Supplementing Copper at the Upper Level of the Adult Dietary Recommended Intake Induces Detectable but Transient Changes in Healthy Adults[1]

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ABSTRACT The health consequences of mild copper excess in humans are unknown. In a previous study, 2 mo of supplementation with up to 6 mg Cu/L in drinking water did not induce detectable changes. Here we assessed a copper supplement at the upper level of dietary recommendations for "healthy" adults. The study was a prospective controlled trial; participants (women and men, 18–50 y old), represented the upper and lower 5% of the ceruloplasmin distribution curve obtained from a community-based sample of 800 healthy adults (n = 41/group, each ~50% men). Individuals received a single daily dose of 10 mg Cu for 60 d. Before and after supplementation, blood [copper, ceruloplasmin protein, homocysteine, liver aminotranferases, Cu-Zn -superoxide dismutase activity in erythrocytes (eSOD), and glutathione in peripheral mononuclear cells] and urine [copper excretion after a 5-h administration of a chelator 2,3-dimercapto-1-propano-sodium sulfonate (DMPS)] analyses were performed. After 2 mo, liver enzyme activities remained below the clinical cutoff value used to diagnose liver dysfunction, but had increased significantly in both groups and genders. These increases were no longer present 12 mo after the copper loading period was completed. Glutathione in mononuclear cells (mmol/g of protein) also increased after the 2-mo copper loading in both groups and in both genders (P = 0.01). eSOD activity, serum homocysteine concentration, and urinary copper excretion 5 h after DMPS administration were not affected. We conclude that copper administered as described induced a transient, mild, but significant elevation of aminotransferases.

KEY WORDS: • *copper* • *liver aminotransferases* • *super oxide dismutase* • *glutathione* • *humans*

We and others recently described aspects of the response to acute copper exposure (1–6), yet there is scant knowledge of the early effects of chronic exposure to higher levels of copper. The biomarkers of copper status available are efficient and helpful in diagnosing copper toxicity at high levels of exposure over a short time period because there are important clinical manifestations and typical changes in blood due to oxidative stress induced by copper. However, traditional markers (mainly blood biochemical and urinary indicators) of copper status are not sensitive enough to reveal small changes in copper status, which may be relevant for long-term health (4,5).

Concern about the limits of copper homeostasis arises first from self-administration of micromineral and vitamin supplements, which has become a common practice in Western countries (6). There is an anecdotal report of a 26-y-old man who required liver transplantation after self-administering 30 mg of copper daily for 30 mo and then increasing the dose to 60 mg/d for 1 y as a "performance enhancer" (7). Although concepts such as dietary recommended intake (DRI)[3] and upper level (UL) have been defined and recommend a daily allowance of 0.9 mg Cu and a maximum of 10 mg Cu/d (8), people do not necessarily follow this advice. Second, because the potential beneficial effects of supplemental copper on cardiovascular and bone health are currently under investigation (9–11), it is possible that copper supplementation at levels close to or above the UL may be proposed as a strategy for subgroups with polymorphisms that render them more vulnerable to copper deficiency.

In a previous randomized, controlled, double-blind study, we exposed apparently healthy adults to up to 6 mg Cu/L of water for 2 mo; based on the daily consumption of water, this represented exposures of up to 20 mg Cu/d (1). Copper was ingested at home during the day as plain water or taken as tea, herbal infusions, or soup. Under these conditions, traditional copper biomarkers, including serum copper, serum ceruloplas-

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[3] Abbreviations used: Cp, ceruloplasmin; DMPS, 2,3-dimercapto-1-propano-sodium sulfonate; DRI, dietary recommended intake; EAR, estimated average requirements; eSOD, superoxide dismutase activity in erythrocytes; GGT, γ-glutamyltransferase; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; PMNC, peripheral mononuclear cells; UL, tolerable upper limit of intake.

min (Cp; protein), and erythrocyte Cu-Zn superoxide dismutase total activity (eSOD) were not affected. The objective of the present study was to assess the effect of a copper supplement at the UL of the DRI in healthy adults, divided according to their serum Cp concentration before the initiation of copper exposure. The UL is a level of intake from food, water, and supplements that is unlikely to pose risks of adverse health effects from excess exposure over the long term in apparently healthy individuals, in an age- and sex-specific population group (8).

