

Supplementation with Vitamin C, Vitamin E or β -Carotene Influences Osmotic Fragility and Oxidative Damage of Erythrocytes of Zinc-Deficient Rats¹

Anton Kraus, Hans-Peter Roth and Manfred Kirchgessner²

Institut für Ernährungsphysiologie der Technischen Universität München-Weihenstephan, 85350 Freising, Germany

ABSTRACT Dietary zinc deficiency in rats causes increased osmotic fragility of their erythrocytes. In this study, the influence of supplementary antioxidants (vitamin C, vitamin E or β -carotene) on osmotic fragility, oxidative damage and components of the primary defense system of erythrocytes of zinc-deficient rats was investigated. Indicators of hemolysis *in vivo* were also examined. Five groups of 12 male rats were force-fed a zinc-adequate diet (control rats), a zinc-deficient diet or a zinc-deficient diet enriched with vitamin C, vitamin E or β -carotene. Compared with the control rats, the rats fed the zinc-deficient diet without supplementary antioxidants had greater red blood cell osmotic fragility, higher concentrations of thiobarbituric acid-reactive substances and alanine, higher glutathione S-transferase activity, lower concentration of glutathione and activity of glutathione peroxidase as well as lower activity of superoxide dismutase in plasma ($P < 0.05$). Supplementation with antioxidants generally improved osmotic fragility in zinc-deficient rats without influencing zinc concentration or alkaline phosphatase activity in plasma, indicators of zinc status. At some of the hypotonic saline concentrations tested, vitamin C and β -carotene significantly affected osmotic fragility. The zinc-deficient rats fed a diet without supplementary antioxidants had significantly higher concentrations of alanine in erythrocytes than the zinc-deficient rats supplemented with vitamin C, vitamin E or β -carotene and had significantly higher levels of thiobarbituric acid-reactive substances in erythrocytes than the rats supplemented with β -carotene. There was no indication of hemolysis *in vivo* in rats fed zinc-deficient diets. The results show that supplementary antioxidants decrease osmotic fragility and oxidative damage of erythrocytes in zinc-deficient rats. *J. Nutr.* 127: 1290–1296, 1997.

KEY WORDS: • zinc deficiency • antioxidants • erythrocyte fragility • oxidative damage • rats

It has been suggested that the trace element zinc plays an important role in the structure and function of biological membranes (Bettger and O'Dell 1993). Dietary zinc deficiency in rats is associated with increased hemolysis of erythrocytes in hypotonic saline (O'Dell et al. 1987, Paterson and Bettger 1985, Roth and Kirchgessner 1994) and in the presence of various detergents, alcohols and toxins (Paterson and Bettger 1985). *In vitro* addition of zinc to red blood cells is also protective against hemolysins (Avigad and Bernheimer 1976, Takeda et al. 1977). Alterations in the composition of the erythrocyte membrane have been detected in zinc-deficient rats (Avery and Bettger 1988 and 1992, Driscoll and Bettger 1991, Eder and Kirchgessner 1993, Johanning and O'Dell 1989, Paterson et al. 1987). The effects have been found to be minor and often caused by the reduction of food intake of zinc-deficient animals rather than by zinc deficiency itself. Hence, changes in the composition of the erythrocyte membrane cannot fully explain the dramatic increase of erythrocyte fragility in zinc-deficient rats.

Oxidative modifications of the membrane increase fragility of red blood cells (Stern 1986, Wagner et al. 1988). Because there is some evidence for a physiological role of zinc as an antioxidant (Bettger 1993, Bray and Bettger 1990), greater oxidative damage in zinc deficiency could be responsible for impaired stability of erythrocytes. In a previous study (Kraus et al. 1997), enrichment of the diet with antioxidants in combination (vitamin C, vitamin E and β -carotene) prevented the elevated osmotic fragility of erythrocytes in zinc-deficient rats. Indeed, this suggested an important role of oxidative damage in the impaired stability of erythrocytes in zinc deficiency. The present study was performed to investigate the effects of supplementary antioxidants on erythrocyte fragility when vitamin C, vitamin E and β -carotene were supplied separately. Furthermore, indicators for oxidative damage as well as components of the primary antioxidant defense system of red blood cells were determined. In addition, indicators of hemolysis *in vivo* were examined.

Reduction of voluntary food intake is a common problem in conventional zinc-deficiency experiments. However, reduced food intake and the associated deficiencies of energy and nutrients in general have a strong influence on properties of membranes and on antioxidant systems (Huang and Fwu 1993, Levin et al. 1992, Xia et al. 1995). Therefore, the rats in the present experiment were force-fed. Force-feeding was a

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² To whom correspondence and reprint requests should be addressed.

