Change in the Level of Vitamin C and Lipid Peroxidation in Tissues of the Inherently Scorbutic Rat during Ascorbate Deficiency

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To investigate an accurate profile of vitamin C deficiency, ascorbate deficiency was caused in the inherently scorbutic rat [Osteogenic Disorder Shionogi (ODS)], and changes in the level of the vitamin in 12 tissues of the animals (plasma, liver, stomach, small and large intestines, lung, heart, kidney, adrenal gland, spleen, muscle, and brain) were followed based on the specific method (Kishida et al. *Anal. Chem.* **1992**, *64*, 1505−1507). The level of ascorbate in plasma decreased most rapidly, and the rate of decline of the vitamin was the slowest in the brain among the 12 tissues. Based on the kinetic profile of ascorbate decay, these tissues were classified into four groups. After 25 days of ascorbate deficiency, indicators of oxidative stress changed significantly compared with the control group. The indices included increased lipid hydroperoxide level determined by the specific method (Tokumaru et al. *Anal. Chim. Acta* **1995**, *307*, 97−102) in the brain, elevated thiobarbituric acid-reactive substances (TBARS) and glutathione peroxidase activity in the heart, and the fall of glutathione in plasma and the liver.

Keywords: *Ascorbic acid; vitamin C; ODS rat; lipid hydroperoxide; lipid peroxidation*

INTRODUCTION

Although vitamin C (Seib and Tolbert, 1982; Davies et al., 1991) is a well-known molecule, the biochemistry of L-ascorbic acid in mammals is poorly understood. One of the reasons may be the fact that the method to determine the vitamin is very limited (Washko, 1992). The level of ascorbic acid has still been often determined by the conventional colorimetric assay (Roe et al., 1943) developed over a half century ago. Recently, we developed a novel method (Kishida et al., 1992) involving the oxidation of ascorbic acid into dehydroascorbic acid that was converted into bis((2,4-dinitrophenyl)hydrazone), whose structure was determined by modern spectroscopic analyses, followed by its quantitation with HPLC. This method was sensitive and specific enough to determine ascorbic acid in biological samples. Besides, this method eliminates the possibility to measure ascorbic acid contaminated with water-soluble reductants, which are difficult to separate by the usual HPLC columns because of high hydrophilicity of vitamin C when biological material is applied directly to HPLC equipped with an electrochemical detector that is being widely used recently (Washko, 1992). Based on this method, it was shown that the conventional colorimetric method was three times as high as the true level in the determination of vitamin C in rat plasma (Kishida et al., 1992). This finding demonstrates that the re-evaluation of the most fundamental study of the vitamin is urgently necessary.

In this paper, we report for the first time the change in the level of ascorbate in animal tissues during vitamin C deficiency based on the newly developed specific method (Kishida et al., 1992). The used animal is a ODS rat (Mizushima et al., 1984), which cannot synthesize vitamin C by the lack of a key enzyme in ascorbate biosynthesis (Kawai et al., 1992). This animal may serve as a good model for the investigation of vitamin C deficiency, especially in relation to the role of ascorbate to prevent radical chain reactions in tissues because ascorbate has received much attention as a reducing agent since its discovery and recently it is recognized as an outstanding antioxidant (Frei et al., 1989). We also report results concerning the level of oxidative stress caused by the depletion of vitamin C based on some indices of lipid peroxidation in biological studies.

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MATERIALS AND METHODS

Materials. Dehydro-L-ascorbic acid bis((2,4-dinitrophenyl)-hydrazone) (Kishida et al., 1992), 1-naphthyldiphenylphosphine, and its oxide (Tokumaru et al., 1995) were prepared according to the literature. All other chemicals were purchased from Wako Pure Chem. Co. Ltd. (Osaka, Japan) and were of analytical grade.

Animals and Diets. A notice from the Prime Minister's Office of Japan (No. 6 of March 27, 1980) for the care and use of laboratory animals was followed. The 5-week-old homozygous male ODS rats (od/od) were purchased from Clear Japan Inc. (Tokyo, Japan). Animals were permitted free access to commercially available vitamin C-deficient food (type CL-2, Clear Japan Inc., Tokyo, Japan).

For the first week, all rats were supplied with distilled and autoclaved water containing 1 g of vitamin C/L, which was shown to be sufficient to maintain normal growth (Mizushima et al., 1984). After the week of acclimation, the rats were divided into two groups. The control group received vitamin C (1 g/L) in drinking water. The experimental group received distilled water without ascorbic acid. The animals were housed in a room with a temperature 24 ± 2 °C and a 12-h light/dark cycle.

