium copper concentrations required to reduce cell growth by half are ~ 23 and 18 p.p.m. for control and Menkes' fibroblasts, respectively. At medium copper levels below those present in the untreated medium (21.3 p.p.b.) the growth of Menkes' cells was apparently slightly less affected by the decreased copper availability than control cells.

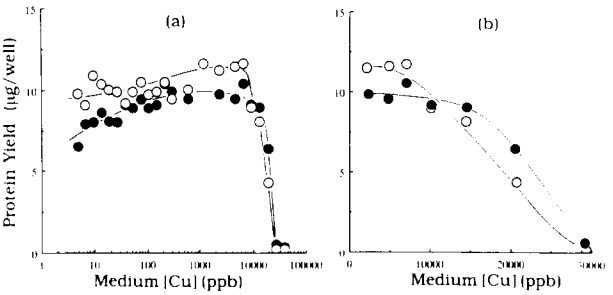

Figure 1. Fibroblast cells were subcultured (1:3 split) into 24-well plates. After 2 h copper was added from a sterile stock solution of $CuCl_2$. Cultures were further incubated for 7 days and cell protein/well was determined after washing cells three times *in situ* with Hanks' solution (4 °C) to remove medium copper. The diagrams show data plotted on a logarithmic *x*-axis scale (**a**) to illustrate trends across the entire concentration range studied and on a normal *x*-axis scale (**b**) to highlight copper toxicity at high copper concentrations. Control normal fibroblast cells (○); Menkes fibroblast cells (●).

After adaptation to low medium copper levels by 7 days growth of both cell types in reduced copper medium (4.8 p.p.b.) growth of Menkes' cells was 19.5% less at the end of the second subculture in the low copper medium compared with the same cells which had medium copper levels supplemented to normal levels in the second subculture (see Table 1). A statistical comparison (*t*-test) of 7 day growth means with and without addition of copper to 21.3 p.p.m. in the second subculture shows that the difference of 19.5% is statistically significant ($P < 0.01$). Under the same conditions the control cells showed only a 1.8% decrease in growth which is clearly not significant. The difference between the mean growth of Menkes' and that of control fibroblasts after two growth cycles of 7 days in DMEM/FCS medium with a very low copper concentration (4.8 p.p.b.) is also significant ($P < 0.01$). Thus, Menkes' fibroblasts are significantly more sensitive to prolonged exposure to physiologically low copper concentrations than normal control cells.

Fibroblast growth and medium copper concentration

Over the entire range of medium copper concentrations tested (4.8–20000 p.p.b.) the intracellular copper load of Menkes' cells was more than that of the control cells (Figure 2a and b) after 7 days growth in medium with depleted/supplemented copper concentrations. Only at very low medium copper concentrations (< 5 p.p.b.) did the intracellular

