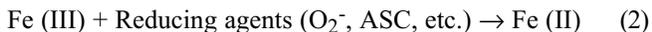
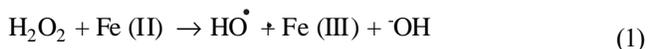


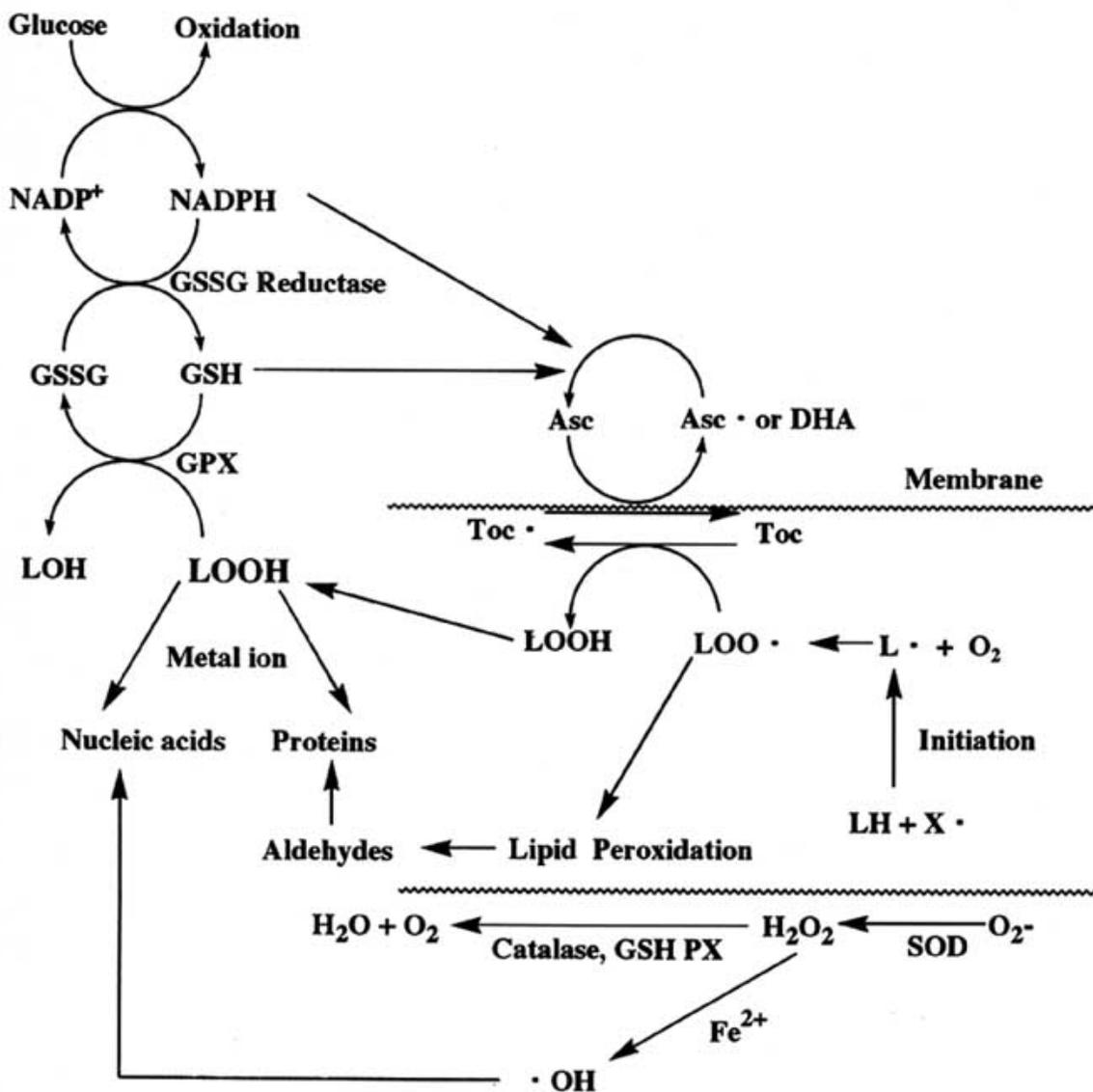


contribute to keep free Fe (III) concentration in the biological fluid low.



Although ROS can react with almost all cellular components such as lipids, DNA, and proteins, membrane lipids are assumed to be the most reactive. The latter possesses a hydrocarbon chain that can undergo facile radical reactions. In an analogous manner, the combustion of gasoline (a mixture of hydrocarbons), usually an explosion, is a typical radical chain reaction. Therefore "lipid peroxidation" has been used as a term to indicate oxidative stress. Radical reactions in the cell are chemically illustrated in Fig. (2).

ROS (designated as X<sup>•</sup> in Fig. 2) initiate radical reactions in the cell by abstracting reactive allylic hydrogen of the lipid. The resulting carbon-centered radical reacts with oxygen rapidly (at a diffusion-controlled rate) forming alkyl peroxy radical (LOO<sup>•</sup>), which is scavenged by vitamin E resulting in the formation of lipid hydroperoxide (LOOH) and α-tocopheryl radical (Toc<sup>•</sup>). LOO<sup>•</sup> also abstracts a hydrogen atom from lipids to generate LOOH. LOOH has a sufficient lifetime to migrate and finally generate reactive radicals by the reaction with metal ions to damage cellular components. In this way, LOOH extends radical reactions to cellular constituents apart from the membrane. In addition, LOOH oxidizes cellular components such as thiol, amine, olefin, and sulfide. Therefore, LOOH is a probable candidate as a functional molecule that transports oxidative power to protein and DNA [7]. In summary, superoxide, hydrogen



**Fig. (2).** Chemical illustration of radical reactions in the cell, In the cell, radical species (designated as X<sup>•</sup>) initiate radical chain reaction. Against the oxidative stress, the cell has antioxidants and antioxidative enzymes. Finally, reducing power is supplied by oxidizing glucose to produce NADPH.

peroxide, hydroxyl radical, alkoxy radical, alkyl peroxy radical, and LOOH are all ROS.

To prevent oxidative stress, the cell has defense mechanisms such as antioxidants and enzymes. Glutathione peroxidase (GPX) decomposes LOOH and hydrogen peroxide using glutathione (GSH) to form oxidized glutathione (GSSG), which is reduced by GSSG reductase using NADPH. The oxidation product,  $\text{NAD}^+$ , is reduced back to NADPH by oxidizing glucose (Fig. 2). This scheme shows that the oxidative stress is finally compensated by the reductive power of glucose.

Oxygen is ambivalent by nature. While oxygen is essential for life, it may also cause extensive tissue damage that may lead to death due to its radical characteristics. For systematic investigations on oxidative stress, a negative aspect of oxygen, it is essential to establish specific and sensitive methods to analyze the key molecules involved in radical reactions and defense mechanisms. We have observed two antioxidative vitamins, C and E, which are consumed by radical reactions. We have paid particular attention to LOOH as a mediator of radical reaction. Reliable methods for the determination of the tissue concentration of ASC and LOOH were not available. Therefore, we developed a specific and sensitive method for the determination of these molecules. A method for the determination of ASC is first explained.

### 3. METHOD TO DETERMINE TISSUE CONCENTRATION OF ASC

For the determination of ASC, a colorimetric method [8] developed over a half century ago was used until recently. In 1992, we developed a specific and sensitive method involving chemical derivatization and HPLC [9]. The principle of this method is shown in Fig. (3). ASC derivatives that exist in biological samples are ASC, dehydroascorbic acid (DHA: oxidized), and 2,3-

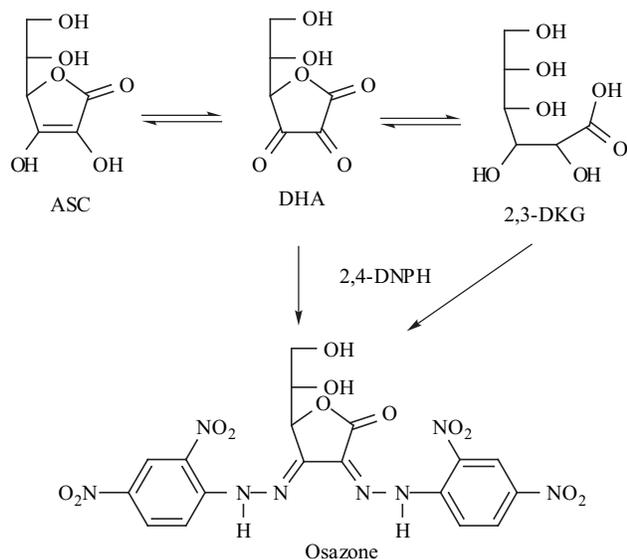


Fig. (3). Reaction for the determination of ASC

ASC is oxidized to DHA, which is quantitatively converted to the osazone with DNPH. The osazone was determined by HPLC.

diketogulonic acid (2,3-DKG: oxidized and ring-opened). In this method, ASC is firstly oxidized with 2,3-dichlorophenol indophenol to DHA, which was reacted with 2,4-dinitrophenyl hydrazine (DNPH). The product is an osazone shown in Fig. (3). This osazone is formed from either DHA or 2,3-DKG. The structure of the osazone was confirmed based on MS and NMR spectra ( $^1\text{H}$ ,  $^{13}\text{C}$ , and COSY) [9]. This osazone was obtained from ASC, DHA, or 2,3-DKG in a quantitative yield, which is a necessary condition for accurate analysis.

The separation of the osazone is made using an octadecyl (C-18) HPLC column, which is useful for the separation of hydrophobic compounds like osazone. Thus, this derivatization contributes to the separation, which assures the specificity of this method. The detection limit of this osazone by absorption at 505 nm is 1 pmol. Recently, direct measurement of ASC using an electrochemical detector has been widely used. However, it is difficult to separate ASC from hydrophilic reductants contained in biological samples.

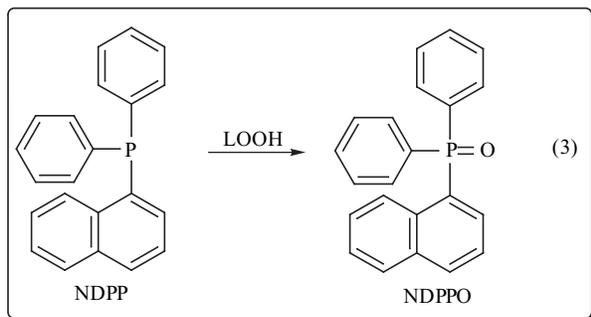
Applying the method, it was shown that the conventional colorimetric method gave a level three times higher than the true level in the determination of ASC in rat plasma [9]. This finding demonstrates that the re-evaluation of fundamental ASC study is urgently required. In the next section, the determination of LOOH, another key substance, is discussed.

### 4. SPECIFIC AND SENSITIVE DETERMINATION OF LOOH WITH CHEMICAL DERIVATIZATION INTO 1-NAPHTHYLDIPHENYLPHOSPHINE OXIDE AND HPLC

LOOH has been postulated to be a mediator of radical reaction. Therefore, it is expected to be a reliable index of oxidative stress. However, the determination of LOOH had been hampered by its instability and extremely low level. Several methods were available for the determination of LOOH in biological materials. The iodometric titration method [10-11] is simple but the sensitivity is at the nanomolar level, which is not sufficient to measure peroxides in animal tissues. Although the chemiluminescent detection method [12-13] of LOOH combined with the separation of the lipid components with HPLC is very sensitive and useful, this method cannot be applied to the most fundamental determination, *i.e.* the quantitation of total LOOH in the lipid, because endogenous compounds such as ubiquinol and tocopherols interfere seriously with the chemiluminescent reaction [14]. In addition, the yield of photons in the chemiluminescent reaction is greatly affected by the structure of the fatty acid bearing hydroperoxide [14].

