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Effect of Zinc Deficiency on Plasma Glutathione in the Rat[1] (41687)

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Abstract. The objective of this study was to determine the effect of zinc status on plasma glutathione concentration. Immature male rats were fed a low-zinc (<1 ppm) diet based on soybean protein. Pair-fed and *ad libitum*-fed controls consumed a similar diet supplemented with zinc (100 ppm). The plasma concentration of total glutathione (GSH plus GSSG), as determined by the glutathione reductase recyling method of Tietze, decreased in all groups when plasma was allowed to stand in air at room temperature. At 5 min approximately 90% of the total glutathione was present as GSH and the measurable concentration of GSH decreased logarithmically during 60 min. Less than one-half of the loss was accounted for by conversion to GSSG; the nature of the remaining loss is unknown. The plasma of rats fed the zinc-deficient diet for 2 weeks contained less total glutathione than that of *ad libitum*-fed controls. There was no difference between zinc-deficient rats and pair-fed controls.

Glutathione occurs primarily within cells where its concentration ranges from about 0.5 to 10 m*M* with greater than 90% of the total existing in the reduced form (1). Zinc status has been shown to affect glutathione concentrations in tissues but there is no information relative to its effect on plasma glutathione concentrations. Hsu *et al.* (2) found elevated levels of reduced glutathione (GSH) in livers of zinc-deficient rats compared to *ad libitum*-fed controls under conditions in which the rats were fasted overnight before samples were taken. On the other hand, Hsu (3) reported a lower concentration of GSH in erythrocytes of zinc-deficient rats compared to pair-fed controls and this has been confirmed recently (4). Both zinc (5) and glutathione (6) have been implicated in membrane stability and zinc may be involved in the maintenance of GSH levels (7). Plasma zinc concentration drops rapidly when rats are fed a low-zinc diet and the sensitivity of platelets to aggregating agents is decreased within 7 days (8). Considering the fact that organs such as liver translocate substantial quantities of glutathione to plasma and there is a significant intravascular phase of glutathione metabolism (9), it is important to determine the effect of zinc status on the concentration of extracellular glutathione.

Until recently, plasma glutathione levels were reported to range from an undetectable level to about 5 μM (9, 10). Anderson and Meister (9) identified the probable cause of the reported low plasma levels when they discovered the rapid disappearance of measurable glutathione from the plasma. When the plasma was rapidly isolated and deproteinized, they found much higher levels (25 μM) than usually reported. The disappearance of measurable glutathione from plasma is not due to the oxidation of GSH to GSSG (9) because the enzymic method of analysis (11) measures both GSH and GSSG. Furthermore, it is not caused by GSH forming a mixed disulfide with plasma protein (9). Apparently GSH reacts with a low-molecular weight plasma component to form an unidentified compound. Glutathione in this complex is undetectable by the glutathione reductase recycling method, but borohydride reduction gives values comparable to those obtained in rapidly isolated and deproteinized plasma.

The form and concentration of glutathione in extracellular spaces may be significant in the maintenance of membrane stability and function. The purpose of this study was to determine the effect of zinc status on the concentration of glutathione in plasma. Since zinc deficiency depresses food intake in rats (12) and fasting lowers tissue glutathione levels (13), a restricted-food-intake control was in-

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cluded. The *in vivo* concentration and form of the total glutathione and the *in vitro* rate of GSH conversion to other forms were determined.

Materials and Methods. Reduced glutathione (GSH), oxidized glutathione (GSSG), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase (GR), triethanolamine (TEA), and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co., St. Louis, Missouri and 2-vinylpyridine (2-VP) from Aldrich Chemical Co., Milwaukee, Wisconsin.

Wistar strain male rats, 130–180 g, were divided into three groups and housed individually in wire-mesh stainless-steel cages. One group was fed *ad libitum* a zinc-deficient (<1 ppm Zn) diet based on soybean protein (14), a second group was pair-fed the same diet supplemented with zinc (100 ppm Zn as $ZnCo_3$), and a third group was fed the zinc-adequate diet *ad libitum*. The groups are referred to as Zn-deficient, pair-fed control, and *ad-lib* control, respectively, and were maintained on the diets for periods of approximately 2 weeks. All rats were given distilled deionized water *ad libitum*. Rats fed the zinc-deficient diet typically gained 27 ± 1.7 g (N = 5) during the first 2 weeks and failed to gain beyond that time. Pair-fed controls gained 34 ± 2.9 g and *ad libitum* controls gained 97 ± 1.7 g. The respective plasma zinc concentrations were 44.9 ± 1.1, 129 ± 7, and 132 ± 4.8 μg/dl. Zinc was determined by flame atomic absorption spectrophotometry on plasma samples diluted fourfold with water.

