PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 187, 38–43 (1988)

Copper Deficiency and Tissue Glutathione Concentration in the Rat (42634)

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Abstract. Copper deficiency in rats increased renal vein and arterial (heart) plasma GSH concentration by approximately 50%. There was no change in plasma GSSG concentration. Renal vein plasma GSSG/GSH ratio was decreased in copper deficiency, which is consistent with previous reports showing a copper-dependant thiol oxidase activity in the renal basement membrane. No change occurred in arterial plasma GSSG/GSH ratio. Hepatic GSH concentrations were also elevated by 50% in copper deficiency, GSSG concentrations were unaffected, but GSSG/GSH ratio was depressed. Renal and cardiac tissue GSH and GSSG were unaffected by copper deficiency. The decreased SOD activity and GSH-Px activity observed in copper deficiency may contribute to increased hepatic and plasma GSH concentrations.

Cellular antioxidant protection is an important area of biochemistry since many of the enzymes and metabolites involved in reactive radical metabolism are dependant on adequate provision of essential nutrients (1). For example Cu, Zn, and Mn are necessary for superoxide dismutase (SOD,[2] EC 1.15.1.1) activity (2, 3), Se for glutathione peroxidase (GSH-PX, EC 1.11.1.9) activity (4), sulfur amino acids for glutathione synthesis (5), and vitamin E for membrane protection (6). Recent studies have indicated an interrelation between some of the systems involved in reactive radical metabolism. For instance, Se-deficient rats have increased hepatic glutathione synthesis and total plasma glutathione (GSH +2GSSG) concentration, perhaps in compensation for the reduced GSH-PX activity of Se deficiency (7, 8). Dietary copper deficiency, in addition to depressing cytosolic SOD activity, results in a decrease in Se-dependant GSH-PX activity in liver and lung (9). These studies suggest that nutritional deficiencies may influence antioxidant protection both directly and indirectly. Copper has recently been shown to be a cofactor for the thiol (glutathione) oxidase of the basolateral region of the plasma membrane of renal and intestinal epithelium and may be important in regulation of the plasma thiol/disulfide ratio (10–12). Changes in tissue thiol, GSH being the major cellular and extracellular thiol (5), and in the thiol/disulfide ratio may be of general metabolic importance (10, 13). The present study was designed to examine the influence of copper deficiency on tissue glutathione concentration.

Materials and Methods. *Reagents.* GR (EC 1.6.4.2), GSH, GSSG, DTNB, NADPH, vitamins, choline–HCl, and triethanolamine were purchased from Sigma (Poole, Dorset, UK). Cu-Zn SOD (EC 1.15.1.1) was purchased from Miles (Stoke Poges, Bucks, UK). Dietary amino acids were obtained from Forum Chemicals (Reigate, Surrey, UK). Hydroxycobalamine was obtained from Glaxo Laboratories (Glaxovet, Harefield, Uxbridge, UK). 2-Vinylpyridine was obtained from Aldrich (Gillingham, Dorset, UK). Sucrose was obtained from Tate and Lyle (Bromley, UK), cod liver oil (BP grade) was from Boots (Nottingham, UK), and beef tallow was obtained locally. All other reagents were obtained from BDH (Poole, Dorset, UK).

Animals and diets. Twenty-four male weanling rats, of the Rowett Hooded Lister strain, were randomly assigned to a copper-

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[2] Abbreviations used: GR, glutathione reductase; SOD, superoxide dismutase; DTNB 5,5′-dithiobis-(2-nitrobenzoic acid); GSH-PX, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide.

