A technique to improve the determination of copper metabolism in small animals using stable isotopes

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Overview

A technique to facilitate stable isotope tracer studies of Cu metabolism in small animals has been developed. The two stable isotopes of copper are naturally present in high abundance and the sensitivity of detection of either isotope when used as a tracer is therefore limited. The practicability of reducing the abundance of ^{65}Cu in rats by feeding a diet containing highly enriched ^{63}Cu was assessed. The aim was to increase the sensitivity with which isotope enrichment in rat tissues could be detected following injection of a ^{65}Cu tracer. Male rats were fed a diet containing ^{63}Cu from weaning up to 100 days post-weaning, and liver and muscle tissues were obtained at logarithmic time intervals. Isotope ratios of extracted and purified Cu were determined using thermal ionization mass spectrometry. The rat tissues showed a rapid rate of enrichment with ^{63}Cu over the initial 32 days. The enrichment rate subsequently diminished in an asymptotic manner up to day 100 when the % abundance of ^{63}Cu in the liver was 98.01%, as compared to 98.11% in the diet. Significant improvements in the sensitivity of enrichment detection on administration of a ^{65}Cu spike were predicted at 100 days and above. In a second study, three groups of rats were given a diet enriched with ^{63}Cu for 70 days and a further three groups received a non-enriched diet. Two of the three groups on each diet were injected with a spike of either 9 or 90 μg ^{65}Cu on day 70, and the liver Cu isotope ratios were analyzed 24 hours after tracer administration. Although, due to biological variation in the rate of tissue ^{63}Cu enrichment, no significant enhancement of detection sensitivity was found at 70 days, the data from the first study shows that very significant advantages would be achieved by 100 days. The technique of artificial enrichment with ^{63}Cu offers a way of extending the duration of a tracer study and reducing the amount of ^{65}Cu spike necessary for the study of Cu metabolism.

Introduction

Long-term studies of copper metabolism in man and animals are impracticable because the available radioisotopes, ^{64}Cu and ^{67}Cu, have half-lives

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Table 1 Nutrient composition of the semi-synthetic diet

Major nutrients	Amount ($g \cdot kg^{-1}$)
Casein	200
Sucrose	648
Arachis oil	100
$CaCO_3$	15
Na_2HPO_4	6.6
KH_2PO_4	15.7
KCl	1.1
$MgSO_4 \cdot 7H_2O$	5.1

Trace minerals	Amount ($mg \cdot Kg^{-1}$)
$Na_2SiO_3 \cdot 5H_2O$	755
$FeSO_4 \cdot 7H_2O$	249
$MnSO_4$	203
$ZnSO_4 \cdot 7H_2O$	176
$Cr_2(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$	48
KIO_4	1.69
NaF	5.50
NH_4VO_3	0.46
$NiCl_2 \cdot 6H_2O$	4.10
$SnCl_4 \cdot 5H_2O$	5.90
Na_2SeO_3	0.22

Vitamins	Amount ($mg \cdot Kg^{-1}$)
α-tocopherol acetate	200
Retinyl acetate	8
Caliciferol	0.25
Choline HCl	1000
Vitamin B_{12}	0.025
Menadione	5
Thiamine	10
Pyridoxine	10
Riboflavine	10
p-aminobenzoic acid	10
Nicotinic acid	30
Ca pentothenate	20
Folic acid	5
Biotin	5
Inositol	400

of only 12.7 and 61.9 hours, respectively. Moreover, the use of radioisotopes in humans is becoming ethically unacceptable and alternative methodologies using stable isotopes are now being used,[1,2] as in the measurement of Zn[3,4,7] and Cu[4-7] absorption in human subjects.

