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# A non-radioactive method for measuring Cu uptake in HepG2 cells

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**Abstract**

At present, all data on Cu uptake and metabolism have been derived from radioactive uptake experiments. These experiments are limited by the availability of the radioactive isotopes $^{64}$Cu or $^{67}$Cu, and their short half-life (12.5 and 62 h, respectively). In this paper, we investigate an alternative method to study the uptake of Cu with natural isotopes in HepG2 cells, a liver cell line used extensively to study Cu metabolism. In nature, Cu occurs as two stable isotopes, $^{63}$Cu and $^{65}$Cu ($^{63}$Cu/$^{65}$Cu = 2.23). This ratio can be measured accurately using inductively coupled plasma mass spectrometry (ICP-MS). In initial experiments, we attempted to measure the time course of Cu uptake using $^{65}$Cu. The change in the $^{63}$Cu/$^{65}$Cu ratio, however, was too small to allow measurement of Cu uptake by the cells. To overcome this difficulty, the natural $^{63}$Cu/$^{65}$Cu ratio in HepG2 cells was altered using long-term incubation with $^{63}$Cu. This had a significant effect on Cu concentration in HepG2 cells, changing it from 81.9 ± 9.46 pmol μg DNA$^{-1}$ (week 1) to 155 ± 8.63 pmol μg DNA$^{-1}$ (week 2) and stabilising at 171 ± 4.82 pmol μg DNA$^{-1}$ (week 3). After three weeks of culture with 2 μM $^{63}$Cu the $^{63}$Cu/$^{65}$Cu changed from 2.18 ± 0.05 to 15.3 ± 1.01. Cu uptake was then investigated as before using $^{65}$Cu. Uptake was linear over 60 min, temperature dependent and consistent with previous kinetics data. These observations suggest that stable isotope ICP-MS provides an alternative technique for the study of Cu uptake by HepG2 cells.


*Keywords:* Inductively coupled plasma-mass spectrometry; Cu uptake; HepG2; Isotope dilution

## 1. Introduction

Cu is an essential trace element, acting as a catalytic co-factor in many critical enzymatic reactions. Cu metalloenzymes are required for normal oxidative metabolism, haemoglobin, elastin and collagen synthesis, free radical detoxification and iron metabolism [1,2]. As well as being essential, Cu can be extremely toxic, generating oxygen radicals by Fenton type reactions [3]. Any change in the regulation of Cu, therefore, can have severe physiological consequences. Therefore, it is important to understand the mechanisms of action and regulation of cellular components responsible for the acquisition, distribution and detoxification of Cu.

The liver plays a central role in Cu homeostasis and distribution. Hepatocytes, which are the primary site of Cu uptake and accumulation in the liver, utilise at least one pathway for Cu uptake. This pathway involves a reduction of $Cu^{2+}$ to $Cu^{1+}$ prior to uptake followed by transfer across the membrane through a classic carrier-mediated process [4]. Two candidates for such transporters have been identified as h*CTR1* and h*CTR2*, which have a $K_m$ for Cu in the low micromolar range. h*CTR1* has been shown to be ubiquitously expressed, with the highest levels being found in the liver and kidney [5]. In support of a major role for Ctr1 in mammalian Cu homeostasis, studies using human fibroblasts, transfected with h*CTR1*, showed a dramatic increase in capacity for Cu uptake. Ctr1 knockout homozygotes

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mice die in utero with severe developmental defects at the time of death, while heterozygotes have severely restricted tissue Cu level.

At present, all data on Cu uptake and metabolism have been derived from experiments with radioactive isotopes ($^{64}$Cu and $^{67}$Cu) [6–10]. However, these radioisotopes have inherent problems that include safety hazards, the legal requirements, storage and disposal. Further, their short half lives $^{64}$Cu or $^{67}$Cu (12.5 and 62 h, respectively) limit their use in long term experiments. Clearly, there is a need for a technique allowing the assessment of Cu uptake without the use of radioactive isotopes. We have developed a method using Cu stable isotope that circumvents the limitations of radiocopper studies and allows the accurate measurement of Cu uptake in HepG2 cells. The new approach, described here, takes advantage of the fact that the two stable isotopes of Cu naturally occur in a known ratio ($^{63}$Cu/$^{65}$Cu = 2.235). Thus, the cells and all cuprospecies within all kinetic compartments are uniformly labelled with this ratio. Experimentally, the $^{63}$Cu/$^{65}$Cu ratio can be measured accurately using inductively coupled plasma mass spectrometry (ICP/MS). The benefit of the isotopic ratio analysis is the ability to determine results based on the ratio value rather than the total amount of the measured metal. Therefore, we used this powerful technique as a tool to measure Cu uptake in HepG2 cells, a liver cell line extensively used for the study of Cu metabolism.