Design of the Work. After 1 week of acclimation, a vitamin C-deficient diet was given to the ODS rats as described above. At the start of the experiment (day 0), the total level of vitamin C in 12 tissues was determined. After 3, 10, 20, and 25 days from the start of the deficiency experiment, the level of vitamin C in these tissues was measured and compared with the starting values. After 25 days of the experiment when the effect of the deficiency was prominent (based on our experience, the longest survival of ODS rats during vitamin C deprivation was about 30 days), the levels of lipid peroxidation were evaluated and compared with those of the control group.

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Determination of Vitamin C. Rats were anesthetized with diethyl ether and killed by collecting the blood from the inferior vena cava using a syringe containing sodium heparin as an anticoagulant. After perfusion of ice-cooled saline from the portal vein, the organs were removed. The excised tissue was homogenized in 5 volumes of 10 mmol/L phosphate-buffered saline (pH 7.2) under ice cooling. All determinations were made by duplicated experiments. The determination of ascorbic acid was made based on the previous report (Kishida et al., 1992) with some modifications. One hundred microliters of tissue homogenate or plasma was added to 900 μL of metaphosphoric acid solution (200 g/L) containing stannous chloride (10 g/L) followed by centrifugation at 10000*g* for 10 min at 4 °C to get a deproteinized sample. A 100-μL sample of the supernatant was taken out and mixed with 100 μL of 2,6-dichloroindophenol (2 g/L) to oxidize ascorbic acid. The solution was mixed with 50 μL of stannous chloride (10 g/L) in a metaphosphoric acid solution (50 g/L) and with 120 μL of 2,4-dinitrophenylhydrazine (20 g/L) in 4.5 mol/L of sulfuric acid. The mixture was incubated for 3 h at 37 °C. After cooling in an ice bath, ethyl acetate (1 mL) and water (1 mL) were added to the reaction mixture. After shaking and centrifugation (1000*g*) for 10 min, 600 μL of the ethyl acetate layer was taken out and evaporated to dryness. The residue was dissolved in 100 μL of acetonitrile, and 10 μL of the sample was applied to HPLC. For HPLC analysis, a Shimadzu LC-9A pump was used. The sample was applied to μ-Bondasphere 5-μm C_{18}-100A column (3.9 × 150 mm, Waters), eluted with a 1:1 mixture of acetonitrile and water adjusted at pH 3.5 with triethylamine (10 g/L) and phosphoric acid at the rate of 1 mL/min. The absorption at 505 nm was recorded with a spectrophotometer (type SPD-10A manufactured by Shimadzu, Kyoto, Japan). The detection limit was 1 pmol, which was identical with the literature (Kishida et al., 1992).

Determination of Lipid Hydroperoxide and Thiobarbituric Acid-Reactive Substances (TBARS). The quantitation of the total level of lipid hydroperoxides was made according to the literature (Tokumaru et al., 1995, 1996). TBARS were measured according to the literature and expressed as nanomole equivalent of malondialdehyde/gram of tissue (Buege and Aust, 1978).

Determination of Glutathione. The tissue was treated according to the literature (Anderson, 1985). The levels of total glutathione and oxidized glutathione (GSSG) were determined by the enzymatic method (Anderson, 1985; Brehe and Burch, 1976).

Enzyme Assay. The activity of glutathione peroxidase (EC 1.11.1.9) was measured based on the literature (Flohe and Guenzler, 1984) using cumene hydroperoxide, which was dissolved in 25% (v/v) methanol at 30 mmol/L (i.e., the final concentration in the assay tube was 3 mmol/L) as a substrate. The activity was expressed as the decrease of NADPH (mg of protein)$^{-1}$ min^{-1}.

Protein Assay. Protein concentrations were determined according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Statistical Analysis. The data were expressed as mean ± SD (*n*), where the number in parentheses represented the number of animals and were statistically analyzed by Welch's *t*-test.

RESULTS AND DISCUSSION

Change of Body Weight of ODS Rats during Ascorbate Deficiency. On the first day (the 0th day) of ascorbate depletion, the body weight of ODS rats was 146.9 ± 10.8 g (n = 39). Body weight of both the control group, which was fed with vitamin C-containing water at 1 mg/mL, and the ascorbate-deficient rats increased steadily. On about the 10th day, body weight of the vitamin C-depleted animals began decreasing in conformity with the literature (Kimura et al., 1992). After 25 days of ascorbate deficiency, the body weight of the animals was 149.2 ± 15.3 g (n = 5), which was significantly lower than the value (253.9 ± 6.7 g (n = 4)) of the control group.

