Tissue Vitamin E Status Is Compromised by Dietary Protein Insufficiency in Young Growing Rats^{1,2}

CHING-JANG HUANG³ AND HUEY-MEI SHAW

Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan 10764, Republic of China

ABSTRACT In vitro erythrocyte hemolysis previously has been found to be significantly elevated in rats fed a low protein diet. To investigate the effects of dietary protein insufficiency on tissue vitamin E status, four groups of male weanling Long-Evans rats were given free access to either an 8% (wt/wt) lactalbumin diet containing 50 (L50) or 500 (L500) mg/kg diet of all-*rac*- α -tocopheryl acetate, or a 20% (wt/wt) lactalbumin diet containing 50 (N50) or 500 (N500) mg/kg of all-*rac*- α -tocopheryl acetate for 6 wk. A fifth group (P50) was pair-fed to the L50 group with the N50 diet. The L50 and L500 groups showed signs of protein malnutrition such as growth retardation as well as lower hematocrit and plasma protein and albumin concentrations. The α -tocopherol concentrations of plasma, heart, lung, kidney and muscle were significantly lower in the L50 group compared with the N50 group. The L500 group showed significantly less α -tocopherol in heart, kidney, muscle, brain and adipose tissue compared with the N500 group. The α -tocopherol concentrations in liver, testis, spleen and adrenal gland did not significantly differ either between the L50 and N50 groups or between the L500 and N500 groups. Supplementation with 500 mg/kg diet of all-*rac*- α -tocopheryl acetate resulted in significantly higher tissue α -tocopherol concentration in all tissues except muscle. The tissue α -tocopherol concentrations of the P50 group were not significantly different from those of the N50 group. The results indicated that protein insufficiency resulted in significantly lower α -tocopherol concentrations in most tissues in young growing rats. *J. Nutr.* 124: 571-579, 1994.

INDEXING KEY WORDS:

- rats • vitamin E
- dietary protein • antioxidative enzymes

Vitamin E is the most important lipid-soluble antioxidant in the body; it works to quench free radicals and acts as a terminator of lipid peroxidation (Burton and Ingold 1989, Ingold et al. 1987b). Vitamin E deficiency in humans (Sokol 1988) occurs in patients with fat malabsorption and in individuals with such genetic disorders as abetalipoproteinemia, homozygous hypobetalipoproteinemia, and the "familial

isolated vitamin E deficiency" (Kayden et al. 1987, Traber et al. 1990b). Impaired utilization (including digestion, absorption, transportation, tissue uptake and storage) of dietary vitamin E rather than inadequate dietary intake seems to underlie most circumstances of vitamin E deficiency. The detailed processes of the utilization of dietary vitamin E by tissue cells are not fully understood at present, and the relative importance of endogenous as opposed to exogenous factors that may alter the utilization of dietary vitamin E by tissue cells also remains to be explored.

Previously we observed elevated in vitro hemolysis of erythrocytes from rats fed a low protein diet (Huang et al. 1988) and demonstrated subsequently that feeding a high polyunsaturated fat and low protein diet resulted in lower antioxidative enzyme activities and enhanced tissue lipid peroxidation (Huang and Fwu 1992). The role of adequate dietary protein in maintaining the balance between antioxidative defense systems and oxidative stress was confirmed in a subsequent study (Huang and Fwu 1993). The RBC in vitro hemolysis test has long been used as a criterion for the assessment of vitamin E status. Although the lowered antioxidative enzyme activity in RBC may partially contribute to the higher hemolysis, the possibility that feeding a low protein diet may result in inefficient utilization of dietary vitamin E cannot be excluded. Rajaram et al. (1977) reported lowered distribution of orally dosed all-*rac*- α -[5-CH₃-³H]tocopherol in the liver cytosol of rats fed a 5% (wt/wt) casein diet. Mouri et al. (1986) showed that α -tocopherol levels in RBC, kidney, lung and

¹Supported by grant no. NSC 81-0409-B002-575 from the National Science Council, Taiwan, Republic of China.

²The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

³To whom correspondence should be addressed.

muscle were lower in rats fed a 10% casein diet compared with rats fed a 20% casein diet. In our previous report, we found that supplementing a low protein diet (containing 8% lactalbumin and 15% soybean oil) with 150 mg/kg diet of all-*rac*- α -tocopheryl acetate significantly decreased the thiobarbituric acid-reactive substances (TBARS) in plasma, heart and liver (but not in the remaining tissues measured) and restored the depressed activities of RBC superoxide dismutase (SOD, EC 1.15.1.1) and catalase (Huang and Fwu 1992). That some tissues of rats fed a low protein diet did respond to the supplementation of all-*rac*- α -tocopheryl acetate provides further evidence that vitamin E utilization may be impaired in protein malnutrition. To better characterize the utilization efficiency of dietary α -tocopherol by various tissues in protein malnutrition, we have now compared the tissue α -tocopherol levels between rats fed a low protein diet and those fed a normal protein diet at both normal and high dietary levels of all-*rac*- α -tocopheryl acetate. The increment in tissue α -tocopherol in response to the increase in dietary all-*rac*- α -tocopheryl acetate provided further information on the utilization of dietary vitamin E as affected by dietary protein insufficiency.

MATERIALS AND METHODS

Animals and diets. Forty-five male weanling Long-Evans rats, weighing 53.0 ± 9.2 g, were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University. They were housed individually in stainless steel wire cages in a room maintained at $25 \pm 2^\circ\text{C}$ with a controlled 12-h light:dark cycle. The rats were fed a nonpurified diet (Laboratory Rodent Chow, Ralston Purina, St. Louis, MO) for 3 d, when body weight reached 75.8 ± 10.4 g. They were then randomly assigned to five diet groups with nine rats in each group. Tap water was freely available. Body weights were recorded weekly. Animal care and handling conformed to the NIH *Guide for the Care and Use of Laboratory Animals* (NRC 1985).