^{65}Cu tracer methods have been developed to study Cu metabolism in small[8] and large[9,10] animals. However, difficulties arise with the use of stable isotopes in long-term studies of Cu metabolism in animals and in man because the natural abundance of the two existing isotopes, ^{63}Cu and ^{65}Cu, is high (69.1% and 30.9%, respectively). This limits the sensitivity with which isotope enrichment can be measured, even when the less abundant isotope is used as a tracer. The detection of enrichment depends on the amount of tracer (spike) administered and the precision with which isotope ratios can be measured. A very large spike may induce pharmacological changes in copper metabolism, whereas isotope enrichment from a small physiological spike may be difficult to detect since a high degree of analytical precision is required. It follows that if the natural abundance of ^{65}Cu in animal tissues could be reduced artificially, the sensitivity of enrichment detection should be improved.

We decided to determine the practicability of reducing the abundance of ^{65}Cu in rat tissues by feeding the animals on a semi-synthetic diet in which the Cu salt supplement was added in the form of $^{63}CuSO_4$. Since the time required for the tissue abundance of ^{63}Cu to equilibrate with that in the diet could not be predicted accurately, we wanted to establish the rate of tissue enrichment from a diet containing this isotope. The second objective of this work was to assess the sensitivity with which enrichment from an injected spike of ^{65}Cu could be detected in the tissues of rats that had been fed a diet containing ^{63}Cu. Liver and muscle were selected for Cu analysis because the liver plays an important role in Cu metabolism, yet most of the body Cu is present in muscle. Moreover, there are considerable differences in Cu turnover in these tissues.

Methods and materials

Enrichment with ^{63}Cu

Thirty-six weanling male rats (Hooded Lister, Rowett strain) were divided at random into six groups of six animals and provided, ad libitum, with distilled water and the semi-synthetic diet shown in *Table 1*. $^{63}CuSO_4$ (United Kingdom Atomic Energy Authority (UKEAE), Harwell, UK; ^{63}Cu abundance, 99.76%) was added to obtain a nominal concentration of 5 mg $^{63}Cu \cdot Kg^{-1}$ diet. Rats were killed using ether at 0, 1, 3.2, 10, 32, 70, and 100 days from weaning. Liver and muscle (upper hind limb including the tensor fasciae latae, biceps femoris, and gluteus maximus) were excised taking care to remove any adhering fat and coagulated blood. Tissues were sealed in trace element-free polyethylene bags and frozen at −20° C.

^{65}Cu tracer study

A further 36 rats from the same weanling group were divided at random into two groups of 18 animals and were given the semi-synthetic diet containing either 5 mg $^{63}Cu \cdot Kg^{-1}$ as $^{63}CuSO_4$ or the same level of $CuSO_4$ with a natural Cu isotope ratio. The rats receiving the ^{63}Cu-enriched diet are referred to hereafter as the ^{63}Cu-fed rats, whereas the animals given the natural Cu will be referred to as the controls. At 70 days from weaning, when the animals weighed approximately 420 g, the ^{63}Cu-fed rats were divided at random into three equal sub-groups. The control rats were divided similarly into three sub-groups. The animals were sedated with ether, and two sub-groups of ^{63}Cu-fed rats and two control sub-groups were injected i.v. with either 9 or 90 μg of ^{65}Cu as $^{65}CuSO_4$ (UKAEA, Harwell, UK; ^{65}Cu abundance, 99.0%) in 0.9% saline. The remaining sub-groups were injected with saline only. The ^{65}Cu doses were equivalent to 1 or 10% of the total body Cu burden of the rats. After 24 hours, the animals were killed with ether and the liver dissected as described above.

Tissue preparation and purification of Cu

All acids were of highest available grade (AristaR grade, BDH Chemical Co., Poole, UK) and water was obtained from a double distillation system. Glassware was acid washed by soaking overnight in 50% HNO_3 followed by a rinse with concentrated HNO_3 and extensive rinsing with double distilled water. The tissues were defrosted at room temperature and samples of liver (5–20 g) or muscle (2–30 g) were transferred to clean borosilicate glass beakers and dried overnight in an oven at 107° C. 1–2 ml of HNO_3 were then added to the dried tissues and the beakers were placed on a hot sand-bath. Once charred, the tissues were placed in a muffle furnace at 450° C for 12 hours. Samples showing incomplete ashing were again heated on a sand-bath with acid and placed back in the furnace for a further 12 hours.