Change in the Level of Vitamin C in Tissues of ODS Rats during Vitamin C Deficiency. *Plasma.* At the start, the level of vitamin C in plasma was 0.025 ± 0.010 mmol/L (n = 4) as shown in Figure 1a. This value was consistent with the reported value (ca. 23 μmol/L) (Kimura et al., 1992) for the ascorbate level in plasma of control male ODS rats determined by the α,α′-dipyridyl method (Zannoni et al., 1974). After 3 days of vitamin C deficiency, the level of ascorbate in plasma was significantly lower than that of the starting value. The level of the vitamin declined as shown in Figure 1a. The concentration of vitamin C in plasma was the lowest among tissues measured in the present work. This result suggested the presence of a transporter for the vitamin in all these tissues, while the transport system of ascorbate was investigated in limited cell types (Welch et al., 1993; Bergsten et al., 1995). The rate of the decrease of the vitamin was the most rapid in plasma as shown in Figure 1.

Liver. On day 0, the level of vitamin C in the liver was 0.851 ± 0.131 μmol/g of tissue (n = 4) as shown in Figure 1b. This value was of the same order of magnitude as the reported values (Kimura et al., 1992; Horio et al., 1987; Kosaka et al., 1990; Kawaguchi et al., 1993). After 3 days of vitamin C deficiency, the level of ascorbate in the liver fell significantly compared with that of the starting value. The level of the vitamin decreased thereafter as shown in Figure 1b.

Stomach, Small and Large Intestines, Lung, Heart, and Muscle. On day 0, the levels of vitamin C in the stomach, small and large intestines, lung, heart, and muscle (femoral) were 0.663 ± 0.107, 1.15 ± 0.186, 0.789 ± 0.161, 1.37 ± 0.233, 0.378 ± 0.035, and 0.235 ± 0.055 μmol/g of tissue (n = 4), respectively. The ascorbate levels of these tissues declined as shown in Figure 1c−g and k, respectively.

Kidney and Spleen. The ascorbate levels of the kidney and the spleen on day 0 were 0.488 ± 0.128 and 2.06 ± 0.25 μmol/g of tissue (n = 4), respectively. These values were similar to the level reported for female ODS rats (Kosaka et al., 1990). Vitamin C in the kidney and the spleen diminished thereafter as shown in Figure 1h and j, respectively.

Adrenal Gland. The concentration of vitamin C in the adrenal gland on day 0 was 5.24 ± 2.10 μmol/g of tissue (n = 4), which was the highest among tissues studied in this work and was the same order of magnitude as the value reported for female ODS rats (Kosaka et al., 1990). The level of ascorbate fell as shown in Figure 1i. The level on day 25 was still even higher than that of plasma at the start.

Brain. The concentrations of vitamin C in the brain at the start was 2.06 ± 0.10 μmol/g of tissue (n = 4). After 10 days of the deficiency, the ascorbate level in the brain decreased significantly as shown in Figure 1l. Even on day 25, the level of the vitamin in the brain was retained at the highest among all organs studied in this work. The rate of decrease of the vitamin was also exceptionally slow in the brain. This result suggested the presence of an unknown mechanism to maintain the level of ascorbate in the brain, where enzymes synthesizing vital hormones and neurotransmitters using vitamin C as a cofactor are present.

Tissue Level of Vitamin C in Control Animals That Were Supplemented with the Vitamin for 25 Days. The ascorbate level in plasma of the control ODS