TABLE 1

Composition of the experimental diet

Ingredient	Amount
	g/kg
Casein	200
Cornstarch	300
Sucrose	323
Corn oil ¹	87
Cellulose	30
Mineral mixture ²	40
Vitamin mixture ³	20

¹ Mazola corn oil (Maizena, Heilbronn, Germany).

² Supplied per kg diet (in g): NaCl, 1.148; NaHCO₃, 1.224; CaCO₃, 4.4; Ca(C₃H₅O₃)₂·5H₂O, 8.7; Ca₅(PO₄)₃·OH, 6.0; MgSO₄·7H₂O, 4.04; KH₂PO₄, 10.247; NaH₂PO₄·H₂O, 3.84; (in mg): FeCl₃·6H₂O, 257; KI, 3.6; MnCl₂·4H₂O, 36.03; NaF, 2.21; Na₂SeO₃·5H₂O, 0.5; Na₂MoO₄·2H₂O, 0.5; CrCl₃·6H₂O, 1.02; CuSO₄·5H₂O, 39.4; NiSO₄·6H₂O, 2.24. All compounds were analytical grade.

³ Supplied per kg diet (in mg): retinol, 8; cholecalciferol, 0.6; *dl*- α -tocopherol acetate, 30 (group +Zn, -Zn, -ZnC, -Zn β C), 300 (group -ZnE); menadione, 5; thiamine·HCl, 5; riboflavin, 10; pyridoxine·HCl, 6; calcium D-(+)-pantothenate, 50; nicotinic acid, 20; choline chloride, 1000; folic acid, 0.2; vitamin B-12, 0.005; ascorbic acid, 650 (only group -ZnC); all-*trans*- β -carotene, 70 (only group -Zn β C); cornstarch to 20 g.

convenient method for supplying rats with sufficient energy and nutrients and for investigating biochemical changes specifically due to zinc deficiency (Schülein et al. 1992).

MATERIALS AND METHODS

Animals and diets. Sixty male Sprague-Dawley rats (Savo GmbH, Kisslegg, Germany) with an average weight of 120 g were divided into five groups of 12. The experiment was divided further into two sections with 30 rats each (six rats per group in each case). The second section of the experiment, identical to the first concerning composition of diet and experimental design, was conducted 1 wk after the termination of the first section. The first group (+Zn)³ was fed a control diet (30.0 mg Zn/kg diet), the second group (-Zn) was fed a zinc-deficient diet (1.1 mg Zn/kg diet), the third group (-ZnC) the zinc-deficient diet enriched with vitamin C (650 mg/kg diet), the fourth group (-ZnE) the zinc-deficient diet enriched with vitamin E (*dl*- α -tocopherol acetate, 270 mg/kg diet; 30 mg/kg was the basal level in all diets) and the fifth group (-Zn β C) the zinc-deficient diet enriched with β -carotene (all-*trans*- β -carotene, 70 mg/kg diet). The rats were housed in plastic cages, had free access to drinking water (double deionized water, supplemented with 0.14 g/L sodium chloride to obtain osmolarity of tap water) and were maintained in a room at 23°C, 60% humidity and a 12-h light:dark cycle. Care and treatment of rats followed recommended guidelines (NRC 1985). The rats received a purified diet with casein as the protein source. The composition of the diet is shown in Table 1.

All rats were force-fed by intragastric tube four times daily (800, 1200, 1800 and 2200 h) as described by Schülein et al. (1992). The intragastric tube consisted of a 10-mL syringe connected to a slide catheter (Braun, Melsungen, Germany). During tube feeding the conscious rat was held by one hand; the other hand moved the catheter into the stomach to inject the slurry. The gentle handling made it unnecessary to accustom the rats to intragastric feeding before the experiment. Each rat received 4 mL of the diet slurry containing 2.9 g dry matter per feeding, resulting in a total of 11.6 g dry matter/d.

³ Abbreviations used: TBARS, thiobarbituric acid-reactive substances, +Zn, Zn-adequate diet; -Zn, Zn-deficient diet; -ZnC, Zn-deficient diet enriched with vitamin C; -ZnE, Zn-deficient diet enriched with vitamin E; -Zn β C, Zn-deficient diet enriched with β -carotene.

After 12 d of feeding, the rats were anesthetized with diethyl ether and decapitated. Blood was collected into heparinized tubes.