Samples ashed to a uniform white powder were dissolved in 1–2 ml of 6.0 M HCl. An aliquot was removed to determine the Cu concentration by atomic absorption spectrophotometry and the remainder was added to the bed surface of a 0.8 × 4 cm anion-exchange column (Bio-Rad AG 1-X8, Cl^- form, 200–400 mesh), which had been prepared by washing with double distilled water and with 6.0 M HCl. Unbound components were eluted with 5 × 2 ml of 6.0 M HCl, and the bound metals were separated by sequential elution with 5 × 2 ml of 4.0 M HCl, 5 × 2 ml of 2.5 M HCl, and 1 × 10 ml of double distilled water. Contrary to the resin manufacturer's specifications, Cu eluted in the 4.0 M HCl fractions and was separated completely from other metals such as Fe and Co. Fractions with the highest Cu concentrations were pooled and the Cu content was determined by flame atomic absorption spectrophotometry (flame AAS). The samples were then evaporated to dryness under heat lamps. Tissue Cu concentrations were typically 5 μg per g fresh weight of liver and 1 μg per g of muscle.

Contamination of samples by extrinsic Cu was evaluated by measuring the change in isotope ratio of 50 μg ^{65}Cu spikes that were taken through the complete preparation procedure from the charring and ashing stage.

Determination of Cu isotope ratios

The $^{63}Cu/^{65}Cu$ ratios were measured by thermal ionization mass spectrometry (TIMS) using a VG 354 instrument equipped with 5 Faraday and one Daly collector. Single rhenium filaments, pre-cleaned at 5 A for 15 min, were loaded with sample using a silica gel technique. The filaments were heated at 1 A and silica gel suspension (1 μl), 0.25 M H_3PO_4 (2 μl), and sample solution (1 μl, 2 μl.μg^{-1} in 0.5 M H_2SO_4) were evaporated on the filament in small drops. The first drop was allowed to dry, the remaining drops were applied just before the filament dried out. Fresh silica gel suspension was prepared on a regular basis by shaking pure silica (1 g) with distilled H_2O (15 ml) for a number of hours. After application of the last drop, the filament current was raised very slowly until it reached about 2 A at which stage acid fumes had been removed, but the filament had not reached red heat. The filament current was then turned down and the filament was ready for loading into the mass spectrometer. The magnetic field and collectors of the mass spectrometer were set with the ^{63}Cu, the more abundant isotope, on the axial collector and the ^{65}Cu on the high mass collector. All operations of the instrument were under control of the computer. The filament current was increased to 1.80 A over 10 min and, after holding for 5 min, the current was increased slowly until an ion beam of pre-determined strength was acquired. Following focusing and optimization of the beam, the $^{63}Cu/^{65}Cu$ ratio was measured about 20 times and recorded. This was repeated at increasing ion current, starting at 1×10^{-13} A and reaching 3×10^{-12} A at which current the most stable beam was obtained. A higher beam current could be achieved but this could lead to instability and short life-time of the beam. At a beam current of 3×10^{-12} A, the ratio was measured in blocks of 20 ratios until the sample was exhausted. The beam was re-focused and reset to 3×10^{-12} A following

each block of ratios. The overall mean of the ratio, with the exclusion of ratios outside 2 σ was corrected for mass discrimination using a correction factor (1.0059) calculated from the replicate analysis of copper sulphate solution.

Calculating the mass of isotope spike in the tissue samples

The mass of ^{65}Cu spike that was present in the tissue samples 24 hours after spike injection was calculated according to the method of Turnlund et al.[6] using the Equations 1 and 2:

$$M^s/M^n = \frac{W^s\,(A^n_{63} - R_{63/65} \cdot A^n_{65})}{W^n\,(R_{63/65} \cdot A^s_{65} - A^s_{63})} \quad (1)$$

$$M^s = \frac{F \cdot M^{AA}}{(1 + F)} \quad (2)$$

F = M^s/M^n
W^s = Atomic weight of spike
W^n = Atomic weight of naturally occurring Cu
M^s = Mass of spike in sample
M^n = Mass of naturally occurring Cu in sample
M^{AA} = Total mass of Cu in sample ($M^s + M^n$)
A^n_{63} = Natural atomic abundance of ^{63}Cu
A^n_{65} = Natural atomic abundance of ^{65}Cu
A^s_{63} = Atomic abundance of ^{63}Cu in spike
A^s_{65} = Atomic abundance of ^{65}Cu in spike
$R_{63/65}$ = Measured ratio of $^{63}Cu/^{65}Cu$