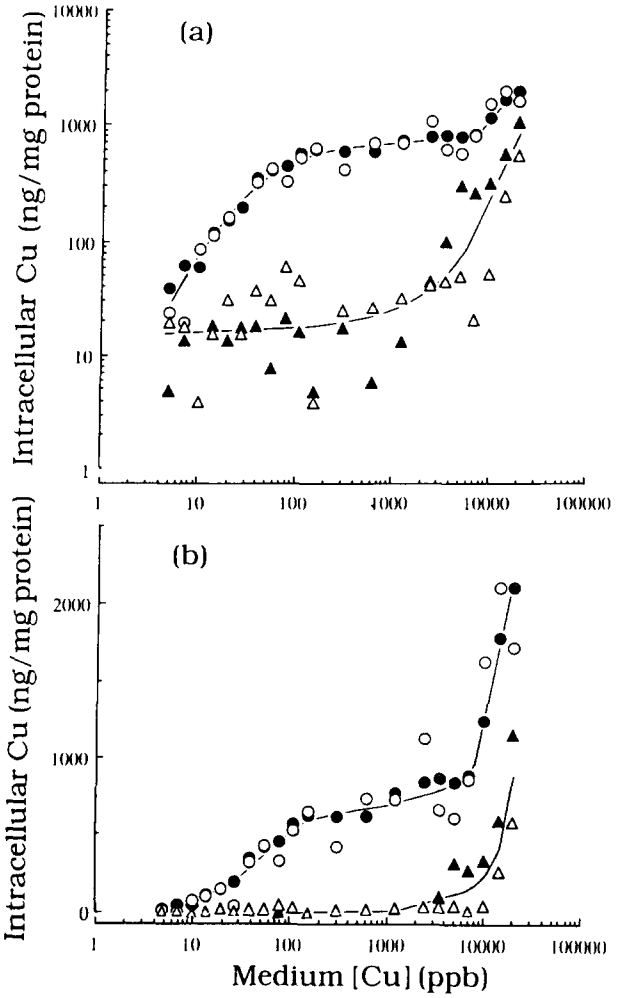

Figure 2. Cells were grown in DMEM/FCS medium in the presence of the copper concentrations indicated for 7 days. Intracellular copper was solubilized by the addition of 100 μl of 0.5 M HNO_3 at 4 °C. After 15 min incubation at 4 °C the acid was removed and analyzed for copper by graphite furnace atomic absorption spectrophotometry (see Materials and methods), protein was determined and copper content (ng mg protein^{-1}) calculated. Data are shown plotted on a logarithmic y-axis (**a**) clarifying trends in the intracellular pools not appreciable with a normal y-axis plot (**b**). Control normal fibroblast cells △ and ▲ (data set 1 and 2); Menkes' fibroblast cells ○ and ● (data set 1 and 2).

copper concentration of Menkes' cells begin to approach that of the control cells. A plateau of apparently stable intracellular copper contents is noted for both cell lines. For Menkes' cells the concentrations of copper at the lower and higher inflection points are ~ 0.08 and 8.0 p.p.m., respectively, giving a fairly stable region of internal copper content over two orders of magnitude of medium copper concentration. Although data for control

Table 1. Effect of two subcultures in DMEM/FCS medium with a very low copper concentration

Fibroblasts	Relative growth (based on cell protein per well) at medium copper concentrations of	
	21.3 p.p.b.	4.8 p.p.b.
Menkes'	100.0 ± 3.0% (6)	80.5 ± 8.1% (17)
Control	100.0 ± 15.2% (3)	98.2 ± 14.6% (13)

Following pre-culture of both cell lines for 7 days in medium containing 4.8 p.p.b. copper (in order to substantially reduce large copper pools present in Menkes' cells under normal growth conditions) each cell line was divided equally into two groups. The first group was subcultured into fresh medium containing 4.8 p.p.b. copper and the second group into the same medium supplemented with copper to complete medium levels (21.3 p.p.b.). After a further 7 days culture fibroblasts were washed three times *in situ* with Hanks' solution (200 μl, pH 7.4 at 4 °C) and cellular protein was determined (see Materials and methods). For each cell line growth of the cells from each group is expressed as a percentage of the growth achieved when the medium copper concentration was supplemented to 21.3 p.p.b. Values are mean ± SD (number of wells in each group).

cells are more variable an upper inflexion point of ~ 6 p.p.m. can be approximated; no lower inflexion point is determinable. The difference in intracellular copper content between the plateau regions of both cell lines is a factor of ~ 30.

Intracellular copper/zinc ratios

Both Menkes' and control cells displayed a similar trend in the maintenance of intracellular zinc/copper ratios (Figure 3). Basal intracellular zinc concentration was ~ 140 ng mg^{-1} protein. The intracellular zinc content was proportional to intracellular copper content above the basal zinc concentration. Although Menkes' fibroblasts clearly showed an elevated intracellular copper content at all medium copper concentrations tested (Figure 2), the relative intracellular zinc content of these cells showed a similar dependence on intracellular copper as the control cells.

Kinetics of copper balance

During incubation (40 min) of diseased and normal fibroblasts with elevated medium copper concentrations a time-dependent net accumulation of intracellular copper can be clearly seen (Figure 4). The kinetics of net uptake for both 1 and 20 p.p.m. medium copper concentration are superimposed on pre-existing intracellular copper pools. After 40 min incubation the total copper content of Menkes' cells was less than twice the initial copper content. Over