Dietary surveys to evaluate total daily copper intake from food and water in Chile revealed that 16.4% of men and 33.3% of women between 20 and 60 y old consumed less than the estimated average requirement (EAR) (12); thus, we considered that this group could have a differential response to copper supplementation. We hypothesized that the distribution of serum Cp concentrations in our subjects would depend on chronic copper exposure and that lower values would represent a chronic low level of exposure; we further hypothesized that the response of individuals would depend on their position on the Cp distribution curve.

SUBJECTS AND METHODS

Design and study groups. The study was a prospective, controlled trial in which individuals received a single daily dose of 10 mg Cu (8) for 60 d. Following the hypothesis that individuals with high and low serum Cp concentrations would respond differently to the copper UL, the study groups were formed on the basis of the highest and lowest deciles of Cp concentrations in a group of 800 apparently healthy individuals. Inclusion criteria were to be free of acute infectious/inflammatory processes [blood C-reactive protein (Array Protein System, Beckman Instruments) and whole blood count within normal], absence of anemia or known chronic illnesses, or drug treatment that could interfere with the study. **Fig. 1** shows the algorithm used to create the groups (*Panel A*) and the design and measurements performed (*Panel B*). Potential participants received detailed information about the protocol and those who agreed to participate signed an informed consent and formed the study groups: 98 individuals represented the ~5% higher and ~5% lower values in the Cp distribution curve. Subjects were 18–50 y and ~50% were >30 y old. Women were not pregnant and did not become pregnant during the study. Of the 87 individuals who finished the supplementation protocol, 82 had complete data for analysis. Supplementation consisted of two 5-mg copper gelatin capsules (as copper sulfate), administered under direct supervision 1 time/d, with plain tap water, between meals. Before and after supplementation, blood and urine studies were conducted. Dietary information was inferred from data obtained previously in the same population (12). Sample size was calculated using α error at 5% and power at 80%; 35–45 individuals per group were required to detect a Δ of 0.5 SD in the biochemical measurements that were planned. The protocol was approved by the Committee on Ethics for Human Research, INTA, University of Chile, which complies with the Helsinki Declaration. INTAs Committee on Ethics is certified by the Office for Human Research Protections (IRB00001493).

Serum and blood cell assays. As part of a long-term effort to identify markers revealing early yet biologically relevant changes in copper metabolism, under the conditions of this study, we tested serum copper and Cp (protein) as traditional copper indicators, liver aminotransferase activities as liver function indicators, and homocysteine, eSOD activity, and glutathione as potential indicators of early oxidative effects. Urinary copper excretion in response to a chelator was explored as an index of copper load, to define possible adverse effects of excess copper in terms of an excess labile copper pool that could be removed by chelation.

Serum copper was measured by atomic absorption spectrometry (Perkin Elmer Model 2280) and Cp protein by nephelometry (Array Protein System, Beckman Instruments). Liver aminotransferases glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and γ-glutamyltransferase (GGT) were determined in serum with routine techniques using commercial kit reagents from Boehringer Mannheim. Homocysteine concentrations were determined by an Abbott Kit (IMX system homocysteine Abbott Laboratories, Diagnostic Division). eSOD activity (total activity in erythrocytes) was measured using a commercial kit (Bioxytech SOD-525 Assay, OXIS International). Glutathione was measured in peripheral mononuclear cells (PMNC) using a Glutathione Assay Kit (Calbiochem, Cayman Chemical).