Statistics

Unless otherwise stated, the data were analyzed by a one-way ANOVA and Tukey's multiple comparisons.

Results

Determination of Cu isotopes

A distinct mass fractionation effect is observed in the termination of copper isotope ratios. For an enriched sample, the $^{63}Cu/^{65}Cu$ ratio will start higher than the expected 2.235,[11] usually at about 2.250, and fall to about 2.20. Because the starting point and the shape of this fall are not entirely reproducible, probably due to loading conditions and beam acquisition, samples were run to extinction, thereby effectively measuring all the ^{63}Cu and ^{65}Cu atoms in the sample. The average ratio should then be a true measure of the $^{63}Cu/^{65}Cu$ ratio. However, in replicate analyses (n = 63) of copper sulphate, a ratio of 2.222 ± 0.007 (1 σ) was obtained. This is lower than the expected 2.235 as some material is lost in the initial stages of beam acquisition and setting-up. Hence, the correction factor of 2.235/2.222 (1.0059) was used to correct measured ratios. For a normal distribution, the 2 σ range for the measurement of normal copper ratios would therefore be 2.221-2.249.

A copper sulphate sample was included with each batch of 14 determinations in order to check the overall performance of sample loading and instrument operation. A typical example of 10 determinations is given in *Table 2*, and shows that the performance of the procedure was satisfactory within the limits of about 0.5%, although there appears to be a bias in the mean ratio.

Samples were analyzed in duplicate and the average ratio used. Examples of individual analyses at different stages of enrichment are given in *Table 3*. The variability between individual rats on the same regime was much greater than the analytical variability, as shown in *Table 4* for six individual rats on the same regime. In view of the biological variation, the analytical precision achieved by this procedure was more than adequate for the technique employed.

Table 2 Replicate $^{63}Cu/^{65}Cu$ isotope ratios for $CuSO_4$

	63/65 ratio
	2.220
	2.234
	2.238
	2.232
	2.224
	2.245
	2.238
	2.200
	2.225
	2.224
x	2.228
SD	0.012
RSD	0.5%

Table 3 Examples of $^{63}Cu/^{65}Cu$ analytical replication for liver Cu in Study 1 (a) and Study 2 (b and c)

a) Enrichment of rats with ^{63}Cu

Time interval (days)	First determination	Second determination	Average
0	2.245	2.230	2.237
1	2.331	2.330	2.331
3.2	2.693	2.705	2.699
10	4.767	4.768	4.767
32	15.590	15.512	15.551
70	33.01	33.44	33.23
100	43.67	43.67	43.67

b) Administration of ^{65}Cu to ^{63}Cu-enriched rats (70 days)

^{65}Cu spike (μg)	First determination	Second determination	Average
0	34.21	34.27	34.24
9	16.12	16.08	16.10
90	3.562	3.552	3.557

c) Administration of ^{65}Cu to non-enriched rats

^{65}Cu spike (μg)	First determination	Second determination	Average
0	2.233	2.247	2.240
9	2.029	2.025	2.027
90	1.0998	1.0994	1.0996

Table 4 Example of overall variability (biological plus analytical) for six individual rats on the same regime (fed ^{63}Cu-enriched diet for 10 days)

Rat	63/65 ratio
1	4.77
2	4.14
3	3.92
4	4.19
5	4.17
6	4.66
$\bar{x}$	4.31
SD	0.30
RSD	7%