0037-9727/88 $1.50

deficient group (8 animals) and a control group (16 animals) with equal mean weight (51.6 g). All animals were housed individually in plastic cages with stainless steel mesh flooring and provided with distilled water *ad libitum*. Animals were maintained at 22°C with a 12-hr light/dark cycle and were weighed weekly. Rats in the copper-deficient group were fed *ad libitum* a purified diet containing <0.2 μg Cu/g diet. Control rats were provided *ad libitum* the same diet supplemented to 10.0 μg Cu/g diet. After 28 days the control group was randomly divided into two groups, with equal mean weight, of 8 animals each—a pair-fed control group and an *ad libitum* fed control group. Pair-fed control rats were provided an amount of control diet equal, to the nearest 0.1 g, to the weight of food consumed by the member of the pair in the copper-deficient group; control diet was fed *ad libitum* to the *ad libitum* control group. The rats were continued on this feeding protocol for a further 3 weeks. The composition of the diet is shown in Table I, and meets or exceeds the recommendations of the National Academy of Sciences (14). The diet is based on Abdel Rahim *et al.* (15) and differs only in the protein (amino acid), carbohydrate, and lipid components.

Methods. Rats fasted for 12 hr were killed by exsanguination under diethyl ether anaesthesia on Days 50, 51, and 52. Blood samples were collected in acid-washed Beckman microfuge tubes (Beckman, Palo Alto, CA) containing 1 mg Na_4EDTA/ml blood. Renal vein blood, 1 ml, was obtained by cannulation of the inferior vena cava after clamping the vena cava above and below the points of entry of the renal veins. Following, renal vein sampling blood was obtained from the left ventricle of the heart by cardiac puncture and was assumed to be representative of arterial blood. The liver was flushed with 0.15 *M* KCl, 4°C, via the portal vein to remove the blood. Liver, heart, and kidneys were excised, frozen in liquid nitrogen, and weighed while frozen. Blood samples were promptly centrifuged for 1.5 min at 4°C in a Beckman microfuge to obtain plasma, which was deproteinized with 0.5 vol 10% (w/v) 5-sulfosalicylic acid. All assay solutions for plasma thiol and GSH determinations contained 5 m*M* EDTA. There was no detectable hemolysis in plasma samples. All plasma samples had less than 0.2% hemolysis as determined using the characteristic Soret band of hemoglobin (16). Plasma thiol concentrations were determined by the DTNB colorimetric assay at 412 nm (17). Total plasma glutathione (GSH +2GSSG) of deproteinized plasma was measured within 5 min of blood collection by the DTNB GR recycling procedure of Griffith (18). GSSG was measured following a 1-hr derivatization of GSH with 2-vinylpyridine (18), and GSH by difference. Care was taken to ensure that the concentration of 5-sulfosalicylic acid and triethanolamine was identical in samples and assay standards (19). Aliquots of liver, heart, and kidney were weighed and homogenized while still frozen in 0.2 *M* citrate, pH 5.0, 5 m*M* EDTA, and centrifuged. Supernatant protein was determined by the Biuret method. Liver and heart homogenates were deproteinized as described for plasma samples and GSH and GSSG were determined as described within 4 min of homogenization. Kidney homogenates were further homogenized in 0.5 vol 10% 5-sulfosalicylic acid in order to disrupt mitochondria, since renal tissue GSH is predominately of mitochondrial origin in contrast to liver and heart (20). GSH and GSSG of renal homogenates were determined as described.

Liver homogenate SOD activity was determined by the cytochrome reduction inhibition assay (21) and GSH-PX activity by the

TABLE I. COMPOSITION OF THE PURIFIED DIET

Component	g/100 g
Amino acid mix[a]	18.00
Sucrose	71.80
Beef tallow	3.50
Cod liver oil	1.50
Choline–HCl	0.10
Vitamin mix	0.12
Mineral mix	4.98

[a] The amino acid mix comprised the following (g): arginine–HCl, 6.0; asparagine, 4.0; monosodium glutamate, 30.0; histidine, 3.0; isoleucine, 5.0; leucine, 7.5; lysine–HCl, 7.0; methionine, 4.0; cystine, 2.0; phenylalanine, 5.0; tyrosine, 3.0; proline, 4.0; threonine, 5.0; tryptophan, 1.5; valine, 6.0; glycine, 5.0; alanine, 5.0; serine 5.0; aspartic acid, 5.0. The mix was based on the National Research Council, National Academy of Sciences, recommendations (14).

method of Paglia and Valentine (22). Diet and tissue Cu concentrations were determined by flame atomic absorption spectrophotometry following wet digestion with concentrated $HC10_4$, H_2SO_4, and HNO_3.