The composition of the four test diets is shown in Table 1. The dietary protein was either 20% (normal protein, N50 and N500 diets) or 8% (low protein, L50 and L500 diets) lactalbumin. The N50 and L50 diets contained an adequate level of vitamin E (50 mg all-*rac*- α -tocopheryl acetate/kg diet; AIN 1977). The N500 and L500 diets contained a high level of vitamin E (500 mg all-*rac*- α -tocopheryl acetate/kg). One group of rats was given free access to each of the four test diets. Food intake was recorded every other day (daily for the L50 group). The fifth group (P50) of rats was fed the N50 diet and pair-fed with the mean consumption of the L50 group. The powdered ingredients were mixed in advance and stored at -30°C ,

TABLE 1
Composition of test diets

Component	Diet			
	L50	N50	L500	N500
	g/kg diet			
Lactalbumin ¹	80.0	200.0	80.0	200.0
Cornstarch ¹	690.0	570.0	690.0	570.0
Soybean oil ¹	150.0	150.0	150.0	150.0
Cellulose ¹	30.0	30.0	30.0	30.0
AIN-76 vitamin mixture (vitamin E free) ²	10.0	10.0	10.0	10.0
AIN-76 mineral mixture ²	35.0	35.0	35.0	35.0
Choline chloride ¹	3.0	3.0	3.0	3.0
all- <i>rac</i> - α -Tocopheryl acetate	0.05	0.05	0.5	0.5

¹Ingredient sources: lactalbumin and choline chloride, Sigma Chemical (St. Louis, MO); α -cellulose, ARBOCEL®, type BE 600/300, J. Bettenmaier, & Söhne, Germany; soybean oil, Taiwan Sugar Company (Taipei, Taiwan); cornstarch, Roquette, France.

²American Institute of Nutrition (1977).

and the oil portion was blended in each week before feeding. The soybean oil usually used had a typical peroxide value (POV) of 1.30 mEq/kg oil and typical vitamin E concentrations of 67, 485 and 21 $\mu\text{g/g}$ oil for α -, γ - and δ -tocopherol, respectively.

Tissue sampling and preparation. After 6 wk of feeding, food was withheld overnight and the rats were killed by carbon dioxide asphyxiation in the morning. Blood was collected from the abdominal vena cava into a EDTA-containing tube. Liver, kidney, heart, spleen, lung, brain, adrenal gland, testis, epididymal fat pad and gastrocnemius muscle were excised, weighed, quick-frozen in liquid nitrogen and stored at -30°C . Aliquots of blood samples were centrifuged at $1000 \times g$ for 10 min, and the separated plasma was stored at -30°C . A portion of liver was immediately homogenized in ice-cold 0.01 mol/L phosphate buffer (pH 7.4, containing 11.5 g/L KCl) using a Potter-Elvehjem-type homogenizer with a Teflon pestle. Aliquots of the crude homogenate (250 g/L) were centrifuged at $12,000 \times g$, 4°C for 20 min (model 1900 centrifuge RA-80, Kubota Co., Tokyo, Japan), and the post-mitochondria supernatants (PMS) were stored at -30°C .

Analysis. Blood samples were analyzed for hematocrit by centrifugation in capillary tubes and for hemoglobin by the cyanomethemoglobin method (Oser 1965). Plasma albumin was determined spectrophotometrically with bromocresol green dye

⁴Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; SOD, superoxide dismutase; PMS, post-mitochondria supernatant; TBARS, thiobarbituric acid-reactive substances.

(Dumas et al. 1971). Commercially available reagent kits were used to analyze plasma samples for triglyceride (Randox Lab-Ltd., Crumlin, North Ireland, U.K.), cholesterol (Randox Lab-Ltd.) and phospholipid (Nissui Pharmaceutical, Tokyo, Japan). Reduced glutathione was estimated by determining nonprotein sulfhydryls as described by Sedlak and Lindsay (1968). In this procedure, freshly prepared liver homogenates were reacted with Ellman's reagent after protein was precipitated by trichloroacetic acid. The frozen liver PMS were thawed and treated with Drabkin's reagent, as described by Paglia and Valentine (1967), and assayed for the activity of glutathione peroxidase (GPX, EC 1.11.1.9) by coupling to glutathione reductase reaction according to the method of Lawrence and Burk (1976). Hydrogen peroxide (final concentration, 88.2 $\mu\text{mol/L}$) and cumene hydroperoxide (final concentration, 526 $\mu\text{mol/L}$) were used as substrates for the assay of Se-dependent and total GPX activities, respectively. Aliquots of the thawed liver PMS were treated with chloroform-ethanol as described by McCord and Fridovich (1969) and assayed for the activity of SOD by the inhibition of cytochrome c reduction initiated by superoxide produced by the xanthine-xanthine oxidase system as described by Flohe and Otting (1984). The thawed liver PMS were also assayed for the activity of catalase by the method of Luck (1963) and for glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity by the increase of absorbance at 340 nm of NADPH as described by Deutsch (1983). Protein contents of PMS and plasma were analyzed by the method of Lowry et al. (1951) using bovine serum albumin as a standard. In the measurement of enzyme activities, the amount of enzymes was adjusted to ensure the linearity of the reaction over the period of measurement and to ensure that the concentrations of substrates were not rate-limiting.