## 2. Materials and methods

### 2.1. Stable isotopes

$^{65}Cu_2NO_3$ (7.46 mg, 99.8% enrichment, AERE Harwell, Oxfordshire, UK) was dried in an oven (50 °C overnight) and dissolved in 2% v/v $HNO_3$ (Primar grade, Fisher Scientific, Loughborough, UK). The isotope purity of the $^{65}$Cu solution was determined accurately by ICP-MS using a Cu standard solution (Spectrosol, BDH) and the concentration verified by reverse isotope dilution mass spectrometry. The Cu histidine ([$^{65}$Cu]$His_2$) stock solution was made by the addition of 11 mg histidine into 50 mL of $^{65}$Cu (105 μM [$^{65}$Cu]$His_2$ final concentration). The solution was adjusted to pH 7.0 with 1 M NaOH. The [$^{63}$Cu]$His_2$ ($^{63}Cu_2NO_3$ 99.7% enrichment AERE Harwell, Oxfordshire, UK) stock solution was made according to the same procedure with a final Cu concentration of 5 mM.

### 2.2. Uptake of $^{65}$Cu by HepG2 cells

#### 2.2.1. Cell culture

HepG2 cells were obtained from the European Type Culture Collection. They were cultured in William's E medium (Gibco, Life technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco, Life technologies, Paisley, UK), penicillin and streptomycin (Gibco, Life technologies, Paisley, UK) using standard aseptic techniques. Cells were maintained in continuous culture at 37 °C in an atmosphere of 20% $O_2$ and 5% $CO_2$. The medium was changed every 3 days and the cells subcultured every week. Experiments were performed on 90% confluent cells. In experiments where the cells were pre-loaded with $^{63}$Cu, cells were incubated in culture medium with 2 μM $^{63}CuHis_2$ for 1–3 weeks.

#### 2.2.2. Cu uptake experimental methods

Culture medium was removed and cells were washed 3 times with ice-cold BSS (136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 18 mM HEPES ((*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), pH 7.4). Following washing, 8 mL of BSS supplemented with $CuHis_2$ (final concentration as given in figures, 37 °C, pH 7.0) was added to each plate, incubated in a 37 °C water bath and removed after the required time. The cells were washed 3 times with ice-cold BSS prior the addition of pronase (1 mg $mL^{-1}$, 4 °C, 10 min). The cells were aspirated and centrifuged (1000*g*, 5 min at 4 °C). The pellet, representing surface bound Cu, was discarded, and the pellet, which contains internalised Cu, was resuspended in 8 mL of ultra-pure water. The pellet was disrupted by sonication and 0.5 mL of the disrupted cells was used for DNA quantification. The remaining cell homogenate was further spiked with 160 μL of a 1:50 dilution $^{65}$Cu (105 μM) and processed for the measurement of the $^{63}$Cu/$^{65}$Cu ratio.

### 2.3. DNA quantification

DNA was quantified according to published methods [11] using Hoechst dye 33258 (Polysciences Ltd, Northampton, UK) and calf thymus DNA as a standard. Samples, standard and Hoechst Dye were diluted appropriately with TNE buffer (10 mM Tris (tris-(hydroxymethyl)aminomethane)–HCl, 1 mM EDTA-(ethylenediamine tetraacetic acid), 0.2 M NaCl, pH 7.4). Equal volume of sample and Hoechst dye (2 μg $mL^{-1}$) were mixed on a 96-well plate (Microfluor black plates, Dynatech, Billinghurst, UK) and read at extinction 356 nm/emission 458 nm on a Fluorlite 1000 plate reader (Dynatech, Billinghurst, UK.)

### 2.4. Preparation of samples for analysis by ICP-MS and AAS

Cell homogenates were freeze-dried in a Savillex digestion vessel (Savillex Corporation, Minnesota, USA) and 1.8 mL of concentrated $HNO_3$ and 0.2 mL $H_2O_2$ (AristaR grade, BDH, Poole, UK) were added to each vessel. They were sealed, incubated overnight