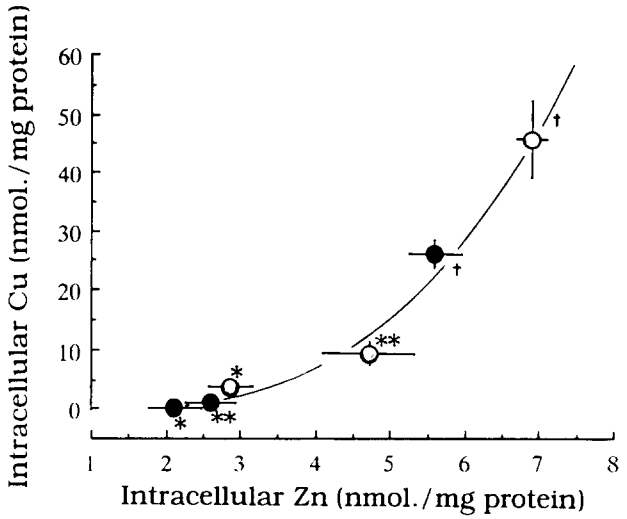

Figure 3. Fibroblasts were grown in DMEM/FCS medium with copper concentrations of 21.3 p.p.b. (*), 1.0 p.p.m. (**) and 20 p.p.m. (†) for 7 days. The intracellular copper and zinc contents of cells from individual wells were simultaneously determined from aliquots of 0.5 M HNO_3 pipetted into wells (previously washed with Hanks' solution) and incubated for 15 min at 4 °C. Corresponding cell protein was determined as described (see Materials and methods). Control normal fibroblast cells (●); Menkes' fibroblast cells (○).

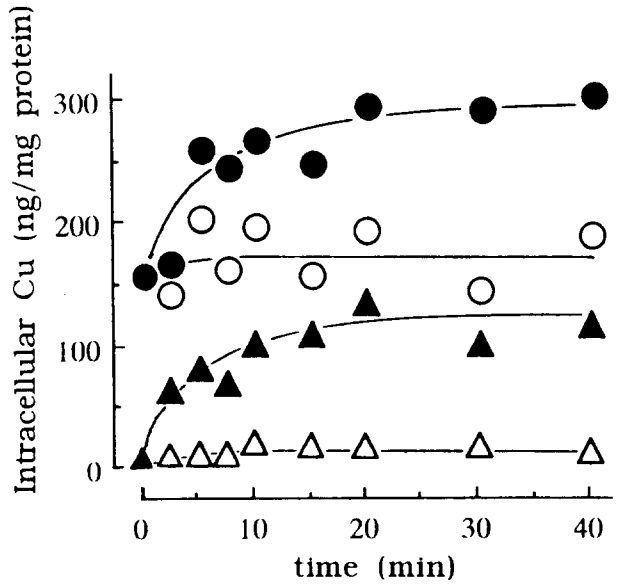

Figure 4. The medium from confluent cultures of fibroblasts (days 6–8 in 96-well plates) was replaced with fresh medium and the plates were temperature equilibrated in a water bath at 37 °C for 1 h. At the times indicated copper was added such that the incubation periods for each measurement were complete at 40 min at which time the plates were placed on ice. Cellular protein and copper were determined as described in Materials and methods. Control normal fibroblast cells △ (1.0 p.p.m.) and ▲ (20 p.p.m.); Menkes' fibroblast cells ○ (1.0 p.p.m.) and ● (20 p.p.m.).

the same time period control cells exhibited indistinguishable net uptake kinetics but achieved a relative increase in total intracellular copper of more than 10-fold. Nevertheless, even this relatively substantial copper uptake by control cells was insufficient to reach the internal copper levels of Menkes' cells before copper loading.

With incubation periods up to 7 days (Figure 5) differences in net copper accumulation between Menkes' and control cells became very clear at both 1 and 20 p.p.m. medium copper concentrations. In agreement with data presented in Figure 3 after 7 days incubation the difference in total cellular copper content between both cell lines is very pronounced at 1 p.p.m. (> 10-fold difference) and relatively small at 20 p.p.m. (~ 2-fold difference).

In the case of both short (0–40 min) and long (0–7 days) term copper uptake studies it is possible to accurately follow net copper uptake using graphite furnace atomic absorption spectroscopy for copper detection without the necessity for labeled copper isotopes. Additionally, important information with respect to pre-existing copper pools (of considerable consequence in both time domains but more important in short-term uptake studies) is by definition of the method accessible for analysis.