Meguro *et al.* [15-16] reported a simple and very sensitive method to analyze LOOH, which was first reacted with aromatic phosphines that react with LOOH quantitatively producing fluorescent phosphine oxide. The fluorescence level of the reaction mixture gave the amount of LOOH. However, we found that a great many fluorescent side products were formed during the reaction of aromatic phosphine with cumene hydroperoxide as analyzed with HPLC. In addition, the stability of the aromatic phosphine was not satisfactory for the determination of LOOH of picomole order only based on the fluorescence of the reaction

mixture. We improved their method and used the reaction of LOOH with 1-naphthyl-diphenylphosphine (NDPP) to form its oxide (NDPPO) (reaction (3)). The resulting NDPPO was determined by HPLC [17]. We selected NDPP, which was the most stable to air oxidation among the aromatic phosphines reported [16]. NDPP was converted into NDPPO quantitatively by the reaction with hydrogen peroxide or cumene hydroperoxide. The detection limit of NDPPO was 1 pmol, namely, 10  $\mu$ l of aliquot in 0.1  $\mu$ M solution.



In the next sections, changes in ASC and LOOH are determined in pathological conditions.

## 5. CHANGE IN THE ASC LEVEL IN TISSUES OF INHERENTLY SCORBUTIC RATS DURING ASC DEFICIENCY

In investigations of the functions of a micronutrient, the effects of a deficiency of the nutrient are usually studied. At first, changes in the tissue concentration of the micronutrient must be clarified during its deficiency. Therefore, the decrease in the ASC level in animal tissues during its deficiency was evaluated using an ODS (Osteogenic Disorder Shionogi) rat [18]. This rat is inherently scorbutic, because as with the human, monkey and the guinea pig, this rat lacks L-gulonolactone oxidase [19] necessary for ASC synthesis from glucose. This animal serves as a good model for the investigation of ASC deficiency, especially in relation to the role of ASC in preventing radical chain reactions in tissues, because ASC is recognized as an outstanding antioxidant [20].

ASC deficiency was induced in ODS rats and changes in the ASC level in 12 tissues were followed utilizing the specific method described above. The results are shown in Fig. (4). The characteristic profile of each tissue is described below [21].

### 5-1. Plasma

At the start, the mean ASC concentration in plasma was 0.025 mmol/l as shown in Fig. (4). The tissue ASC concentration on day (d) 0 represents the normal value of each tissue of the ODS rat. Since the axis of the ordinate of each chart in Fig. (4) is almost the same unit (mmol/l =  $\mu$ mol/g tissue), the tissue ASC concentrations can be clearly compared with each other. The ASC concentration of plasma was the lowest among 12 tissues. This result clearly shows the presence of a transporter of ASC from plasma in these 11 tissues, although the transport system of ASC has been investigated only in limited cell types [22-23].

After 3 d of ASC-deficiency, the ASC level in plasma was significantly lower than the starting value. The rate of the decrease in ASC was the most rapid in plasma among 12 tissues (Fig. 4).

### 5-2. The Brain

The ASC concentration in the brain at the start was  $2.06 \pm 0.10$   $\mu$ mol/g tissue, namely about 2 mM, which was 2 orders higher than that in plasma. The rate of decrease in ASC was also exceptionally slow in the brain, and on d 25, the ASC level in the brain was retained at the highest among 12 tissues (Fig. 4). These results indicate the presence of an unknown mechanism to maintain the ASC level in the brain, where enzymes synthesizing vital hormones and neurotransmitters using ASC as a cofactor are present.

### 5-3. The Difference in the Kinetics of the Decrease in ASC Among Tissues

The decreasing profiles of ASC in 12 tissues in Fig. (4) are classified into 4 groups on the basis of two kinetic parameters. The parameter P1 (the 1st half-life) is defined as the period during which the ASC level becomes half of the starting value and the second parameter P2 (the 2nd half-life) is designated as the period during which the ASC level decreases to one fourth of the starting level after P1. For plasma, P1 and P2 are both 2.5 d (*i.e.* 1st order kinetics) and the decrease is the most rapid. For the liver, stomach, the small and large intestines, and the heart, P1 is 2.5 and P2 is about 5 d; the decrease is biphasic (for the heart, P2 is about 7 d and longer than other tissues of this group). For the lung, kidney, adrenal gland, spleen, and muscle, both P1 and P2 are 5 d, namely ASC decreases by 1st order kinetics. In the brain, P1 is 14 and P2 is 11 d and ASC is retained the longest. Although it is difficult to explain even the tissue specificity in the decreasing profile of ASC during its deficiency, these analyses are the beginning of more detailed future studies.

### 5-4. The Change in the Level of LOOH in ODS Rats During ASC Deficiency

The tissue level of LOOH is measured according to the method described above [17]. In the experimental group, ODS rats fed without ASC for 25 d were used. The LOOH level in the brain of the deficient group was significantly higher than that of the control rats. No significant difference between the deficient and the control groups in the LOOH contents of the heart, liver, lung and the kidney was observed. Although ASC was retained the most, an increase in LOOH was detected only in the brain. This result suggests that the brain is highly susceptible to oxidative stress caused by ASC deficiency.

### 5-5. The Level of GSH in Tissues During ASC Deficiency

A link between two water-soluble antioxidants, *i.e.* ASC and GSH was suggested [24]. Guinea pigs given a scorbutic diet for 9 d exhibited higher than normal levels of GSH in

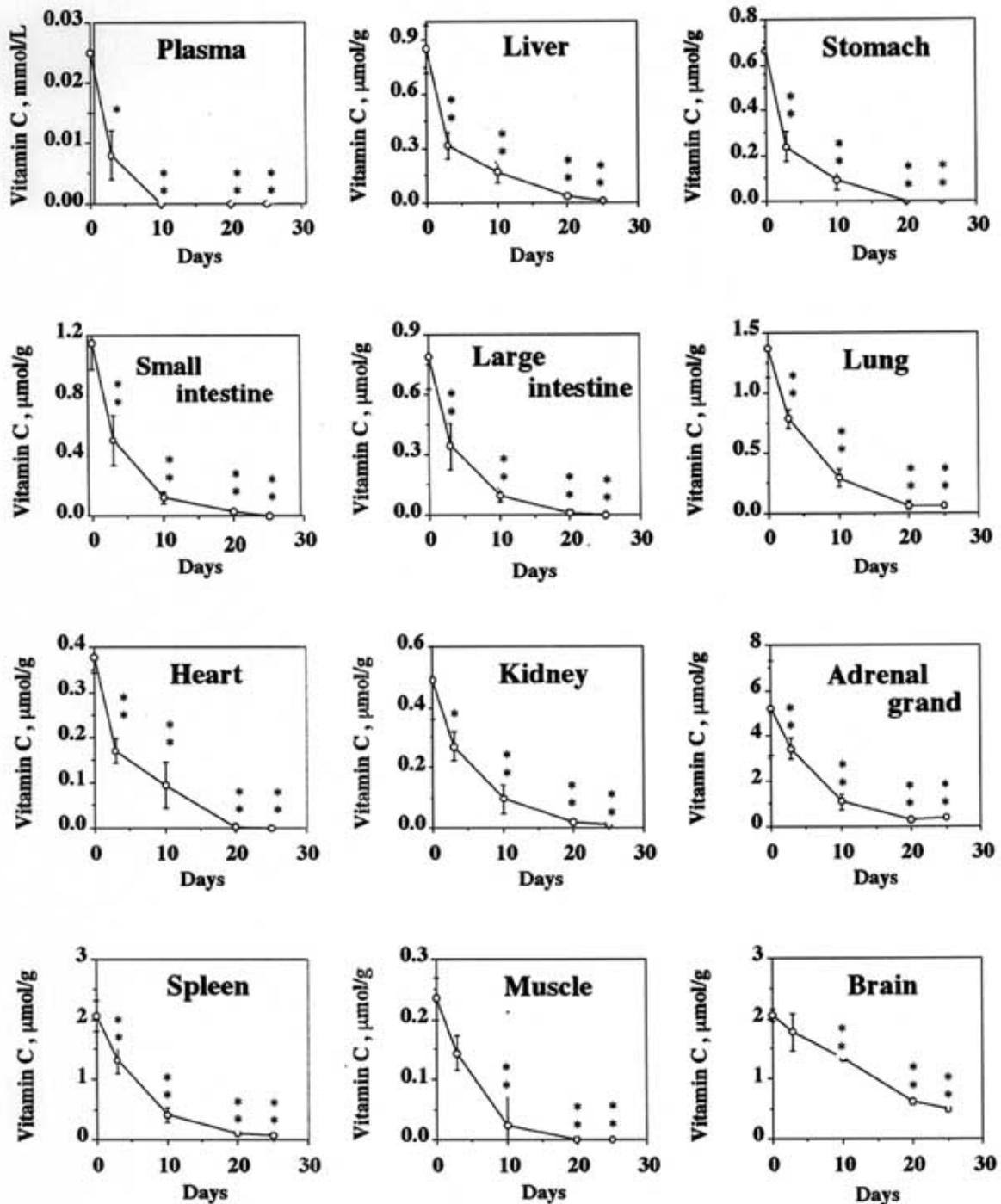


Fig. (4). Change in tissue ASC concentrations during ASC deficiency in ODS rat.

ODS rats were fed a ASC-free diet. The ASC concentrations in 12 tissues were determined. Since the axis of the ordinate of each chart is the same unit ( $\text{mmol/l} = \mu\text{mol/g tissue}$ ), the tissue ASC concentration and decreasing kinetics can be clearly compared to each other.

the liver and kidney [24] and GSH deficiency increased hepatic ASC synthesis in mice [25]. We measured the total GSH level in ODS rats during ASC deficiency. On the 25th d of deficiency, the plasma level of GSH was significantly lower than that of the control. The GSH level in the liver of the experimental group was also significantly lower than that of the corresponding control. These results indicate that the GSH level in plasma and the liver decreases gradually during

the depletion of ASC. Although the consumption of GSH may increase during ASC deficiency because of increased recycling of ASC and oxidative stress, GSH synthesis in the liver of ODS rats does not seem to be enhanced.

The total GSH levels in the brain, heart, lung, and the kidney of the ASC-depleted rats after 25 d were not significantly different from the corresponding control.

## 5- 6. The Change in the Activity of GPX

The activity of GPX is elevated by oxidative stress [26-28]. The total activity of GPX was measured utilizing cumene hydroperoxide as a substrate. The GPX activity in the heart of the experimental group (d 25 of deficiency) was significantly higher than that of the control animals. This elevation may be a defensive action to counteract the increase in LOOH. No change in the GPX activity by ASC deficiency for 25 d was observed in the plasma, brain, liver, lung, and kidney.

## 5-7. Oxidative Stress Profile in Normal ODS Rat

To provide an outline, indices of oxidative stress in normal ODS rat [21] were calibrated, and approximate values are shown in Table 1. The characteristics of these parameters except in plasma are:

- 1) The concentration of LOOH is 10-80  $\mu\text{M}$ , which is comparable to that of vitamin E.
- 2) The concentration of ASC is higher than that of LOOH or vitamin E by 1-2 orders of magnitude.
- 3) The concentration of GSH is at mM order, and all tissues have sufficient GPX activity to maintain a low tissue LOOH concentration.

In plasma, the LOOH concentration is negligible, despite the concentrations of ASC and GSH being much lower than those in other tissues. Therefore, plasma should possess strong peroxidase activity as suggested in albumin [29], apolipoprotein A-I [30], and paraoxonase [31] in addition to GPX.