Sample preparation. To obtain erythrocytes, heparinized blood was centrifuged (1000 \times g at 4°C for 10 min). The buffy coat was discharged and plasma was removed and stored at -80°C. Red blood cells were washed three times with PBS. Erythrocytes were then diluted with PBS and stored at -80°C for further analysis.

Assessment of zinc status. Plasma zinc concentration was determined after dilution with double-distilled water (1:5) directly in the flame of an atomic absorption spectrophotometer (model 5100, Perkin Elmer, Überlingen, Germany). Activity of alkaline phosphatase (EC 3.1.3.1) in plasma was measured with an automatic analyzer (model 704, Hitachi, Tokyo, Japan) using a commercial reagent kit (test kit number 816388, Boehringer, Mannheim, Germany).

Osmotic fragility measurement. In vitro osmotic fragility of erythrocytes was determined according to a method of Cartwright (1963), modified by O'Dell et al. (1987), using different saline solutions from 3.00 to 4.00 g/L. Freshly obtained heparinized whole blood was pipetted in PBS (pH 7.4), followed by careful mixing and incubation for 15 min at room temperature. After centrifugation (500 \times g at 4°C for 10 min), the concentration of hemoglobin in the supernatant was measured colorimetrically with a photometer (Kontron Instruments, Zürich, Switzerland). The percentages of maximal hemolysis (at 0.00 g/L saline) were plotted vs. the respective salt concentrations.

Antioxidative enzymes. Antioxidative enzymes were all measured colorimetrically with a photometer (Kontron Instruments). Superoxide dismutase (EC 1.15.1.1) activity was assayed by the method of Marklund and Marklund (1974). Catalase (EC 1.11.1.6) activity was measured according to the method of Aebi (1986). Activity of glutathione reductase (EC 1.6.4.2) was determined after the method of Goldberg and Spooner (1986). Glutathione peroxidase (EC 1.11.1.9) activity was determined with *tert*-butyl-hydroperoxide as the substrate (Levander et al. 1983). For measuring the activity of glutathione S-transferase (EC 2.5.1.18) a method of Beutler (1985) was used. No differentiation was made between various isoforms of glutathione S-transferase. The activity of antioxidant enzymes in red blood cells was expressed per unit protein content of the erythrocyte hemolysate. Protein was measured after a method of Smith et al. (1985) with bovine serum albumin as the standard.

Indicators for oxidative damage. Thiobarbituric acid-reactive substances (TBARS) in erythrocytes were determined following a method of Fukunaga et al. (1993) with HPLC. Briefly, protein of the erythrocyte hemolysate was precipitated with 1.6 mol/L perchloric acid. After centrifugation (10,000 \times g at 4°C for 5 min) a portion of the supernatant was mixed with BHT and thiobarbituric acid and heated at 100°C (45 min). After cooling, TBARS were extracted with *n*-butanol. Before injection into HPLC, the butanol extract was passed through a 0.22- μ m filter (Millipore, Eschborn, Germany). The HPLC system consisted of a Merck-Hitachi apparatus (Darmstadt, Germany) with autosampler and fluorescence detection, a guard column (Polyspher OA HY) (Merck) and a RP 18 (LiChrospher) as the analytical column (250 mm \times 3 mm, 18 μ m particle size) (Merck). Elution was performed with acetonitrile/water (2:8, vol/vol) at a flow rate of 1 mL/min at room temperature. The volume of injection was 20 μ L. TBARS were detected fluorometrically. The concentration of TBARS was calculated using 1,1,3,3-tetraethoxypropane as an external standard.

Alanine release from erythrocytes was assayed after a method of Davies (1988), modified by Zamora et al. (1991). Alanine was determined fluorometrically (apparatus Shimadzu RF 5000, Duisburg, Germany) by the alanine dehydrogenase-catalyzed reduction of NAD⁺ to NADH. Alanine concentration was quantified using alanine standards. Concentration of TBARS and alanine was expressed per unit protein content of the erythrocyte hemolysate.