Except for preliminary experiments, plasma for glutathione determination was collected after the rats had consumed the diets for approximately 2 weeks. Unless otherwise indicated, the rats were anesthetized with sodium pentobarbital (50 mg/kg) and blood collected into a heparinized syringe from the distal aorta between 1000 and 1500 hr unless otherwise specified. Immediately after collection, the blood was centrifuged for 1 min at 8000*g* in an Eppendorf microfuge and the plasma allowed to stand at 25°C. Aliquots of the plasma (0.5 ml) were deproteinized at intervals of 5, 10, 20, 30, and 60 min by addition of 0.5 ml of 10% trichloracetic acid (TCA); protein was removed by centrifugation for 1 min at 8000*g*.

Total glutathione (GSH + 2GSSG) was determined by the DTNB-glutathione reductase recycling method of Tietze (11). GSSG was determined by Griffith's (15) 2-VP modification of the Tietze method. The 2-VP reacts with GSH so that the method measures only GSSG. GSH was calculated by difference.

For total glutathione determination, 100 μl of TCA supernatant was placed in a cuvette and the following reagents added: 3 μl of H_2O, 10 μl of neat TEA, 700 μl of 0.3 mM NADPH (in 125 mM phosphate buffer, pH 7.5, containing 6.3 mM EDTA), 100 μl of 6.0 mM DTNB (made fresh daily), and 10 μl of GR (150 units/ml). The reaction was followed by measuring the change in absorbance at 412 nm (ΔA_{412}) for 20 min; the slope of the curve was proportional to the total amount of glutathione (GSH + 2GSSG) present in the original sample. Plasma GSH concentration was calculated from standard curves which related the rate of ΔA_{412} to the concentration of pure GSH. These curves were prepared at the time of each assay.

For the GSSG determination, 100 μl of the TCA supernatant was placed in a small test tube followed by 10 μl of neat TEA and 3 μl of neat 2-VP. TEA was used to adjust the pH for optimal reaction between 2-VP and GSH. The tube was incubated for 60 min at room temperature whereupon 700 μl of 0.3 mM NADPH and 100 μl of 6.0 mM DTNB were added and mixed well. The contents of the tube were transferred to a cuvette and 10 μl of GR (150 units/ml) was added to start the reaction. All GSSG levels were reported as GSH equivalents. The data were treated statistically by the student's t test.

Results and Discussion. Preliminary studies included the effect on glutathione concentration of the type of anesthesia, food intake, and time of sampling during the day. Significantly higher levels (20.2 ± 1.3 vs 16.4 ± 1.6 μM at 5 min) were measured in *ad libitum* control rats when pentobarbital rather than ether was used. Pentobarbital was used for the remainder of the study. To determine diurnal variation, rats were fed the control diet *ad libitum* and blood was collected at 0800, 1500 and 2000 hr. Total glutathione was determined at the 5-min time point as described in "Methods." The results gave strong evidence for a diurnal variation in plasma glutathione; levels in controls were high in the morning and low in the

evening (0800 hr = 21.6 ± 0.8 μM, 1500 hr = 18.1 ± 1.5 μM and 2000 hr = 14.1 ± 0.4 μM). A 12-hr fast (2000 to 0800 hr) of controls lowered plasma glutathione significantly (20.0 ± 1.5 vs 16.0 ± 1.6 μM, at 5 min).

Given the fact that zinc-deficient rats exhibit cyclic food intake (12), plasma glutathione levels were measured in zinc-deficient rats during periods of maximum and minimum food intake. As shown in Table I, there was no significant difference in plasma glutathione between the days of maximum and minimum food intake in either zinc-deficient or pair-fed control rats. The concentration in zinc-deficient plasma was less than that in *ad libitum* controls, but there was no difference between zinc-deficient and pair-fed control rats during either phase of their feeding cycles.