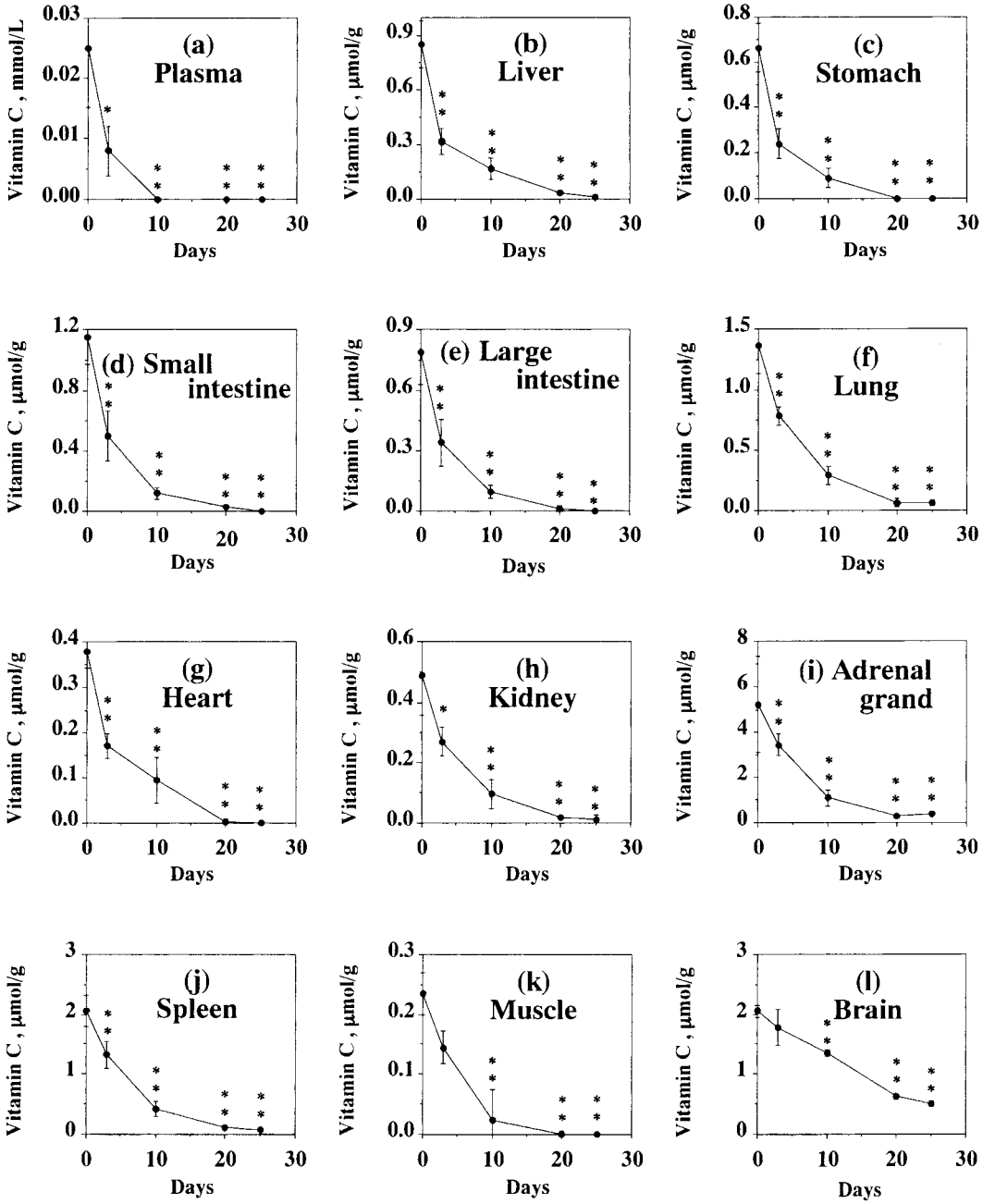

Figure 1. Change of vitamin C level in tissues of ODS rats during the deficiency of ascorbate. ODS rats were fed a vitamin C-deficient diet. On the starting day (the 0th day), the level of the vitamin was measured for 12 tissues as described in Materials and Methods. After 3, 10, 20, and 25 days, the concentration of ascorbate in these tissues was determined. The axis of abscissa means days from the starting day, and the vertical axis represents the level of vitamin C. Data points show mean ± SD (n = 4−6). The level of ascorbate is shown (as mmol/L) for (a) plasma and (as μmol/g of tissue) for all other tissues from (b) liver to (l) brain. Asterisks indicate a significant difference from the corresponding starting value (Welch's t-test: *, $P < 0.05$; **, $P < 0.01$).

rats (n = 4) that were supplemented with vitamin C for 25 days was 0.024 ± 0.007 mmol/L, which was almost identical to that at the start. This observation was common for all other tissues. The ascorbate levels of the liver, stomach, small and large intestines, lung, heart, muscle, kidney, adrenal gland, and brain of the control group (n = 4) were 0.829 ± 0.128, 0.600 ± 0.139, 1.06 ± 0.19, 0.783 ± 0.213, 1.44 ± 0.317, 0.313 ± 0.071, 0.159 ± 0.051, 0.554 ± 0.092, 5.58 ± 1.46, and 2.18 ± 0.26 μmol/g of tissue, respectively. These levels were almost identical to the corresponding starting value. These results demonstrated that the tissue level of vitamin C was maintained constantly by the dose of vitamin C given to the control group (1 g/L of drinking water). This result is consonant with the report (Mizushima et al., 1984) indicating that the dose was sufficient to sustain normal growth.

