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Vitamin E prevents the elevation of thiobarbituric acid–reactive substances but not hemolytic anemia in rats fed excess methionine

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Supplementing an 8% casein (8C) diet with 3% methionine (8C3M) resulted in a 13% reduction in hemoglobin in rats compared with control (8C). The NaCl concentration which caused 50% hemolysis of erythrocytes incubated at 37°C for 24 hr was 0.85% for 8C and 1.05% for 8C3M. The plasma levels of thiobarbituric acid–reactive substances (TBARS) and Cu, Zn-superoxide dismutase (SOD) activity of erythrocytes in the 8C3M group were 15% higher and 35% lower than those of the 8C group, respectively. Supplementation with vitamin E to 8C3M (all-rac-α-tocopheryl acetate, 4,240 μmol/kg of diet, 10 times the amount in other diets) prevented the elevation in plasma TBARS and the decrease in SOD activity of erythrocytes but did not affect the development of anemia. Alteration of the cytoskeletal protein composition of erythrocyte membranes in the 8C3M group was observed. Those hematological alterations caused by excess methionine ingestion were prevented by the addition of 3% glycine to the 8C3M diet. (J. Nutr. Biochem. 7:77–84, 1996.)

Keywords: methionine toxicity; hemolytic anemia; erythrocyte; vitamin E supplementation; rat

Introduction

Methionine, an essential amino acid for adequate growth and development of mammals, is one of the most highly toxic amino acids, and excess amounts can retard growth and damage tissue.[1,2] Methionine is reportedly metabolized by two pathways, trans-sulfuration and transamination, and measurement of the various metabolic products and enzyme activities suggests that excess methionine is metabolized by the trans-sulfuration pathway.[3–5] The limited capacity of this pathway may lead to high levels in the plasma and tissues and may be responsible for the adverse effects of methionine, at least on growth and food intake.[6] Although transamination pathway also appears to produce toxic metabolites,[1,7] the quantitative importance of the transamination pathway remains unknown.[4]

We previously compared the patterns of components in the plasma, various tissues, and urine of rats fed a low casein diet and a low casein diet supplemented with excess methionine and characterized one of the transamination pathway products, 2-keto-4-methylthiobutyric acid, in the urine of rats fed an excess methionine diet for the first time.[8] However, our findings regarding whether or not excess methionine was metabolized via the transamination pathway were inconclusive because the amount of 2-keto-4-methylthiobutyric acid was very small compared with the amount of methionine ingested and because no metabolic intermediates other than 2-keto-4-methylthiobutyric acid (such as methylthiopropionic acid and methanethiol) were detected. Although we also observed accumulation of ophthalmic acid and a decreased glutathione in the liver of rats fed a low-casein diet, we found accumulation of glutathione and γ-aminobutyric acid and a decrease of ophthalmic acid when this diet was supplemented with excess methionine. We therefore proposed that ophthalmic acid accumulates in the liver during deficiencies of sulfur-containing amino acids and is used for the synthesis of glutathione after excess methionine ingestion, resulting in decreased ophthalmic acid and increased glutathionine and γ-aminobutyric

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acid.[9,10] This study was conducted to investigate the effect of vitamin E supplementation to rats given an excess methionine diet on the elevation of plasma thiobarbituric acid–reactive substances. (TBARS) and on the development of anemia.

Methods and materials

General methods

The hemoglobin concentration (Hb) was measured using a commercial kit (Hemoglobin B-Test Wako, Wako Pure Chemical Industries, Osaka, Japan). The hematocrit value (Hct) was measured by centrifugation (Hitachi Centrifuge Hematocrit MC-20 (Tokyo, Japan), speed 8, 7 min) of blood in a microhematocrit tube. The red blood cell count (RBC), Hb, Hct, and reticulocytes in blood collected on the last day of feeding were measured. Iron and copper in some organs were measured with an Atomic Absorption Spectrometer Spectr AA-20 (Varian, Techtron, Mulgrave, Australia) after wet ashing with mixed acid (HNO_3–$HClO_4$, 3:1) unless otherwise noted.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and Duncan's multiple range test to determine whether the differences between means were significant ($P < 0.05$).