In agreement with earlier observations (9), the total glutathione concentration measured by the reductase, recycling method decreased rapidly after plasma was collected. As shown in Fig. 1 differences in the plasma level due to diet occurred only at the 5- and 10-min points. The *ad libitum*-fed control rats had the highest measurable plasma concentration and it decreased at a faster initial rate than the zinc-deficient plasma. The concentration of GSSG increased in the plasma during the 60-min period so that finally it accounted for virtually all of the measurable glutathione. Nevertheless, the measurable concentration was less than one-half that observed at 5 min. A major proportion of the total glutathione

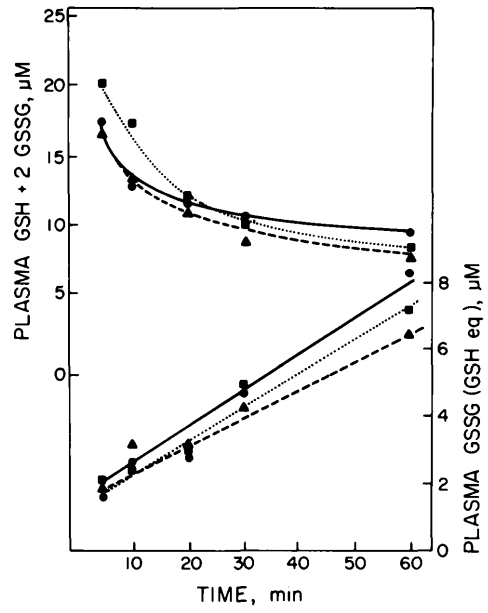

FIG. 1. Time-dependent loss of measurable total glutathione (GSH + 2GSSG) and increase in GSSG in zinc-deficient (2 weeks) and control rat plasma at 25°C. The data points are means of Zn-deficient (▲) (N = 10); pair-fed control (●) (N = 6); and *ad lib*-fed control (■) (N = 5) groups. Only the 5- and 10-min means for total glutathione are statistically different ($P < 0.05$) between the deficient and *ad libitum* controls. The respective 5- and 10-min values (mean ± SEM) were: for the zinc-deficient group 15.6 ± 1.2 and 13.2 ± 1.1; for the pair-fed group, 16.2 ± 1.6 and 12.8 ± 1.4; and for the *ad libitum* group, 20.2 ± 1.8 and 16.0 ± 1.5. The differences in GSSG concentrations between groups were not statistically significant at any time point.

TABLE I. TOTAL PLASMA GLUTATHIONE AS A FUNCTION OF ZINC STATUS AND THE FOOD INTAKE CYCLE

Dietary group	Day of maximum intake (μM)	Day of minimum intake (μM)
Zn deficient	16.0 ± 2.1[a]	15.4 ± 2.0[a]
Pair-fed control	18.0 ± 1.8[a,b]	16.0 ± 2.1[a]
Ad lib control	20.2 ± 1.8[b]	

Note. Values ± SEM (N = 5) are total glutathione (GSH + 2GSSG) in plasma 5 min after collection. Rats were killed on either day of maximum or minimum food intake during their food intake cycle after being on the respective diets 13–15 days. Minimum intake occurred at approximately 5-day intervals after consumption of the low-zinc diet. Different superscripts indicate statistical significance at $P < 0.05$.

was converted to a form not readily reducible by NADPH and glutathione reductase. Dietary status had no effect on the GSSG concentrations at any time.

In an attempt to estimate the *in vivo* plasma concentrations of glutathione, the GSSG concentrations were subtracted from the total (GSH plus GSSG) and the values plotted logarithmically versus time (Fig. 2). The disappearance of GSH appears to follow a pseudo-first-order reaction. On the assumption that virtually all of the *in vivo* plasma glutathione is in the form of GSH, extrapolation to zero time would provide a measure of its *in vivo* concentration. The extrapolated value for *ad libitum* control plasma was 23.5 μM and for the zinc-deficient plasma 16.4 μM. The high