The spleen level of ascorbate of the control animal was 2.66 ± 0.276 μmol/g, which was significantly higher than that at the start. The reason of this difference remained unexplained, but this observation indicated that the spleen of the control animal was not vitamin C deficient.

Difference in the Kinetics of the Decrease of Ascorbate among Tissues. Among 12 tissues whose levels of ascorbate were followed during vitamin C deficiency, the decreasing profiles shown in Figure 1 may be classified into four groups on the basis of two kinetic parameters. The parameter P1 (the first half-life) is defined as the period during which the level of ascorbate becomes half of the starting value, and the

Table 1. Comparison of Lipid Hydroperoxides, Glutathione, Glutathione Peroxidase Activity, and TBARS in the Brain, Heart, Liver, Lung, and Kidney between Vitamin C-Deficient and Control ODS Rats[a]

		brain	heart	liver	lung	kidney
lipid hydroperoxides (pmol/mg of protein)	deficient	166.9 ± 19.4**	331.0 ± 77.2	289.0 ± 59.0	233.7 ± 137.4	323.0 ± 71.9
	control	73.5 ± 37.5	453.7 ± 81.6	237.0 ± 50.0	101.1 ± 91.7	249.2 ± 57.7
total glutathione (μmol/g of tissue)	deficient	1.56 ± 0.24	1.69 ± 0.18	5.41 ± 0.19**	1.52 ± 0.17	2.40 ± 0.19
	control	1.66 ± 0.05	1.84 ± 0.08	7.89 ± 0.69	1.82 ± 0.26	2.39 ± 0.26
GSSG (nmol/g of tissue)	deficient	8.01 ± 3.10	70.7 ± 2.7	56.4 ± 12.0**	27.1 ± 9.9	68.3 ± 33.4
	control	7.56 ± 1.89	58.4 ± 7.6	157.9 ± 43.1	34.4 ± 5.8	115.7 ± 37.1
glutathione peroxidase activity (nmol of NADPH oxidized (mg of protein)$^{-1}$ min^{-1})	deficient	25.33 ± 9.21	137.3 ± 15.7**	628.4 ± 76.3	177.4 ± 40.0	301.8 ± 58.5
	control	13.21 ± 6.86	102.8 ± 5.2	593.5 ± 56.0	170.2 ± 28.5	258.8 ± 33.0
TBARS (nmol MDA/g of tissue)	deficient	31.2 ± 3.16	27.1 ± 4.14**	30.0 ± 3.4	25.8 ± 7.5	42.7 ± 3.9
	control	28.3 ± 3.05	15.8 ± 3.17	29.0 ± 13.1	18.8 ± 7.14	38.7 ± 1.7

[a] ODS rats were fed a vitamin C-deficient diet, and the control group was supplied with vitamin C-contained water (1 g/L) for 25 days. Levels of lipid hydroperoxides, glutathione, glutathione peroxidase activity, and TBARS were determined for the brain, heart, liver, lung, and kidney as described in the text. Values are means ± SD of four or five rats. Asterisks indicate significant difference from the corresponding control (Welch's *t*-test: *, $P < 0.05$; **, $P < 0.01$).

second parameter P2 (the second half-life) is designated as the duration during which the level of ascorbate decreases to one-fourth that of the starting level after P1. For plasma, P1 and P2 were both 2.5 days (i.e., first-order kinetics) and the decrease was the most rapid. For the liver, stomach, small and large intestines, and heart, P1 was 2.5 and P2 was about 5 days, i.e., the decrease was biphasic (for the heart, P2 was about 7 days and longer than other tissues of this group). For the lung, kidney, adrenal gland, spleen, and muscle, both P1 and P2 were 5 days. In the brain, P1 was 14 days and P2 was 11 days, and the vitamin was retained the longest.

Change in the Level of Lipid Hydroperoxide in ODS Rats during Vitamin C Deficiency. It is widely accepted that activated oxygen species initiate radical reactions in the cell by abstracting reactive allylic hydrogen of the lipid. Resulting carbon-centered and hydroperoxy radicals are highly reactive, and they rapidly react with neighboring molecules. For the extention of radical reactions to cellular constituents apart from lipid, "active mediators", which are formed by radical reactions and have a sufficient lifetime to migrate and generate finally reactive radicals, may be postulated. Lipid hydroperoxide is a probable candidate for such a functional molecule to transport the oxidative power to protein and DNA (Halliwell and Gutteridge, 1990). Therefore, the level of lipid hydroperoxides may be an effective indicator for the evaluation of oxidative stress rather than TBARS, which are final products of lipid peroxidation.

