Development of the stable isotope tracer approach for studies of copper turnover in the rat and mouse

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The stable isotope tracer approach was explored for long-term investigations of copper turnover in the adult rat and mouse, with inductively coupled plasma mass spectrometry for isotope measurements. The isotopic measurement method permitted precision and accuracy of <1.0%, with an overall sample blank of <0.05 μg copper. Rats were fed a copper-deficient diet and deionized water with (+Cu) or without (−Cu) copper (20 μg/ml). Both groups underwent a single-day replacement of drinking water with 20 μg/ml of ^{65}Cu. Compared with the baseline isotope ratio ($^{65}Cu/^{63}Cu$) of 0.462 ± 0.002, blood plasma ratios for the +Cu group on days 2, 7, and 14 postdosing were 0.702 ± 0.021, 0.557 ± 0.004, and 0.474 ± 0.001, respectively. The corresponding data for liver were 0.652 ± 0.018, 0.560 ± 0.005, and 0.482 ± 0.001, respectively. For the −Cu group, respective plasma ratios were 1.580 ± 0.04, 0.917 ± 0.02, and 0.664 ± 0.01 for days 2, 7, and 14 postdosing, and the ratios for liver were 0.978 ± 0.02, 0.876 ± 0.04, and 0.739 ± 0.03. Mice previously made copper deficient to varying degrees were given a single-day replacement with the label. When the 24-hour postdosing isotope ratios in the livers of these mice were correlated with the activity of plasma ceruloplasmin, a negative correlation (r = −0.85) was observed. Isotope enrichment in both rats and mice was greater in the copper-deficient animals compared with the controls.

Keywords: Stable isotopes; copper; rat; mouse

Introduction

Studies of copper turnover requiring long-term observations with isotopic tracers have not been possible due to the short half-life of the two available radioisotopes (^{64}Cu, half-life = 12.7 hours; ^{67}Cu, half-life = 61.7 hours). The alternative approach, use of stable isotopes as tracers, has recently been explored in a number of studies with human subjects.[1,2] However, these studies have focused only on issues of absorption from the gastrointestinal tract.[1,2] There are no reports of whether this approach is feasible and useful for investigation of issues of copper turnover in animal models, a problem of uncertain outcome due, in large part, to the high natural abundance of the two stable isotopes of copper (^{63}Cu 68.4 wt%; ^{65}Cu 31.6 wt%).

We have examined the question of whether this approach can be successful, from a methodologic perspective, in both adult rats and mice. For this purpose, we have developed the method of inductively coupled plasma mass spectrometry (ICP-MS)[3] for precise measurement of the $^{65}Cu/^{63}Cu$ ratio in tissues and fluids derived from such experiments. We have also carried out preliminary experiments in both adult rats and mice to examine the resultant degree of isotopic enrichment following administration of a physiologic dose of enriched ^{65}Cu. These experiments have led to the following new developments: methodology of ICP-MS for animal model studies with stable isotopes of copper and the demonstration that the stable isotope tracer approach can be used effectively to investigate copper turnover in the rat and mouse.

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Materials and methods

The overall objectives of this feasibility study were (1) to develop a precise method for the measurement of the stable isotope $^{65}Cu/^{63}Cu$ ratio in tissues from animals, and (2) to explore the feasibility of achieving sufficient isotopic enrichment compared with the precision of the isotope measurement method over an extended period in small laboratory animals following the administration of physiologically relevant doses of highly enriched ^{65}Cu.

Two types of experiments were carried out. First, analytic methods were explored for precise and accurate measurement of the $^{65}Cu/^{63}Cu$ ratio. The aim was to achieve sufficient precision in animals, compared with isotope enrichment, while permitting a high enough sample throughout to make animal model investigations possible. The recently developed method of ICP-MS[3] was explored for the measurements. Second, three small experiments were performed in adult rats and mice to explore the degree of isotope enrichment that could be achieved and the initial time course of isotope distribution in liver and plasma.