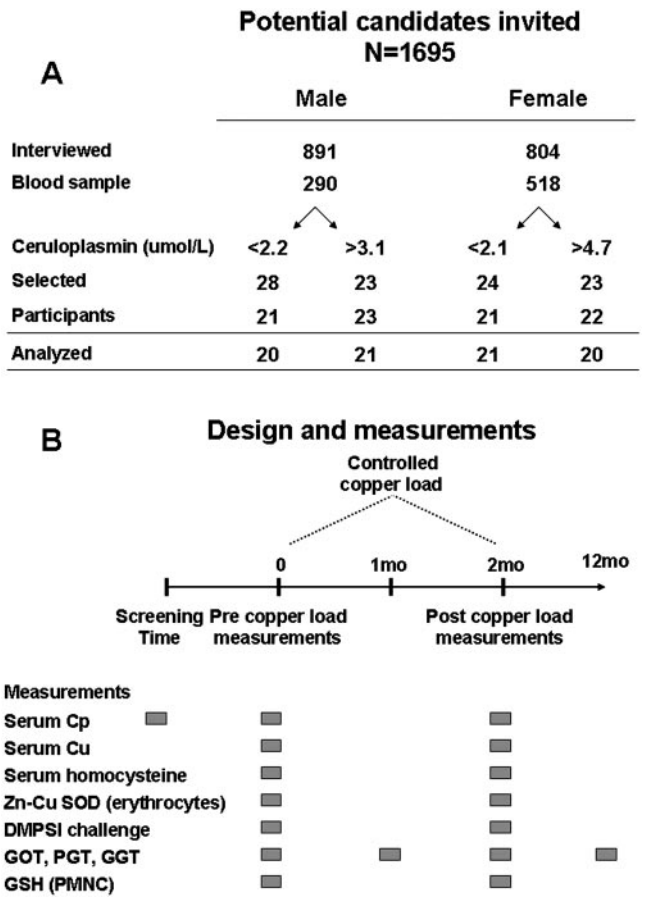

FIGURE 1 Flowchart used to form the study groups (*Panel A*) and the design and measurements performed at the different times of assessment (*Panel B*).

Two additional determinations were included in the protocol, i.e., serum Cp was measured immediately before supplementation started, giving information about the variability of Cp values between the time of screening and the beginning of supplementation (~6 wk later), and aminotransferase activities were also measured after 1 mo of supplementation.

Urine assays. Urinary copper excretion was measured before and after a chelator challenge, both before and after supplementation. An additional preliminary study using normal individuals and a patient with Wilson's disease was performed to establish an operational protocol for this challenge. The dose of 2,3-dimercapto-1-propano sodium sulfonate (DMPS, Dimaval®, Heyl Laboratory) used in adult patients with chronic arthritis or Wilson's disease ranges between 750 mg/d (250 mg 3 times/d) and 2000 mg/d (500 mg 4 times/d) (13–15). Based on results of this preliminary study, a single dose of 300 mg/individual and a 4-h urinary collection period were chosen to assess labile copper available for chelation and excretion in urine. After an overnight fast, individuals arrived at the clinical research facilities. Urine passed first thing in the morning (time 0) at home was kept in a special container provided by the researchers. After arrival at INTA, subjects urinated again and then were administered 3 capsules of 100 mg of DMPS in 200 mL of tap water. They remained

under direct medical supervision for the next 4 h, during which urine was collected. They then received a snack (chicken sandwich and a piece of fruit), after which they resumed their customary activities. Containers for urinary collection were trace element free. A 15-mL aliquot was obtained from each urinary collection and kept at −20°C. Urinary copper was measured by atomic absorption spectrometry (Perkin Elmer, Model SIMAA 6100). Creatinine in urine was determined with a method modified by Jaffé. Data are presented as μmol Cu/mol creatinine.

Statistical analysis. Data were analyzed for the 2 Cp groups (lowest and highest 5% of each decile) at different times and levels of exposure (time of screening, time before beginning copper supplementation; time when supplementation was completed and 12 mo after supplementation was completed). In some subjects, Cp concentration measured immediately before supplementation began differed from initial values (measured at screening) and was no longer within the lowest decile in the distribution curve. We assessed how this affected the definition of groups and the interpretation of results. In the group whose serum Cp category changed, GGT activity, but not that of other enzymes, was already different when the supplementation period began, thus explaining the significant differences for GGT activity in the high and low Cp groups at the time of initiating copper supplementation (U test; $P < 0.01$); these differences were affected by gender (U test; $P < 0.05$). Therefore, results were analyzed as originally planned based on serum Cp concentration and also after categorizing the data by gender. Statistical analysis was performed using SAS 8.02® for Windows (SAS Institute). The nonparametric U test was used to analyze each marker for time of exposure, using group as the independent category. A second analysis assessed Δ values between time of screening and the rest of the sampling times (before starting supplementation, immediately after finishing it, and 12 mo after the beginning of the protocol). Finally, we used 2-way ANOVA PROC MIXED for repeated measures to evaluate the effect of group (subjects with low and high Cp concentrations), the response to each treatment during the time of exposure, and the interactions between group and time of exposure. Because concentrations of glutathione had a skewed distribution, values are presented as geometric means and ±1 SD ranges.