Table 5 Contamination by extrinsic Cu during the preparation and purification of liver and muscle Cu. The amount of contamination is expressed as a percentage of Cu in the liver or muscle samples from rats at the different sampling times

Sample time (days)	Liver (%)	Muscle (%)
0	0.151	3.18
1	0.132	2.72
3.2	0.173	2.29
10	0.210	1.22
32	0.100	0.458
70	0.074	0.228
100	0.065	0.220

Evaluation of contamination

Contamination of ^{65}Cu spikes by extrinsic Cu during the ashing and ion exchange procedure was computed from the change in Cu isotope ratio using Equation 1 to calculate M^x/M^n. For 5 replicate spikes, the amount of contaminating Cu ranged from 45.1–88.1 ng (mean = 57.2 ng). This amount of contamination was a small proportion of the total copper extracted from the tissues at each time interval in Study 1 (*Table 5*). The % contamination for liver was lower at weaning than at 10 days because although the quantity of tissue was small, the Cu concentration in livers of weanling rats is high. At the latter sampling times, the amount of starting material, and therefore the total amount of Cu, was much greater than with the younger weanling rats, which accounts for a steady decline in the value of % contamination above 10 days.

Tissue enrichment with ^{63}Cu

The enrichment of rat liver and muscle Cu with ^{63}Cu from the diet was very rapid up to day 32, at which point the abundance of ^{63}Cu was approximately 93% in the liver. The rate of increase in enrichment in both tissues thereafter diminished in an asymptotic manner. Enrichment of liver and muscle progressed at a similar rate up to day 10 but as the animals reached maturity, enrichment tended to be greater in the liver.

The measured abundance of ^{63}Cu in the ^{63}Cu-enriched diet was 98.11%, and non-linear regression analysis indicated a ^{63}Cu equilibration abundance of 98.01% for liver and 96.20% for muscle. At 70 days weaning, the abundance of ^{63}Cu in liver and muscle was 97.18% and 95.44%, respectively, equivalent to 98.21% and 99.15% of the equilibration values predicted from their regression curves. The relative standard deviation (RSD) of the mean

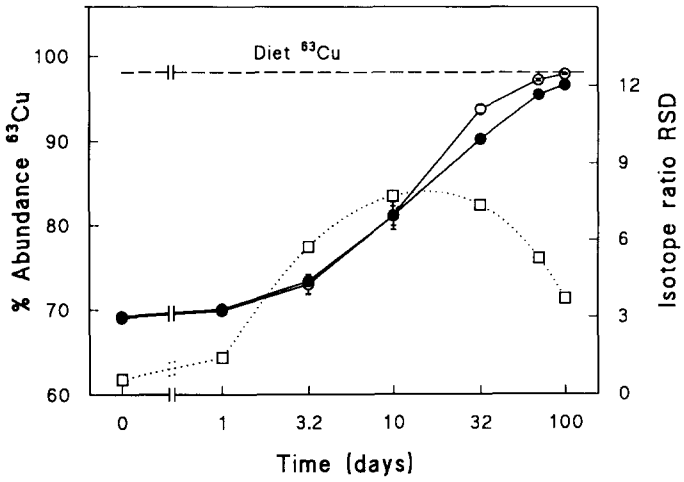

Figure 1 The change with time in the abundance of ^{63}Cu in the liver (○) and muscle (●) of weanling rats (0 days) given a semisynthetic diet containing 5 mg ^{63}Cu·kg^{-1}. The relationship of the liver Cu isotope ratio RSD with time is also shown (□). Percent abundance is presented as the mean ± SD.

isotope ratios for liver Cu increased with time to a maximum of about 7% at 10–32 days and thereafter decreased (*Figure 1*).

Tissue enrichment with ^{65}Cu

When ^{63}Cu-fed rats and controls were injected with a spike of 90 μg ^{65}Cu, the total Cu content of the liver increased by 6–8 μg after 24 hours, although this change was not significant ($P > 0.1$, *Table 6*). Injection of 9 μg of ^{65}Cu had no effect on liver Cu content.