Definitions

Natural copper consists of two stable isotopes: ^{63}Cu (68.4 wt%) and ^{65}Cu (31.6 wt%). Both are available (Oak Ridge National Laboratory, Oak Ridge, TN, USA) as highly enriched preparations (^{63}Cu: 99.9%, $0.8/mg; ^{65}Cu: 99.7%, $1.9/mg). In the present investigation, we used enriched ^{65}Cu as the in vivo label and ^{63}Cu as measure of organ natural copper. Following the accepted terminology with radiotracers, we have defined the following expression:

$$(\text{specific activity})_{65/Cu} = (SA)_{65/Cu} = {}^{65}Cu^*/Cu = 0.684[{}^{65}Cu^*/{}^{63}Cu] = 0.684(R_{65/63} - R^\circ_{65/63}) \quad (1)$$

where $(SA)_{65/Cu}$ is the number of micrograms of ^{65}Cu from the label ($^{65}Cu^*$) to the number of micrograms of natural copper in the organ; ^{63}Cu is the number of micrograms of ^{63}Cu in the organ; 0.684 is the natural abundance of ^{63}Cu (wt fraction); and $R^\circ_{65/63}$ and $R_{65/63}$ are the ratios of $^{65}Cu/^{63}Cu$ in the organ before and after the administration of label (wt/wt), respectively.

When measurement of the isotope ratios is carried out with ICP-MS, the measured ion beam intensity ratio (corrected for blank) ($I_{65,c}/I_{63,c}$) is not always numerically equal to $R_{65/63}$. This measured ratio is referred to as $MR_{65/63,c}$, and is then related to the true expected ratio ($R_{65/63}$, $R^\circ_{65/63}$) by use of stable isotope calibration standards.[3]

Instrumentation

The instrument used in these experiments, an Elan Model 250 system (SCIEX/Perkin-Elmer, Norwalk, CT, USA), was equipped with a Meinhard nebulizer (TR-30C, Meinhard Associates, CA, USA). The details of instrument operation have been given previously.[4] Prior to each experiment, we optimized the MS instrument settings (ion optics) and the ICP parameters for signal level using a solution of 0.05 μg Cu/ml. The ICP operating parameters used in these measurements were rf forward power, 1,120 W; nebulizer flow rate, 1.1 l/min; plasma and auxiliary flow rates, 11 and 2.3 l/min; and solution flow rate, 0.9 ml/min.

Chemicals

All chemicals used in this study were analytic reagent grade, purchased from various chemical supply houses, and used without further purification.

Enriched stable isotope ^{65}Cu (99.7 wt%) was purchased from Oak Ridge National Laboratory (Oak Ridge, TN, USA) as ^{65}CuO. The material was dissolved in a minimum amount (a few drops) of concentrated HCl and volume adjusted with deionized water to the desired level; the copper content was measured by atomic absorption spectrophotometry. This stock solution was used in all subsequent experiments. Stable isotope calibration solutions were prepared from this stock solution and atomic absorption elemental copper standards (EM Science, Cherry Hill, NJ, USA) by spiking appropriate concentrations of the latter with incremental levels of the former. This was done to achieve a series of standard solutions with a final copper concentration of 0.05 μg/ml, but with an isotope ratio ($^{65}Cu/^{63}Cu$, wt/wt basis) that varied over the 0.4616 to 4.499 range. These standards were used to convert the blank-corrected measured ion beam intensity ratios ($MR_{65/63,c}$) in samples to the expected true isotope ratio ($R_{65/63}$).

Chemical separation procedures

Three approaches to selective separation of copper from interfering species were evaluated: precipitation with the chelating agent ammonium pyrrolidindithiocarbamate (APDC),[5] APDC precipitation followed by ion exchange chromatography, and solvent extraction with 8-hydroxyquinoline.[5] The general procedures for these methods are given in *Figure 1*. These approaches evolved from observations of matrix-specific interfering molecular species present when the APDC procedure was used. The major interfering species were traced to the presence of variable levels of sulfur in different tissues.