As discussed in this section, an accurate analysis provides basic information. In the next two sections, the interaction of vitamins C and E, and the biological activity of DHA are evaluated solely based on the accurate determination of these vitamins.

## 6. INTERACTION BETWEEN ASC AND VITAMIN E IN TISSUES OF ODS RATS

### 6-1. Background

The regeneration of vitamin E from tocopheryl radical (shown as Toc $\cdot$  in Fig. 2) with hydrogen transfer by ASC is well characterized by *in vitro* studies [32-33]. However, the

interaction between these vitamins *in vivo* is still controversial [34-35]. Many experiments have been performed to resolve this problem by measuring the change in these vitamins in animal models [36-42]. The determination of ASC in these studies was made based on the widely used colorimetric method developed over a half century ago [8]. We attempted to resolve this problem using a specific and sensitive method [9].

### 6-2. One Model Study

How can we show the regeneration of tocopherol from tocopheryl radical by ASC *in vivo*? First, an assumption has to be made. If ASC rescues vitamin E, this rescuing effect should disappear during ASC deficiency. Therefore, the tissue vitamin E concentration should be lowered during ASC deficiency, if such a rescuing effect exists. Therefore, we studied the nature of interaction of ASC with vitamin E by evaluating the change in tissue levels of these vitamins caused by depletion of ASC, vitamin E, or vitamins C and E simultaneously in ODS rats [43].

ODS rats were divided into 4 groups: the control, vitamin C-deficient (designated as [-C]), vitamin E-deficient (designated as [-E]) and simultaneous vitamins C and E-deficient (defined as [-C, -E]) groups. Rats were fed for 14 and 21 d.

### 6-3. Saving Effect of ASC on Vitamin E

As shown in Fig. (5), the  $\alpha$ -tocopherol concentration in plasma of the -C group on 21 d was significantly lower than that of the control. Similar results were obtained in the heart, lung, liver and kidney on 21 d and in the plasma, brain, lung and liver on 14 d (Fig. 5). These results demonstrate that the decrease in  $\alpha$ -tocopherol is enhanced by ASC deficiency, *i.e.* ASC rescues vitamin E *in vivo*, supporting the presence of interactions including direct reaction of tocopheryl radical with ASC as shown by *in vitro* studies [32-33]. This is consistent with the study reporting that chronic ASC deficiency decreased the concentration of  $\alpha$ -tocopherol in the liver and lung of guinea pig [41].

The vitamin E levels of the [-E], and the [-C, -E] groups were significantly lower than those in the control and the [-C] rats for all tissues on 14 as well as on 21 d (Fig. 5). No significant difference was observed between the [-E], and the

**Table 1. Approximate Levels of Molecules Involved in Radical Reactions in Tissues**

	LOOH ( $\mu\text{M}$ )	Vitamin E ( $\mu\text{M}$ )	ASC ( $\mu\text{M}$ )	GSH ( $\mu\text{M}$ )	GPX mmol/kg tissue, min
Plasma	Not detected	17	30	10	4
Brain	13	25	2,100	1,700	2
Heart	83	30	380	1,700	14
Lung	21	39	1,400	1,800	10
Liver	50	35	850	7,900	94
Kidney	33	17	490	2,500	35
Muscle	18	19	240	640	3

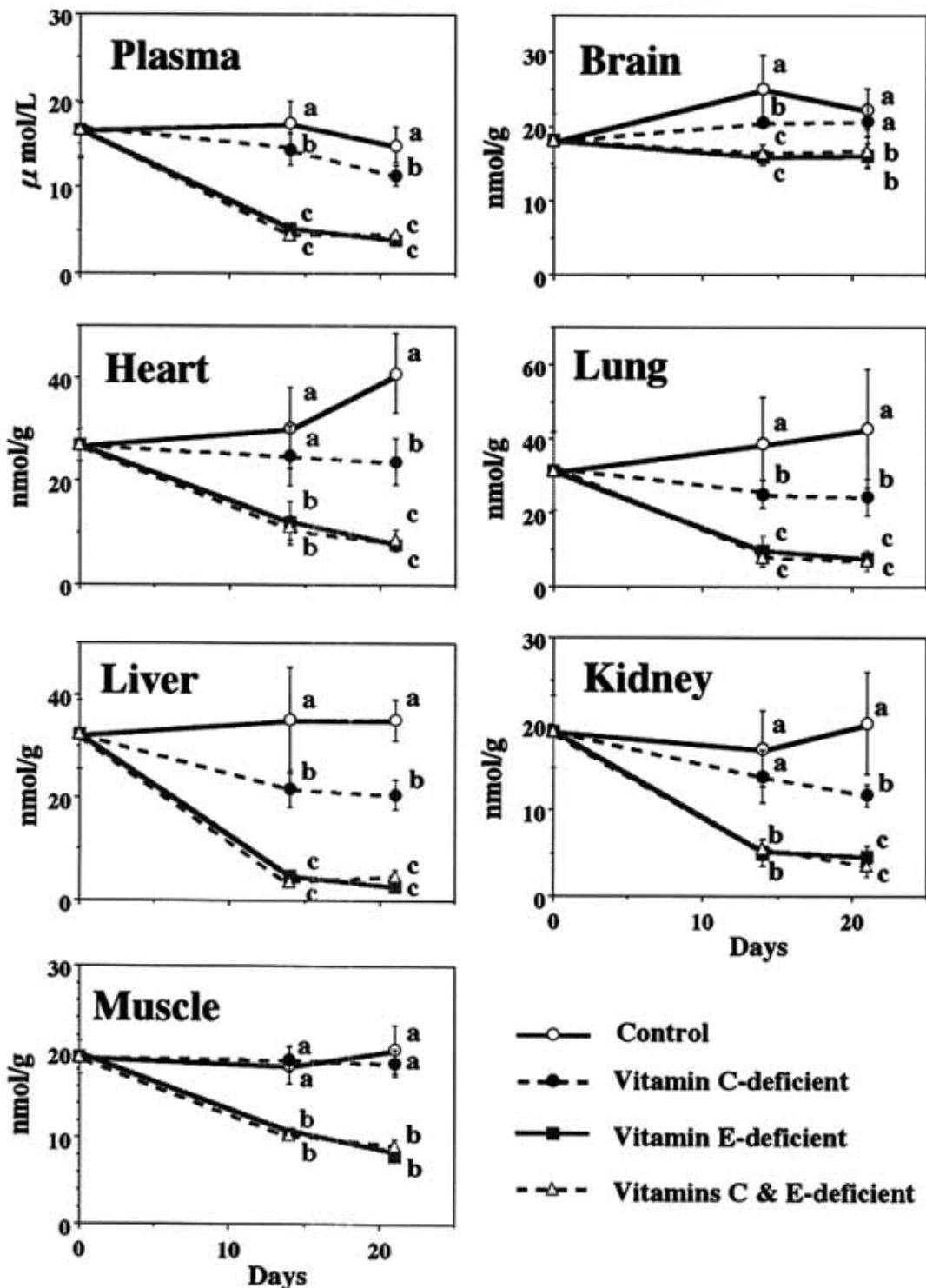
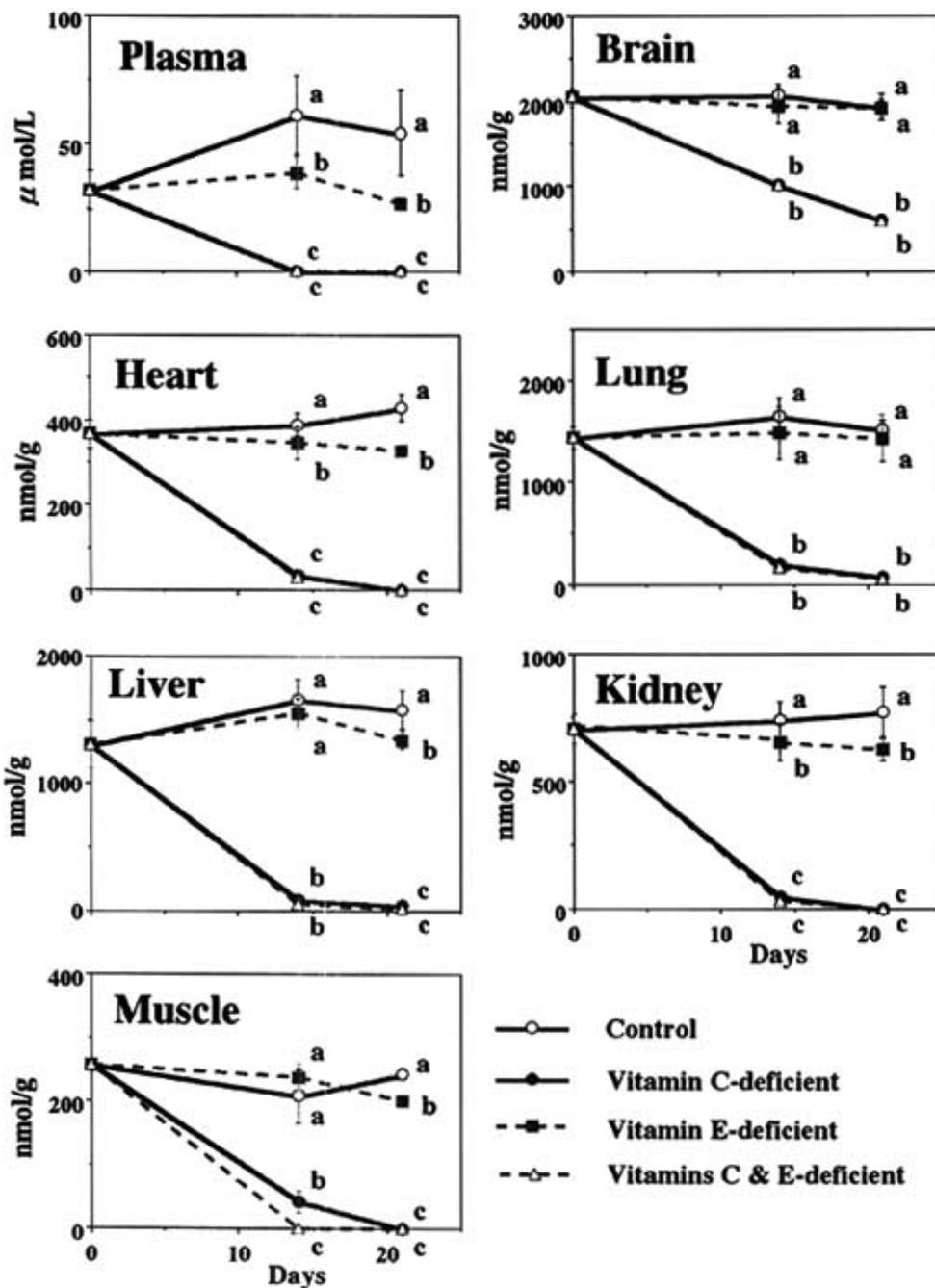


Fig. (5). Change in the tissue level of vitamin E in the inherently scorbutic rats fed control, ASC-deficient, vitamin E-deficient and simultaneous vitamin C and E-deficient diets. On the d 0, 14 and 21, the rats were sacrificed and the vitamin E levels in the tissues were determined. Values are means ± SD of 4 or 5 rats. Different letters indicate significant differences between groups by the Fisher's protected least significant difference test (P < 0.05). This figure shows the rescuing effect of ASC on vitamin E [40].

[-C, -E] groups for all tissues. This may be explained on the grounds that the effect of ASC was masked by the low vitamin E level, caused by its deficiency except in the case of the brain (see below).