Tissue vitamin E was extracted as described by Buttriss and Diplock (1984) and analyzed by reverse-phase HPLC as reported by Hatam and Kayden (1979) with some modifications. Frozen tissues (liver, kidney, heart, spleen, lung, adrenal gland, epididymal fat pad and muscle) were thawed and homogenized in 0.01 mol/L phosphate buffer (pH 7.4). Two milliliters of absolute ethanol (containing 10 g/L pyrogallol) and 0.3 mL of saturated KOH were added to 1 mL of the tissue homogenates (for adipose tissue, 2 mL of saturated KOH and 2 mL of homogenate were used) in screw-capped tubes. Tubes were capped, vortexed and incubated in a 70°C water bath for 30 min. After saponification, tubes were cooled in an ice bath, and then 1 mL of distilled water and 4 mL of *n*-hexane were added. For plasma vitamin E analysis, 0.5 mL of plasma was mixed with 2 mL of absolute ethanol (containing 10 g/L pyrogallol), 0.1 mL of HCl and 6 mL of *n*-hexane. Tubes containing plasma or saponified tissues were vortexed for 2 min, and the organic

layer was removed after separation. Solvent was removed in a freeze-dryer and the residue solubilized in 0.1 mL of methanol for HPLC analysis of vitamin E. The reverse-phase HPLC analyses of α -tocopherol were performed with a Jasco model 880-PU pump, model 870-UV/Vis detector, model 851-AS autosampler and a computerized data processor. A 4 $\times$ 125 mm Lichrosphere 100RP-18 column (Merck, Darmstadt, Frankfurt, Germany) containing 5- μm particles protected by a guard column was used. The eluant (pure methanol) was pumped at a rate of 1 mL/min. The tocopherols were detected by UV detector (292 nm wavelength), and the retention time of a typical α -tocopherol peak was 5.2 ± 0.3 min. The Chemlab software package (Shiunn-Hwa Co., Taipei, Taiwan) was used to integrate and process the data.

Statistical analysis. Data were expressed as means $\pm$ SD. The significance of differences among the five groups was analyzed by one-way ANOVA and Duncan's multiple range test using the General Linear Model of SAS (SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$. Because the variance of the tissue α -tocopherol of the five groups was not homogeneous, these data were transformed to the log value before analysis by one-way ANOVA and Duncan's multiple range test. Because there was no significant difference in the tissue α -tocopherol values between the N50 and P50 groups, the data of the P50 group was excluded, and two-way ANOVA were conducted among the N50, L50, N500 and L500 groups to confirm the effects of dietary protein, vitamin E and their interaction.

RESULTS

General nutrition indicators. Rats fed the low protein diets (L50 and L500) showed growth retardation. Their final body weight, body weight gain, food intake and feed efficiency were significantly lower than those of rats fed normal protein diets (N50 and N500) (Table 2). Other than feed efficiency, these values were slightly but significantly lower in the P50 group than in rats given free access to normal protein diets (N50 and N500) but were significantly higher than the values for rats fed low protein diets (L50 and L500). The feed efficiency of the P50 group was significantly higher than those of the other four groups (Table 2). The growth, food intake and feed efficiency were not significantly different between the L50 and L500 groups or between the N50 and N500 groups. Rats fed the low protein diets (L50 and L500) had significantly lower relative liver weight than the three groups fed a normal protein level (N50, N500 and P50) (Table 2).

The L50 group generally had the lowest hematocrit and plasma albumin and protein concentrations among the five groups of rats (Table 2). Rats given free

TABLE 2

Body weight gain, food intake, feed efficiency, hematocrit, and plasma albumin and protein concentrations in rats fed two levels of dietary protein and vitamin E¹

	Dietary group ²				
	L50	N50	P50	L500	N500
Final body weight, g	198.4 ± 44.3 ^c	326.1 ± 34.9 ^a	258.5 ± 49.9 ^b	185.5 ± 30.2 ^c	318.6 ± 48.7 ^a
Body weight gain, g/d	4.73 ± 1.05 ^c	7.76 ± 0.83 ^a	6.15 ± 1.19 ^b	4.41 ± 0.72 ^c	7.58 ± 1.16 ^a
Food intake, g/d	11.94 ± 2.40 ^b	15.66 ± 1.65 ^a	11.94 ± 2.41 ^b	11.79 ± 2.01 ^b	15.49 ± 2.13 ^a
Feed efficiency, g gain/g food intake	0.39 ± 0.02 ^c	0.50 ± 0.02 ^b	0.52 ± 0.02 ^a	0.37 ± 0.01 ^d	0.49 ± 0.01 ^b
Relative liver weight, g/100 g body wt	3.2 ± 0.2 ^b	3.8 ± 0.4 ^a	3.8 ± 0.4 ^a	3.4 ± 0.6 ^b	3.7 ± 0.4 ^a
Hematocrit	0.425 ± 0.012 ^c	0.483 ± 0.033 ^a	0.462 ± 0.008 ^b	0.443 ± 0.002 ^{bc}	0.463 ± 0.018 ^b
Plasma albumin, g/L	38.1 ± 0.4 ^b	41.3 ± 2.4 ^a	40.8 ± 2.2 ^a	39.3 ± 2.1 ^{ab}	41.9 ± 2.1 ^a
Plasma protein, g/L	62.9 ± 21.5 ^b	82.0 ± 13.3 ^a	75.0 ± 10.3 ^{ab}	70.2 ± 9.2 ^{ab}	82.8 ± 2.7 ^a

¹Values are means ± SD for groups of nine rats. The significance of differences among the five groups was analyzed by one-way ANOVA and Duncan's multiple range test. Values within a row with different superscript letters are significantly different ($P < 0.05$).

²Rats were fed for 6 wk an 8% (wt/wt) lactalbumin diet containing 50 (L50) or 500 (L500) mg all-*rac*- α -tocopheryl acetate/kg diet or a 20% (wt/wt) lactalbumin diet containing 50 (N50) or 500 (N500) mg all-*rac*- α -tocopheryl acetate/kg diet. The P50 group was pair-fed to the L50 group and consumed the N50 diet.

access to normal protein diets (N50 and N500) had significantly ($P < 0.05$) higher values for these blood measurements than the L50 group. Although the rats fed the low protein diet with the high vitamin E level (L500) had slightly higher hematocrit and plasma albumin and protein concentrations, the values were not significantly different from those of the N500 group, nor were the differences between the L50 and L500 groups significant. The plasma protein and albumin concentrations of the P50 group were not significantly different from those of the N50 group. Although the hematocrit of the P50 group was lower than that of the N50 group, it was significantly higher than that of the L50 group.