Ceruloplasmin assays

Plasma ceruloplasmin (EC 1.16.3.1) activity was determined in the mice experiments on blood samples obtained from the retroorbital plexus. The assay was performed on plasma obtained from microhematocrit tubes using o-dianisidine as substrate at 37°C in 0.1 M sodium acetate (pH 5.5) containing 10 μM diethylenetriaminepentaacetic acid.[6]

Animals and diets

Adult male Fischer rats (F344, Charles River, Portage, WI, USA) weighing 258 ± 12 g (SD, n = 33) were used in one experiment. They were housed in polycar-

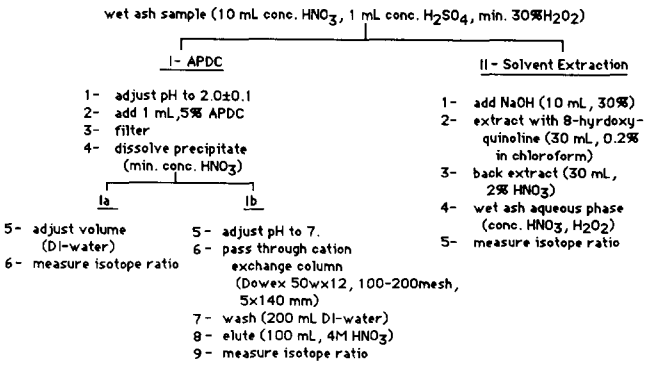

Figure 1 General scheme of sample preparation for measurement of the $^{65}Cu/^{63}Cu$ ratio with ICP-MS.

bonate cages with stainless steel wire bottoms and kept under standard lighting and temperature conditions. They were fed a purified diet ad libitum (TD80388, Teklad, Madison, WI, USA) with copper content (by analysis) 0.43 ± 0.04 μg/g. The composition of the diet (modified AIN-76A)[7] was sucrose, 500 g/kg; casein, 200 g/kg; cornstarch, 150 g/kg; corn oil, 50 g/kg; cellulose, 50 g/kg; mineral mix, 35 g/kg; and vitamin mix, 10 g/kg. Deionized water with or without copper (20 μg/ml) was supplied ad libitum.

Swiss albino mice, Hsd: (ND/4) (S)BR, purchased at weaning (Harlan Sprague Dawley, Indianapolis, IN, USA), were used in two experiments. They were fed, ad libitum, a nonpurified diet (Purina Mouse Chow, Ralston-Purina Company, St. Louis, MO, USA; [Cu] = 15.2 ± 1.4 μg/g) and deionized water (20 ng Cu/l) until used in the experiments, when they were fed the purified diet described above.

Results

Because of the two identified objectives of this investigation, the development of analytic chemistry of stable isotopes of copper and the feasibility of their use in animal studies, the results will be discussed in two separate sections.

Analytic chemistry of copper isotopes

The central issue with respect to the feasibility of applying the stable isotope approach to studies of the metabolism of copper in animals is the relationship between the expected level of isotopic enrichment following the administration of a physiologically relevant dose of ^{65}Cu and the precision with which the $^{65}Cu/^{63}Cu$ ratio can be measured.

The basic analytic performance data of the ICP-MS method for the two isotopes of copper are given in *Table 1*. The ion beam intensity data demonstrate that in solutions containing 0.05 μg Cu/ml, a minimum of 9,000 c/s could be obtained so that accumulation of the requisite 1×10^6 counts for the achievement of 0.1% measurement precision (relative standard deviation) could be met for total integration times less than 100 s. The routinely achieved precision in the isotope ratio was always <1.0%. The detection limit of the instrument was <0.5 ng of copper. When the instrument was operated continuously over an 8-hour period, the measured ratio was always within ±1 SD of the mean of the measurements. When stable isotope calibration standards containing about 0.05 μg Cu/ml but with varying isotope ratios over the 0.462 to 2.500 range were used, linear ($r^2 \geq 0.999$) calibration plots of $MR_{65/63}$ versus $R_{65/63}$ were obtained.