The DTNB GR recycling assay was found to have sensitivity in the nanomolar range while tissue glutathione was in the millimolar to micromolar range. Prior to GSSG determinations 2-vinylpyridine derivatization of GSH was complete by 30 min; GSH was undetectable in standard solutions following derivatization. Standard additions of either GSH or GSSG gave a recovery (mean ± SD, $N = 4$) of 102.3 ± 8.1%. Replicate analyses of rat liver gave a coefficient of variation of 5.8% ($N = 5$).

One copper-deficient rat died during the feeding protocol, and the blood samples from another copper-deficient rat were lost during processing.

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA), and the significance of differences between means by the least significant difference test.

Results. With the exception of rat weight, there was no significant difference in the measured parameters between pair-fed and *ad libitum* fed control animals. Growth of the rats was normal. Copper deficiency produced the characteristic significant cardiac hypertrophy (23). Heart weight as a percentage of body weight (mean ± SD) was 0.52 ± 0.09, copper deficient; 0.35 ± 0.02, pair-fed control; 0.33 ± 0.01, *ad libitum* fed control ($P < 0.05$ copper deficient in comparison to either pair-fed or *ad libitum* fed control).

Arterial and renal vein plasma thiol were not significantly affected by copper deficiency. Both renal vein and arterial plasma total glutathione and GSH were significantly elevated, by approximately 50%, in copper deficiency. There was no significant change in circulating GSSG. However, GSSG/GSH ratio of renal plasma tended to be lower in copper-deficient animals with a significant difference between copper-deficient and pair-fed control animals, though not between copper-deficient and *ad libitum* control animals. This trend was not evident in arterial plasma (Table II).

Hepatic total glutathione and GSH were increased by copper deficiency by approximately 50%. Hepatic GSSG concentrations were unchanged; however, GSSG/GSH ratios were significantly reduced in copper deficiency (Table III).

Cardiac and renal tissue total glutathione, GSH, and GSSG were unchanged by copper deficiency, and were between 16.0 and 25.0 nmole GSH/mg protein and 0.5 and 0.7 nmole GSSG/mg protein, respectively. Copper deficiency produced the expected reduction in hepatic copper concentration and cytosolic SOD activity, and also significantly reduced hepatic cytosolic GSH-PX activity (Table IV).

Discussion. The study indicates that dietary copper deficiency results in 50–60% increase in plasma and hepatic total glutathione and GSH concentrations. These increases cannot be attributed to food intake differences since total glutathione, GSH, and GSSG concentrations did not differ signifi-

TABLE II. RENAL VEIN AND ARTERIAL PLASMA THIOL, GSH AND GSSG AT 51 DAYS

	Copper deficient ($N = 6$)	Pair-fed control ($N = 8$)	*Ad libitum* fed control ($N = 8$)
Renal vein thiol (mM)	0.30 ± 0.03	0.26 ± 0.04	0.25 ± 0.05
Arterial thiol (mM)	0.32 ± 0.04	0.27 ± 0.08	0.33 ± 0.03
Renal vein GSH + 2GSSG (μM)	10.20 ± 2.21[a]	6.78 ± 2.30[b]	7.19 ± 1.63[b]
Renal vein GSH (μM)	9.35 ± 2.04[a]	5.82 ± 2.05[b]	6.30 ± 1.59[b]
Renal vein GSSG (μM)	0.43 ± 0.17	0.48 ± 0.17	0.45 ± 0.22
Renal vein GSSG/GSH (mole %)	4.62 ± 1.80[c]	9.04 ± 4.41[d]	7.48 ± 4.01[c,d]
Arterial GSH + 2GSSG (μM)	24.40 ± 11.22[c]	16.37 ± 2.95[d]	15.59 ± 1.59[d]
Arterial GSH (μM)	20.26 ± 7.57[c]	13.73 ± 2.67[d]	13.09 ± 2.41[d]
Arterial GSSG (μM)	2.07 ± 1.95	1.32 ± 0.33	1.25 ± 0.74
Arterial GSSG/GSH (mole %)	9.07 ± 5.30	9.97 ± 3.58	10.77 ± 9.52

Note. $\bar{X}$ ± SD. Means within a row with different superscripts are significantly different: [a,b] $P < 0.01$; [c,d] $P < 0.05$.