Plasma lipids and α -tocopherol. There were no significant differences in the plasma cholesterol and phospholipid concentrations among the five groups of rats (Table 3). The P50 group had slightly lower plasma triglyceride concentration than the remaining four groups of rats, but the difference was significant only between this group and the L500 group. The plasma α -tocopherol concentration of the L50 group was about half of that of the N50 and P50 groups ($P < 0.05$, Table 3). There were no significant differences in plasma α -tocopherol between the N50 and P50 groups or between the L500 and N500 groups. Although the plasma α -tocopherol concentrations of the N500 and L500 groups were slightly higher than those of the N50 and P50 groups, the difference was

TABLE 3

Plasma triglyceride, cholesterol, phospholipid, and α -tocopherol concentrations in rats fed two levels of dietary protein and vitamin E¹

	Dietary group ²				
	L50	N50	P50	L500	N500
Triglyceride, mmol/L	0.833 ± 0.229 ^{ab}	0.855 ± 0.335 ^{ab}	0.648 ± 0.234 ^b	1.060 ± 0.293 ^a	0.809 ± 0.242 ^{ab}
Cholesterol, mmol/L	2.12 ± 0.81	2.20 ± 0.85	2.10 ± 0.56	2.04 ± 0.67	2.29 ± 0.57
Phospholipid, mmol/L	41.8 ± 10.7	46.7 ± 12.0	35.3 ± 9.0	48.5 ± 12.2	45.3 ± 7.4
α -Tocopherol, μ mol/L	4.51 ± 2.25 ^c	8.86 ± 2.71 ^{ab}	8.23 ± 0.99 ^b	10.03 ± 1.70 ^{ab}	10.63 ± 2.28 ^a
α -Tocopherol: triglyceride ratio, μ mol/mmol	5.32 ± 2.12 ^c	11.46 ± 4.21 ^{ab}	14.53 ± 5.43 ^a	9.38 ± 2.92 ^{bc}	14.17 ± 5.22 ^a

¹Values are means ± SD for groups of eight or nine rats. The significance of differences among the five groups was analyzed by one-way ANOVA and Duncan's multiple range test. Values within a row with different superscript letters are significantly different ($P < 0.05$).

²Rats were fed for 6 wk an 8% (wt/wt) lactalbumin diet containing 50 (L50) or 500 (L500) mg all-*rac*- α -tocopheryl acetate/kg diet or a 20% (wt/wt) lactalbumin diet containing 50 (N50) or 500 (N500) mg all-*rac*- α -tocopheryl acetate/kg diet. The P50 group was pair-fed to the L50 group and consumed the N50 diet.

significant only between the N500 and P50 groups. The α -tocopherol:triglyceride ratio in plasma was significantly lower in the L50 group compared with the N50, P50 and N500 groups (Table 3). Although the L500 and N500 groups had similar concentrations of plasma α -tocopherol, the plasma α -tocopherol:triglyceride ratio was significantly lower in the L500 group ($P < 0.05$).

Tissue vitamin E. The tissue α -tocopherol concentrations are shown in Table 4. Among all tissues measured, there were no significant differences in the α -tocopherol concentrations between the P50 and the N50 groups, indicating that lowered food intake per se did not affect tissue α -tocopherol levels. The L50 group showed significantly lower α -tocopherol concentrations in muscle, heart, lung and kidney compared with the N50 group, in contrast to similar α -tocopherol concentrations in liver, testes, spleen, adrenal gland, brain and adipose tissue in these two groups.

On the other hand, when the dietary all-*rac*- α -tocopheryl acetate level was raised to 500 mg/kg, rats fed the low protein diet (L500) showed significantly lower α -tocopherol in their muscle, brain, adipose tissue, heart and kidney compared with the corresponding group receiving a normal protein level (N500). The α -tocopherol concentrations in spleen, adrenal gland, liver, testes and lung were not significantly different between the L500 and the N500 groups.

Results from two-way ANOVA (Table 4) confirmed that dietary protein level had a significant effect on α -tocopherol concentration in muscle, brain, adipose tissue, heart, lung and kidney but not in liver, testis, spleen or adrenal glands. On the other hand, a high dietary vitamin E level resulted in significantly higher α -tocopherol concentrations in all tissues examined except muscle. The interaction between dietary protein and dietary vitamin E level was significant in heart, brain and adipose tissue.

The level of γ -tocopherol detected in tissues was lower than that of α -tocopherol. Generally, γ -tocopherol levels were only about 10 to 15% of those of α -tocopherol except for adrenal gland and kidney, in which the differences between the α - and γ -form vitamers were less (data not shown). In addition, the tissue γ -tocopherol concentrations were not lower in the low protein diet-fed groups compared with the corresponding normal protein-fed controls.

Other antioxidant components in liver. Other antioxidative components, including antioxidative enzyme activities and reduced glutathione in liver, are shown in Table 5. Significantly lower hepatic activities of G6PDH, catalase, SOD, total and Se-dependent GPX as well as the reduced glutathione level were observed in the L50 group compared with the N50 and/or P50 groups. The L500 group also had significantly lower G6PDH, Se-dependent GPX activities and reduced glutathione concentration than

TABLE 4
Tissue α -tocopherol concentration in rats fed two levels of dietary protein and vitamin E^{1,2}