Because of the potential presence of interfering molecular species,[3] the reproducibility of isotope ratio measurements was explored in various tissues of rats. This was done for the three procedures that were developed (*Figure 1*). Results of these experiments are given in *Table 2*. These data demonstrate that neither the APDC nor the APDC-solvent extraction procedure was suitable for accurate isotopic analysis with respect to the wide range of matrices involved. For example, with the APDC procedure, the range of measured values was 0.463 (liver) to 0.544 (muscle) compared with 0.454 for the copper standard (second column, *Table 2*). The addition of an ion exchange separation step (third column) improved the results, but not to the necessary extent (same as precision of the method). Subsequent experiments (data not shown) indicated that sulfate interference at m/z = 65 may be responsible, possibly $^{33}S^{16}O_2^+$ and/or $^{32}S^{33}S^+$. Attempts to remove sulfate with precipitating agents like $BaCl_2$ were not entirely effective, and this led to the development of the solvent extraction procedure (fourth and fifth columns). The data obtained with the solvent extraction procedure indicated that the measured ratios for all matrices could be reproduced with a relative standard deviation of 0.4% (0.4639 ± 0.0018, *Table 2*), and the mean of all the measurements agreed with the value for the copper standard also to within 0.4%. When the precision (relative standard deviation of several independent measurements) and accuracy (deviation from the ratio measured for copper standard) were tested for plasma and liver from rats, the following values were obtained for $MR_{65/63}$: plasma (n = 9), 0.4662 ± 0.0027 compared with 0.4559 ± 0.0007 for copper standard; and liver (n = 11), 0.4656 ± 0.0024 compared with 0.4638 ± 0.0022 for standard. Liver and plasma samples were measured on different occasions so that the differences between them are related to instrument operating conditions. These data taken collectively demonstrate that precision and accuracy of the method was always better than 1.0%.

Finally, we investigated the issue of copper contamination arising from the overall procedure. This was done on three occasions over a period of 2 months. On each occasion, three complete blanks (complete procedure given for solvent extraction of *Figure 1* performed with 10 ml deionized water as sample) were prepared. Total copper content of the blanks was estimated by comparison with a solution of 0.05 μg Cu/ml using either of the two isotopes. The total copper content of the blanks on the three occasions was 0.045 ± 0.007, 0.037 ± 0.002, and 0.014 ± 0.006 μg Cu/sample.

Table 1 Data related to the sensitivity of inductively coupled plasma mass spectrometry for precise measurement of the $^{65}Cu/^{63}Cu$ ratio

Date	0.05 μg Cu/ml		$MR_{65/63}$	Deionized water		Detection limit (ng Cu)[b]
	I_{63}[a]	I_{65}[a]		I_{63}[a]	I_{65}[a]	
2/14/89	19,290	8,950	0.464 ± 0.002	20	10	0.5
1/23/89	28,040	13,080	0.466 ± 0.004	35	8	0.3
12/2/88	30,140	14,030	0.466 ± 0.002	22	11	0.2
11/3/88	35,920	16,730	0.466 ± 0.002	9	8	0.1
9/8/88	22,480	10,330	0.459 ± 0.003	85	48	0.6
8/5/88	103,170	47,350	0.459 ± 0.003	16	11	0.1
8/1/88	87,100	40,370	0.464 ± 0.002	64	26	0.1
7/5/88	22,850	10,550	0.462 ± 0.001	20	11	0.3

All data are the averages of 10 sequential measurements on the same solution. $MR_{65/63}$: ratio of I_{65}/I_{63} (mean ± SD for 10 measurements).
[a] Counts per second. A solution of 0.05 μg Cu/ml or deionized water was measured for ion intensities at m/z = 63 (I_{63}) and m/z = 65 (I_{65}).
[b] Detection limit was estimated as the copper content corresponding to $3\sqrt{B}$ where B is the ion intensity for I_{63} or I_{65} when deionized water was the analyte.