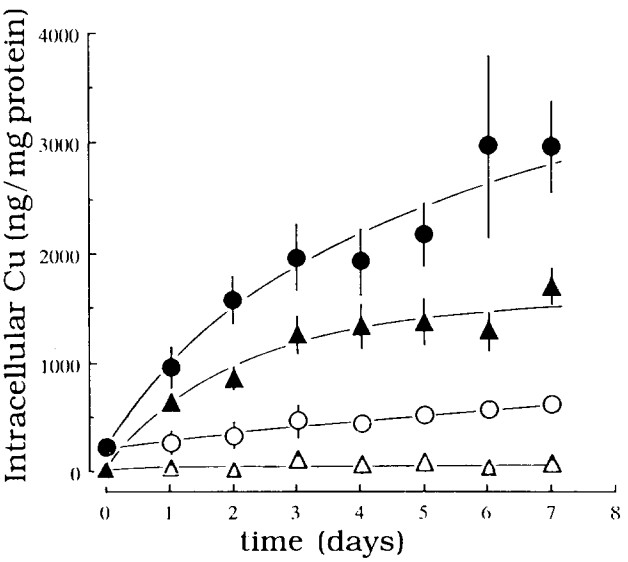

Figure 5. Two hours after subculturing cells into 96-well plates aliquots of sterile $CuCl_2$ were pipetted into the wells to give copper concentrations of 1.0 and 20 p.p.m. Each day up to 7 days strips were removed from the plates and put on ice. Protein content and intracellular copper were determined as described (see Materials and methods). Control normal fibroblast cells △ (1.0 p.p.m.) and ▲ (20 p.p.m.); Menkes' fibroblast cells ○ (1.0 p.p.m.) and ● (20 p.p.m.).

Discussion

Because of the extremely wide range of copper concentrations (at least four decades) in which both fibroblasts types were capable of growing (<4.8 p.p.b. to >20 p.p.m.) experiments had to be designed using logarithmically based increments in copper concentrations (Figures 1 and 2). This allowed identification of growth trends at the extremes of high and low copper availability as well as enabling elucidation of cellular response where copper levels were not growth limiting.

The growth characteristics of Menkes' and control cells at high medium copper concentrations (Figure 1) confirmed previous observations that Menkes' cells are slightly more sensitive to toxic levels of copper than control cells (Chan *et al.* 1978, Leone *et al.* 1985). However, the concentrations of copper involved (~ 20 p.p.m. in this study) are not physiologically relevant and the difference in sensitivity is not likely to account for clinical symptoms in Menkes' disease where copper deficiency not excess is suspected as the root of the disease (Danks 1989). Further, as Figure 2 shows, the upper inflexion point of ~ 7 p.p.m. for both cell lines (above which cells accumulate excess copper) confirms the similarity of response to high copper challenge.

Interestingly, under conditions of adequate copper supply Menkes' fibroblasts achieve a relatively stable intracellular copper pool albeit ~ 30-fold greater than their control cell counterparts (Figure 2). This plateau region of relatively stable internal copper content is observable over a 100-fold concentration change in medium copper (0.08–8.0 p.p.m.) clearly demonstrating that Menkes' cells can control internal copper status. The implications for Menkes' disease are that cells must receive an abundant supply of copper in order to achieve a stable copper content even though much above the requirements for non-diseased cells. Furthermore, Menkes' cells with an intracellular copper content the same as normal cells are copper deficient in as much as they are well below the plateau copper concentration. Similarly, the untreated DMEM/FCS medium (21 p.p.b.) is not capable of supplying Menkes' cells with sufficient copper to reach the 'desired' plateau level of intracellular copper content.

The less pronounced decrease in growth of Menkes' cells compared with control cells at the very low medium copper concentration end of the response continuum after 7 days culture was unexpected. This might, however, be explained by the additional intracellular pools present in Menkes' cells grown in untreated medium. One well of a 24-well plate with 300 μl medium with a copper concentration of 4.8 p.p.b. copper will contain ~ 1.4 ng of copper. Assuming a total of 10 μg cell protein in one well (Figure 1), an intracellular copper content for Menkes' cells of ~ 200 ng Cu mg^{-1} cell protein (Figure 2) and a 1:3 split, the addition of Menkes' cells to the medium will add more than 40% extra copper to the total well copper content. Bearing in mind the intracellular location of this copper the continued good growth of Menkes' cells (in comparison to the control cells) for short periods, i.e. for one subculture, at very low copper concentrations is perhaps not unreasonable.