Tissue	Dietary group ³				Dietary factor ²		
	L50	N50	P50	nmol/g tissue	N500	Protein	Vitamin E
Liver	35.31 $\pm$ 7.06 ^b	34.29 $\pm$ 3.93 ^b	30.94 $\pm$ 10.99 ^b	89.59 $\pm$ 26.40 ^a	88.44 $\pm$ 20.22 ^a	0.8233	0.0001
Testis	13.43 $\pm$ 2.00 ^c	14.24 $\pm$ 1.13 ^{bc}	15.78 $\pm$ 2.71 ^b	20.19 $\pm$ 2.76 ^a	19.94 $\pm$ 3.50 ^a	0.2100	0.0001
Muscle	3.40 $\pm$ 1.75 ^c	8.37 $\pm$ 2.05 ^a	7.82 $\pm$ 3.93 ^{ab}	5.17 $\pm$ 2.58 ^{bc}	9.02 $\pm$ 1.79 ^a	0.0001	0.1800
Spleen	7.29 $\pm$ 4.76 ^b	11.45 $\pm$ 6.03 ^{ab}	12.60 $\pm$ 8.51 ^{ab}	19.14 $\pm$ 6.81 ^a	18.33 $\pm$ 9.55 ^a	0.1591	0.0006
Adrenal gland	195.7 $\pm$ 71.8 ^b	271.5 $\pm$ 60.2 ^{ab}	245.2 $\pm$ 54.5 ^{ab}	345.6 $\pm$ 153.5 ^a	334.6 $\pm$ 214.2 ^a	0.7497	0.0233
Brain	5.29 $\pm$ 1.91 ^b	5.54 $\pm$ 1.13 ^b	6.19 $\pm$ 2.60 ^b	7.38 $\pm$ 2.48 ^b	17.73 $\pm$ 4.62 ^a	0.0001	0.0001
Adipose tissue	1.10 $\pm$ 0.25 ^b	1.15 $\pm$ 0.12 ^b	0.90 $\pm$ 0.25 ^b	1.17 $\pm$ 0.44 ^b	5.38 $\pm$ 3.20 ^a	0.0006	0.0001
Heart	0.90 $\pm$ 0.32 ^d	2.74 $\pm$ 1.63 ^{bc}	2.90 $\pm$ 1.36 ^b	1.54 $\pm$ 0.58 ^c	9.78 $\pm$ 7.98 ^a	0.0010	0.0057
Lung	18.63 $\pm$ 6.58 ^c	25.23 $\pm$ 2.00 ^b	25.05 $\pm$ 3.80 ^b	28.64 $\pm$ 3.96 ^{ab}	36.57 $\pm$ 11.59 ^a	0.0077	0.0001
Kidney	5.22 $\pm$ 2.00 ^c	12.01 $\pm$ 1.43 ^{ab}	9.57 $\pm$ 3.31 ^b	10.81 $\pm$ 3.68 ^b	14.44 $\pm$ 1.36 ^a	0.0001	0.0001
							<i>P value</i>
							0.9910
							0.5581
							0.5378
							0.3124
							0.3408
							0.0001
							0.0002
							0.0241
							0.7733
							0.0895

¹Values are means $\pm$ SD for groups of nine rats. Data were transformed to the log values and analyzed by one-way ANOVA and Duncan's multiple range test. Values within a row with different superscript letters are significantly different ($P < 0.05$).

²The effects of dietary protein, vitamin E and their interaction were analyzed by two-way ANOVA, excluding data of the P50 group.

³Rats were fed for 6 wk an 8% (wt/wt) lactalbumin diet containing 50 (L50) or 500 (L500) mg all-*rac*- α -tocopheryl acetate/kg diet or a 20% (wt/wt) lactalbumin diet containing 50 (N50) or 500 (N500) mg all-*rac*- α -tocopheryl acetate/kg diet. The P50 group was pair-fed to the L50 group and consumed the N50 diet.

TABLE 5

Antioxidative enzyme activities and reduced glutathione (GSH) concentration in liver of rats fed two levels of dietary protein and vitamin E^{1,2}

	Dietary group ³									
	L50		N50		P50		L500		N500	
	$\mu\text{g protein}$									
G6PDH	6.86 ±	10.96 ^b	11.47 ±	3.30 ^a	11.69 ±	3.15 ^a	7.64 ±	1.78 ^b	12.45 ±	4.21 ^a
Catalase	119.7 ±	22.9 ^b	133.2 ±	19.1 ^{ab}	158.6 ±	49.1 ^a	104.8 ±	11.0 ^b	118.1 ±	43.6 ^b
SOD	884.9 ±	173 ^b	1051.5 ±	200 ^{ab}	1168.5 ±	306 ^a	858.0 ±	129 ^b	1023.6 ±	174 ^{ab}
Total GPX ⁴	3.95 ±	1.39 ^b	5.72 ±	1.51 ^{ab}	6.60 ±	3.22 ^a	3.89 ±	1.23 ^b	5.47 ±	1.37 ^{ab}
Se GPX ⁴	2.16 ±	0.95 ^c	4.03 ±	1.09 ^{ab}	4.97 ±	2.36 ^a	1.97 ±	0.66 ^c	3.53 ±	0.95 ^b
Non-Se GPX ⁴	1.78 ±	0.59	1.68 ±	1.12	1.68 ±	1.39	1.91 ±	0.73	1.94 ±	1.08
	$\mu\text{mol/g liver}$									
GSH	2.46 ±	0.37 ^b	3.54 ±	0.84 ^a	3.88 ±	1.37 ^a	2.46 ±	0.87 ^b	4.14 ±	0.93 ^a
	mg/g liver									
Protein ⁵	77.09 ±	11.89 ^a	67.54 ±	10.48 ^{ab}	63.43 ±	12.16 ^b	76.45 ±	9.71 ^a	75.36 ±	6.86 ^a

¹Values are means $\pm$ SD for groups of eight or nine rats. After ANOVA, classification of the group means was performed using Duncan's multiple range test. Values within a row with different superscript letters are significantly different ($P < 0.05$).

²One unit of glucose-6-phosphate dehydrogenase (G6PDH) or 1 unit of glutathione peroxidase (GPX) is defined as 1 μmol NADPH produced or consumed per minute. One unit of catalase is defined as moles per minute. One unit of superoxide dismutase (SOD) is defined as 50% of inhibition, which is equivalent to a decrease in absorbance of 0.025/min under the assay condition.