Thiol-compounds and amino acids. For the simultaneous measurement of thiol-compounds and amino acids in erythrocytes, the HPLC method of Fariss and Reed (1987) was used. The method is based on the derivatization of thiols with iodoacetic acid to form S-carboxymethyl derivatives followed by chromophore derivatization of primary amines with Sanger's reagent (1-fluoro-2,4-dinitrobenzene). As in the measurement of TBARS, erythrocytes were treated with 1.6 mol/L perchloric acid and the supernatant was used in the

TABLE 2

Zinc concentration and activity of alkaline phosphatase in plasma and weight gain of rats force-fed a zinc-adequate diet (+Zn), a zinc-deficient diet (-Zn), or zinc-deficient diet enriched with vitamin C (-ZnC), vitamin E (-ZnE) or β -carotene (-Zn β C)¹

	+Zn	-Zn	-ZnC	-ZnE	-Zn β C	Pooled SEM
Zinc, $\mu\text{mol/L}$	20.95 ^a	6.73 ^b	7.34 ^b	6.73 ^b	6.42 ^b	0.61
Alkaline phosphatase, U/L ²	686 ^a	404 ^b	384 ^b	422 ^b	395 ^b	36
Weight gain, g/12 d	62 ^a	48 ^b	45 ^b	47 ^b	47 ^b	2

¹ Values are expressed as means, $n = 12$. Means in a row with different superscript letters differ significantly ($P < 0.05$).

² One unit is defined as the conversion of 1 μmol substrate per minute at 37°C.

analytical procedure. The HPLC system (Merck-Hitachi) was equipped with an autosampler, a UV detector and a 3-aminopropyl column (Spherisorb-NH₂, 5 μm , 250 mm \times 4 mm, MZ-Analysentechnik, Mainz, Germany). After a 100- μL injection of the derivatization solution, the mobile phase was maintained at 80% A (80% methanol in metal-free water) and 20% B (0.5 mol/L sodium acetate in 64% methanol) for 5 min, followed by a 10-min linear gradient to 1% A and 99% B (flow rate 1.5 mL/min). Then the mobile phase was held at 99% B for another 10–15 min, all at room temperature. The quantification was carried out with γ -glutamyl glutamate as an internal standard. Total glutathione was calculated as the sum of reduced glutathione and glutathione disulfide. The concentration of thiol-compounds and amino acids was expressed per unit protein content of the erythrocyte hemolysate.

Indicators for hemolysis in vivo. Activity of acid phosphatase and concentration of potassium were measured with an automatic analyzer (model 704, Hitachi) and commercial available test kits (test kit nos. 1360469 and 1298011, respectively, Boehringer). Free hemoglobin in plasma was assayed colorimetrically using a test kit (procedure no. 527) from Sigma (Deisenhofen, Germany). The test measures only free hemoglobin, not hemoglobin bound to haptoglobin or hemopexin. The concentration of haptoglobin in plasma was determined with HPLC and UV detection according to the method of Schröder et al. (1990). As before, the HPLC system consisted of a Merck-Hitachi apparatus with autosampler, a UV detector (254 nm), a column heater, a Zorbax diol GFC/PTH, Bioseries (Du Pont, Wilmington, MA) as the guard column and a GF-250 Zorbax, Bioseries (9.4 mm \times 25 cm) as the analytical column. Elution was performed with 0.2 mol/L Na₂HPO₄ at a flow rate of 1 mL/min (40°C). Before injection (volume, 40 μL), plasma was diluted with the eluent (1:10) and filtered through a 0.22- μm polycarbonate membrane filter (Millipore). The concentration of haptoglobin in samples was identified using a haptoglobin standard (Sigma). No differentiation was made between different isoforms of haptoglobin.

Statistical analysis. The results were statistically evaluated using ANOVA with a PC-version of SAS (Release 6.04, SAS Institute, Cary, NC). The division of the study into two sections was considered as a second factor in the ANOVA. Therefore, the model included two factors, i.e., diet and the experiment section. Only differences due to the factor diet were described. When a P -value in the ANOVA was < 0.05 , Tukey's test (Holzer et al. 1994) was used for multiple comparisons. In the case of TBARS, some observations were compared separately using linear contrasts. Differences were considered significant if $P < 0.05$.

RESULTS

Weight gain and zinc status of the rats. Despite identical food intakes, the rats fed the zinc-deficient diet gained significantly less weight (25%) than the rats fed the zinc adequate diet (+Zn, Table 2). Zinc-deficient rats had a lower concentration of zinc and activity of alkaline phosphatase in plasma. In the zinc-deficient rats, typical symptoms such as sparse and rough hair, mild skin lesions and lethargy were noted. Thus, supplementary antioxidants had no influence on growth rate, zinc status or symptoms of zinc deficiency.

Osmotic fragility. The in vitro osmotic fragility of red blood cells is illustrated in Figure 1. Hemolysis in hypotonic saline was highest in cells from group -Zn. Group +Zn showed the lowest fragility of erythrocytes. The supplementation with either vitamin C (-ZnC), vitamin E (-ZnE) or β -carotene (-Zn β C) generally improved osmotic fragility in zinc-deficient rats, although the only significant effects were due to vitamin C and β -carotene. There were some marked differences between groups -Zn and -ZnC, -ZnE or -Zn β C which were not significant because of the large variation associated with the analytical procedure. The trend for antioxidant supplementation to improve osmotic fragility in cells from zinc-deficient rats was clear.