Table 2 Comparison of measured ratio ($MR_{65/63}$) in different biologic materials

Matrix	APDC—8/19/88	APDC-ion exchange—8/19/88	Solvent extraction	
			12/2/88	12/15/88
0.05 μg/ml	0.4540	0.4540	0.4658	0.4486
Liver	0.4628	0.4556	0.4648	
Red blood cells	0.5320	0.4689		0.4404
Kidney	0.4797	0.4553		
Kidney	0.4764	0.4556	0.4661	
Plasma	0.5011	0.4598		0.4437
Heart	0.4665	0.4557	0.4622	
Brain	0.4921	0.4574	0.4645	
Muscle	0.5444	0.4659	0.4620	
Mean ±1 SD			0.4639 ± 0.0018 (0.4%)	

Samples from each matrix (n = 3 to 5) were processed according to *Figure 1* using either of the three separation procedures. The resultant solution was analyzed for I_{63} and I_{65} and their ratio, $MR_{65/63}$.

Achieved in vivo isotope enrichment

Three experiments were performed to establish the degree of achieved isotope enrichment under physiologically relevant dosing conditions. In one experiment, adult rats were randomized into two groups (copper-restricted, −Cu; and copper-supplemented, +Cu). Both were fed the purified diet ad libitum for 4 weeks. The +Cu group received deionized water containing natural copper (20 μg/ml) during the experiment, except for the test day (day 7 of experiment). The −Cu group received only deionized water during the 4 weeks, except on the test day. On the test day, both groups received water containing 20 μg ^{65}Cu/ml, which they were allowed to consume ad libitum.

Three animals were killed on each of the following days: −7, 0 (prior to dosing), 2 (48 hours after start of dosing), 7, 14, and 21. Blood (7 ml) was collected and plasma was separated by centrifugation. The livers were removed intact. All samples were placed in acid-washed plastic containers and stored (−20°C) for isotopic analysis.

The results of isotope ratio measurements in plasma and liver for this experiment are summarized in *Table 3*. The data are given as $R_{65/63}$ and also as the ratio

Table 3 Observed changes in isotope ratio in adult rats given a single-day replacement of copper as ^{65}Cu

Time (d)	$R_{65/63,plasma}$	$R_{65/63,liver}$	$(SA)_{liver/plasma}$
+Cu group			
−7	0.461 ± 0.002	0.462 ± 0.002	
0	0.463 ± 0.002	0.460 ± 0.002	
2	0.702 ± 0.021	0.652 ± 0.018	0.80 ± 0.01
7	0.557 ± 0.004	0.560 ± 0.005	1.07 ± 0.07
14	0.474 ± 0.001	0.482 ± 0.001	1.96 ± 0.09
21		0.469 ± 0.001	
−Cu group			
−7	0.461 ± 0.002	0.462 ± 0.002	
0	0.463 ± 0.001	0.459 ± 0.001	
2	1.580 ± 0.04	0.978 ± 0.02	0.46 ± 0.02
7	0.917 ± 0.02	0.876 ± 0.04	0.92 ± 0.10
14	0.664 ± 0.01	0.739 ± 0.03	1.41 ± 0.21
21	0.544 ± 0.02	0.590 ± 0.04	1.67 ± 0.41

Adult rats were fed a copper-deficient diet and given either deionized water or 20 μg Cu/ml for 4 weeks. On day 7, both groups received a solution of 20 μg ^{65}Cu/ml instead of their drinking water. Three animals were killed at each indicated time: $(SA)_{65/Cu} = {}^{65}Cu^*/Cu = 0.6842 \times (R_{65/63} - R°_{65/63})$; $^{65}Cu^*$, organ content of ^{65}Cu originating from the label; Cu, organ content of natural copper; $R_{65/63}$, organ isotope ratio for $^{65}Cu/^{63}Cu$ (wt/wt); $R°_{65/63}$, baseline isotope ratio (wt/wt). Data represent mean ± SEM values (n = 3).

of the specific activity (Eq. 1) for liver to the corresponding value for plasma. The values of $R_{65/63}$ are mean ± SEM for group averages (n = 3); those for the specific activity ratios are the mean ± SEM of the individual ratios.

In the +Cu group, the isotope ratio in plasma increased by 52% of its baseline value at 48 hours. It then declined to 21% above baseline level at 7 days, and 3% at 14 days. The increments for liver were 41%, 21%, 4.6%, and 1.7% for days 2, 7, 14, and 21, respectively. The experimentally determined overall variation, including interanimal variability, was within the 0.2% to 3% range for the postadministration samples of plasma and liver, indicating a high level of reproducibility in organ retention of copper among the animals.