The tissue level of hydroperoxides is measured according to the specific and sensitive method (Tokumaru et al., 1995, 1996). As the experimental group, ODS rats which were fed without the vitamin for 25 days were used. As shown in Table 1, the lipid hydroperoxide level in the brain of the deficient group was significantly higher than that of the control rats. A significant difference between the deficient and the control groups in the contents of lipid hydroperoxides of the heart, liver, lung, and kidney was not observed. Although ascorbic acid was retained most, the increase of lipid hydroperoxide was detected only in the brain. This result suggests that the brain is highly susceptible to oxidative stress caused by the deficiency of the vitamin.

Level of Glutathione in Tissues during Vitamin C Deficiency. The link between two water-soluble antioxidants, i.e., vitamin C and glutathione, was suggested (Meister, 1992). It was reported that guinea pigs given a scorbutic diet for 9 days exhibited higher than normal levels of glutathione in the liver and kidney (Meister, 1992) and that glutathione deficiency increased hepatic ascorbate synthesis in mice (Martensson, 1992). In this work, we determined the level of total glutathione in ODS rats during vitamin C deficiency. On the 25th day of the deficiency, the plasma level of glutathione was 0.0068 ± 0.0016 mmol/L (n = 5), which was significantly lower than that of the control (0.0129 ± 0.0013 (n = 4)). The level of glutathione in the liver of the experimental group was also significantly lower than that of the corresponding control as shown in Table 1.

For comparison, the time course of the decrease of glutathione in plasma and the liver was followed. Levels of glutathione in plasma of ascorbate-depleted rats on the 0th, 3rd, 10th, and 20th days were 0.0144 ± 0.0013 (n = 6), 0.0138 ± 0.0024 (n = 4), 0.0135 ± 0.0035 (n = 5), and 0.0058 ± 0.0004 mmol/L (n = 5), respectively, and the last value was significantly lower than the starting level. Contents of glutathione in the liver of the 0-, 3-, 10-, and 20-day ascorbate-deficient animals (n = 5) were 7.86 ± 0.333, 7.70 ± 0.68, 7.54 ± 0.58, and 5.21 ± 0.33 μmol/g, and again the last value was significantly lower than the starting level. The levels of glutathione in plasma and the liver of the 25-day deficient group were also significantly lower than the corresponding starting level. These results indicate that the level of glutathione in plasma and liver decreases gradually during the depletion of the vitamin. Although the consumption of glutathione may increase in the deficiency of vitamin C because of increased recycling of vitamin C and oxidative stress, glutathione synthesis in the liver of ODS rats did not seem to be enhanced. The levels of total glutathione in the brain, heart, lung, and kidney of the ascorbate-depleted rats for 25 days were not significantly different from the corresponding control as shown in Table 1.

It is conceivable that the level of oxidized glutathione (GSSG) increases by ascorbate deficiency. However as shown in Table 1, the GSSG level in the liver of vitamin C-deficient rat decreased unexpectedly concomitantly with the decrease of total glutathione as compared to the control animals, while the GSSG contents in other organs did not change significantly on the 25th day of the deficiency. The GSSG level in the plasma of the vitamin C-deficient rats was 1.42 ± 0.21 μmol/L (n = 4), which was also significantly lower than that of the control (2.24 ± 0.51 (n = 4)). These results indicated that the level of GSSG did not increase by the oxidative stress caused by the vitamin deficiency.

Change in the Activity of Glutathione Peroxidase. The activity of glutathione peroxidase is known

to elevate with the increase of oxidative stress (Harris, 1992; Cowan et al., 1993; Ji and Fu, 1992). The total activity of glutathione peroxidase was measured utilizing cumene hydroperoxide as a substrate. The enzymatic activity in the heart of the experimental group (day 25 of deficiency) was significantly higher than that of the control animals as shown in Table 1. The change in the activity of the enzyme by vitamin C deficiency for 25 days was not observed in other tissues such as plasma, brain, liver, lung, and kidney. The elevation of the activity in the heart may be a defensive action to counteract the increase of lipid hydroperoxides.