Indicators for oxidative damage. Concentration of TBARS in erythrocytes was significantly lower in group +Zn than in groups -Zn and -ZnC and was the same as in groups -Zn β C and -ZnE (Table 3). Group -Zn β C had a significantly lower concentration of TBARS than groups -Zn and -ZnC. Group -ZnE had lower TBARS concentration than group -ZnC and also less than group -Zn when groups -ZnE and -Zn were compared separately by linear contrasts. The concentration of alanine in erythrocytes in group -Zn was significantly higher than in group +Zn. The supplementation with vitamin C, vitamin E or β -carotene in zinc-deficient rats led to a significant reduction of erythrocyte alanine to the level of control rats.

Antioxidative enzymes. Rats of groups -Zn and -Zn β C had lower erythrocyte glutathione peroxidase activity than group +Zn (Table 4). Activity of erythrocyte glutathione S-transferase was significantly higher in group -Zn than in

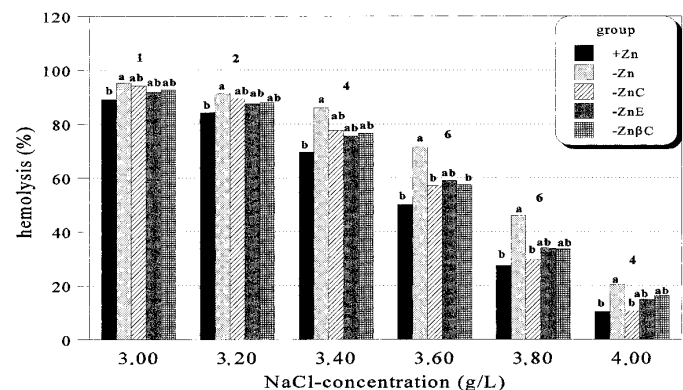


FIGURE 1 Osmotic fragility of erythrocytes from rats fed a zinc-adequate diet (+Zn), a zinc-deficient diet (-Zn), or zinc-deficient diet enriched with vitamin C (-ZnC), vitamin E (-ZnE) or β -carotene (-Zn β C). Values are means, $n = 12$. Numbers above each set of data are pooled SEM. Means within a NaCl concentration not sharing a superscript letter differ significantly ($P < 0.05$).

TABLE 3

Concentration of thiobarbituric acid-reactive substances (TBARS) and alanine in erythrocytes of rats force-fed a zinc-adequate diet (+Zn), a zinc-deficient diet (-Zn), or zinc-deficient diet enriched with vitamin C (-ZnC), vitamin E (-ZnE) or β -carotene (-Zn β C)¹

	+Zn	-Zn	-ZnC	-ZnE	-Zn β C	Pooled SEM
TBARS, $\mu\text{mol/g protein}^2$	0.37 ^c	0.55 ^{ab}	0.60 ^a	0.46 ^{bc}	0.37 ^c	0.03
Alanine, $\mu\text{mol/g protein}^2$	0.85 ^b	1.24 ^a	0.98 ^b	0.91 ^b	0.79 ^b	0.06

¹ Values are expressed as means, $n = 12$. Means in a row with different superscript letters differ significantly ($P < 0.05$).

² Erythrocyte hemolysate protein.

group +Zn. Groups -ZnC, -ZnE and -Zn β C had intermediate activities that were not significantly different from either group -Zn or +Zn. Superoxide dismutase activity in plasma of group -Zn was lower than in group +Zn. Groups -ZnC, -ZnE and -Zn β C were not significantly different than either group +Zn or group -Zn. Activity of superoxide dismutase, glutathione reductase and catalase in erythrocytes was unaffected by dietary treatment.

Thiol-compounds and amino acids. The zinc-deficient rats had significantly lower concentrations of reduced glutathione, total glutathione and aspartate in erythrocytes than the +Zn group of rats (Table 5). Group -ZnE had significantly higher concentrations of reduced glutathione and total glutathione than group -Zn and group -ZnC. The various treatments had no influence on the concentrations of glutathione disulfide (oxidized glutathione), cysteine, cystine or glutamate.

Indicators of hemolysis in vivo. Activity of acid phosphatase, as well as concentration of potassium in plasma, was significantly lower in -Zn rats, whereas concentration of haptoglobin in plasma tended to be higher than in +Zn rats ($P = 0.085$) (Table 6). There was no influence of dietary Zn on the concentration of free hemoglobin in plasma. Supplementation of the -Zn diet with antioxidants did not affect indicators of hemolysis measured in vivo.