#### 6-4. Rescuing Effect of Vitamin E on ASC

The change in the ASC level in these tissues was also determined. As shown in Fig. (6), on d 14, the plasma ASC level of the control rats was significantly higher than that of



**Fig. (6).** Change in the tissue level of ASC in the inherently scorbutic rats fed control, ASC-deficient, vitamin E-deficient and simultaneous vitamin C and E-deficient diets. On the d 0, 14 and 21, the rats were sacrificed and the ASC levels in the tissues were determined. Values are means ± SD of 4 or 5 rats. Different letters indicate significant differences between groups by the Fisher's protected least significant difference test ( $P < 0.05$ ). This figure shows the rescuing effect of vitamin E on ASC [40].

the [-E] group. A similar difference was observed for the heart and kidney on 14 d and for the heart, liver, muscle, kidney, and plasma on 21 d. These results indicate that the lack of vitamin E accelerates the consumption of ASC in these tissues. Since the direct regeneration of ASC from monodehydroascorbate and DHA by tocopherol is unlikely based on redox potential, these observations indicate that the vitamin E deficiency enhances radical reactions in the

hydrophobic region resulting in the elevated oxidative stress in the aqueous phase of the cell to consume more ASC. It is generally considered that radical reactions of lipid and aqueous phases take place independently. However, this result indicates that interactions between the hydrophobic and water phases occur *in vivo*, and that these reactions are detectable by measuring key antioxidants.

In the [-C] group, the ASC level decreased (Fig. 6). The ASC levels of the [-C], and the [-C, -E] groups were significantly lower than those of the control and the -E groups for all tissues on 14 as well as 21 d. In plasma, the ASC level on d 14 in these groups was under the detection limit. In the plasma, heart, kidney and muscle, the ASC levels of both the [-C], and the [-C, -E] groups on 21 d were under the detection limit, therefore no significant difference was found between these groups. In the muscle, the concentration of ASC of the -C, -E group was significantly lower than that of the -C group on 14 d, indicating again a rescuing effect of vitamin E on the tissue ASC.

In the brain, where both vitamins C and E decreased most slowly during their deficiencies [21, 44], no interaction of these vitamins was observed (Fig. 5 and 6). No rescuing effect of vitamin E on ASC was detected in the lung. These results indicate that the interaction of these vitamins depends on the metabolic nature of tissues and is not always observed clearly in all tissues.

In the next section, the biological activity of DHA is also evaluated using accurate determination of ASC.

## 7. VERY LOW VITAMIN C ACTIVITY BY ORALLY ADMINISTERED DHA

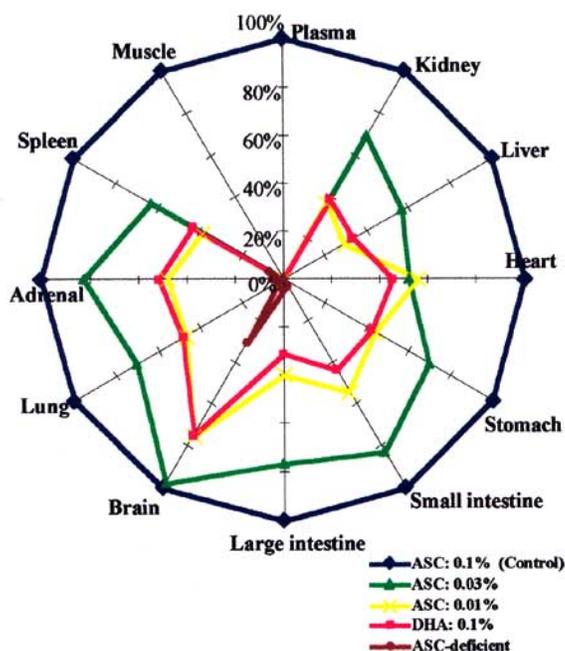
The recommended daily intake of ASC according to public health information is 60 mg for adults [2]. The biological activity of DHA, which is easily formed from ASC during the storage and the cooking process, has been considered to be equivalent to ASC [2] based on studies [45-47] made several decades ago. Although interesting studies concerning the molecular mechanisms of DHA transport

have been reported [48-49], the nutritional activity of DHA as ASC has not yet been determined by modern analytical methods.

We evaluated the nutritional activity of orally administered DHA [50] based on accurate determination of the tissue ASC content. In the study, ODS rats were also used. The design of the study is as follows:

ODS rats were divided into 5 groups designated as the control, 0.03% ASC-fed, 0.01% ASC-fed, 0.1% DHA-fed, and C-deficient groups. The control group received drinking water containing 0.1% ASC as described previously [18]. The 0.03% ASC- and 0.01% ASC-fed groups were given drinking water containing 0.03% and 0.01% of ASC, respectively. The 0.1% DHA-fed group was given drinking water containing 0.1% DHA. Since DHA is immediately converted into 2,3-diketogulonate in a neutral aqueous solution, DHA was dissolved in 1 mM HCl. The stability of DHA in the cell and in plasma clearly indicates the presence of some stabilizing agent(s) for DHA in biological fluids.

The nutritional activity of orally ingested DHA was evaluated by comparing the ASC concentrations in 12 tissues of rats administered four different doses of ASC as described above, because tissue ASC concentrations, which directly reflect absorption, distribution, and utilization of DHA, are the most fundamental information in evaluating the nutritional activity of DHA. To evaluate the activity of DHA, determinations were made on the 21 d, when the ASC level in all tissues except the brain, adrenal gland, and spleen of C-deficient group decreased to a null level as shown in Fig. (4). The tissue level of the oxidized form of ASC, namely the sum of DHA and 2,3-diketogulonate, was



**Fig. (7).** Tissue total ASC in the control (0.1% ASC-fed), 0.03% ASC-fed, 0.01% ASC-fed, 0.1% DHA-fed, and ASC-deficient rats after 21 d.

The ASC level of the control was taken as 100% for each tissue. This figure shows that the nutritional activity of DHA is 10% of ASC.

always less than 5% of the total ASC in all groups [21, 43]. Therefore, the total ASC concentration was defined as the ASC level. These results indicate that absorbed DHA is reduced and utilized as ASC. The results are shown in Fig. (7). The tissue level of ASC in the 0.1% DHA-fed group was significantly higher than that of the C-deficient group in all tissues except the plasma and muscle, but it was significantly lower than that of the control (0.1% ASC-fed) group in all tissues and still significantly lower than that of 0.03% ASC-fed groups in all tissues except the plasma, heart, and muscle (Fig. 7). These findings demonstrate that DHA has a definite activity as ASC but that the efficiency of DHA is significantly lower than 30% of ASC on the weight and the molar bases. No significant difference was observed between the tissue ASC concentration in the 0.1% DHA-fed group and that of the 0.01% ASC-fed group in all 12 tissues (Fig. 7). These findings clearly show that the activity of orally ingested 0.1% DHA solution is equivalent to 0.01% ASC solution; the efficiency of DHA as ASC is equivalent to 10% of ASC. This is a reasonable result considering the extremely unstable nature of DHA.

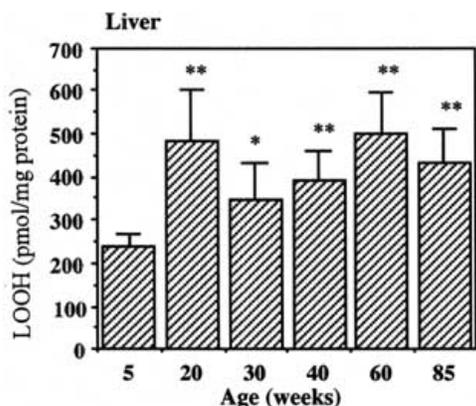
Based on these observations, we conclude that the anti-scorbutic activity of DHA is almost 10% of ASC and we propose that it is necessary to reevaluate the nutritional requirement of ASC based on both ASC and DHA contents of foods.

In the next sections, the efficiency of LOOH as an index of oxidative stress along with ASC is evaluated in model systems.

## 8. CHANGE IN LOOH CONCENTRATION IN PATHOLOGICAL MODELS

### 8-1. Change in LOOH Level in Mouse Tissues with Aging

In this section, the change in the LOOH level in mouse tissues is discussed during aging, in which radical reactions play an essential role. The LOOH level in the liver of 5-



**Fig. (8).** Change in the hydroperoxide (LOOH) level in the liver with aging [49].

Each value is the mean  $\pm$  standard deviation. Asterisks indicate significant differences from 5-week-old mice (\* $P < 0.05$ , and \*\* $P < 0.01$ ).

week-old mice was  $239 \pm 31$  pmol/mg protein. The LOOH levels of 20-, 30-, 40-, 60- and 85-week-old groups were  $487 \pm 115$ ,  $348 \pm 87$ ,  $395 \pm 65$ ,  $498 \pm 98$  and  $431 \pm 81$  pmol/mg protein, respectively and these values were significantly higher than the LOOH content of the 5-week-old animals (Fig. 8) [51]. The LOOH concentrations did not significantly differ among the 20, 30, 40, 60 and 85-week-old groups. Thus, the total LOOH level in the liver increases until 20 weeks old and retains the increased level thereafter without a significant change until 85 weeks old. This observation demonstrates that LOOH does exist continuously at a constant concentration and exerts a damaging effect on the cells, although LOOH is a labile molecule and its concentration is determined dynamically by a balance among the formation, and spontaneous and enzymatic decompositions. This result also suggests that the ability of the liver to maintain the level of deleterious LOOH does not change significantly until 85 weeks of age after maturation.

The LOOH concentration in the kidney and heart showed a similar change to the liver [51]. These results indicate again that the LOOH level in mouse tissues also elevates during maturation and thereafter remains constant with aging.

### 8-2. Senescence-Accelerated Mouse (SAM)

SAM, a group comprising nine inbred strains of accelerated senescence-prone mice (SAMP) and three of accelerated senescence-resistant mice (SAMR), were first established by Takeda *et al.* [52]. SAMP individuals show an irreversible advancement of senescence that is manifested by clinical signs and gross lesions such as alterations in general behavior, degenerative arthritis, hair loss, and increased skin coarseness. In addition, SAMP-8 exhibits impaired learning and memory [53-55]. The LOOH level in the brain was significantly higher in SAMP-8 than that in SAMR-1 at 3, 6, and 9 months of age. The LOOH levels in the heart, liver and lung showed increases with aging in both strains, and they were significantly higher in SAMP-8 than in SAMR-1 at both 3 and 6 months of age. These results indicate that increased oxidative stress in the brain and peripheral organs is a cause of the senescence-related degeneration and impairments observed in SAMP-8 [56].

In the next section, the change in LOOH level during deficiency of vitamin E, a typical antioxidant, is elucidated.

### 8-3. Increase in LOOH in Tissues of Vitamin E-deficient Rats

The LOOH level was determined in vitamin E-deficient rat tissues, which should be a good *in vivo* model of enhanced radical reactions. In the heart, lung, and kidney, the LOOH level increased significantly as early as 4 weeks after feeding a tocopherol-deficient diet compared with that of the control group. After 8 weeks of the deficiency, similar results were obtained. These results indicate that LOOH is available as a sensitive indicator of lipid peroxidation in these organs, because it takes several months to detect manifestations of the vitamin deficiency based on conventional indices [44].

In the brain, the LOOH concentration was not augmented and the decrease in vitamin E was the slowest among the tissues studied [44]. This is consistent with the report that the brain is protected against vitamin E loss compared with other tissues [57-59]. Considering the result that the decrease in ASC in the brain during ASC deficiency in the ODS rat was the slowest among tissues (Fig. 4), the brain must have an unknown mechanism to retain antioxidant vitamins. The GSH level and activities of GPX and SOD in the brain, heart, lung, liver, kidney, and muscle were not affected by the deficiency of vitamin E for even 8 weeks [44] as reported previously [58]. This result indicates that the GSH level and induction of GPX are not so sensitive as LOOH as an index of oxidative stress.