Change in the Level of TBARS during Vitamin C Deficiency. TBARS is commonly used as an index of lipid peroxidation in biological systems, although the reliability of the parameter has been questioned even in the oxidation of a simple oil (Kishida et al., 1993a) and fatty acids (Kishida et al., 1993b). Our papers (Kishida et al., 1993a,b) demonstrate that the elevation of TBARS closely reflects the increase of lipid peroxidation in an early stage of peroxidation. As one indicator, TBARS in the tissues of ODS rats in the deficiency of ascorbate were measured. The TBARS level of the heart of the experimental animals (deficiency for 25 days) was significantly higher than that of the control group as shown in Table 1. This observation shows the enhancement of lipid peroxidation in the heart by the lack of ascorbate.

Significant differences in TBARS between the deficient and the control groups were not observed in the brain, liver, lung, and kidney as shown in Table 1. These results do not exclude the possibility of the increase of oxidative stress in these tissues by the deficiency of the vitamin, because it is probable that TBARS (mainly aldehydic products of lipid peroxidation) are metabolized rapidly in these tissues. Kimura et al. (1992) reported an increase of hepatic and plasma TBARS values in vitamin C-depleted ODS rats. The discrepancy with our work is not clear and may be accounted for by the difference of the determination method.

Effect of Vitamin C Deficiency in ODS Rats. It is well established (Burns et al., 1987) that vitamin C has an essential role as a cofactor of enzymes that synthesize neurotransmitters and hormones, which are vital for the maintenance of life. The high mortality of the scorbutic rat in the deficiency of ascorbate for only 30 days may be mainly due to the lack of these indispensable factors localized in the brain and adrenal gland.

On the other hand, the enhancement of radical reactions after 25 days of the deficiency of the vitamin in the brain, heart, liver, and plasma is demonstrated as described above on the basis of indicators of lipid peroxidation, which are classified in four categories, namely, *increase of products of lipid peroxidation* such as lipid hydroperoxide and TBARS, *enhanced activity of antioxidant enzymes* such as glutathione peroxidase, *decrease of antioxidants* such as glutathione, and *accumulation of a mediator of peroxidation* such as lipid hydroperoxides. In the study of radical reactions in biology, the oxidative stress is assumed to be enhanced in a qualitative sense if any one of these various indicators shows a positive result. This situation is explained on the grounds that quantitative relationships among the parameters have not been investigated well and the efficiency of each index should depend on both the metabolic nature of the tissue and the functional mechanism of the stress. Thus, the present results suggest that the enhanced radical reaction induced by the vitamin deficiency is one of factors causing the high mortality of the scorbutic animals.

ABBREVIATIONS USED

GSSG, oxidized glutathione; ODS, Osteogenic Disorder Shionogi; TBARS, thiobarbituric acid-reactive substances.

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LITERATURE CITED

Anderson, M. E. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.* **1985**, *113*, 548−555.

Bergsten, P.; Yu, R.; Kehrl, J.; Levine, M. Ascorbic acid transport and distribution in human B lymphocytes. *Arch. Biochem. Biophys.* **1995**, *317*, 208−214.

Brehe, J. E.; Burch, H. B. Enzymatic assay for glutathione. *Anal. Biochem.* **1976**, *74*, 189−197.

Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302−310.

Burns, J. J., Rivers, J. M., Machlin, L. J., Eds. Third conference on Vitamin C. *Ann. N.Y. Acad. Sci.* **1987**, *498*.

Cowan, D. B.; Weisel, R. D.; Williams, W. G.; Mickle, D. A. G. Identification of oxygen responsive elements in the 5′-flanking region of the human glutathione peroxidase gene. *J. Biol. Chem.* **1993**, *268*, 26904−26910.

Davies, M. B.; Austin, J.; Partridge, D. A. *Vitamin C: Its Chemistry and Biochemistry*; Royal Society of Chemistry: Cambridge, U.K., 1991.

Flohe, L.; Guenzler, W. A. Assays of glutathione peroxidase. *Methods Enzymol.* **1984**, *105*, 114−121.

Frei, B.; England, L.; Ames, B. N. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6377−6381.

Halliwell, B.; Gutteridge, J. M. C. Role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol.* **1990**, *186*, 1−85.

Harris, E. D. Regulation of antioxidant enzymes. *FASEB J.* **1992**, *6*, 2675−2683.