DISCUSSION

The observation that dietary zinc deficiency leads to greater erythrocyte fragility was confirmed in the present study. The results of this study indicate that oxidative damage is responsible for impaired erythrocyte stability. First, zinc-deficient rats without antioxidant supplementation (-Zn) had higher concentration of thiobarbituric acid-reactive substances

(TBARS) and higher levels of alanine in erythrocytes than the control rats (+Zn). TBARS and alanine concentrations are used as indicators of lipid peroxidation and protein breakdown, respectively (Davies 1988, Halliwell and Gutteridge 1989). Hence, dietary zinc deficiency caused greater oxidative damage in erythrocytes. Second, supplementation with antioxidants, substances possessing the ability to inhibit oxidative processes, improved osmotic fragility of erythrocytes in zinc-deficient rats without influencing zinc status, growth rate or symptoms of zinc deficiency. Certainly, the improvement of erythrocyte fragility was smaller than in a previous experiment (Kraus et al. 1997) in which antioxidants were given in combination.

Vitamin C, vitamin E and β -carotene are well-known antioxidants (Beyer 1994, Packer 1993, Palozza and Krinsky 1992). However, only for vitamin E is there clear evidence for membrane-stabilizing properties (Niki et al. 1991, Palozza and Krinsky 1992). Surprisingly, supplementation with vitamin C exerted the strongest effect on osmotic fragility. Vitamin C as a hydrophilic antioxidant is unable to prevent lipid peroxidation in membranes effectively (Niki et al. 1991, Sato et al. 1995). According to the study of Davies and Goldberg (1987), alanine concentration is a more sensitive indicator of oxidative damage in erythrocytes than TBARS. Breakdown of proteins and lipid peroxidation are reported to be events that can take place independently of one another (Davies and Goldberg 1987), and lipid peroxidation in erythrocyte membranes is not necessarily associated with hemolysis (Sato et al. 1995, Van den Berg et al. 1991). In the present experiment, supplementation of the -Zn diet with vitamin C had no influence on the concentration of TBARS in erythrocytes but resulted in a significant reduction of alanine concentration. These results suggest a causal role of protein breakdown in the impaired

TABLE 4

Activity of antioxidative enzymes in erythrocytes or plasma of rats force-fed a zinc-adequate diet (+Zn), a zinc-deficient diet (-Zn), or zinc-deficient diet enriched with vitamin C (-ZnC), vitamin E (-ZnE) or β -carotene (-Zn β C)¹

	+Zn	-Zn	-ZnC	-ZnE	-Zn β C	Pooled SEM
Superoxide dismutase, $\text{kU/L}^{2,3}$	29.8 ^a	24.1 ^b	24.9 ^{ab}	24.8 ^{ab}	24.2 ^{ab}	1.4
Superoxide dismutase, $\text{kU/g protein}^{3,4}$	25.4	24.9	25.0	25.3	25.1	1.0
Glutathione reductase, $\text{U/g protein}^{3,4}$	13.2	14.1	13.7	11.7	12.1	0.9
Glutathione peroxidase, $\text{U/g protein}^{3,4}$	1313 ^a	1134 ^b	1243 ^{ab}	1204 ^{ab}	1085 ^b	61
Glutathione S-transferase, $\text{U/g protein}^{3,4}$	13.9 ^b	18.7 ^a	18.5 ^{ab}	14.9 ^{ab}	15.5 ^{ab}	1.4
Catalase, $\text{U} \cdot 10^6/\text{g protein}^{3,4}$	32.0	36.3	37.5	34.5	35.8	2.4

¹ Values are expressed as means, $n = 12$. Means in a row with different superscript letters differ significantly ($P < 0.05$).

² Superoxide dismutase was measured in plasma and values of activity are calculated per liter of plasma.

³ Definition of unit (U): one unit is defined as the conversion of 1 μmol substrate per minute at 37°C, except for superoxide dismutase (1 unit is defined as the amount of enzyme necessary to inhibit the autoxidation of pyrogallol by 50% at 25°C) and catalase (1 unit is defined as the amount of enzyme necessary to decompose half of the hydrogen peroxide in the sample in 15 s at 20°C).

⁴ Erythrocyte hemolysate protein.