Composition of the diets

The composition of the basal diet (8C) was as follows (%): casein (Alacid™, New Zealand Dairy Board, New Zealand), 8; corn oil, 5; mineral mixture, 4; vitamin mixture, 1; choline chloride, 0.2; vitamin E granules (Eisai, Tokyo, Japan) that contained 424 μmol all-*rac*-α-tocopheryl acetate/g, 0.1; and sucrose, up to 100. Retinyl palmitate and ergocalciferol (Eisai) were also added to levels of 7.66 and 0.0504 μmol/kg of diet, respectively. The test diets were prepared by adding methionine to a level of 3% (8C3M), methionine and glycine to levels of 3% each (8C3M3G), or methionine and vitamin E to levels of 3% and 4,240 μmol/kg of diet (10 times the amount in the other three diets), respectively (8C3MVE). These supplements were added at the expense of sucrose. The mineral mixture was based on the report from the AIN-76 Diet Workshop[11] except for Co, F, Sn, I, Mg, Ca, P, and K, which were not mentioned in the report and for which Ebihara et al.[12] was followed. The vitamin mixture was prepared according to the specification of the AIN-76 vitamin mixture[13] except that menadione and ascorbic acid were adjusted to 5.81 and 284 μmol/kg of diet according to AIN[14] and Harper,[15] respectively.

Experiment 1

Feeding experiments with an excess methionine diet for hematological studies were performed three times under essentially the same conditions unless otherwise noted.

Animals and diet

Male Sprague–Dawley rats (mean body weight 99 g; Japan SLC Inc., Japan) were housed in individually suspended cages in a temperature-controlled room (23 ± 1°C) with 12 hr of light (0800–20000 hr). They were fed a 25% casein diet for 4 days before experimental feeding and were divided into three groups of six rats each with a mean weight of 120 g. Groups were allowed free access to the three diets of 8C, 8C3M, and 8C3M3G for 30 days. Blood was collected from a caudal vein on days 6, 13, and 20 for Hb and on days 8, 15, and 22 for Hct measurements. Blood was obtained through the abdominal aorta under pentobarbital anesthesia (Nembutal, 5 mg/100 g of body weight) on day 30. Rats were killed by exsanguination, and the liver, spleen, and kidneys were immediately excised and weighed. The blood was used for the erythrocyte osmotic fragility test and measurements of hematological parameters.

Erythrocyte osmotic fragility test

The osmotic fragility test of fresh erythrocytes and erythrocytes kept for 24 hr at 37°C was done in various concentrations of NaCl (2–5.5 g/L for fresh erythrocytes and 1–12 g/L for erythrocytes kept for 24 hr at 37°C) as described by Ray and Noteboon.[16] Twenty-five microliters of blood was incubated with 2.5 mL of graded NaCl solution for 30 min at room temperature and centrifuged (3,000 rpm, 15 min), and the optical density of the supernatant was measured at 540 nm.

Splenic iron

Splenic iron was measured by atomic absorption spectroscopy after wet ashing of a whole spleen with 20 mL of mixed acid.

Liver triglycerides

Lipids were extracted with a chloroform-methanol mixture (2:1, 1.7 mL) from lyophilized liver (20 mg) by the method of Folch et al.,[17] and triglycerides were measured with a kit for measurement of neutral lipids (TG-EN Kainos, Kainos Laboratories, Tokyo, Japan).

Experiment 2

Experiment 2 was done to investigate the influence of excess methionine ingestion on plasma TBARS and the antioxidation capacity of erythrocytes. The effect of supplementation with a large amount of vitamin E, an oxygen radical scavenger, to excess methionine diet was also studied.

Animals and diet

Four groups of six rats each with a mean weight of 120 g were allowed free access to the four diets of 8C, 8C3M, 8C3M3G, and 8C3MVE for 31 days. The blood withdrawn on day 28 was centrifuged (3,000 rpm, 15 min), and the activities of catalase, glutathione peroxidase (GSH-PX), and Cu, Zn-superoxide dismutase (SOD) in erythrocytes were determined. Plasma was analyzed for TBARS, ceruloplasmin, 1,1-dephenyl-2-picrylhydrazyl (DPPH)-reactive substances, and water-soluble fluorescent substances.