The proportion of spike found in the liver of ^{63}Cu-fed rats was lower than that in the control animals (*Table 6*). However, it was not affected by the amount of ^{65}Cu injected.

Discussion

A high degree of tissue enrichment with ^{63}Cu, and therefore a very low abundance of ^{65}Cu, was achieved by feeding rats a diet containing 5 mg ^{63}Cu·Kg^{-1} for 70–100 days. The degree of enrichment in liver and muscle was initially similar but, latterly, was significantly higher ($P = 4.4 \times 10^{-6}$ at 70 days) in liver, perhaps reflecting the difference in Cu turnover between these tissues in adult rats. Nevertheless, the high abundance of ^{63}Cu attained in both tissues by 100 days shows that enrichment of rats by dietary means is a practical proposition.

In addition to isotope abundance, however, the sensitivity of enrichment detection following injection of a ^{65}Cu spike is dependent on the precision of isotope ratio determination. As seen in *Figure 1*, the ratio RSD for liver Cu changed quite considerably throughout this study. In contrast to

Table 6 Hepatic deposition of Cu, 24 hours after administration of a 9 or 90 μg spike of ^{65}Cu to non-enriched rats or animals enriched with ^{63}Cu

Rat group	^{65}Cu spike (μg)	Total liver Cu (μg)	% Abundance ^{65}Cu	Liver Cu from spike (μg)	% Spike in liver
Control	0	45.3 ± 8.2	30.62 ± 0.17	—	—
	9	43.7 ± 7.0	32.97 ± 0.29[a]	1.35 ± 0.25	14.9 ± 2.8
	90	51.0 ± 9.1	46.53 ± 1.41[a]	11.9 ± 2.2	13.2 ± 2.4
^{63}Cu-fed	0	45.1 ± 7.6	2.97 ± 0.46	—	—
	9	43.9 ± 10.8	5.32 ± 0.37[a]	1.04 ± 0.14	11.6 ± 1.6
	90	53.4 ± 9.5	22.23 ± 1.84[a]	10.8 ± 1.6	11.9 ± 1.8

[a] Significant differences from 0 spike by one-way ANOVA ($P < 0.05$).

elements of lower atomic mass, naturally occurring fractionation of Cu isotopes is negligible, and it can therefore be assumed that the isotope ratio RSD reflects a combination of manipulation and instrumentation imprecision. However, when an enriched isotope is administered to animals, a third source of error, biological variation, is introduced because the isotope may be metabolized at a slightly different rate in different individual animals. Thus, it is not surprising that the isotope ratio RSD for the rats in this study was found to increase up to about 7% when the animals were fed a diet containing ^{63}Cu for 10–32 days (*Figure 1*). As the abundance of ^{63}Cu in the rat tissues approaches that in the diet, the contribution of error from biological sources diminishes and ultimately disappears altogether when the tissue and diet isotope ratios are in equilibrium. In the current study, a rapid decrease in RSD above 32 days was associated with the approaching equilibration of diet and tissue isotope ratios and an RSD of 3.7% was recorded at 100 days (*Figure 1*). Although this RSD value was over 6 × that of the control, the sensitivity of enrichment detection following administration of a ^{65}Cu spike is also considerably influenced by the abundance of ^{65}Cu in the animal tissues. Improvements in sensitivity can therefore be assessed by calculating the proportion of spike ^{65}Cu:naturally occurring tissue Cu (M^s/M^n), which will change the isotope ratio by an amount that would achieve statistical significance. In the example below, we have chosen to determine M^s/M^n for a change in isotope ratio of 2 × SD, because this level of error is used commonly to identify significant deviations from the mean of normally distributed data. The data for the 100-day liver in Study 1 is used as an example:

Mean isotope ratio (^{63}Cu/^{65}Cu) for 6 rats = 46.487
Standard deviation of mean isotope ratio = 1.729
RSD = 3.72%
Reduce isotope ratio by 2 standard deviations = 46.487 − (2 × 1.729) = 43.029