³Rats were fed for 6 wk an 8% (wt/wt) lactalbumin diet containing 50 (L50) or 500 (L500) mg all-*rac*- α -tocopheryl acetate/kg diet or a 20% (wt/wt) lactalbumin diet containing 50 (N50) or 500 (N500) mg all-*rac*- α -tocopheryl acetate/kg diet. The P50 group was pair-fed to the L50 group and consumed the N50 diet.

⁴Total GPX activity was assayed by using cumene hydroperoxide as a substrate, whereas Se GPX was assayed by using hydrogen peroxide as a substrate; non-Se GPX activity is the difference between total and Se GPX.

⁵Post-mitochondria supernatant protein.

the N500 group. There were no significant differences in the non-Se GPX activity among the five groups of rats.

DISCUSSION

In the present study, soybean oil (15%, wt/wt) was incorporated into the experimental diet because we observed very little difference in the tissue TBARS between the low protein diet group and normal protein group when the dietary polyunsaturated fat level was as low as 2 to 5 g/100 g soybean oil (Huang and Fwu 1992). The same regimen was also employed in our previous study, the results of which showed decreased antioxidative enzyme activities in liver and RBC and elevated tissue TBARS and in vitro hemolysis (Huang and Fwu 1993). The reductions in the antioxidative enzyme activities observed in the present study were generally similar to our previous results (Huang and Fwu 1993), although SOD activity was less affected by protein malnutrition. Because the SOD assay used in this study (inhibition of the cytochrome c oxidation, which is induced by the superoxide generated by xanthine-xanthine oxidase) was different from that of the previous study (inhibition of the photooxidation of *o*-dianisidine; Misra

and Fridovich 1977), it is not known whether the slight difference between the two studies reflects the different methods.

Although the various forms of tocopherols in the soybean oil used in this study were not stripped, the main tissue tocopherol detected was the α -form. Generally, the peaks corresponding to γ -tocopherol in the HPLC chart were much lower than that of the α -vitamer. Furthermore, it is known that the tissue retention of γ -tocopherol is relatively low (Behrens and Madère 1983 and 1987). Therefore, we focused only on the changes in tissue α -tocopherol concentrations in the present study.

The responses of tissue α -tocopherol to protein malnutrition varied among different tissues. Liver, testis, adrenal gland and spleen were not affected, whereas heart, lung, kidney, muscle, plasma, brain and adipose tissue were significantly lowered by dietary protein inadequacy. These data agree with the study of Mouri et al. (1986), in which lung, kidney and muscle α -tocopherol concentrations were lower in rats fed a 10% casein diet than in rats fed a 20% casein diet. However, not only was our study more extensive, because we measured more tissues, but also the 2×2 factorial design enabled us to observe the effects of dietary protein insufficiency at both normal and high levels of dietary vitamin E. Two-way ANOVA revealed a significant interaction between

dietary protein and vitamin E level in their effects on tissue α -tocopherol concentrations in heart, brain and adipose tissue. In these tissues, the elevations of α -tocopherol in response to a 10-fold increase in dietary vitamin E were considerably lower in the animals fed the low protein diet compared with normal protein-fed controls. Data obtained in this study provide evidence in support of our hypothesis that dietary protein inadequacy compromises the tissue utilization of dietary α -tocopherol.

It has been shown that the absorbed dietary α -tocopherol is transported on the chylomicron particles (Bjørneboe et al. 1986) and eventually enters the liver with the chylomicron remnants (Bjørneboe et al. 1987a). It is then re-packaged into the VLDL and secreted (Bjørneboe et al. 1987b and 1990, Cohn et al. 1988). Accordingly, the redistribution of α -tocopherol from liver to peripheral tissue depends largely on the VLDL secretion from the liver. In the extreme form of protein malnutrition, kwashiorkor, there is inadequate synthesis of lipoprotein fractions necessary for the transport of triglycerides and other lipids from the liver, and this leads to accumulation of lipid in the liver (Whitehead and Alleyne 1972). Mouri et al. (1986) speculated that a low protein intake may decrease the efficiency of tocopherol transport to tissue cells secondarily to a reduction in circulating lipoprotein containing α -tocopherol. However, in the present study it is unlikely that the 8% lactalbumin diet had such an extreme effect. We did not observe a significant increase in liver triglyceride concentration in our previous study using the same feeding regimen (Huang et al. 1988), and in the present study, the lowest plasma triglyceride concentration was observed in the P50 group rather than in the L50 group. This result can be attributed to the lower or insufficient energy intake of this group, which diminished hepatic endogenous lipogenesis and decreased the export of VLDL from liver. If lower plasma α -tocopherol in the L50 group occurred secondarily to a reduction in the secretion of lipoprotein from the liver, then one would expect to see lower plasma α -tocopherol in the P50 group and an unaltered α -tocopherol:triglyceride ratio in the L50 group. However, we found neither. The significantly lower plasma α -tocopherol concentration and α -tocopherol:triglyceride ratio in the L50 group suggested that the redistribution of α -tocopherol from liver to the peripheral tissues may be impaired in rats fed a low protein diet containing a normal level of α -tocopheryl acetate, but the impaired redistribution is not readily attributable to a reduction in the lipoprotein secretion from the liver.