Activities of antioxidation enzymes

Blood collected from a caudal vein (100 μL) was diluted with 2 mL of 9 g/L phosphate-buffered saline (PBS) (pH 7.4) and centrifuged (3,000 rpm, 5 min). Plasma thus obtained was used for the measurement of lipid peroxides as described below. The precipitate was suspended in 2 mL of 9 g/L PBS and centrifuged (3,000 rpm, 5 min). This process was repeated twice. The erythrocyte hemolysate was prepared by adding 2 mL of H_2O to the precipitate. Catalase activity of the hemolysate was determined using a hydrogen peroxide solution as the substrate by the method of Abei.[18] GSH-PX activity of the hemolysate was measured using GSH as the substrate according to the method of Gunzler et al.[19] SOD activity was measured by its ability to prevent the reduction of iodonitrotetrazolium violet by superoxide produced by the xanthine-xanthine oxidase system.[20] One milliliter of hemolysate was mixed with ethanol (0.25 mL) and chloroform (0.25 mL), and the

Table 1 Body weight gain, food intake, and relative weights of liver, kidneys, and spleen in rats fed the experimental diets*

Diet	Weight gain (g)	Food intake (g)	Liver	Kidney left (g/100 g of body weight)	Spleen
8C†	44.2 ± 2.3^{b}‡	363.3 ± 5.5^{a}	4.53 ± 0.28^{b,c}	0.37 ± 0.01^{c}	0.19 ± 0.11^{c}
8C3M	−11.2 ± 5.9^{c}	175.6 ± 12.8^{b}	4.94 ± 0.28^{b}	0.68 ± 0.02^{a}	0.34 ± 0.02^{a}
8C3M3G	71.6 ± 4.7^{a}	337.2 ± 19.2^{a}	5.78 ± 0.17^{a}	0.48 ± 0.01^{b}	0.23 ± 0.02^{b}
8C3MVE	−16.4 ± 5.3^{c}	152.4 ± 13.0^{b}	5.34 ± 0.21^{a,b}	0.66 ± 0.02^{a}	0.35 ± 0.02^{a}

*Experiment 2 (for 31 days). Initial body weight, 120 g. Data of Experiments 1 and 3 are not shown, because they are essentially the same as those of the 8C, 8C3M, and 8C3M3G groups in Experiment 2.
†Abbreviations used: 8C, 8% casein; 8C3M, 8% casein supplemented with 3% methionine; 8C3M3G, 8% casein supplemented with 3% methionine and 3% glycine; 8C3MVE, 8% casein supplemented with 3% methionine and vitamin E of 10 times the amount in the other three diets.
‡All values are mean ± SEM ($n = 6$). Means in the same column with a different superscript are significantly different by Duncan's multiple range test ($P < 0.05$).

mixture was centrifuged (3,000 rpm, 10 min). The supernatant was used for the enzyme activity assay. A standard curve was obtained with authentic SOD (bovine erythrocyte, Wako Pure Chemical Industries) treated by the same procedures.

Plasma TBARS

The plasma TBARS level was measured by the method of Yagi[21] and expressed as the malonaldehyde concentration.

Plasma DPPH-reactive substances and water-soluble fluorescent substances

DPPH, a purple radical, is reduced to a colorless substance by radical scavengers such as GSH and vitamin E. DPPH-reactive substances were measured as described by Blois.[22] The OD_{517} of each plasma sample was measured and expressed relative to that of H_2O as a blank. Water-soluble fluorescent substances in plasma as a parameter of lipid peroxidation were determined by excitation at 350 nm and emission at 460 nm according to the method of Tsuchida et al.[23] Quinine was used as a standard.

Iron and copper in liver, kidney, and spleen

Iron and copper in the liver, kidney, and spleen were measured after wet ashing of the liver (1 g), whole right kidney, and whole spleen with 20 mL of mixed acid. Plasma copper was determined by the measurement of OD_{600} after reaction with 2-(2-thiazorylazo)-4-methyl-5-(sulfomethyl-amino) benzoic acid using a Cu Neo "Cino test" (Cino Test, Tokyo, Japan).[24]

Plasma ceruloplasmin

Plasma ceruloplasmin was determined by its oxidase activity with o-dianisidine as the substrate according to the method of Schosinsky et al.[25]

Experiment 3

Experiment 2 showed that vitamin E did not protect against the development of anemia caused by excess methionine ingestion even though the plasma level of TBARS, an indicator of lipid peroxidation, decreased significantly. Therefore, Experiment 3 was done to investigate the changes in the protein components in erythrocytes caused by excess methionine ingestion.