#### 8-4. Increase in LOOH in the Rat Liver and Kidney After Administering Ferric Nitrilotriacetate (Fe-NTA)

Fe-NTA causes radical reactions in the liver and kidney, resulting in hemochromatosis [60] and renal adenocarcinoma [61] by repeated injections. Two hours after an intraperitoneal (i.p.) injection of Fe-NTA to rats at a dose of 15 mg of Fe/kg body weight, the LOOH level transiently increased significantly in the liver (from  $190.1 \pm 58.8$  of the control to  $467.1 \pm 175.8$  pmol/mg protein) and kidney (from  $181.8 \pm 52.3$  of the control to  $405.9 \pm 22.7$  pmol/mg protein) [62]. These results demonstrate that oxidative stress was increased by an iron overload in these tissues. This idea is supported by reports that an increase in radical-derived products such as thiobarbituric acid reactive substances (TBARS) [63-67], 4-hydroxy-2-nonenal-modified proteins [65], 8-hydroxydeoxyguanosine [68], and aldehyde-modified proteins [66] occurs in the kidney within a few hours of the administration of Fe-NTA.

These results also indicate that radical reactions are rapidly initiated after the administration of Fe-NTA and do not last for a long time. This may be the reason why repeated injections are necessary to cause renal adenocarcinoma. These observations support the idea that Fe-NTA raises oxidative stress, which is a cause of hemochromatosis and renal adenocarcinoma.

### 9. IMPORTANCE OF ASC AMONG INDICES OF OXIDATIVE STRESS IN ANIMAL TISSUES

#### 9-1. Change in Oxidative Stress in the Streptozotocin Diabetic Rat

Diabetes mellitus is characterized by a series of complications that affect many organs. ROS have been implicated in the pathogenesis of diabetes mellitus as well as its complications [69]. Glucose autoxidation and non-enzymatic protein glycation produce oxygen free radicals [70-71]. In this section, we discuss the oxidative stress in diabetic rat tissues on the basis of the tissue concentrations of ASC, vitamin E, and LOOH.

A decrease in the ASC concentration in the plasma, liver, and kidney of diabetic rats is reported [72-79]. Concerning the level of vitamin E in the plasma, kidney, liver, and heart ventricle during experimental diabetes, controversial results have been reported [75, 77-83]. We therefore determined the

concentration of ASC and vitamin E in seven tissues of rats with experimentally induced diabetes [84].

Diabetes was induced in Wistar rats by injection of streptozotocin (40 mg/kg body weight, i.p.).

#### Concentration of Tissue LOOH in Diabetic Rats

The LOOH levels in the liver and kidney of the diabetic group were already significantly higher at the 4th week compared to those of control rats. The LOOH concentrations of the liver, kidney, heart, and muscle of diabetic rats were significantly higher than those of the control group at the 8th week [84]. These results demonstrate that an enhanced oxidative stress is caused in these tissues by diabetes. These results coincide with a view that radical reactions are involved in diabetic complications such as nephropathy and cardiomyopathy.

#### ASC Concentration in Diabetic Rats

The tissue ASC concentrations in diabetics were significantly decreased in the plasma, heart, lung, liver, and kidney compared to the control group 4 weeks after the injection of streptozotocin. After 8 weeks, the brain ASC level in the diabetic rats also decreased significantly compared to the control group. These results indicate that tissue ASC in diabetic rats decreases in the whole body, although ASC is not an essential vitamin to Wistar rats in contrast to the ODS rats [18]. The decrease in ASC is ascribed to the enhanced consumption of the vitamin by elevated oxidative stress caused by diabetes, as evidenced by augmentation of tissue LOOH as described above. The decrease in ASC may also be due to the reduced activity of glucose-6-phosphate dehydrogenase, which produces NADPH to regenerate ASC from DHA [85], impaired hepatic biosynthesis of ASC [86], and increased urinary excretion [86].

#### Vitamin E Concentration in Diabetic Rats

At the 4th week, the  $\alpha$ -tocopherol concentration of diabetic rats increased significantly in the plasma, brain, heart, liver, kidney, and muscle compared to that of the control rats. After 8 weeks, significant differences between the control and diabetic groups in tissue vitamin E were observed for the plasma, heart, liver, and muscle.

Different from the case of ASC, contradictory results have been reported concerning the plasma vitamin E concentration in diabetes. For example, the vitamin E level in diabetic rat plasma was reported to increase [80, 83], decrease [77], or to be unchanged [75]. This is also the case for the plasma vitamin E concentration determined for diabetic patients [87, 88-90]. The discrepancies among reports seem to be explained on the grounds that the vitamin E concentration depends on the experimental conditions such as duration and stage of diabetes.

The elevation of tissue tocopherol may be due to the mobilization of the vitamin with lipids from the liver and adipose tissues resulting in hyperlipidemia accompanied by diabetes. In conclusion, in diabetes, tissue LOOH and vitamin E increased, and ASC decreased as shown later in Table 4.

In the following sections, oxidative stress caused by chemicals is discussed. Toxicological study often provides a

typical pathological condition, which is a valuable model to study the relationship between oxidative stress and cell damage. It also allows us to evaluate the relative efficiency of indices of oxidative stress.

## 9-2. Evaluation of Oxidative Stress Based on LOOH, ASC and Vitamin E During Apoptosis and Necrosis Caused by Toxicants in Rat Liver

It is well established that carbon tetrachloride (CCl<sub>4</sub>) [91], thioacetamide [91-93], and D-galactosamine [94] are typical hepatotoxins causing centrilobular necrosis by radical reactions [91-93, 95-99]. Histological studies show that all these toxicants induce apoptosis in the liver [100-104]. To explore the mechanism of cell death and oxidative stress by these chemicals that seem to share a common mechanism, we have made a series of studies, where a dose of each chemical is selected to cause maximal necrosis 24 h after administration of these drugs on the basis of plasma GOT.

### *Liver Necrosis Caused by D-Galactosamine*

A necrogenic dose [99, 103] of D-galactosamine (1 g/kg, i.p.) was administered to Wistar rats. After 12 h, plasma GOT was not significantly different from that of the control group, which was administered saline. After 18 h, plasma GOT was increased to a significantly higher level than that of the control group (Table 2) [105]. After 24 h, the plasma GOT activity increased dramatically compared to that of the control group (Table 2). These results showed that the necrotic process was initiated at around 18 h, and progressed thereafter.

### *Estimation of Apoptosis by Caspase-3 Activity in the Liver*

Muntane *et al.* [102] and Stachlewitz *et al.* [104] reported that apoptosis in the rat liver was observed histochemically after D-galactosamine administration. To elucidate the mechanism of apoptosis, the activity of caspase-3-like protease, a cysteine protease specifically involved in apoptosis [106], was determined.

Caspase-3-like protease activity in the liver determined using a specific peptide substrate acetyl-Asp-Glu-Val-Asp- $\alpha$ -(4-methylcoumaryl-7-amide) (Ac-DEVD-MCA) increased significantly compared to that in the control group 18 and 24 h after D-galactosamine administration (Table 2). The maximal activity (59.6 pmol/mg protein/min) of the enzyme

was considerably higher than that caused by CCl<sub>4</sub> (24.2 pmol/mg protein/min) [107] and thioacetamide (31.2 pmol/mg protein/min) [108]. These results indicate that D-galactosamine causes a higher apoptosis/necrosis ratio in the liver than CCl<sub>4</sub> (a mixture of CCl<sub>4</sub> and mineral oil (1:1) was applied orally at 4 ml/kg body weight) and thioacetamide (500 mg/kg body weight, i.p.), since the caspase-3 activity may reflect the extent of apoptosis. The high incidence of apoptosis in D-galactosamine intoxication is partly explained on the grounds that the toxicity of D-galactosamine is mediated through serum TNF- $\alpha$  [102, 109], which causes apoptosis in the liver cells by activating caspases [110, 111].

Since Ac-DEVD-MCA is also a substrate of caspase-7 [112], the DEVDase activity arises from both caspases 3 and 7, which are proteases specifically activated in apoptosis. Therefore, the activity of caspase-3-like protease is a good indicator of apoptosis in animal tissues.

Jaeschke *et al.* [111] reported that endotoxin, a hepatotoxin leading to necrosis, causes 17-fold activation of caspase-3-like protease in the mouse liver 7 h after administration. The activation caused by endotoxins is much higher than that observed in toxicological studies described above, although the animal species are different. The extent of apoptosis caused by these toxicants may be much less than that caused by endotoxin. Therefore, it is suggested that these chemicals mainly cause necrosis and partial apoptosis, rather than apoptosis and necrosis in a sequential fashion.

### *Caspase-3 Activity in Plasma*

In plasma, caspase-3 activity was barely detectable in the control rats and was increased significantly 24 h after drug administration along with a dramatic increase in GOT (Table 2). It is surprising that caspase-3, a protease, showed activity in plasma, which has a strong anti-protease activity. These results indicate that the activity of caspase-3 in the liver and plasma is a reliable biochemical indicator of apoptosis under pathological conditions. Increases in caspase-3 activity in the liver and plasma were also observed when thioacetamide [108, 113] or CCl<sub>4</sub> [107] was applied to rats, as well as when liver regeneration after two-thirds partial hepatectomy was inhibited by  $\alpha$ -blocker [114].

These results demonstrate that D-galactosamine, CCl<sub>4</sub>, and thioacetamide cause apoptosis in the liver involving the

**Table 2. The Activities of Caspase-3-Like Protease of the Liver and Plasma and GOT in D-Galactosamine-Treated Rats After 12, 18, and 24 h and the Control Rats [102]**

	12 h	18 h	24 h	Control
Caspase-3-like protease (pmol/mg protein/min)				
Liver	18.1±3.3	49.3±5.3**	59.8±12.9**	18.3±2.3
Plasma	0.17±0.07	3.79±0.95	16.7±9.1*	0.06±0.02
Plasma GOT (Karmen units)				
	246±132	1445±191*	2454±721**	83.7±3.6

D-galactosamine (1 g/kg, i.p.) was administered to rats. After 12, 18, and 24 h, the activities of caspase-3-like protease in the liver and plasma and plasma GOT were determined. Control rats received saline and the enzyme activity was determined after 24 h. Values are means±S.E. for 6-7 rats and asterisks indicate significant differences from the corresponding control group (ANOVA Fisher's protected least significant difference test (PLSD), \*P<0.05 and \*\*P<0.01).

activation of caspase-3 and that the apoptosis and necrosis proceed simultaneously in the liver, from which caspase-3 is released by secondary necrosis taking place around 24 h after as shown by the high plasma caspase-3 and GOT activities.

#### Contribution of Liver Apoptosis to Plasma Caspase-3 Activity

Since measurable caspase-3 activity is detected in the control rat liver, it may be argued that the increase in plasma caspase-3 activity is not caused by apoptosis but is the result of leakage of basal levels of caspase-3 from damaged hepatocytes into the plasma in the same way as GOT is released. To evaluate the relative contribution of apoptosis to plasma caspase-3 activity, the activity ratio of plasma caspase-3 to GOT was calculated based on the data in Table 1. If plasma caspase-3 activity originates solely from necrotic cells and not from apoptotic cells, this ratio should be constant. The ratio  $\times 10^4$  is 6.9, 26.2, 68.1, and 7.2, in 12, 18, and 24-h D-galactosamine-treated rats and the control rat, respectively [105]. As this ratio after 18 and 24 h was significantly higher than that of the control group, it is concluded that plasma caspase-3 activity is derived from apoptotic hepatocytes in rats that were given D-galactosamine. Similar results were obtained in the intoxication of rats with  $\text{CCl}_4$  [107] and thioacetamide [108, 113].