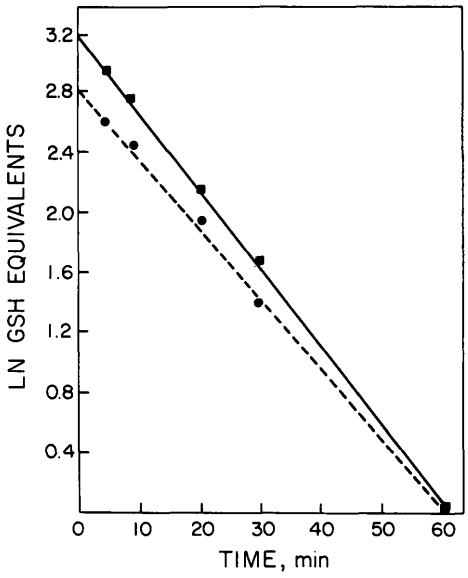

FIG. 2. Semilogarithmic (natural log) plots of calculated GSH concentrations versus time at 25°C. Values are for zinc deficient (●) and *ad lib* controls (■). The curve for the pair-fed controls essentially overlapped that of the zinc-deficient group and was omitted. The slopes of the curves, -5×10^{-2} for the control and -4.4×10^{-2} for the deficient groups, are the pseudo first-order rate constants for GSH disappearance.

proportion of GSH in plasma is consistent with the observation of Anderson *et al.* (16) that GSH is the form translocated from tissue to plasma.

While the plasma glutathione level in zinc-deficient rats was lower than that of *ad libitum*-fed controls, it did not differ significantly from the pair-fed control. This fact does not discount the significance of zinc deficiency on glutathione because it is not evident that pair-feeding provides the best control. The plasma glutathione values presented here agree with those measured by Meister *et al.* (9, 16) in rat blood obtained from the aorta and the time dependent loss of measurable glutathione is consistent with their results (9). It is not known whether or not the nonmeasurable component exists *in vivo*. If so, it might account for the lower level of glutathione in zinc-deficient plasma.

The decreased concentration of extracellular GSH in zinc-deficient plasma may be of physiological significance but this is a moot question. Since liver GSH is elevated by zinc deficiency, the data suggest either a decreased rate of translocation to plasma or a more rapid removal of GSH from plasma *in vivo*.

1. Meister A, Tate SS. Glutathione and related γ-glutamyl compounds: biosynthesis and utilization. Annu Rev Biochem **45**:599–604, 1976.
2. Hsu JM, Anthony WL, Buchanan PJ. Incorporation of glycine-1-^{14}C into liver glutathione in zinc deficient rats. Proc Soc Exp Biol Med **127**:1048–1051, 1968.
3. Hsu JM. Zinc as related to cystine metabolism. In: Prasad A, ed. Trace Elements in Human Health and Disease. New York, Academic Press, pp 295–310, 1976.
4. Mills BJ, Lindeman RD, Lang CA. Effect of zinc deficiency on blood glutathione levels. J Nutr **111**: 1098–1102, 1981.
5. Bettger WJ, Fish TT, O'Dell BL. Effect of copper and zinc status of rats on erythrocyte stability and superoxide dismutase activity. Proc Soc Exp Biol Med **158**:279–282, 1978.
6. Schulman JD. Genetic disorders of glutathione and sulfur amino acid metabolism. Ann Intern Med **93**:330–346, 1980.
7. Chvapil M, Ludwig JC, Sipes IG, Misiorowski RL. Inhibition of NADPH oxidation and related drug oxidation in liver microsomes by zinc. Biochem Pharmacol **25**:1787–1791, 1979.
8. Gordon PR, O'Dell BL. Rat platelet aggregation impaired by short-term zinc deficiency. J Nutr **110**:2125–2129, 1980.
9. Anderson ME, Meister A. Dynamic state of glutathione in blood plasma. J Biol Chem **255**:9530–9533, 1980.
10. Hagenfeidt L, Arvidsson A, Larsson A. Glutathione and γ-glutamylcysteine in whole blood, plasma and erythrocytes. Clin Chim Acta **85**:167–173, 1978.
11. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem **27**:502–522, 1969.
12. Chesters JK, Quarterman J. Effect of zinc deficiency on food intake and feeding patterns of rats. Brit J Nutr **24**:1061–1069, 1970.
13. Leaf G, Neuberger A. The effect of diet on the glutathione content of liver. Biochem J **41**:280–287, 1947.
14. Gordon PR, O'Dell BL. Short term zinc deficiency and hemostasis in the rat. Proc Soc Exp Biol Med **163**:240–244, 1980.
15. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem **106**:207–212, 1980.
16. Anderson ME, Bridges RJ, Meister A. Direct evidence for interorgan transport of glutathione and that the nonfiltration renal mechanism for glutathione utilization involves γ-glutamyl transpeptidase. Biochem Biophys Res Commun **96**:848–853, 1980.

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