Animals and diet

Three groups of six rats each with a mean weight of 120 g were allowed free access to the three diets of 8C, 8C3M, and 8C3M3G for 34 days. Blood was centrifuged (3,000 rpm, 15 min), and erythrocytes were used to prepare ghost (hemoglobin-free membrane).

Preparation of the erythrocyte membrane

Erythrocytes were hemolyzed in hypotonic phosphate buffer (20 mOsm, pH 7.4), and a hemoglobin-free erythrocyte membrane was prepared as described by Dodge et al.[26] and lyophilized.

Table 2 Hematological parameters of rats fed the experimental diets*

Diet	RBC† ($\times 10^6/\mu L$)	Hb (g/dL)	Hct (%)	Reticulocyte (%)
8C†	7.37 ± 0.08^{a}‡	14.95 ± 0.29^{a}	40.13 ± 0.53^{a}	3.01 ± 0.28^{c}
8C3M	5.73 ± 0.09^{c}	13.00 ± 0.22^{b}	33.97 ± 0.60^{c}	6.69 ± 0.33^{a,b}
8C3M3G	6.81 ± 0.10^{b}	14.50 ± 0.18^{a}	37.45 ± 0.61^{b}	5.99 ± 0.72^{b}
8C3MVE	5.66 ± 0.17^{c}	13.57 ± 0.35^{b}	33.23 ± 0.96^{c}	8.22 ± 0.71^{a}

*Experiment 2. Data of Experiments 1 and 3 are not shown because they are essentially the same as those of the 8C, 8C3M, and 8C3M3G groups in Experiment 2.
†Abbreviations used: RBC, red blood cell count; Hb, hemoglobin concentration; Hct, hematocrit value; 8C, 8% casein; 8C3M, 8% casein supplemented with 3% methionine; 8C3M3G, 8% casein supplemented with 3% methionine and 3% glycine; 8C3MVE, 8% casein supplemented with 3% methionine and vitamin E of 10 times the amount in the other three diets.
‡All values are mean ± SEM ($n = 6$). Means in the same column with a different superscript are significantly different by Duncan's multiple range test ($P < 0.05$).

Table 3 Iron content in spleen, liver, and kidneys of rats fed the experimental diets*

	Spleen		Liver		Kidney	
	(mg/whole)	(mg/g)	(mg/whole)	(mg/g)	(mg/whole)	(mg/g)
8C†	0.08 ± 0.00^{c}‡	0.25 ± 0.02^{c}	0.68 ± 0.03^{b}	0.09 ± 0.01^{b}	0.03 ± 0.00^{b}	0.06 ± 0.00^{b}
8C3M	0.46 ± 0.04^{a}	1.23 ± 0.08^{a}	0.92 ± 0.07^{a}	0.18 ± 0.02^{a}	0.05 ± 0.01^{a}	0.07 ± 0.01^{a}
8C3M3G	0.17 ± 0.03^{c}	0.37 ± 0.03^{c}	$0.74 \pm 0.04^{a,b}$	0.07 ± 0.00^{b}	0.06 ± 0.01^{b}	0.06 ± 0.01^{a}
8C3MVE	0.33 ± 0.04^{b}	0.92 ± 0.13^{b}	$0.83 \pm 0.10^{a,b}$	0.15 ± 0.02^{a}	0.08 ± 0.01^{a}	0.08 ± 0.01^{a}

*Experiment 2. The splenic iron contents in Experiment 1 are not shown because they are essentially the same as those of the 8C, 8C3M, and 8C3M3G groups in Experiment 2.
†Abbreviations used: 8C, 8% casein; 8C3M, 8% casein supplemented with 3% methionine; 8C3M3G, 8% casein supplemented with 3% methionine and 3% glycine; 8C3MVE, 8% casein supplemented with 3% methionine and vitamin E of 10 times the amount in the other three diets.
‡All values are mean ± SEM ($n = 6$). Means in the same column with a different superscript are significantly different by Duncan's multiple range test ($P < 0.05$).