A^n_{63} = 46.487/(46.487 + 1) = 0.9789
A^n_{65} = 1 − 0.9789 = 0.0211
$R_{63/65}$ = 43.029
W^n = (Atomic weight of ^{63}Cu × A^n_{63}) + (Atomic weight of ^{65}Cu × A^n_{65}) = (62.94 × 0.9789) + (64.94 × 0.0211) = 62.98
A^s_{63} = 0.0100
A^s_{65} = 0.9900
W^s = (Atomic weight of ^{63}Cu × A^s_{63}) + (Atomic weight of ^{65}Cu × A^s_{65}) = (62.94 × 0.0100) + (64.94 × 0.9900) = 64.92

The value of M^s/M^n can be calculated using Equation 1:

$$M^s/M^n = \frac{64.92 \times (0.9789 - (43.029 \times 0.0211))}{62.98 \times ((43.029 \times 0.9900) - 0.0100)}$$

$$= 0.0017$$

The equivalent value of M^s/M^n predicted for the control liver (Day 0) on the basis of starting isotope ratio of 2.232 and an RSD of 0.582% is 0.0038. It is therefore quite clear that the sensitivity of enrichment detection in 100-day, ^{63}Cu-fed rats has been improved twofold. Predictions of M^s/M^n for intermediate sample times have also been calculated, and the relationship between M^s/M^n and the duration of ^{63}Cu ingestion is shown by the "Empirical" curve in *Figure 2*. The "Theoretical" curve of *Figure 2* indicates the relationship that could be obtained in the absence of biological variation. In this case, the RSD for isotope ratios at all sample times would be the same as the control value, namely 0.582%. Assuming that all biological variation resulting from feeding ^{63}Cu to rats diminishes to zero as equilibrium is reached between diet and tissue isotope ratios, we can predict that the "Empirical" curve for M^s/M^n in *Figure 2* will eventually meet the "Theoretical" curve. This shows that further and substantial improvements in sensitivity are inevitable as the ingestion period for ^{63}Cu is increased above 100 days.

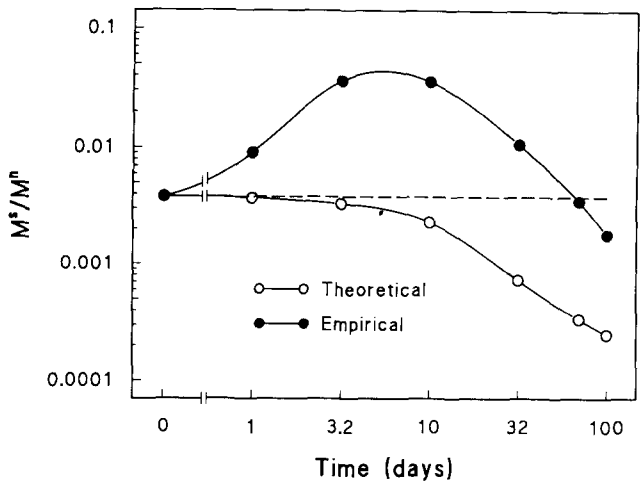

Figure 2 The proportion of spike to "natural" liver Cu (M^s/M^n) which would change the mean Cu isotope ratio by 2 standard deviations at each sample time. The theoretical values are calculated taking into account error from sources such as sample manipulation and analysis. Empirical values are based on measured ratio RSD values that include biological variation. The dashed line shows the control (0 day) level for (M^s/M^n), and points below this line indicate improved sensitivity of enrichment detection.