#### Changes in the LOOH Level in Rats Treated with These Chemicals

Twelve hours after D-galactosamine administration, the liver LOOH concentration did not differ from that in the control group (Table 3) [105]. Eighteen hours after injection of D-galactosamine, when apoptosis and necrosis of the liver cells started based on the increase in liver caspase-3 and plasma GOT levels (Table 2), the liver LOOH concentration still did not differ from that in the control group (Table 3). After 24 h, when extensive necrosis had developed, the liver LOOH concentration was increased to 1.5-fold of that of the control rats (Table 3). By thioacetamide intoxication, the concentration of liver LOOH was also increased 2.2-fold [113] that in the control rats after 24 h, when extensive necrosis had developed. These observations indicate that radical reaction, *i.e.* lipid peroxidation, is caused by D-galactosamine, and thioacetamide and that necrosis is mediated by radical reaction.

#### Changes in the ASC Level in Rats Treated with These Chemicals

Twelve hours after D-galactosamine administration, the hepatic ASC level did not differ from that in the control group (Table 3) and it dropped to 60% of the level in the control group after 18 h (Table 3). After 24 h, the ASC level in the liver remained at 58% of the level in the control group (Table 3). Although ASC is not an essential vitamin for Wistar rats, excessive liver damage and oxidative stress caused by D-galactosamine may deplete ASC as in the case of diabetes. The decrease in the ASC concentration in the liver was also observed after administration of thioacetamide [113] or  $\text{CCl}_4$  [107]. These results indicate that the tissue ASC concentration is a reliable indicator of oxidative stress even in Wistar rats. The plasma ASC concentration was not changed by D-galactosamine administration (Table 3).

It may be argued that the decrease in liver ASC is not caused by oxidative stress but by damage in ASC synthesis resulting from the liver intoxication. However, the deficiency of ASC in ODS rats for 24 h did not result in such a severe loss of ASC as shown in Fig. (4). Therefore, it is concluded that oxidative stress is the main cause of the decrease in the liver ASC during D-galactosamine intoxication.

#### Changes in the Vitamin E Level in Rats Treated with These Chemicals

The hepatic  $\alpha$ -tocopherol level increased 18 h after D-galactosamine administration (Table 3). Since the liver ASC was largely decreased by D-galactosamine, oxidative stress should have increased in the liver. However, liver vitamin E increased. The elevation of the liver tocopherol level may be explained on the grounds that vitamin E is mobilized [115-116] from plasma and adipose tissues to alleviate the oxidative stress as evidenced by the significant decrease in the plasma tocopherol level 18 h after D-galactosamine administration (Table 3). Twenty-four hours after D-galactosamine injection, oxidative stress increased further based on the elevated level of LOOH and decrease in ASC (Table 3). However, the hepatic concentration of  $\alpha$ -tocopherol did not change (Table 2). These results also show that the tissue concentration of ASC reflects oxidative stress more directly than that of vitamin E.

**Table 3. The Concentrations of Vitamin C and Vitamin E of the Liver and Plasma in D-Galactosamine-Treated Rats After 12, 18, and 24 h and the Control Group [102]**

	Liver LOOH (pmol/mg protein)	Vitamin C (nmol/g tissue)		Vitamin E (nmol/g tissue)	
		Plasma	Liver	Plasma	Liver
D-galactosamine-treated					
After 12 h	216.2±23.7	50.6±3.0	1514±191	10.1±1.6	27.1±1.2
After 18 h	247.6±15.1	42.1±1.8	807±50**	6.2±0.6**	29.8±2.4*
After 24 h	322.6±25.0**	54.8±6.1	784±41**	8.5±1.4	28.3±2.4
Control	212.7±13.0	44.9±3.7	1344±40	11.9±1.1	23.6±1.5

D-galactosamine (1 g/kg, *i.p.*) was administered to rats. After 12, 18, and 24 h, the concentrations of LOOH in the liver, vitamin C and vitamin E in the plasma and liver were determined. Twenty-four hours after the administration of saline, determinations were made for control rats. Values are means±S.E. of 6-7 rats and asterisks indicate significant difference from the control group (ANOVA Fisher's protected least significant difference test (PLSD), \* $P < 0.05$  and \*\* $P < 0.01$ ).

**Oxidative Stress, Apoptosis, and Necrosis**

Eighteen hours after the administration of D-galactosamine, the activation of caspase-3 and the elevation in plasma GOT took place. Simultaneously, ASC was decreased in the liver. The involvement of ROS in apoptosis has been suggested. Recently, we reported [117-118] that hydroxyl radicals were involved in the activation of caspase-3 during apoptosis of human leukemic HL-60 cells induced by anticancer drugs based on systematic evaluation of different kinds of antioxidants. We also reported that oxidative stress during the apoptotic process was not as extensive and did not markedly decrease the cellular concentration of vitamin E [117]. This result is consistent with the study that low concentrations (around 100  $\mu$ M) of hydrogen peroxide induce apoptosis, while hydrogen peroxide at mM order causes necrosis [119].

Therefore, the decrease in ASC in the liver 18 h after D-galactosamine administration may be related to necrosis judging from a large increase in GOT at 18 h. Twenty-four hours after D-galactosamine injection, plasma GOT increased further and LOOH in the liver significantly increased. This is consistent with the case of thioacetamide intoxication [113]. Whether oxidative stress is a cause or a result of necrosis is an important issue. Necrosis caused by  $CCl_4$  intoxication did not involve extensive lipid peroxidation in sharp contrast to the well-accepted idea. This point is discussed in the next section. Regardless, the observation that necrosis does not always involve extensive radical reactions indicates that necrosis by D-galactosamine and thioacetamide is not a cause of oxidative stress, but a result of radical reactions.

**Evaluation of Oxidative Stress During Apoptosis and Necrosis Caused by  $CCl_4$  in Rat Liver**

A number of studies have established that the initial event in the rat given  $CCl_4$  is the lipid peroxidation of the endoplasmic reticulum of the liver cells initiated by trichloromethyl radical generated by the reaction of  $CCl_4$  with cytochrome P450 [91, 95]. Based on these studies,  $CCl_4$  is known as a typical poison causing severe oxidative stress. However, the LOOH concentration was increased by  $CCl_4$  only in the mitochondria, and that in the microsomal fraction or whole liver was not changed [120]. It is known [120-122] that TBARS do not increase significantly in the liver of rats treated with  $CCl_4$ . These observations indicate

that  $CCl_4$  does not cause extensive lipid peroxidation as is widely accepted.

We also demonstrated [120] that  $CCl_4$  inactivated cytochrome oxidase in the mitochondria. Recent studies show a key role of the mitochondria in apoptosis [123]. It is well documented [124] that caspase-3 is activated by cytochrome c released from the mitochondria.  $CCl_4$  damages the liver mitochondria by inhibition of cytochrome oxidase as well as by enhanced oxidative stress as evidenced by the increase in LOOH [120]. Therefore, mitochondrial damage caused by  $CCl_4$  may be involved in both apoptosis and necrosis.

These results indicate that  $CCl_4$  causes apoptosis in the liver by activating caspase-3 and that the apoptosis and necrosis proceed simultaneously in the liver, from which caspase-3 is released by secondary necrosis taking place around 24 h after as shown by the high plasma caspase-3 and GOT activities as in the case of D-galactosamine [105] and thioacetamide [113].

Along with radical reactions, the increase in calcium content in the liver [125-126] and liver mitochondria [127] by  $CCl_4$  was reported. Since the cellular calcium concentration is transiently augmented in cell death [128], it is probable that damage of the respiratory chain in the mitochondria [120] and the resulting disorder of calcium metabolism [126-127, 129] by  $CCl_4$  are the main cause of apoptosis and necrosis of the liver cells.

**Comparison Among Oxidative Stress Indices**

In Table 4, indices of oxidative stress in the liver are compared in 4 pathological conditions. The increase in LOOH is always accompanied by the decrease in ASC. However, the change in vitamin E does not coincide with these two parameters. Since the change in ASC precedes that in LOOH, the tissue concentration of ASC is concluded to be the most sensitive index of oxidative stress.

**10. OXIDATIVE STRESS IN APOPTOSIS OF CULTURED CELLS**

Since the first appearance of the term "apoptosis" [130], great advancements have been achieved in elucidating the signaling systems of apoptosis [131]. Among cellular processes, a large body of evidence has accumulated to

**Table 4. Change in Indices of Oxidative Stress in Rat Liver**

	Vitamin C	Vitamin E	LOOH	Maximal GOT
Diabetes (STZ)	↓	↑	↑	
$CCl_4$	↓	→	→ (whole liver) ↑ (Mitochondria)	8250
Thioacetamide	↓	↓	↑	4220
D-Galactosamine	↓	↑	↑	2500

Arrows ↑, ↓, and → mean increase, decrease, and no change, respectively.

suggest that ROS play a key role as a common mediator of apoptosis [132-134]. ROS cause mitochondrial permeability transition [123, 135] resulting in the release of caspase activators such as Apaf-1 [136] and cytochrome c [124]. Among ROS, hydroxyl radicals were involved during apoptosis of HL-60 cells induced by anticancer drugs like actinomycin D, H7, or daunorubicin on the basis of effective inhibitions by catalase, a spin-trap agent (PBN, *N*-*t*-butyl- $\alpha$ -phenylnitron), *N*-acetylcysteine, and vitamin E [117-118]. Catalase inhibited effectively the activation of caspase-3-like protease by these inducers [118], indicating that hydrogen peroxide, which is a common mediator of apoptosis of HL-60 cells induced by these chemicals, is also involved in the activation of caspase-3, because catalase is a specific enzyme that decomposes hydrogen peroxide.

A spin-trap agent, PBN, significantly inhibited the activation of caspase-3 caused by these drugs [118]. This result indicates that hydroxyl radicals are involved in the process leading to the activation of caspase-3, since hydrogen peroxide is suggested to be a mediator as described above, and it easily generates hydroxyl radical by a Fenton-type reaction. Therefore, hydrogen peroxide and hydroxyl radical are common mediators of caspase-3 activation caused by these chemicals with apparently different functional mechanisms.

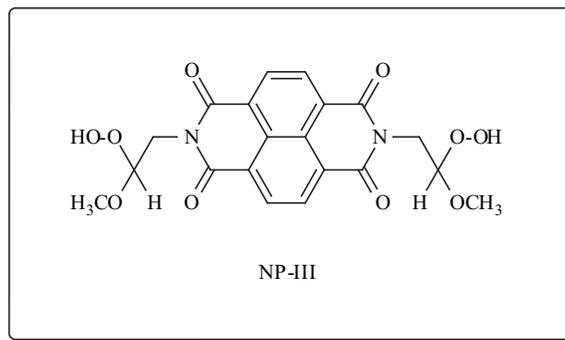
Based on the mitochondrial activity determined by oxygen consumption, complex IV was commonly inhibited by these drugs, and the inhibitions of complex IV were almost fully restored by the addition of cytochrome c [118]. This result shows that the inhibition of complex IV is not due to the damage in this enzyme itself but due to the release of cytochrome c from the mitochondria. The release of cytochrome c by these drugs was also demonstrated by Western blot analysis. The addition of catalase inhibited the depression of complex IV activity induced by actinomycin D and H7. These observations indicate a direct relationship between hydrogen peroxide and the release of cytochrome c resulting in the activation of caspase-3 during apoptosis caused by these inducers [118].