Methemoglobin

The methemoglobin concentration was determined by the difference in OD_{630} of hemolyzed blood before and after the addition of KCN.[27]

Free thiol and methionine sulfoxide in erythrocyte membrane

The thiol content in erythrocyte membranes was assayed by the measurement of OD_{412} after reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) as described by Haest et al.[28] GSH was used as a standard. For determination of the methionine sulfoxide content, the erythrocyte membrane was hydrolyzed with 140 g/L of $Ba(OH)_2$ at 120°C for 5 hr in a sealed tube[29] and the hydrolysate was analyzed with a Hitachi 835 high-speed amino acid analyzer. Protein in erythrocyte membranes was determined as described by Lowry et al.[30]

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of erythrocyte membrane protein

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of erythrocyte membrane protein on a disc gel was carried out by the method of Fairbanks et al.[31] as modified by Ikehara et al.[32] on a 5.6% gel (60 × 5 mm) using an 8 mA current on a gel in a glass tube (70 × 5 mm). The developed gel was stained with 0.4 g/L of Coomassie brilliant blue and scanned with Shimazu Dual-Wavelength Flyingspot Scanner CS-9000, Kyoto, Japan, at 590 nm.

Results

The significant suppression of body weight gain and food intake and the enlargement of the kidneys and spleen caused by the excess methionine ingestion were alleviated significantly by the addition of 3% glycine to the diet, but the enlarged liver in the 8C3M group was not improved by feeding with the 8C3M3G diet (*Table 1*). Liver triglycerides increased 2.6 fold in the excess methionine group and did not decrease with the addition of glycine as in the case of liver weight. The hepatic triglyceride contents of the 8C, 8C3M, and 8C3M3G groups (mg/g of liver, Experiment 1) were 27.4 ± 3.3,[b] 70.0 ± 16.9,[a] and 82.6 ± 15.7,[a] respectively.

A significant decrease in RBC, Hb, and Hct in the 8C3M groups (78, 87, and 85% of the 8C group, respectively) indicated the development of anemia in rats fed the excess methionine diet (*Table 2*). Those parameters in the 8C3M3G group also returned to the control levels, but those in the 8C3MVE group were not different from those of the 8C3M group. The osmotic fragility of erythrocytes incu-

Table 4 Plasma TBARS concentration in rats fed the experimental diets

Diet	Plasma TBARS (malonaldehyde nmol/mL)
8C*	$4.0 \pm 0.2^{a,b}$†
8C3M	4.6 ± 0.3^{a}
8C3M3G	$3.9 \pm 0.4^{a,b}$
8C3MVE	3.6 ± 0.2^{b}

Data are for experiment 2.
*Abbreviations used: 8C, 8% casein; 8C3M, 8% casein supplemented with 3% methionine; 8C3M3G, 8% casein supplemented with 3% methionine and 3% glycine; 8C3MVE, 8% casein supplemented with 3% methionine and vitamin E of 10 times the amount in the other three diets.
†All values are mean ± SEM ($n = 6$). Means in the same column with a different superscript are significantly different by Duncan's multiple range test ($P < 0.05$).

Table 5 Activity SOD in erythrocytes and DPPH-reactive substance in plasma of rats fed the experimental diets (Experiment 2)

Diet	SOD (U*/g Hb)	DPPH-reactive Substance (%)
8C†	820.02 ± 43.65^{a}‡	97.93 ± 2.42^{a}
8C3M	532.03 ± 79.07^{b}	$92.70 \pm 2.12^{a,b}$
8C3M3G	$598.59 \pm 101.05^{a,b}$	$90.29 \pm 1.33^{b,c}$
8C3MVE	796.67 ± 67.68^{a}	84.64 ± 2.62^{c}

*See Ref. 20 for the expression of activity.
†Abbreviations used: 8C, 8% casein; 8C3M, 8% casein supplemented with 3% methionine; 8C3M3G, 8% casein supplemented with 3% methionine and 3% glycine; 8C3MVE, 8% casein supplemented with 3% methionine and vitamin E of 10 times the amount in the other three diets.
‡All values are mean ± SEM ($n = 6$). Means in the same column with a different superscript are significantly different.