RESULTS

Of the 98 potential candidates that represented the upper and lower 5% of the Cp distribution curve, 41 individuals of each gender finished the protocol. They remained healthy throughout the study period; none of the dropouts were due to adverse effects of copper and the characteristics of those who left the study did not differ from those who completed the protocol.

Serum copper and Cp concentrations differed between groups with high and low Cp and between men and women ($P < 0.001$, **Table 1**, ANOVA). The time of exposure was significant only for men ($P = 0.0013$, U test), and there was no interaction between group and time of exposure.

Liver enzyme activities (**Table 2**) did not change after 1 mo of controlled copper exposure. At the end of the 2-mo supplementation period, activities of the 3 enzymes remained below the cutoff value used for diagnosing liver dysfunction or disease (16), but all 3 had increased significantly in the 2 study groups in both women and men (ANOVA, $P < 0.001$). In those individuals identified as having higher GTT activity before copper supplementation, this activity remained significantly higher after 2 mo (U test, $P < 0.001$), but not 12 mo after copper loading was completed. There was a significant effect of time of exposure (ANOVA, $P < 0.001$), but there was no effect of Cp.

Glutathione in PMNC increased significantly after supplementation in the total study group and also in women and men when analyzed separately ($P = 0.01$). Glutathione concentrations [geometric means (± range of 1 SD)] were 5.6 (1.9–17.1) and 12.5 (7.6–20.8) μmol/mg protein before and after 2 mo of supplementation, respectively ($P < 0.01$). The 2 Cp groups did not differ. eSOD activity and serum homocysteine concentration did not differ before and after controlled copper exposure; eSOD was 77.3 ± 25.6 IU/g Hb (before) and 67.3

TABLE 1

Serum copper, Cp, and urinary copper excretion 5 h after challenge with a chelator (DMPS) in healthy women and men at the time of screening and before and after 2-mo of controlled copper exposure[1]

	Men		Women		
Percentile[2]	<5th	>95th	<5th	>95th	Significance $P < 0.001$
			%ile Cp		
n	20	21	21	20	
Serum Cu, *μmol/L*					
Screening	12.4 ± 1.9	19.0 ± 2.7	14.2 ± 3.8	31.8 ± 4.8	Cp,[3] by gender
Before	13.1 ± 2.3	17.7 ± 2.4	15.0 ± 2.5	27.5 ± 5.6	Cp,[3] by gender
After	13.9 ± 2.8	19.5 ± 1.9	16.0 ± 3.2	27.7 ± 6.6	Time, all groups[4]
Serum Cp, *μmol/L*					
Screening	1.92 ± 0.15	3.48 ± 0.31	1.97 ± 0.23	5.35 ± 0.54	Cp,[3] by gender
Before	2.10 ± 0.65	2.83 ± 0.34	2.21 ± 0.49	4.24 ± 0.85	Cp,[3] by gender
After	1.80 ± 0.29	2.56 ± 0.29	2.05 ± 0.42	3.77 ± 0.88	Cp,[3] by gender
Urinary Cu, *μmol Cu/mol creatinine*					
Basal (before)	0.07 ± 0.05	0.08 ± 0.06	0.07 ± 0.04	0.08 ± 0.05	
Basal (after)	0.05 ± 0.01	0.07 ± 0.06	0.11 ± 0.07	0.10 ± 0.03	
0–4 h (before)	1.14 ± 0.59	1.10 ± 0.45	1.29 ± 0.63	1.36 ± 0.69	
0–4 h (after)	1.09 ± 0.67	1.02 ± 0.41	1.48 ± 0.39	1.39 ± 0.52	Time, all groups[4]

[1] Values are means ± SD.
[2] Percentiles in a serum ceruloplasmin distribution curve obtained in 800 healthy adults.
[3] Nonparametric U test: $P < 0.001$ between low and high Cp, by gender.
[4] ANOVA repeated measurements: $P < 0.001$ considering all categories (Cp and gender) and times (screening, before and after copper supplementation). There were no interactions between groups and exposure.