In the −Cu group, isotope ratio in plasma increased by 242% on day 2, and declined to 98%, 44%, and 18% above baseline value on days 7, 14, and 21, respectively. The increments for liver were 112%, 90%, 60%, and 28% above baseline for days 2, 7, 14, and 21, respectively. The overall reproducibility for each group was always better than 7% (liver on day 21).

Two experiments were performed with adult mice. In one, six 7-month-old animals were divided into two groups: −Cu (n = 4) and +Cu (n = 2). Both groups were fed the purified diet for 8 weeks. The −Cu group received deionized water (20 ng/l) while the +Cu group was given water containing 20 μg Cu/ml. At the end of the 8-week period, both groups were provided with deionized water containing 19 μg ^{65}Cu/ml for 24 hours. Blood was drawn into heparinized microhematocrit tubes under light ether anesthesia from the retroorbital plexus, and the animals were killed by cervical dislocation. The livers were removed and frozen for isotopic analysis. Two age-matched mice were fed the nonpurified diet and used for determination of baseline isotope ratios.

In the second experiment, six mice were fed the −Cu diet and deionized water for 7 weeks. At the end of this period, they were given ^{65}Cu-water as in the previous experiment. Blood was drawn both 24 hours before dosing and immediately before death for the measurement of ceruloplasmin activity. The livers were removed and saved for isotopic analysis.

In *Table 4*, we have summarized the results of both isotopic analyses and ceruloplasmin activity measurements for the two experiments with mice. The range of $R_{65/63}$ observed for the mice in both experiments was 0.650 to 0.821 compared with the ratio for baseline animals of 0.465 ± 0.009 (footnote of *Table 4*). The isotope enrichment data for these two experiments are given in the form of $(SA)_{65/Cu}$ (Eq. 1) to permit comparison between achieved isotope enrichment and ceruloplasmin levels. In the second experiment, there was a significant negative linear correlation ($r = -0.85$, $P < 0.05$) between the observed liver specific activity and the corresponding ceruloplasmin activity measured prior to the administration of the label. No estimate of ceruloplasmin activity was obtained prior to isotope administration in the first experiment. However, $(SA)_{liver}$ was higher for all −Cu mice compared with the two +Cu animals (*Table 4*).

Table 4 Observed changes in specific activity and ceruloplasmin activity in adult mice

Mouse No.	Treatment	$(SA)_{65/Cu,liver}$	Ceruloplasmin activity (U/l) Before	After
First experiment				
1	+Cu	0.154		36.6
2	+Cu	0.132		39.4
3	−Cu	0.233		15.2
4	−Cu	0.249		35.9
5	−Cu	0.226		20.8
6	−Cu	0.209		38.6
Second experiment				
1	−Cu	0.233	0	14.1
2	−Cu	0.244	0	19.9
3	−Cu	0.166	4.3	30.2
4	−Cu	0.160	25.5	33.7
5	−Cu	0.156	22.8	36.0
6	−Cu	0.141	28.6	43.1

Adult mice were fed a copper-deficient diet and given either deionized water or 20 μg Cu/ml for 8 weeks (first experiment) or 7 weeks (second experiment). They were then given a solution of 19 μg ^{65}Cu/ml instead of drinking water for 24 hours. Plasma and liver samples were analyzed for isotopes of copper. In the second experiment, plasma was also obtained prior to feeding ^{65}Cu. See Eq. 1 for definition of specific activity. Range of observed $R_{65/63}$: 0.650 to 0.821, R° = 0.465 ± 0.009. Treatment refers to the dietary procedures prior to ^{65}Cu administration.