Table 6 The plasma ceruloplasmin and copper content in plasma, liver, and kidneys of rats fed the experimental diets (Experiment 2)

Diet	Plasma ceruloplasmin (U*/mL)	Copper content				
		Plasma (mg/dL)	Liver (μg/g)	Liver (μg/whole)	Kidney (μg/g)	Kidney (μg/whole)
8C†	0.11 ± 0.01[a]‡	114.41 ± 5.20[a]	6.347 ± 1.704[a]	44.63 ± 10.33[a]	2.160 ± 0.169[b]	1.330 ± 0.112[a]
8C3M	0.08 ± 0.01[b]	90.69 ± 6.72[a,b]	1.500 ± 0.183[b]	7.93 ± 0.72[b]	7.542 ± 2.006[a]	5.425 ± 1.270[b]
8C3M3G	0.05 ± 0.02[b]	67.57 ± 21.37[b]	0.917 ± 0.201[b]	10.02 ± 2.14[b]	1.968 ± 0.489[b]	1.853 ± 0.514[b]
8C3MVE	0.08 ± 0.01[b]	96.58 ± 11.53[a,b]	0.668 ± 0.106[b]	3.75 ± 0.68[b]	5.227 ± 1.316[a,b]	3.468 ± 0.849[b]

*See Ref. 24 for the expression of activity.
†Abbreviations used: 8C, 8% casein; 8C3M, 8% casein supplemented with 3% methionine; 8C3M3G, 8% casein supplemented with 3% methionine and 3% glycine; 8C3MVE, 8% casein supplemented with 3% methionine and vitamin E of 10 times the amount in the other three diets.
‡All values are mean ± SEM ($n = 6$). Means in the same column with a different superscript are significantly different.

bated at 37°C for 24 hr in the 8C3M group was higher than those in the 8C and 8C3M3G groups. The NaCl concentration which resulted in 50% hemolysis was 0.85% for 8C, 1.05% for 8C3M, and 0.92% for 8C3M3G. The methemoglobin content in the 8C3M group was not significantly different from that of the 8C group. The methemoglobin levels in the 8C, 8C3M, and 8C3M3G groups (%, Experiment 3) were 1.87 ± 0.3,[b] 2.67 ± 0.3,[a,b] and 2.93 ± 0.24,[a] respectively. The iron content in tissues, especially in the spleen, in the 8C3M group was significantly higher compared with the control and 8C3M3G groups (*Table 3*). Higher plasma TBARS levels in the excess methionine group were reduced 15 and 22% by the addition of glycine and vitamin E, respectively (*Table 4*), but the 8C3MVE group developed anemia. There were no significant differences in the plasma level of DPPH-reactive substances between the 8C3M and 8C3M3G groups and the plasma level of DPPH-reactive substances did not necessarily reflect the level of TBARS (*Table 5*). No significant difference in the water-soluble fluorescent substance value was observed among the four diet groups (Experiment 2, data not shown).

SOD activity of erythrocytes in the 8C3M group was significantly lower than that in the 8C group (*Table 5*), but there was not significant difference in catalase and GSH-PX activities among the four diet groups (Experiment 2, data not shown).

The mean value of the plasma ceruloplasmin and copper content in the 8C3M3G group was low because the values of three rats were extremely low compared with those of the other three rats in the group (less than one-tenth). The kidneys in the 8C3M and 8C3MVE groups contained higher amounts of copper than those of two of the other groups (*Table 6*). The content of copper in the spleen was under the detection limit in all diet groups.

No significant difference in either protein or thiol content in erythrocyte membranes was observed among the 8C, 8C3M, and 8C3M3G groups (data not shown). The methionine sulfoxide content in erythrocyte membranes was below the detection limit in all three diet groups.

The densitometry profile of SDS–PAGE of erythrocyte membranes revealed that the ratio of the band corresponding to spectrin, which is a main component of the cytoskeleton of erythrocytes (*Figure 1,* corresponding to C1, 2, M1, 2, and G1, 2 in the 8C, 8C3M, and 8C3M3G groups, respectively) decreased, and several bands with molecular weights less than 65,000 increased in the 8C3M group (corresponding to M7~). The lower molecular weight bands that increased in the 8C3M group decreased remarkably in the 8C3M3G group (corresponding to G8~).