To further examine fibroblast growth at very low medium copper concentrations without interference from high intracellular copper contents cells were pre-cultured for 7 days in medium containing 4.8 p.p.b. copper. This should result in a reduction of intracellular copper content for Menkes' cells of ~ 4-fold (Figure 2). Subsequent subculture of these Menkes' cells with highly reduced intracellular copper pools into medium with 4.8 p.p.b. copper revealed that Menkes' cells are significantly more sensitive to prolonged exposure to low medium copper concentrations than control cells treated identically (Table 1). Thus, long-term exposure of Menkes' cells to environments with very low copper availability will lead to impaired cellular function under conditions where the function of normal cells would not be affected, suggesting the clinical symptoms are aggravated by an increased sensitivity to low copper environments typically found in many organs of diseased patients.

As is clear from Figure 3 the intracellular copper/zinc balance of Menkes' cells is apparently normal, the zinc content being dependent upon the copper load above a basal zinc level. This dependence of zinc concentration on copper content might be explained by the presence of a high concentration of intracellular MT usually associated with Menkes' cells under normal medium copper conditions (Labadie *et al.* 1981, Riordan & Jolicoeur Paquet 1982, Leone *et al.* 1985) and, indeed, under the normal medium conditions (21.3 p.p.b. copper) used here (result not shown). Metallothionein is a protein with a well-documented avidity for and normally contains a heterogeneous mixture of heavy metals within its two metal cluster sites per molecule (Kägi & Kojima 1987).

In determining the kinetics of copper balance across the cell plasma membrane techniques using labeled copper should be carefully interpreted. Thus, as is clear from Figure 4, on addition of 20 p.p.m. copper (a concentration near the limit of

fibroblast tolerance for 7 days growth) copper uptake was superimposed on pre-existing intracellular copper pools. Even after 40 min incubation the intracellular copper content only doubled. Over the same time period that of the control cells increased by a factor of ~10. However, the kinetics of copper uptake and the total copper absorbed over the 40 min period was essentially the same in both cases. Since it is well documented that control and Menkes' cells are capable of effluxing copper (Beratis *et al.* 1978, Onishi *et al.* 1980, Packman & O'Toole 1984, Herd *et al.* 1987) a considerable amount of caution must be taken when analyzing results from kinetic studies performed with labeled copper. When intracellular copper pools are small, as in the case of control cells, an equilibrium between exogenously labeled copper and the internal copper pool may be fast but this does not necessarily hold true for Menkes' cells where copper pools are large (Figure 2) under normal growth conditions. Thus, the following assumption must be made cautiously:

$$*Cu_{(out)} \overset{\text{plasma membrane}}{\Leftrightarrow} *Cu_{(in)} \qquad (1)$$

This simple model does not take into account pre-existing volatile pools of intracellular copper. Copper MT in Menkes' cells could well provide such a pre-accumulated reservoir of copper available for exchange and efflux.

Determination of copper accumulated over an extended incubation period (7 days) with medium copper concentrations near the toxic limit of fibroblast copper tolerance (Figure 5) provides details of long-term kinetics for copper uptake. As can be predicted from Figure 2, the primary difference in accumulation is not at near lethal copper concentrations (20 p.p.m.) but at much lower concentrations (1 p.p.m.) where Menkes' cells accumulate large amounts of copper and control cells are able to maintain intracellular copper at low levels.

In conclusion the copper requirement of Menkes' cells is apparently so high that intracellular copper balance is only achievable when their internal copper pool is ~30-fold greater than that of control cells, resulting in the well-documented presence of MT (Labadie *et al.* 1981, Riodan & Jolicoeur Paquet 1982, Leone *et al.* 1985) and a significant increase (Figure 3) in intracellular zinc. Both MT and increased zinc are indicators of copper overload stress. However, cells display simultaneous copper deficiency in the form of low activity of certain enzymes (Rennert *et al.* 1980, Royce *et al.* 1980, Lui *et al.* 1982, Kodama *et al.* 1989) whereas other copper enzymes appear normal (Packman *et al.* 1984). This paradox of specific copper deficiency in the presence of apparent copper excess is the central biological enigma of Menkes' disease in which the use of non-radioactive copper has yet to play the significant role it deserves.

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