TABLE 5

Concentration of thiol-compounds and amino acids in erythrocytes of rats force-fed a zinc-adequate diet (+Zn), a zinc-deficient diet (-Zn), or zinc-deficient diet enriched with vitamin C (-ZnC), vitamin E (-ZnE) or β -carotene (-Zn β C)¹

	+Zn	-Zn	-ZnC	-ZnE	-Zn β C	Pooled SEM
	$\mu\text{mol/g protein}^2$					
Total glutathione	11.54a	8.33c	8.36c	10.08b	8.97bc	0.36
Reduced glutathione	11.01a	7.89c	7.88c	9.57b	8.57bc	0.35
Glutathione disulfide	0.53	0.44b	0.48b	0.51	0.40bc	0.05
Cysteine	1.06	1.06	1.22	1.21	1.37	0.25
Cystine	0.27	0.28	0.35	0.33	0.24	0.04
Glutamate	4.64	4.40	4.00	3.93	3.51	0.44
Aspartate	0.72a	0.58b	0.49b	0.50b	0.58b	0.04

¹ Values are expressed as means, $n = 12$. Means in a row with different superscript letters differ significantly ($P < 0.05$).

² Erythrocyte hemolysate protein.

stability of erythrocytes in zinc-deficient rats. This is compatible with the concept that zinc protects essential sulfhydryl groups of proteins in plasma membranes from oxidation to disulfides. Reduction of disulfide bonds would require electron donors such as reduced glutathione (O'Dell et al. 1987). However, zinc deficiency was associated with depletion of glutathione, as will be discussed later. Zinc deficiency failed to affect osmotic fragility when a diet high in sulfur amino acids was provided (O'Dell et al. 1985 and 1987). In addition, zinc is present in the body mainly bound to proteins.

It is remarkable that the effects of supplementation with vitamin C were achieved with a dosage of 650 mg/kg diet. Rats are able to synthesize 26–58 mg ascorbic acid per kilogram body weight per day (Pauling 1986). Assuming a food intake of 11.6 g/d, as in this experiment, and a body weight of 150 g, this is equivalent to 336–750 mg ascorbic acid per kilogram diet. Thus, supplementation with vitamin C was in the physiological range. The minimum requirement of rats that are unable to synthesize ascorbic acid because of a genetic disorder was reported to be 300 mg per kilogram diet (Horio et al. 1985).

Supplementation with vitamin E (300 mg per kg diet) was chosen to be 10 times the minimum requirement for growing rats (NRC 1978). The basis of this was the consideration that a 10-fold vitamin E intake in humans would improve erythrocyte stability. β -Carotene is not considered to be an essential nutrient. Supplementation with β -carotene was chosen to be 70 mg per kilogram diet. Relative to metabolic body weight (body weight^{0.75}), this corresponds to a daily intake of 80 mg β -carotene in humans (with regard to the body weight and feed intake of rats in this experiment).

Protective effects of supplementary antioxidants in zinc de-

fiency have also been shown in chicks (Bettger et al. 1980). Supplementation with vitamin E, as well as with various synthetic antioxidants, improved skin lesions caused by zinc deficiency, whereas vitamin C had no influence. Supplementary vitamin E also led to a reduction of TBARS in the skin of zinc-deficient chicks (Bettger et al. 1980).

The data presented here suggest an increase in oxidative processes during zinc deficiency and therefore an antioxidant action of zinc in vivo. The proposed role for zinc as a physiological antioxidant has been reviewed (Bettger 1993, Bray and Bettger 1990). A possible mechanism for an antioxidant action of zinc is the competition of zinc with metals that are able to catalyze reactions of free radical generation, for example, iron or copper. Zinc does not change valence state. Therefore, replacement of zinc by iron or copper in biological structures could result in oxidation of corresponding ligands such as sulfhydryl groups (Bray and Bettger 1990, Willson 1989). Another possibility for an antioxidant effect of zinc is its function in copper- and zinc-dependent superoxide dismutase which removes superoxide anion radicals. Nevertheless, superoxide dismutase activity in erythrocytes has been shown to remain unchanged during zinc deficiency (Coudray et al. 1992, Roth and Kirchgessner 1994, Roussel et al. 1993) as shown in the present experiment as well. However, a reduction of superoxide dismutase activity in plasma caused by zinc deficiency has been shown by others (Coudray et al. 1992, Olin et al. 1995). A plausible reason for this is that dietary zinc deficiency causes a reduction of the zinc concentration in plasma but not in erythrocyte cytoplasm; there is a slight reduction of membrane-bound zinc (Johanning and O'Dell 1989, Roth and Kirchgessner 1994). Superoxide dismutase is supposed to be a first line of defense against radicals generated in plasma (Olin et al.