Many similar studies are reported. It is well documented that hydrogen peroxide serves as a mediator of apoptosis [132, 134], and that hydrogen peroxide itself induces apoptosis in many types of cells including HL-60 cells [137]. Hydroxyl radicals, easily generated in the cells by Fenton and/or Haber-Weiss reactions, have been suggested as a cause of apoptosis in many studies [117-118, 132, 134]. However, no direct experimental evidence has been presented to show that hydroxyl radical itself causes apoptosis. In the next section, experimental evidence is presented.

### 10. 1 Apoptosis Caused by Selectively Generated Hydroxyl Radicals

*N,N'*-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthal-diimide (NP-III) [138] selectively generates hydroxyl radical on UVA irradiation with a quantum yield of 0.03 [139] (reaction 4). NP-III oxidizes DNA [138] and protein [140-141] and also causes DNA damage [142] in V79 cells and growth inhibition [143] of *Acanthamoeba* by photochemical generation of hydroxyl radical. We have demonstrated that photochemically generated hydroxyl radicals from NP-III do

cause apoptosis in HL-60 cells involving the activation of caspase-3.



The rate of apoptosis by 1  $\mu\text{M}$  of NP-III was comparable to that by 100  $\mu\text{M}$  of hydrogen peroxide [137], indicating that hydroxyl radical caused apoptosis in HL-60 cells more effectively than hydrogen peroxide by at least two orders of magnitude.

There are many studies showing ROS, especially hydrogen peroxide, as an intermediate of apoptosis utilizing the fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This compound is easily converted into fluorescent fluorescein by hydrogen peroxide [144]. This method is very convenient and has become a standard to show the involvement of ROS, especially hydrogen peroxide. However, the specificity of the method has been challenged by Taketani *et al.* [145], who have shown that oxidation of DCFH is not always caused by ROS but related to the heme content in cells. The importance of the specific determination of key molecules is reconfirmed. More specific and sensitive methods to detect ROS are required to evaluate the role of ROS in apoptosis.

In the next section, oxidative stress is discussed as a cause of atherosclerosis, a representative disease of the elderly. In addition, the possibility of its prevention by antioxidative food factors including vitamin C is discussed.

## 11. ROLE OF RADICAL REACTION IN ATHEROGENESIS

### 11-1. Background

Atherosclerosis is a chronic arterial disease causing myocardial and cerebral infarcts, which are serious life-threatening complications for the elderly. Ample evidence supports the view that oxidatively modified low-density lipoprotein (oxLDL) plays a key role in the onset of atherogenic processes [5, 146-148]. It exerts many effects such as promotion of apoptosis, adherence of monocytes, and their migration into the subendothelial space of blood vessels, and foam cell formation. It is also involved in inducing migration, proliferation, and transformation of smooth muscle cells. However, the chemical nature of oxLDL has not been well characterized.

Although LDL is composed of lipid, sugar chain, and protein, studies on the oxidation of LDL have focused mainly on lipid peroxidation [148-149] and the resulting

modification of apolipoprotein B-100 (molecular mass of 512 kDa) (apoB) by the aldehydes produced [147-148]. Palinski *et al.* [147] reported that malondialdehyde- or 4-hydroxynonenal-modified LDL was detected immunohistochemically in the atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbit aorta and in human sera using antibodies against LDL modified with these aldehydes.

As for the sugar moiety of LDL, Tertov *et al.* [150] showed that a part of LDL isolated from patients with coronary artery atherosclerosis bound to a Sepharose-linked *Ricinus communis* agglutinin, a lectin which interacts with galactose residues, and suggested that desialylated LDL was increased in serum of those patients. We reported [151] that sialic acid moieties of LDL were decreased by oxidation with  $\text{Cu}^{2+}$ , and radical reaction was a possible mechanism for the increase in desialylated LDL in serum of atherosclerotic patients.

Sialic acid was significantly more reactive in radical reaction than other sugar components such as mannose, galactose and N-acetylglucosamine in the radical reaction of transferrin [152]. The reactive nature of sialic acid to oxidative stress may be reflected in the fact that the brain content of sialic acid was decreased by vitamin C and E deficiencies for 3 weeks in ODS rats [153]. It is possible that the degradation of only one sialic acid leads to catabolism of LDL through asialoglycoprotein receptor or other recognition mechanisms. Thus, it is important to investigate the role of sialic acid as a controlling factor of the lifetime of glycoproteins, which have terminal sialic acid moieties that are attacked by ROS at first. Therefore, oxidative modification of sialic acid is a possible mechanism in atherogenesis.

### 11-2. Radical Reaction of the Protein Part of LDL in Atherogenesis

On the other hand, studies on radical reactions of the protein are limited to *in vitro* chemical reactions using purified proteins such as albumin and LDL [154-155]. Studies on radical reactions of isolated LDL [155-165] and albumin [166-167] demonstrated that cleavage of peptide bonds and cross-linkage occurred. In these *in vitro* studies, the products were analyzed by SDS-PAGE with dye staining of proteins. Therefore, it remained unknown whether such fragmentation and cross-linkage occurred in serum, because no appropriate method had been available to follow the change in each protein in the presence of a great many other proteins.

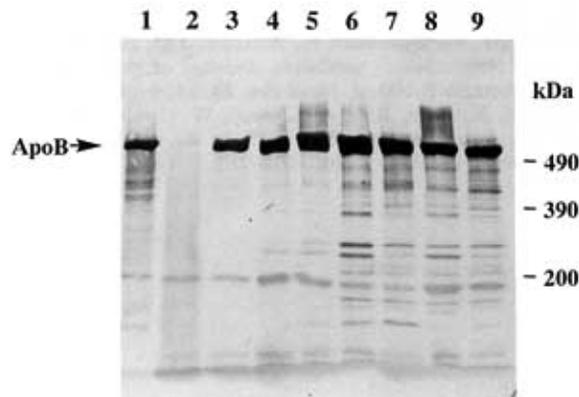
We reported [168] that immunoblotting was an effective method to follow each protein during radical reaction and that human serum albumin and transferrin underwent fragmentation by radical reactions in isolated solution as well as in plasma based on immunoblot assay. This study shows that fragmentation of protein, namely cleavage of peptide bonds by radical reaction, occurs effectively in aqueous solution at 37°C, pH 7.4. This was unexpected, because facile cleavage of the amide bond by a radical mechanism was not well known.

Western blot analysis allows us to show that the radical reaction of LDL, caused by  $\text{Cu}^{2+}$ , gives a characteristic

pattern of fragmented apoB [169]. The radical nature of the reaction was unambiguously shown by the results that radical scavengers such as  $\beta$ -mercaptoethanol, BHT, and probucol inhibited the reaction and that a radical initiator AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride] initiated apoB fragmentation.

Degradation of apoB in human serum was investigated. After dialysis, serum was reacted with  $\text{Cu}^{2+}$  and the change in apoB was followed with Western blotting. In the absence of  $\text{Cu}^{2+}$  or in the presence of 10  $\mu\text{M}$   $\text{Cu}^{2+}$ , degradation of apoB was negligible even after 5 h at 37°C. This observation showed that protease was not involved in the fragmentation of apoB because in the presence of 50  $\mu\text{M}$   $\text{Cu}^{2+}$ , apoB in the serum was degraded progressively and the apoB band at 512 kDa disappeared completely within 2 h. The fragmentation pattern of the products was similar to that obtained from the reaction of isolated LDL. In addition, the band pattern obtained by the immunoblot analysis was almost identical with that obtained from the oxidation reaction of isolated LDL by protein staining using Coomassie brilliant blue [169]. This result demonstrates that the antibody used in this study reacts with polypeptides derived from apoB almost equally and that each band density corresponds to the protein content derived from apoB.

Furthermore, both fragmented and cross-linked apoB proteins similar to the *in vitro* oxidation products of LDL and serum were detected in normal human sera [169]. In addition, these products tended to increase with age [169]. Immunoblots of sera of seven volunteers were carried out using anti-apoB antiserum. As shown in Fig. (9), sera of females in their twenties contain lower amounts of the degradation products (lanes 3-5) than those of older volunteers in their forties (lanes 6 and 7) and seventies (lanes 8 and 9). It is worthwhile to note that the band pattern of serum is similar to that of the *in vitro* oxidation products of LDL and serum indicating that radical reactions actually take place *in vivo*.

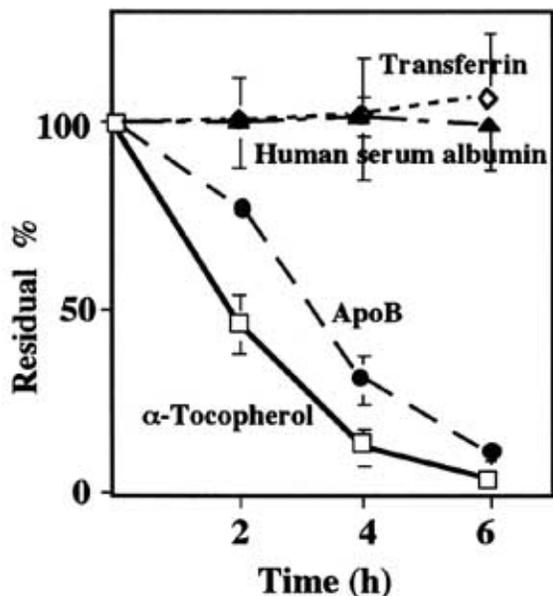


**Fig. (9).** Immunoblots of human sera using anti-apoB antibody. Lane 1, LDL treated with 1.67  $\mu\text{M}$   $\text{Cu}^{2+}$  at 37°C for 1 h; lane 2, serum of a 21-year-old female treated with 50  $\mu\text{M}$   $\text{Cu}^{2+}$  at 37°C for 1 h; lanes 3 to 9, sera of females aged 21, 22, 24, and 41, a 49-year-old male, a 74-year-old female, and a 75-year-old male, respectively.

In lanes 4-9 of Fig. (9), bands of higher molecular weight than apoB were observed, a finding consistent with *in vitro* studies describing that cross-linked apoBs were formed by oxidation of apoB [155, 159-160]. Since the concentration of apoB in serum is about 1 mg/ml [169], which is much higher than that used in our *in vitro* reaction (50  $\mu$ g/ml) where degradation predominated, intermolecular reactions take place leading to the formation of higher molecular weight products [169]. Immunoblot study also shows that the conjugated products derived from apoB oxidation are present in serum.

These observations indicate that the calibration of fragmentation and conjugation pattern of apoB in the serum can be a reliable indicator of oxidative stress and atherosclerosis. In the next section, immunoblot analyses of apoB proteins in human sera are performed, and the results are compared with widely used clinical parameters of atherosclerosis including plasma ASC level.

Fragmentation and conjugation of proteins may be caused by radical reactions. Then, the fragmentation and aggregation should take place exclusively in albumin, because it is the most abundant protein in serum and undergoes fragmentation by radical reaction of plasma [168]. Here, a very simple question may be asked. Why do radical reactions occur selectively in apoB? To address this important issue, we compared the reactivity of plasma proteins in radical reactions.



**Fig. (10).** Change in  $\alpha$ -tocopherol, apoB, human serum albumin, and transferrin by the reaction of human plasma with  $\text{Cu}^{2+}$

Human plasma, diluted with PBS four-fold, was treated with 400  $\mu\text{M}$  of  $\text{Cu}^{2+}$  at 37°C. From the oxidized plasma solutions, samples were withdrawn at 0, 2, 4, and 6 h after the addition of  $\text{Cu}^{2+}$  and the  $\alpha$ -tocopherol, apoB, HSA (human serum albumin), and TF contents were measured. Each point was mean  $\pm$  S.D. Where no bars are shown, S.D. was smaller than the symbol. This figure shows that apoB is unusually reactive to radical reactions compared to other plasma proteins and its reactivity is even comparable to vitamin E.