TABLE III. LIVER GSH AND GSSG AT 51 DAYS

	Copper deficient ($N = 7$)	Pair-fed control ($N = 8$)	*Ad libitum* fed control ($N = 8$)
GSH + 2GSSG (μmole/g wet)	2.41 ± 0.69^a	1.47 ± 0.32^b	1.60 ± 0.49^b
GSH (μmole/g wet)	2.28 ± 0.68^a	1.35 ± 0.30^b	1.46 ± 0.47^b
GSSG (μmole/g wet)	0.06 ± 0.02	0.06 ± 0.02	0.07 ± 0.02
GSSG/GSH (mole %)	2.84 ± 1.00^a	$4.30 \pm 1.66^{b,*}$	5.14 ± 1.44^b

Note. $\bar{X} \pm$ SD. Means within a row with different superscripts are significantly different, $P < 0.05$. Data were also calculated as nmole/mg cytosolic protein. The significance of the differences remained unchanged. Liver cytosolic protein was 68.5 ± 14.9 mg/g wet liver (mean $\pm$ SD), with no differences between groups.

* $P < 0.1$ and >0.05 in comparison to copper deficient group.

cantly between pair-fed and *ad libitum* fed control animals. A diurnal variation in hepatic glutathione content occurs in response to food intake, with maximum hepatic glutathione values occurring during the hours of feeding (24). In this study glutathione was measured following a 12-hr (overnight) fast, and hence dietary precursor sulfur amino acid flux would not be a contributing factor to glutathione synthesis. As a consequence of fasting the control liver glutathione values were approximately 50% of the values normally reported in fed animals (25).

Recent studies have indicated an interorgan cycle of GSH metabolism (5, 26, 27). The liver is the major organ of GSH synthesis and, owing to the lack of γ-glutamyl transpeptidase in hepatic tissue, GSH is mainly exported to the plasma (5, 27). Renal and other tissues utilize GSH from plasma via γ-glutamyl transpeptidase as a mechanism for amino acid delivery, and for intracellular processes that require GSH (5, 27). Renal tissue is the major site of plasma GSH removal, and accounts for approximately 50% of GSH removed from the plasma (5). The values obtained for renal vein and arterial (heart) plasma and hepatic GSH are in agreement with published values (5).

Apart from its involvement in kidney and intestinal basement membrane thiol oxidase (10–12), no role is known for copper in the synthesis and metabolism of glutathione (5, 27), and thus the experimental differences we have observed are difficult to interpret.

Recent studies have shown that copper deficiency, in addition to depressing cytosolic Cu-Zn SOD activity, decreases Se-dependent GSH-PX activity by an unknown mechanism (9). Decreased GSH-PX activity, as a consequence of dietary Se deficiency, has been shown to increase renal vein and arterial plasma GSH, hepatic GSH synthesis and turnover, and renal GSH clearance (7, 8). Hence, it is possible that the observed increases in GSH in copper deficiency may be secondary to reductions in Se-dependent GSH-PX. However, Se deficiency decreases liver GSH-PX activity by approximately 90% in comparison to controls (28), whereas these results and others (9) show that Cu deficiency produces an approximate 50% reduction. Furthermore, it has been shown that a small amount of dietary Se (0.03 μg/g)

TABLE IV. LIVER Cu, SOD, AND GSH-PX AT 51 DAYS

	Copper deficient ($N = 7$)	Pair-fed control ($N = 8$)	*Ad libitum* fed control ($N = 8$)
Cu (μg/g wet)	0.72 ± 0.23^c	2.71 ± 0.47^b	3.08 ± 0.46^b
SOD (U/mg protein)[c]	4.50 ± 1.26^a	11.84 ± 0.47^b	12.30 ± 2.20^b
GSH-PX (U/mg protein)[d]	0.79 ± 0.12^a	1.25 ± 0.13^b	1.22 ± 0.10^b

Note. $\bar{X} \pm$ SD. Means within a row with different superscripts (^{a,b}) are significantly different, $P < 0.05$.