A 30–40-kDa rat liver cytosolic protein that binds to α -tocopherol with high affinity and specificity has been reported (Behrens and Madère 1982, Catignani and Bieri 1977). Several studies have demonstrated that a rat liver cytosolic protein with similar molecular weight was highly active in mediating in-

termembrane transfer of α -tocopherol (Murphy and Mavis 1981, Verdon and Blumberg 1988). This α -tocopherol-binding protein has been proposed as an intracellular transporter of α -tocopherol and may be responsible for the preferential retention of α -tocopherol (Behrens and Madère 1987, Traber et al. 1990a). Traber et al. (1990b) showed that the ability to incorporate α -tocopherol into lipoprotein secreted by the liver was impaired in patients with familial isolated vitamin E deficiency. They speculated that this impaired ability may be related to an impaired intracellular transfer function by the α -tocopherol-binding protein. Recently two groups of researchers (Sato et al. 1991, Yoshida et al. 1992) have purified such a protein from rat liver. Further investigation of liver cytosolic α -tocopherol transfer activity as well as the α -tocopherol-binding protein in protein-malnourished rats may disclose its possible role in the impaired α -tocopherol secretion from the liver of protein-malnourished rats.

If the impaired redistribution of α -tocopherol from liver to the peripheral tissues in the L50 group was the major cause of the lower α -tocopherol concentrations in plasma and peripheral tissues, then, from the viewpoint of mass balance, one would expect to see some accumulation of α -tocopherol in the liver unless an enhanced catabolism exists. [Our preliminary data showed no significant difference in fecal α -tocopherol content between the control and protein-malnourished groups (unpublished data).] Because the liver α -tocopherol concentration of the L50 group was not significantly different from those of the P50 and N50 groups, an enhanced α -tocopherol catabolism in the liver is speculated. Because the liver antioxidant capability, including the antioxidative enzyme activities and reduced glutathione, was significantly lower in rats fed the low protein diets, it is quite possible that more α -tocopherol was "consumed" to compensate for the depressed antioxidant capability. This point remains to be verified by measuring hepatic and fecal α -tocopherol quinone.

The levels of α -tocopherol in extrahepatic tissues are determined both by uptake and by catabolism or turnover. The uptake of α -tocopherol by a tissue depends on its plasma concentration and the specific uptake mechanism of that tissue. At normal dietary vitamin E intake (50 mg/kg diet), lowered plasma α -tocopherol concentration may be the major determinant of the reduced α -tocopherol levels in heart, lung, kidney and muscle. However, α -tocopherol levels in these four tissues of the L500 group were lower than for the N500 group despite the fact that the plasma concentrations of these two groups were similar. These data imply either that the uptake mechanism of α -tocopherol was altered or that α -tocopherol catabolism (turnover) was enhanced in these four tissues when rats were protein malnourished. Using deuterium-labeled stable isotope, Ingold et al. (1987a) demonstrated half-lives for RRR-

α -tocopherol of 7.6, 11.2, 13.3, 10.9 and 16.6 d in lung, kidney, heart, muscle and spleen, respectively, in contrast to the longer half-lives (29.4, 34.8 and 33.3 d) in brain, adipose tissue and testis. The shorter half-lives of α -tocopherol in heart, lung, kidney and muscle may render the α -tocopherol in these tissues more vulnerable to a lower plasma α -tocopherol concentration. The relatively long half-lives of α -tocopherol in brain, testis and adipose tissue may partially explain why these tissues were minimally affected by protein malnutrition at normal dietary vitamin E intake. However, an impaired uptake may still exist in the brain and adipose tissue of the protein-malnourished rats, because the α -tocopherol concentrations of these tissues were lower in the L500 group than in the N500 group. As for testis, adrenal gland and spleen, a highly efficient uptake and/or regeneration mechanism for α -tocopherol may exist that protects the α -tocopherol levels in these tissues from being affected by dietary protein insufficiency.

In conclusion, this study demonstrated that the α -tocopherol levels in most tissues were decreased by feeding growing rats a low protein diet with either a normal or high level of dietary vitamin E. This finding may be important for pediatric patients with secondary protein malnutrition.