Discussion

No previous report of the feasibility of using the stable isotope ^{65}Cu for the investigation of copper turnover in rats and mice is available. A suitable method is needed because of the lack of radioisotopes with sufficiently long half-life and ready availability for this trace element. The method of ICP-MS reported here, using the solvent extraction procedure, provides measurement precision and accuracy of about 0.5% in the measurement of $R_{65/63}$. When it is coupled with in vitro isotope dilution, which requires measurement of the ratio before and after spiking with ^{65}Cu, it can provide quantitative isotopic analysis with accuracy of better than 1.0%. This is at least as good as the previously reported methods of thermal ionization MS[2] or neutron activation analysis,[1] and offers the important additional advantages of high sample throughput,[3] less capital investment, and more common availability. This is especially important as ICP-MS instruments are now common in many laboratories focusing on general trace element analysis.

From the viewpoint of feasibility, the lower the achieved level of isotopic enrichment, the larger the uncertainty in the calculation of specific activity. This is an especially important consideration for copper due to the relatively high natural abundance of ^{65}Cu. The magnitude of this error can be readily calculated from the considerations of propagation of errors for the ex-

pression $(SA)_{65/Cu} = 0.684 \times (R_{65/63} - R°_{65/63})$. The expression describing the magnitude of the uncertainty is $[\sigma/SA = \{0.014R°/(R - R°)\}$; for $\sigma/R = \sigma/R° = 0.01R°]$. Under these conditions, when $R = 1.1R°$, the value of σ/SA is 0.14. For $R = 1.3R°$ or $1.5R°$, the respective values of σ/SA are 0.047 and 0.028. Therefore, for the case of 10% isotopic enrichment above baseline, the resultant uncertainty will be 14% if the isotope ratio measurement precision is 1%. For more precise measurements, this decreases appropriately. Thus, given the present state of the analytic development of this technique, viz., measurement precision <1.%, a minimum isotopic enrichment of 10% is required to permit investigations at the <14% level of uncertainty. The data given in *Table 3* show that in the +Cu group, this level was reached sometime between days 7 and 14 for both plasma and liver. The data for the −Cu group show that even after 21 days, the level of isotopic enrichment exceeded the minimum acceptable limit of 10%. Based on these considerations, we conclude that the level of dosing used in this experiment was satisfactory for the conduct of reasonably extended observations (>10 days) under both conditions of dietary copper intake. In fact, should the dose of ^{65}Cu given to the −Cu group be undesirably high, it could be reduced markedly without a major adverse impact on the overall uncertainties of the experiment.

The three animal experiments described were carried out to establish the feasibility of this approach. Some initial insight about turnover of plasma and liver copper was also obtained (*Table 3*). When data for $R_{65/63}$ were expressed in terms of $(SA)_{65/Cu}$, the peak $^{65}Cu*$ content of plasma for the +Cu group corresponded to 17% of its endogenous copper content. The corresponding value for liver was 13%. This decreased with time to approximately 1% for both compartments at 14 days. In the −Cu group, copper from the label constituted a much larger fraction of liver (35%) and plasma (77%) natural copper at 2 days. While the decline was also rapid, at 21 days after dosing, the label was still present at 6% to 9% of natural copper.

When data for $(SA)_{65/Cu}$ in the +Cu group of the rat experiment were plotted on a semilogarithmic scale, linear relations were obtained indicating first-order kinetics with a half-life of 2.8 days for plasma and 4.0 days for liver: for plasma, $\ln(SA_{65/Cu}) = -1.196 - 0.246t$ ($r^2 = 0.987$); for liver, $\ln(SA_{65/Cu}) = -1.642 - 0.173t$ ($r^2 = 0.990$). Since these animals were on constant copper intake, one may assume a constant value for plasma and liver copper contents. If this is the case, the calculated half-lives then reflect the half-lives for the disappearance of $^{65}Cu*$ from plasma and liver compartments. Weiss and Linder[8] reported the half-life of the disappearance of ^{67}Cu from liver to be about 3 days, similar to the whole body disappearance of 4.5 days. Their data indicate a half-life for disappearance of radioactivity present in the gel permeation fraction corresponding to ceruloplasmin of 3.5 days. Hickman et al.[9] injected a preparation of recrystallized native ceruloplasmin labeled with ^{64}Cu simultaneously with reconstituted sialic acid-1-^{14}C-ceruloplasmin into a male albino rabbit and determined half-lives of 56 and 54 hours, respectively. In contrast, when Marceau and Aspin[10] estimated the half-life of ^{67}Cu-ceruloplasmin obtained from donor rat plasma previously injected with $^{67}CuCl_2$, they obtained a value of 13 hours. This is similar to the half-life of 12 to 14 hours obtained by Holtzman and Gaumnitz[11] in rats injected with ^{14}C-holo-ceruloplasmin (compared with 6 hours for apo-ceruloplasmin). These discrepancies could be related to differences in the extent of reutilization of ^{14}C and radiocopper, effects of copper status of the animals, any species differences, or, potentially, the integrity of the labeled ceruloplasmin, as these and other factors may play important roles in the determination of the disappearance rate of radioactivity from plasma[12-15].