TABLE 2

Serum liver enzymes activities in healthy women and men below the 5th percentile and above the 95th percentile of the serum Cp distribution curve, before and after Cu supplementation[1]

Enzyme	Sex	%ile Cp	*n*	Screening	After 1 mo	After 2 mo	After 14 mo	2-way ANOVA[2] $P < 0.01$
					μkat/L			
GOT	M	<5th	20	0.22 ± 0.02	0.26 ± 0.03	0.38 ± 0.03	0.28 ± 0.02	T*
		>95th	21	0.22 ± 0.02	0.24 ± 0.02	0.36 ± 0.03	0.26 ± 0.01	
	F	<5th	21	0.22 ± 0.02	0.24 ± 0.02	0.36 ± 0.03	0.26 ± 0.01	T*
		>95th	20	0.26 ± 0.03	0.24 ± 0.02	0.38 ± 0.03	0.28 ± 0.04	
GPT	M	<5th	21	0.25 ± 0.03	0.31 ± 0.03	0.45 ± 0.04	0.26 ± 0.03	T*
		>95th	20	0.27 ± 0.03	0.34 ± 0.03	0.43 ± 0.04	0.22 ± 0.02	
	F	<5th	21	0.27 ± 0.03	0.34 ± 0.03	0.43 ± 0.04	0.22 ± 0.02	T*
		>95th	20	0.27 ± 0.04	0.28 ± 0.02	0.43 ± 0.03	0.25 ± 0.05	
GGT	M	<5th	21	0.20 ± 0.03	0.24 ± 0.05	0.34 ± 0.06	0.29 ± 0.06	T*
		>95th	20	0.41 ± 0.08	0.35 ± 0.06	0.52 ± 0.08	0.40 ± 0.07	
	F	<5th	21	0.41 ± 0.08	0.35 ± 0.06	0.52 ± 0.08	0.40 ± 0.07	T*
		>95th	20	0.23 ± 0.04	0.27 ± 0.05	0.32 ± 0.05	0.35 ± 0.08	

[1] Values are means ± SD.
[2] Two-way ANOVA excluded values 12 mo after finishing copper supplementation. * Significant effects of time (T) are shown.

± 23.9 IU/g Hb (after) in the low Cp group and 83.5 ± 33.5 IU/g Hb (before) and 65.7 ± 21.3 IU/g Hb (after) in the high Cp group. The serum homocysteine concentrations were 8.3 ± 1.9 μmol/L (before) and 8.3 ± 1.8 μmol/L (after) in the low Cp group and 9.1 ± 1.9 μmol/L (before) and 9.1 ± 1.9 μmol/L (after) in the high Cp group. Urinary copper excretion after the DMPS test did not differ between groups or genders (Table 1). In the repeated-measure analysis, the effect of time of exposure was significant among women ($P = 0.0092$) but not men.

DISCUSSION

Presently, the WHO/FAO/International Atomic Energy Agency and the National Academy of Science/Food and Nutrition Board DRIs for trace elements and metals are 9 and 10 mg copper/d as the tolerable UL of intake from food, water, and supplements, respectively (17). The UL is not a precise estimate of safe, chronic copper doses in humans; rather, it is based on estimates derived from usual dietary exposure multiplied by a factor of 10, considered a reasonable default value in the absence of specific dose-response evaluations. As part of a continued effort to define the health consequences of copper deficit and excess, in this study, the provision of a UL equivalent taken as a single dose and between meals was intended to mimic the practice of ingesting supplements but also concentrated the exposure to a few hours rather than spreading it over the day, which would be more representative of the way in which most populations are routinely exposed if they are receiving copper-contaminated drinking water.

The main effect was a significant increase in the activities of 3 liver aminotransferases after 2 mo of controlled copper exposure with the specified dose and regimen. This increase was significant, but all enzyme activities were below the corresponding clinical cutoff values used to diagnose liver dysfunction, and participants did not exhibit symptoms or positive findings on physical examination suggestive of liver disease. Previous studies yielded no demonstrable effects at a similar copper daily dosage, but spread over the day (4). We hypothesize that the difference in this study may be due to the use of a single dose (bolus) taken on an empty stomach; this would favor prompt absorption in contrast with our previous study in which individuals occasionally ingested even larger amounts of copper (up to 20 mg/d) over the entire day, but mainly mixed with dietary components able to bind copper and thus diminish its bioavailability. We measured aminotransferases after 12 mo to confirm the transient nature of the observed elevation and were also able to verify normal liver function as well as no specific signs or symptoms of ill health 1 y after the exposure was completed.