Discussion

This study was conducted to examine the changes in the erythrocytes of rats induced by excess methionine ingestion. The results suggest that lipid peroxidation was not a principal cause of anemia induced by excess methionine but rather it occurred concomitantly with anemia and that there was a correlation between changes in the protein components of erythrocyte membranes and anemia. The development of hemolytic anemia caused by excess methionine ingestion, which was strongly evidenced by increases in reticulocytes (*Table 2*) and in the osmotic fragility of erythrocytes and by enlargement of the spleen (*Table 1*), was prevented by the supplementation of glycine, as in previous reports.[4,33]

A high correlation between the level of TBARS (mainly malonaldehyde) and erythrocyte damage has been reported.[34] Lipid peroxidation, an important feature of methionine toxicity, is proposed to be due to defects in the enzymatic antioxidative defense system.[35] Although significantly higher plasma TBARS in the excess methionine group suggested that peroxidation of erythrocyte lipids was correlated with the development of anemia (*Table 4*), the fact that supplementation of vitamin E to the excess methionine diet prevented the increase in plasma TBARS and the decrease in SOD activity in erythrocytes but failed to prevent the development of hemolysis shows that lipid peroxidation is not a principal cause of anemia induced by excess methionine ingestion.

Cu, Zn-SOD activity in erythrocytes is closely correlated to the copper status,[36,37] and excess methionine ingestion decreases copper availability in the rat.[38,39] The inconsistency between the diet-induced changes in the copper concentration in plasma and tissues and in SOD activities in the erythrocytes in this experiment (*Tables 5 and 6*) suggests, however, that the methionine-induced reduction in SOD ac-

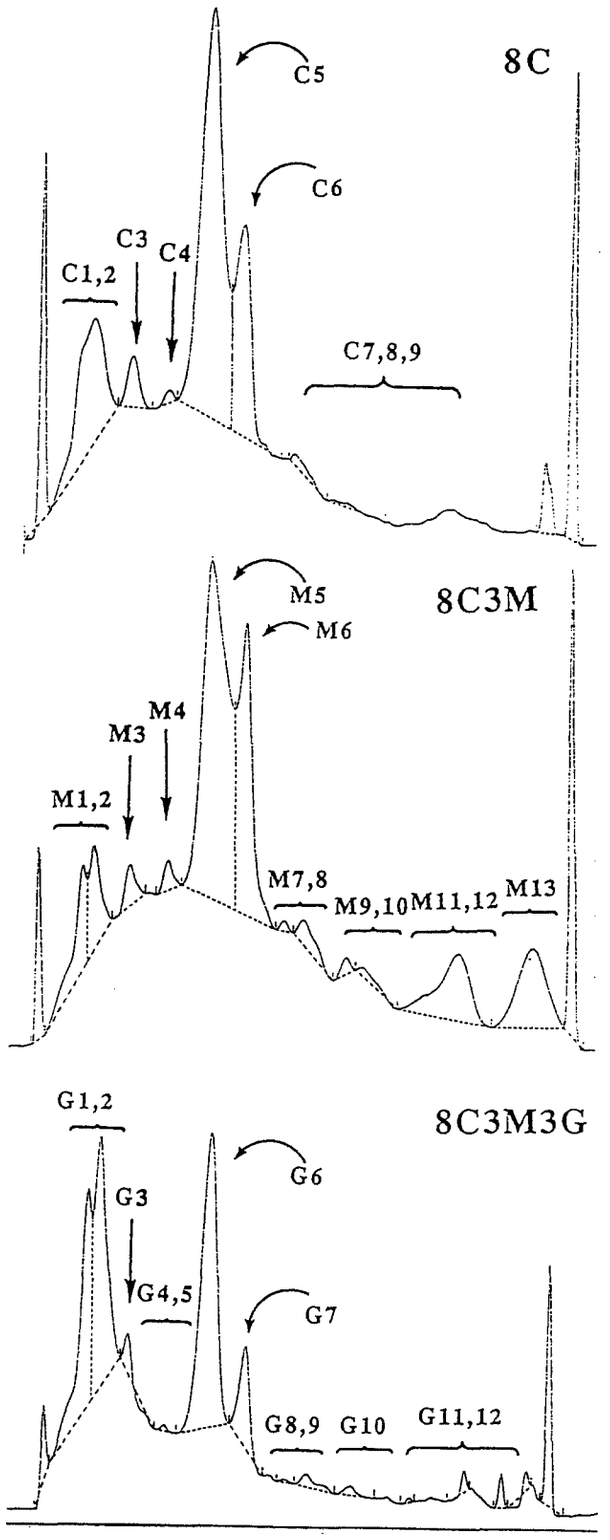

Figure 1 Densitrometry profile by SDS-PAGE of erythrocyte membrane proteins from rats fed the experimental diets (Experiment 3). Ten milligrams of erythrocyte membrane prepared from the pooled blood of six rats in each diet group was extracted with 100 μL of 50 g/L of SDS, and an aliquot of the extract was subjected to SDS-PAGE. 8C, 8% casein; 8C3M, 8% casein supplemented with 3% methionine; 8C3M3G, 8% casein supplemented with 3% methionine and 3% glycine.