LITERATURE CITED

- American Institute of Nutrition (1977) Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. *J. Nutr.* 107: 1340-1344.
- Behrens, W. A. & Madère, R. (1982) Occurrence of a rat liver α -tocopherol binding protein in vivo. *Nutr. Rep. Int.* 25: 107-112.
- Behrens, W. A. & Madère, R. (1983) Interrelationship and competition of α - and γ -tocopherol at the level of intestinal absorption, plasma transport and liver uptake. *Nutr. Res.* 3: 891-897.
- Behrens, W. A. & Madère, R. (1987) Mechanisms of absorption, transport and tissue uptake of RRR- α -tocopherol and *d*- γ -tocopherol in the white rat. *J. Nutr.* 117: 1562-1569.
- Bjørneboe, A., Bjørneboe, G.E.A., Bodd, E., Hagen, B. F., Kveseth, N. & Drevon, C. A. (1986) Transport and distribution of α -tocopherol in lymph, serum and liver cells in rats. *Biochim. Biophys. Acta* 889: 310-315.
- Bjørneboe, A., Bjørneboe, G.E.A. & Drevon, C. A. (1987a) Serum half life, distribution, hepatic uptake and biliary excretion of α -tocopherol in rats. *Biochim. Biophys. Acta* 921: 175-181.
- Bjørneboe, A., Bjørneboe, G.E.A. & Drevon, C. A. (1990) Absorption, transport and distribution of vitamin E. *J. Nutr.* 120: 233-242.
- Bjørneboe, A., Bjørneboe, G.E.A., Hagen, B. F., Nossen, J. & Drevon, C. A. (1987b) Secretion of α -tocopherol from cultured rat hepatocytes. *Biochim. Biophys. Acta* 922: 199-205.
- Burton, G. W. & Ingold, K. U. (1989) Vitamin E as an in vitro and in vivo antioxidant. *Ann. N. Y. Acad. Sci.* 570: 7-22.
- Buttriss, J. L. & Diplock, A. T. (1984) High-performance chromatography methods for vitamin E in tissues. In: *Methods in Enzymology* (Packer, L., ed.), vol. 105, pp. 131-138. Academic Press, New York, NY.
- Catignani, G. L. & Bieri, J. G. (1977) Rat liver α -tocopherol binding protein. *Biochim. Biophys. Acta* 497: 349-357.
- Cohn, W., Loechleiter, F. & Weber, F. (1988) α -Tocopherol is secreted from rat liver in very low density lipoproteins. *J. Lipid Res.* 29: 1359-1366.
- Deutsch, J. (1983) Glucose-6-phosphate dehydrogenase. In: *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. III, pp. 190-197. Academic Press, New York, NY.
- Doumas, B. T., Watson, W. A. & Biggs, H. G. (1971) Albumin standards and the measurement of serum albumin with bromocresol green. *Clin. Chim. Acta* 31: 87-96.
- Flohe, L. & Otting, F. (1984) Superoxide dismutase assays. *Methods Enzymol.* 105: 93-101.
- Hatam, L. J. & Kayden, H. J. (1979) A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood. *J. Lipid Res.* 20: 639-645.
- Huang, C. J., Cheung, N. S. & Lu, V. R. (1988) Effects of deteriorated frying oil and dietary protein levels on liver microsomal enzymes in rats. *J. Am. Oil Chem. Soc.* 65: 1796-1803.
- Huang, C. J. & Fwu, M. L. (1992) Protein insufficiency aggravates the enhanced lipid peroxidation and reduced activities of antioxidative enzyme in rats fed diets high in polyunsaturated fat. *J. Nutr.* 122: 1182-1189.
- Huang, C. J. & Fwu, M. L. (1993) Degree of protein deficiency affects the extent of the depression of the antioxidative enzyme activities and the enhancement of tissue lipid peroxidation in rats. *J. Nutr.* 123: 803-810.
- Ingold, K. U., Burton, G. W., Foster, D. O., Hughes, L., Lindsay, D. A. & Webb, A. (1987a) Biokinetics of and discrimination between dietary RRR- and SRR- α -tocopherols in the male rat. *Lipids* 22: 163-172.
- Ingold, K. U., Webb, A. C., Witter, D., Burton, G. W., Metcalfe, T. A. & Muller, D.P.R. (1987b) Vitamin E remains the major lipid-soluble, chain-breaking antioxidant in human plasma even in individuals suffering severe vitamin E deficiency. *Arch. Biochem. Biophys.* 259: 224-225.
- Kayden, H. J., Sokol, R. J. & Traber, M. G. (1987) Familial vitamin E deficiency with neurologic abnormalities in the absence of fat malabsorption. In: *Clinical and Nutritional Aspects of Vitamin E* (Hayaishi, O. & Mino, M., eds.), pp. 193-197. Elsevier, Amsterdam, The Netherlands.
- Lawrence, R. A. & Burk, R. F. (1976) Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem. Biophys. Res. Commun.* 71: 952-958.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Luck, H. (1963) Catalase. In: *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 885. Academic Press, New York, NY.
- McCord, J. M. & Fridovich, I. (1969) Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049-6055.
- Misra, H. P. & Fridovich, I. (1977) Superoxide dismutase: a photochemical augmentation assay. *Arch. Biochem. Biophys.* 181: 308-312.
- Mouri, K., Hayafune, Y. & Igarashi, O. (1986) Effect of dietary protein on vitamin E levels in erythrocyte and tissues of rats. *J. Nutr. Sci. Vitaminol.* 32: 145-155.
- Murphy, D. J. & Mavis, R. D. (1981) Membrane transfer of α -tocopherol. *J. Biol. Chem.* 256: 10464-10468.
- Oser, B. L. (1965) Blood analysis. In: *Hawk's Physiological Chemistry*, p. 1096. McGraw-Hill, New York, NY.
- National Research Council (1985) Guide for the Care and Use of Laboratory Animals. Publication no. 85-23 (rev.), National Institutes of Health, Bethesda, MD.
- Paglia, D. E. & Valentine, W. N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* 70: 158-169.
- Rajaram, O. V., Fatterpaker, P. & Sreenivasan, A. (1977) Effect of protein deficiency on absorption, transport and distribution of α -tocopherol in the rat. *Br. J. Nutr.* 37: 157-165.
- Sato, Y., Hagiwara, K., Arai, H. & Inoue, K. (1991) Purification and characterization of the α -tocopherol transfer protein from rat liver. *FEBS Lett.* 288: 41-45.

- Sedlak, J. & Lindsay, R. H. (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* 25: 192-205.
- Sokol, R. J. (1988) Vitamin E deficiency and neurologic disease. *Annu. Rev. Nutr.* 8: 351-373.
- Traber, M. G., Burton, G. W., Ingold, K. U. & Kayden, H. J. (1990a) *RRR*- and *SRR*- α -tocopherols are secreted without discrimination in human chylomicrons, but *RRR*- α -tocopherol is preferentially secreted in very low density lipoproteins. *J. Lipid Res.* 31: 675-685.
- Traber, M. G., Sokol, R. J., Burton, G. W., Ingold, K. U., Papus, A. M., Huffaker, J. H. & Kayden, H. J. (1990b) Impaired ability of patients with familial isolated vitamin E deficiency to incorporate α -tocopherol into lipoproteins secreted by the liver. *J. Clin. Invest.* 85: 397-407.
- Verdon, C. & Blumberg, J. (1988) Influence of dietary vitamin E on the intermembrane transfer of α -tocopherol as mediated by an α -tocopherol binding protein. *Proc. Soc. Exp. Biol. Med.* 189: 52-60.
- Whitehead, R. G. & Alleyne, G.A.O. (1972) Pathophysiological factors of importance in protein-calorie malnutrition. *Br. Med. Bull.* 28: 72-78.
- Yoshida, H., Yusin, M., Ren, I., Kuhlenkamp, J., Hirano, T., Stolz, A. & Kaplowitz, N. (1992) Identification, purification and immunochemical characterization of a tocopherol-binding protein in rat liver cytosol. *J. Lipid Res.* 33: 343-350.