Elevations of liver enzyme activities have been historically described in patients with hepatitis or liver damage of various origins. In a recent study of 12,808 Japanese male workers (18) who underwent an annual health checkup, GOT, GPT, and GGT were measured and analyzed for potential associations between liver function and lifestyle. Logistic regression analysis to explain differences in aminotransferase activities revealed that alcohol consumption, hypertriglyceridemia, and diabetes mellitus were related to elevated GOT, whereas elevated GPT was significantly related to obesity, sedentary life, hypercholesterolemia, and hypertriglyceridemia. We are not able to attribute the observed changes solely to copper intake because we did not evaluate the confounders described in the Japanese study; thus a possible interaction effect could account for our results. In addition, results recently obtained (unpublished) showed that indeed aminotransferases vary over time, but not with the magnitude observed in this study. Because we did not include a control group, a potential seasonal or time-related effect cannot be excluded. However, participants in our study had a normal complete blood count and C reactive protein and were apparently "healthy" based on clinical evaluation and physical examination.

It is difficult to interpret the changes in glutathione in peripheral blood cells because most data published refer to glutathione in the liver, an organ that plays a central role in the metabolism of this molecule. The increase of glutathione in PMNC may represent an adaptation of these blood cells to the extra copper. Glutathione prevents the adverse effect of copper excess by binding copper and thus protects various cell components from oxidative stress. In addition, glutathione forms a copper-glutathione complex that is excreted in bile

(the main route for excretion of copper from the body) (19,20). Decreased hepatic glutathione was described in several pathologic conditions including alcoholism (21) and symptomatic Wilson's disease (22). In patients with AIDS, decreased glutathione was reported in peripheral blood cells (23). Integrating these data with our results, our finding of a higher glutathione concentration could be interpreted as a compensatory response of peripheral cells to increased copper availability during the absorption process. Because glutathione is consumed when oxidative damage occurs, our finding of higher concentrations of glutathione after a 2-mo exposure to supplemental Cu suggests a response that is sustained over time. However, the lack of correlation between copper concentration in PMNC and glutathione concentration in the same cells (data not shown) suggests that this hypothesis is either not correct or that the increase in copper intake was within the range of homeostasis, and mechanisms existed that were able to handle the excess copper available.

DMPS, given at a dose that induced a dramatic difference in urinary copper excretion between the patients with Wilson's disease and normal volunteers in the preliminary protocol, did not significantly increase urinary copper in the study participants (data not shown). The 300 mg of DMPS as a single dose is sufficient to chelate the metal when tissue concentrations are elevated, as observed in patients with Wilson's disease (24). This dose is currently recommended for individuals suspected of suffering from chronic metal toxicity (25), e.g., individuals with potential mercury toxicity due to mercury from dental fillings (26–28). In our case, we chose an oral DMPS dose that was documented to be well tolerated by individuals suspected of mercury toxicity. The low dose in addition to the rather moderate copper load given to our subjects may explain the lack of an effect of DMPS on urinary copper excretion. Alternatively, the chronic exposure to copper may have led to copper sequestration in body pools that were less accessible to the chelator. The fact that the effect of time of exposure was significant among women and not among men in the repeated-measures analysis suggests that women may have a different response to DMPS, but present results do not permit further analysis.

In summary, copper administered as a single dose at the UL for 2 mo is associated with a transient elevation of serum aminotransferases and a rise in mononuclear blood cell glutathione. Studies comparing dose regimen as a single dose supplement vs. doses divided throughout the day and ingested with food are required to confirm whether the present UL for copper in human adults is indeed safe and whether the model to evaluate chronic safety should be a single-dose exposure or one distributed in multiple doses over the day, thus mimicking normal food and water consumption. Our results raise the possibility that single-dose supplements have the potential to induce effects with lower daily exposure; this may have implications for the safety of other metals such as iron and zinc.

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