tivity was not caused by changes in plasma copper. Although copper deficiency reportedly causes anemia by lowering ceruloplasmin activity,[40] the hematological parameters of the three rats with very low plasma copper and ceruloplasmin content in the 8C3M3G group were similar to the other three rats in the same diet group (described in the Results) and they did not show sings of anemia. Therefore, no correlation between the development of anemia and the ceruloplasmin content was observed in this study.

Iron accumulation in tissues so far examined (especially in the spleen) in the 8C3M and 8C3MVE groups was correlated to the development of anemia. Although total iron was not determined, the accumulation of iron in all tissues suggests that excess methionine ingestion affects the iron in the body and/or stimulates iron absorption.

Since lipid peroxidation is not a prerequisite for methionine-induced anemia in rats, the effect of excess methionine ingestion on changes in the protein components of erythrocyte membranes was examined. The inside of the lipid bilayer of the erythrocyte membrane is reinforced by networks of cytoskeletal proteins such as spectrin and actin which are presumably needed to maintain cell shape and elastic deformability. Although each cytoskeletal protein could not be accurately measured, the apparent change in the cytoskeletal protein composition in the 8C3M group and the normalization by supplementation with glycine (*Figure 1*) suggest that the changes in the erythrocyte cytoskeletal protein composition is at least partly responsible for the erythrocyte abnormalities. It was assumed that alteration in the composition of erythrocyte cytoskeletal proteins in the excess methionine group was caused by active oxygen species, but the content of methemoglobin, which is formed from oxyhemoglobin and superoxide,[41] was not correlated to the development of anemia. Therefore, it seems unlikely that superoxide produced by the oxidation of oxyhemoglobin was involved in the alteration of erythrocyte membranes.

Although a correlation was reported between the increase of methionine sulfoxide residues with aging and an increase in the protein turnover,[42] the methionine sulfoxide content in the erythrocytes of all diet groups was below the limits of detection, indicating that elevated erythrocyte turnover is not accompanied by an increase of methionine sulfoxide in erythrocyte membrane proteins. Modification of thiol groups of erythrocyte reduces the deformability of erythrocytes and increases hemolysis,[43] but no significant changes in the thiol group content of erythrocyte membranes was seen in any group. Thus, the mechanism by which excess methionine ingestion causes changes in erythrocyte cytoskeletal protein with erythrocyte abnormalities remains obscure.

Recently, methionine toxicity has been linked to hepatic accumulation of S-adenosylmethionine because of a limited number of methyl group receptors, resulting in liver dysfunction.[44] A large amount of adenosine triphosphate (ATP) is consumed in the conversion of methionine to S-adenosylmethionine, and a decrease in erythrocyte ATP is known to increase echinocytes and to decrease erythrocyte deformability and filterability[45] resulting in the enhanced breakdown of erythrocytes and hemolytic anemia. Furthermore, a decrease in the hepatic ATP by ingestion of excess methi-

onine in guinea pigs[46] and rats[47] has been reported. Although no data are available on changes in erythrocyte ATP by excess methionine ingestion, it is interesting to note that the changes in erythrocytes induced by ATP deficiency are very similar to those caused by excess methionine ingestion.

It has been reported that dietary zinc deficiency in rats enhances the osmotic fragility of erythrocytes and the alteration of membrane skeletal protein composition[46,48] and that it affects the polyamine compositions of erythrocyte membranes.[49,50] Thus, the changes in the concentration of membrane-bound polyamines may account for some of the effects of dietary zinc deficiency on the structure and function of erythrocyte membrane. The study on the effect of excess methionine ingestion on the concentrations of ATP, zinc, and polyamines in erythrocytes is currently underway.

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