When these plots were constructed for the −Cu group of the experiment with rats, plasma-specific activity also decreased in a first-order manner (half-life = 5.2 days; $\ln(SA_{65/Cu}) = -0.097 - 0.134t$; $r^2 = 0.992$), but the pattern was clearly nonlinear for liver. Interpretation of the −Cu group is complicated by the fact that both natural copper and $^{65}Cu*$ decreased during the experiment. Therefore, it is not possible to address the question of whether copper restriction would have been responsible for the observed increase in the half-life of plasma-specific activity, as might be expected due to initiation of conservation mechanisms. Had we measured total copper contents of the samples, we would have been able to gain initial information about this important question.

When the data for liver-specific activity were referenced to the corresponding plasma value (*Table 3*), the nonuniform labeling of endogenous copper in the two compartments became apparent. In both groups, the ratio of specific activity for liver to plasma increased with time, indicating the expected rapid incorporation of label into ceruloplasmin and its export into the plasma compartment. At later times in the course of the experiment, the liver was more enriched compared with the plasma, indicating progressive incorporation of the hepatic label into nonceruloplasmin components of liver copper and the lack of its availability for export into plasma. This was the case for both groups (*Table 3*). The higher level of preferential liver enrichment for the +Cu group is consistent with the hypothesis that ceruloplasmin derives its copper from newly absorbed copper and that portion of liver copper is not available for incorporation into this protein.

Based on the measurement of ceruloplasmin activity, the adult mice in these two experiments were not as copper-deficient as young mice subjected to a similar treatment.[16] The copper requirement for adult mice is probably less than that for growing mice. There was a significant linear correlation between the observed liver specific activity $[(SA)_{65/Cu}]$ and ceruloplasmin activity measured in samples obtained prior to administration of the isotope ($r = -0.85$, $P < 0.05$). No estimate of plasma ceruloplasmin activity was obtained prior to the administration of the label in one of the experiments with mice. However, $(SA)_{65/Cu}$ was higher

for all of the animals in the −Cu group compared with the +Cu group (*Table 4*). Therefore, these preliminary data suggest that there may be a quantitative relationship between ceruloplasmin activity in plasma and copper status of the animal as determined by observations on the extent of plasma isotope enrichment, but considerable further research is required to establish the validity of this relationship.

In conclusion, this is the first report of the application of the stable isotope approach to studies of metabolism of copper in laboratory rodents. As these animal models are involved in the major portion of research conducted on this subject and no suitable radiotracers are available, the present alternative may be an attractive option. The data presented here clearly demonstrate that the stable isotope ^{65}Cu can be useful for investigations of the dynamics of metabolism of this element and that, in suitably designed experiments, it permits the conduct of reasonably extended observations. In addition, this approach has the major added feature of permitting investigations of the fate of administered label in reference to organ copper. This should then permit in-depth investigation of differential labeling of various copper compartments.

Finally, because of the need to administer the stable isotope ^{65}Cu at substrate levels, modifications are needed in the traditional designs of kinetic experiments based on the use of radiotracers. This may create difficulties in cases in which the needed dose levels perturb the steady state, as in certain aspects of copper deficiency experiments. However, in many instances, the appropriate modifications of experimental design should permit circumvention of such potential difficulties.

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