[c] Cytochrome *c* reduction inhibition assay, 1 U = 50% inhibition of the uninhibited rate.

[d] Micromoles of NADPH oxidized, min^{-1} with H_2O_2 substrate.

will correct plasma GSH levels in Se deficient rats to those of control animals, but will not increase GSH-PX activity to control values (29).

Cu-Zn SOD plays a major role in the metabolism of oxygen-derived radicals (30) and dietary copper deficiency reduces the activity of this enzyme (2). Since these results, and others (9), show that GSH-PX is also reduced in copper deficiency a single nutritional deficiency compromises two antioxidant systems. The increased GSH concentration observed may be in compensation for this reduced antioxidant capacity, and perhaps suggests increased GSH synthesis in copper deficiency. GSH is an effective radical scavenger at physiological concentrations (30), and the decreased GSH-PX activity and SOD activity of copper deficiency may necessitate increased GSH for the metabolism of cellular free radicals. Increased O_2^- (superoxide) due to decreased SOD activity may increase $OH^\cdot$ (hydroxyl radical) concentrations via the metal-catalyzed Haber Weiss reaction (30), with concomitant increases in lipid hydroperoxides. No increases were observed in kidney and heart GSH in response to copper deficiency. However, the major fate of plasma GSH utilized by renal tissue is via γ-glutamyl transpeptidase delivery of amino acids, with concomitant GSH hydrolysis (5). Consequently, increased GSH utilization by renal tissue would probably not be detected by GSH-specific assays of this tissue.

Renal vein and arterial plasma GSSG comprised 4–10% of GSH concentrations, a value in good agreement with previous studies (5). Recent reports have shown a copper-dependent thiol (GSH) oxidase activity in the basolateral region of renal and intestinal epithelial plasma membranes (10–12). Because of the basolateral localization of this thiol oxidase it has been suggested that it functions to adjust the plasma GSSG/GSH ratio (10). The data (Table III) suggest an effect of dietary copper deficiency on renal plasma GSSG/GSH ratio, which is consistent with the role of copper in thiol (GSH) oxidation in plasma. No changes were observed in GSSG/GSH ratio of arterial plasma due to copper deficiency. This is perhaps not surprising since liver is the major organ contributing to plasma glutathione (as GSH) and copper-dependent thiol oxidase only occurs in renal and intestinal basement membrane (5, 10).

The GSSG/GSH ratio of hepatic tissue was decreased by copper deficiency. As far as is known intracellular thiol oxidation is not a copper enzyme-dependent process (10). Thus the observed changes in hepatic GSSG/GSH may reflect the suggested increase in GSH synthesis in copper deficiency. An increase in GSH synthesis would be expected to decrease GSSG/GSH ratios since hepatic tissue metabolizes GSSG by NADPH-dependent GR, or by expulsion from the cell when GSSG concentrations are high (5). Any increases in GSH oxidation to GSSG by radical-derived species due to the compromised reactive radical metabolism of copper deficiency cannot account for the decreased GSSG/GSH ratio of liver.

Hitherto the only consistent evidence of an involvement of dietary copper in the metabolism and oxidation of thiols has been provided by dramatic structural defects in the keratin of sheep wool (31) and the hair of infants with the genetic defect in copper metabolism, Menkes' disease (32). Although both lesions reflect a failure of disulfide bridge formation from cysteine during the maturation of prekeratin, neither the precise point of involvement of copper in this process nor the reasons for its extreme sensitivity to copper depletion are known (31, 32). Copper deficiency has been shown to produce several significant changes in lipid and carbohydrate metabolism in the rat (23, 33). The role of thiols, dithiols, and changes in thiol/dithiol ratios as modifiers of key metabolic enzymes have recently been reviewed (13). Since GSH is the major intracellular thiol, increases in hepatic GSH and changes in the GSSG/GSH ratio may offer an explanation for the perturbations in lipid and carbohydrate metabolism in copper deficiency.

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Received December 9, 1986. P.S.E.B.M. 1988, Vol. 187.
Accepted September 3, 1987.