The results of the second study confirmed the high degree of enrichment of liver Cu with ^{63}Cu after 70 days on a ^{63}Cu-enriched diet. The ^{63}Cu/^{65}Cu ratio attained was 34.84 ± 2.81, and we were able to predict the M^s/M^n for both control and ^{63}Cu-fed, saline-injected rats, which would be required to decrease the mean isotope ratio by 2 × SD. The calculated values for control and ^{63}Cu-fed rats were 0.00512 and 0.00559, respectively. Assuming a liver Cu content of 45 μg and 12% as an approximate proportion of the total spike that would be recovered in the liver after 24 hours, we can estimate that 1.9 μg and 2.1 μg of ^{65}Cu, as an injected spike, is required to achieve a change in isotope ratio of 2 × SD in control and ^{63}Cu-fed rats, respectively. In practice, the administration of 9 μg of ^{65}Cu to ^{63}Cu-fed rats resulted in an almost 80% increase in the abundance of ^{65}Cu in the liver after 24 hours. This compares with only 8% in control rats. However, the greater variability in isotope ratios of saline-injected, ^{63}Cu-fed rats at day 70 compared to control animals eliminated any advantage obtained by artificial enrichment with ^{63}Cu. The benefits of artificial enrichment are increasingly apparent over and above 70 days, when the isotope ratio RSD decreases towards control levels.

The total liver Cu was elevated in both control and ^{63}Cu-fed rats given a 90 μg injection of spike, although this increase was not statistically significant ($P > 0.1$). The amount of spike that was recovered in the liver was, not surprisingly, proportional to the dose of spike administered. However, when expressed as a proportion of the injected spike, some differences between control and ^{63}Cu-fed rats were apparent. The proportion of spike recovered in the liver was lower in the ^{63}Cu-fed rats than in control animals, although the difference was only marginally significant ($P < 0.1$). In addition, the precision of the estimate was improved in the enriched animals and was similar for both the 9 and 90 μg tracer dose.

The study shows that long-term artificial enrichment with ^{63}Cu can provide a practical solution, at least in small animals such as the rat, to the problems associated with the use of stable isotopes of high natural abundance. The isotope costs are not excessive and an average intake of 70–100 μg ^{63}Cu/day/rat resulted in a doubling of the total cost of the diet. The biological variation in the rate of artificial enrichment with ^{63}Cu is a disadvantage if Cu metabolism in the developing rat (up to 70 days post-weaning) is to be studied by this method. This problem could be circumvented either by using second generation ^{63}Cu-enriched rats or by using each animal as its own control. The latter option is an attractive one because by eliminating inter-animal variation, the value M^s/M^n would be

reduced to the theoretical value shown in *Figure 2*. The proportion of injected spike to total body Cu after 100 days artificial enrichment with ^{63}Cu could therefore be reduced by a factor of 15 as compared to that at 0 days. The value of this method would depend on obtaining sequential samples from the animals, which would favor the use of, for example, plasma in preference to liver. Earlier[12] and recent[8] studies indicate that the kinetics of Cu turnover in liver and in plasma are different, but the improvement in enrichment discrimination brought about by sequential sampling of individual animals would be evident in both liver and plasma.

Several micrograms of Cu are required for TIMS and since rat plasma contains only about 1 $\mu g\ Cu \cdot ml^{-1}$, this instrument would be unsuitable for the analysis of isotope ratios in plasma samples of <1 ml. However, inductively coupled plasma source mass spectrometry (ICP/MS) can be used to measure Cu isotope ratios using smaller amounts of metal. Indeed, in rats of normal Cu status, it would be possible to determine the isotope ratio of Cu from only 100 μl of plasma using this technique. The precision that can be achieved for the determination of isotope ratios of plasma or serum Cu by ICP/MS is reported to be <1% and generally <0.5%,[8,13] although it would be influenced by the amount of Cu available for analysis. The speed of ratio analysis by ICP/MS is also a great advantage and compares favorably with the slow sample throughput time using TIMS.

Although biological variability in the rate of ^{63}Cu-enrichment eliminates the potential advantage of artificial enrichment in the short-term, we conclude that significant advantages of this technique become apparent at 100 days when rats are fed a diet containing 5 mg $^{63}Cu \cdot Kg^{-1}$ continuously from weaning. The technique of artificial enrichment with ^{63}Cu offers a way of extending the duration of a tracer study and reducing the amount of ^{65}Cu spike necessary for the study of Cu metabolism in adult rats.

Acknowledgments

The authors wish to thank Dr. I. Bremner and Dr. B. McGaw for their critical review of the manuscript, and Mr. I. Nevison for statistical advice.

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