Horio, F.; Ozaki, K.; Oda, H.; Makino, S.; Hayashi, Y.; Yoshida, A. Effect of dietary ascorbic acid, cholesterol and PCB on cholesterol concentrations in serum and liver in a rat mutant unable to synthesize ascorbic acid. *J. Nutr.* **1987**, *117*, 1036−1044.

Ji, L.; Fu, R. Responses of glutathione system and antioxidant enzymes to exhaustive exercise and hydroperoxide. *J. Appl. Physiol.* **1992**, *72*, 549−554.

Kawaguchi, M.; Hayakawa, F.; Kamiya, Y.; Fujii, T.; Ito, J.; Sakuma, N.; Fujinami, T. Effect of ascorbic acid on pituitary prolactin secretion in the non-ascorbate synthesizing osteogenic disorder Shionogi (ODS) rat. *Life Sci.* **1993**, *52*, 975−979.

Kawai, T.; Nishikimi, M.; Ozawa, T.; Yagi, K. A missense mutation of L-gulono-γ-lactone oxidase causes the inability of scurvy-prone osteogenic disorder rats to synthesize L-ascorbic acid. *J. Biol. Chem.* **1992**, *267*, 21973−21976.

Kimura, H.; Yamada, Y.; Morita, Y.; Ikeda, H.; Matsuo, T. Dietary ascorbic acid depresses plasma and low density lipoprotein lipid peroxidation in genetically scorbutic rats. *J. Nutr.* **1992**, *122*, 1904−1909.

Kishida, E.; Nishimoto, Y.; Kojo, S. Specific determination of ascorbic acid with chemical derivatization and high-performance liquid chromatography. *Anal. Chem.* **1992**, *64*, 1505−1507.

Kishida, E.; Kamura, A.; Tokumaru, S.; Oribe, M.; Iguchi, H.; Kojo. S. Re-evaluation of malondialdehyde and thiobarbituric acid-reactive substances as indices of autoxidation

based on the oxygen consumption. *J. Agric. Food Chem.* **1993a**, *41*, 1−4.

Kishida, E.; Tokumaru, S.; Ishitani, Y.; Yamamoto, M.; Oribe, M.; Iguchi, H.; Kojo, S. Comparison of the formation of malondialdehyde and thiobarbituric acid-reactive substances from autoxidized fatty acids based on oxygen consumption. *J. Agric. Food Chem.* **1993b**, *41*, 1598−1600.

Kosaka, H.; Terada, N.; Ito, Y.; Uozumi, M. Decrease of nitrate biosynthesis in scorbutic mutant rats unable to synthesize ascorbic acid. *Life Sci.* **1990**, *46*, 1249−1254.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−276.

Martensson, J.; Meister, A. Glutathione deficiency increases hepatic ascorbic acid synthesis in adult mice. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11566−11568.

Meister, A. On the antioxidant effects of ascorbic acid and glutathione. *Biochem. Pharmacol.* **1992**, *44*, 1905−1915.

Mizushima, Y.; Harauchi, T.; Yoshizaki, T.; Makino, S. A rat mutant unable to synthesize vitamin C. *Experientia* **1984**, *40*, 359−361.

Roe, J. H.; Kuether, C. A. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J. Biol. Chem.* **1943**, *147*, 399−407.

Seib, P. A., Tolbert, B. M., Eds. *Ascorbic acid: Chemistry, Metabolism, and Uses*; American Chemical Society: Washington, DC, 1982.

Tokumaru, S.; Tsukamoto, I.; Iguchi, H.; Kojo, S. Specific and sensitive determination of lipid hydroperoxides with chemical derivatization into 1-naphthyldiphenylphosphine oxide and high performance liquid chromatography. *Anal. Chim. Acta* **1995**, *307*, 97−102.

Tokumaru, S.; Iguchi, H; Kojo, S. Change of the lipid hydroperoxide level in mouse organs on ageing. *Mech. Ageing Dev.* **1996**, *86*, 67−74.

Washko, P. W.; Welch, R. W.; Kuldeep, R. D.; Wang, Y.; Levine, M. Ascorbic acid and dehydroascorbic acid analyses in biological samples. *Anal. Biochem.* **1992**, *204*, 1−14.

Welch, R. W.; Bergsten, P.; Butler, J. D.; Levine, M. Ascorbic acid accumulation and transport in human fibroblasts. *Biochem. J.* **1993**, *294*, 505−510.

Zannoni, V.; Lynch, M.; Goldstein, S.; Sato, P. A rapid micromethod for the determination of ascorbic acid in plasma and tissue. *Biochem. Med.* **1974**, *11*, 41−48.

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