TABLE 6

Concentration of potassium, free hemoglobin, haptoglobin and activity of acid phosphatase in plasma of rats force-fed a zinc-adequate diet (+Zn), a zinc-deficient diet (-Zn), or zinc-deficient diet enriched with vitamin C (-ZnC), vitamin E (-ZnE) or β -carotene (-Zn β C)¹

	+Zn	-Zn	-ZnC	-ZnE	-Zn β C	Pooled SEM
Potassium, mmol/L	6.87a	6.01b	5.68b	6.14b	6.04b	0.14
Free hemoglobin, g/L	0.19	0.19	0.22	0.20	0.21	0.02
Haptoglobin, g/L	12.6	15.2	14.9	14.0	14.3	0.8
Acid phosphatase, U/L ²	40.0a	29.8b	30.6b	28.4b	31.1b	1.8

¹ Values are expressed as means, $n = 12$. Means in a row with different superscript letters differ significantly ($P < 0.05$).

² One unit is defined as the conversion of 1 μmol substrate per minute at 37°C.

1995). The importance of the reduction of extracellular superoxide dismutase activity in the oxidative damage of erythrocytes is unclear.

In spite of the unchanged zinc concentration in red blood cells of zinc-deficient rats (Johanning and O'Dell 1989, Roth and Kirchgessner 1994), some alterations in the primary antioxidant system of erythrocytes were observed in the present experiment. Glutathione functions as cosubstrate for glutathione peroxidase and glutathione S-transferase and shows antioxidant properties itself (Beutler and Dale 1989). Glutathione acts synergistically with zinc in protecting sulfhydryl groups (Kosower and Kosower 1989, Wilson 1989). The reason for depletion of glutathione in zinc-deficient rats is unknown although it has been observed by others (Hsu 1982, Mills et al. 1981). Possibly, zinc deficiency causes higher consumption of glutathione. But because zinc-deficient rats supplemented with antioxidants also had a lower concentration of glutathione, depletion of glutathione is not likely to be responsible for the increased fragility of erythrocytes. The elevated glutathione S-transferase activity in erythrocytes of zinc-deficient rats ($-Zn$) indicates an increase in oxidative processes because activity of glutathione S-transferase increased in response to oxidative stress in rats (Aniya and Naito 1993), mice (Van Canegham 1984) and humans (Ramdath and Golden 1993). Lower activity of glutathione peroxidase in erythrocytes of zinc-deficient rats was also observed by Roth and Kirchgessner (1994). Antioxidant supplementation in zinc-deficient rats had only minor influence on components of the antioxidant system of erythrocytes. Reduction of glutathione concentration and glutathione peroxidase activity as well as higher activity of glutathione S-transferase are supposed to be secondary effects of zinc deficiency. Depletion of glutathione and decline of glutathione peroxidase activity may have contributed to the higher oxidative damage and osmotic fragility of erythrocytes in zinc-deficient rats. The lower aspartate concentration in zinc-deficient rats likely reflects a general disturbance in amino acid metabolism due to zinc deficiency.

To examine whether the higher osmotic fragility of red blood cells in zinc-deficient rats ($-Zn$) led to hemolysis in vivo, some indicators of hemolysis were determined. Hemolysis is associated with the release of potassium, hemoglobin and acid phosphatase from erythrocytes into the plasma. In the case of haptoglobin, a decreased concentration in plasma is considered to indicate hemolysis (Thomas 1992). Haptoglobin is an acute-phase protein. Hence, the slight increase ($P = 0.085$) of haptoglobin was possibly caused by an intensified acute-phase reaction due to zinc deficiency. The lower acid phosphatase activity in plasma of zinc-deficient rats likely was a direct consequence of zinc deficiency because acid phosphatase is a zinc-dependent enzyme (Fujimoto et al. 1993). The reduction of plasma potassium concentration may reflect an indirect effect of zinc depletion because zinc deficiency is associated with general disturbances in the balance of water and minerals (O'Dell 1981, Song 1987). Thus, there is no indication of hemolysis in vivo during dietary zinc deficiency as already proposed by O'Dell et al. (1987).

In conclusion, dietary zinc deficiency caused higher fragility of erythrocytes in zinc-deficient rats as shown in previous studies and also caused greater oxidative damage. The supplementation of zinc-deficient rats with vitamin C, vitamin E or β -carotene improved erythrocyte fragility and led to lower oxidative damage of erythrocytes. This is consistent with antioxidant actions of these substances and suggests that oxidative modifications of proteins are responsible for impaired erythrocyte stability in zinc-deficient rats.

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