When radical reactions were initiated in plasma with  $\text{Cu}^{2+}$ , apoB underwent extensive fragmentation concurrently with the decrease in  $\alpha$ -tocopherol, while human serum albumin and transferrin were not decreased at all (Fig. 10) [170]. When radical reactions were initiated with  $\text{Cu}^{2+}$  with hydrogen peroxide or AAPH,  $\alpha$ -tocopherol and apoB were also decreased steadily while albumin and transferrin were not decreased at all. These results clearly show that apoB is extremely reactive, even comparable to  $\alpha$ -tocopherol, toward radical reactions. This unusually high reactivity of apoB may be explained on the grounds that different from albumin and transferrin, apoB is covered by lipids, which are reactive to radicals. A similar profile of the decrease in apoB to vitamin E supports this idea.

These results also explain why radical reaction products of apoB are present in human sera. The unusually sensitive nature of LDL to oxidation may allow us a mechanism-based diagnosis of the oxidative stress and atherosclerosis in an individual by its pattern analysis. In addition, this character of apoB shows the importance of oxLDL in atherogenesis.

### 11-3. Evaluation of apoB Fragmentation and Cross-Linkage in Serum as an Index of Atherosclerosis

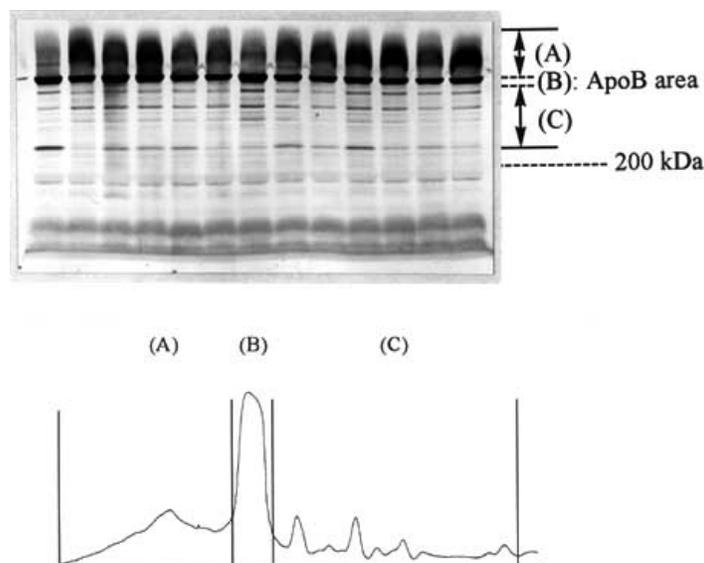
Based on the discussion above, the fragmentation and cross-link pattern of apoB reflects the oxidative stress in an individual and this pattern is probably a mechanism-based index of atherosclerosis. A method was developed to evaluate the fragmentation and conjugation pattern of apoB.

### 11-4. Determination of Oxidative Index of apoB

Human sera from 249 Japanese inhabitants who participated in the annual local health-check program in Kisei Town (a rural area in Mie Prefecture, Japan) were analysed. When human serum was subjected to similar immunoblot analysis, cross-linked apoB proteins with a higher molecular mass than apoB and fragmented apoB proteins were visualized (Fig. 11). For all subjects, ladders were observed at similar positions (Fig. 11). The band pattern of these sera was similar to that of the *in vitro* oxidation products of LDL and serum [169].

The density of these protein bands was calibrated using software (NIH Image). The band showing a higher molecular weight than apoB is defined as band (A), and the apoB band is defined as band (B). The sum of fragments with a molecular mass smaller than apoB and larger than approximately 250 kDa (above which 2 bands were always detected) is defined as band (C), which represents fragmented apoB. Band (C) is selected as shown in Fig. (11) on the criterion that it does not contain fragments with a molecular mass lower than 250 kDa, because nonspecific staining including apolipoprotein B-48 may be contained in the low-molecular-weight bands in the immunoblot.

To minimize the difference among membranes, serum from the same person was always placed in the middle of each gel as a control. One parameter is defined as B-ox for each person [171]. B-ox is calibrated from each membrane as  $((A)+(C) \text{ of each sample}) / [(A)+(C) \text{ of the control}]$ , which



**Fig. (11).** Immunoblot of human serum with anti-apoB [168]

Sera were subjected to electrophoresis and immunoblot with anti-apoB. The density of stained bands in the area shown by (A), (B) and (C) was calibrated. Area (B) corresponds to apoB. A typical calibration of the central band using NIH Image 1.61 is shown below the immunoblot.

represents the ratio of radical reaction products of each sample to the control subject. If this value increases, it means that radical reaction products increase, *i.e.*, the oxidative stress increases in the subject.

#### 11-5. Comparison of the B-ox Value with Clinical Parameters of Atherosclerosis

The B-ox value of male subjects ( $n=88$ ) is  $1.16\pm 0.49$ , which is significantly ( $P < 0.05$ ) higher than that ( $1.00\pm 0.44$ ) of females ( $n=161$ ), while the age ( $59.9\pm 12.3$ ) of the male subjects is not significantly different from that ( $57.2\pm 11.9$ ) of the female group. Among female subjects, the B-ox ( $0.79\pm 0.38$ ) of subjects younger than 50 ( $n=34$ ) is significantly ( $P < 0.01$ ) lower than that ( $1.05\pm 0.44$ ) of the 50 and over group ( $n=127$ ) *i.e.* mainly post-menopausal females. These characteristics of B-ox coincide with the widely known properties of atherosclerosis.

B-ox shows a significant positive correlation with intima-media thickness of the carotid artery (IMT), age, and

LDL cholesterol (Table 5), while B-ox exhibits a significant negative correlation with high-density lipoprotein (HDL) cholesterol (Table 5). The correlation coefficient of B-ox with IMT is even larger than that of LDL cholesterol with IMT. These results show that B-ox is a good atherosclerosis index, *i.e.* a radical mechanism-based indicator, and is superior to LDL cholesterol, which does not correlate with age. Age significantly correlates with all these indices except HDL-, and LDL-cholesterols (Table 5). HDL cholesterol gives a significant negative correlation with IMT, and LDL cholesterol as is known well (Table 5).

B-ox shows a significant positive correlation with total cholesterol, and triglyceride, and inversely correlates with the plasma ASC concentration (Table 6). Although the plasma concentration of  $\alpha$ -tocopherol significantly correlates positively with LDL cholesterol, triglyceride, and total cholesterol [171], it does not give a significant correlation with either B-ox (Table 6) or IMT. The ratio ( $\alpha$ -tocopherol/total cholesterol) does not correlate significantly with either B-ox (Table 6) or IMT. Plasma ASC does not

**Table 5.** Correlation Coefficients Between Parameters [168]

	LDL cholesterol	HDL cholesterol	IMT	Age
B-ox	0.36** (242)	-0.29** (242)	0.23* (117)	0.26** (249)
LDL cholesterol		-0.15* (245)	0.20* (119)	0.05 (245)
HDL cholesterol			-0.19* (119)	-0.09 (245)
IMT				0.57** (119)

The number of subjects is shown in parenthesis. (\* $P < 0.05$ , \*\* $P < 0.01$ )

**Table 6.** Correlation coefficients of B-ox with other parameters [168]

	Total Cholesterol	Triglyceride	Vitamin C	$\alpha$ -Tocopherol	$\alpha$ -Tocopherol/ Total cholesterol
B-ox	0.30** (242)	0.16* (242)	-0.15* (229)	0.08 (229)	-0.10 (229)

The number of subjects is shown in parenthesis. (\*P < 0.05, \*\*P < 0.01)

correlate with IMT either. In conclusion, B-ox, the oxidation index of apoB, correlates significantly with indices of atherosclerosis. This observation shows that B-ox is a reliable index of atherosclerosis, and also supports the view that the oxidative stress, *i.e.* radical reaction, plays a key role in atherogenesis.

It is well known that the apoB concentration is a risk factor of atherosclerosis. However, this apoB concentration has been determined by ELISA and the thus obtained value is the sum of all immunoreactive apoB proteins (apoB itself, and conjugated and fragmented apoBs). ApoB itself (band (B)) correlated with B-ox, and LDL cholesterol [171]. As an index of atherosclerosis, a factor defined as [(A)+(C)/(B)] of each subject is a possible candidate instead of B-ox. The correlation of this value with IMT is slightly lower ( $r=0.21$ ,  $P < 0.05$ ) than that of B-ox. This may be explained on the grounds that apoB itself increases in atherosclerosis and the increase in oxidized apoB expressed by [(A)+(C)] is masked when it is divided by the increased apoB (B). Therefore, it is conceivable that the ratio of ((A)+(C)) to a common denominator [((A)+(C)) of the control, in this study] gives a better correlation with IMT.

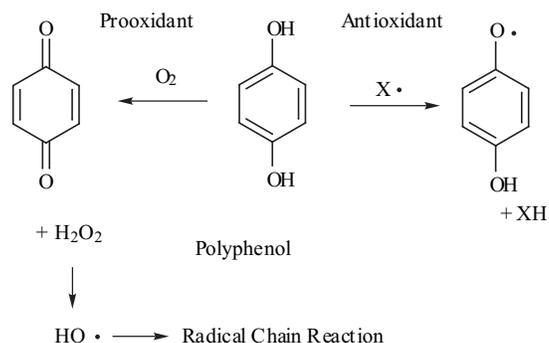
Although there are many studies concerning the beneficial effect of antioxidative vitamins C and E on atherosclerosis and cardiovascular disease [172-174], this issue is not yet conclusive. ASC shows a significant negative correlation with B-ox, while neither vitamin E nor the ratio ( $\alpha$ -tocopherol/total cholesterol) shows a correlation with B-ox (Table 6). These observations indicate an important role of ASC in the prevention of apoB oxidation *in vivo*, while the contribution of vitamin E as an antioxidant for apoB was not confirmed. These results may be supported by discussions made in previous sections that the concentration of tissue ASC more sensitively reflected oxidative stress than that of vitamin E, and also a study [20] showing that ASC decreased firstly during radical reaction of human plasma.

Atherosclerosis progresses with aging and causes dangerous complications. It is desirable if these processes are halted by food factors as evidenced by the favorable effect of ASC. Recently, diseases of the elderly such as atherosclerosis, diabetes, and cancer are referred to as "lifestyle-related diseases" in Japan. Among lifestyles, diet may be the most important factor in the regulation of these diseases. However, it is very difficult to evaluate the effect of food factors *in vivo*, because an appropriate method is not available. Although many *in vitro* studies have been made concerning the antioxidative effect of food factors, these studies cannot estimate the effect *in vivo*. Furthermore, different from medicines, the effect of food factors should be very slight even if present, making their study difficult. In the next section, the effect of antioxidative food factors is discussed.

## 12. ANTIOXIDATIVE EFFECT OF FOOD FACTORS *IN VIVO*

### 12-1. Problems Involved in the Study of Food Factors

Firstly, we must discuss the problems associated with the study of antioxidants in foods. For example, polyphenols are aromatic compounds with multiple hydroxyl groups. The simplest molecule is hydroquinone, shown in Fig. (12). This compound is a radical scavenger or antioxidant if it scavenges a radical by hydrogen atom transfer. However, these antioxidants are capable of reducing oxygen to produce ROS such as superoxide and hydrogen peroxide. In this case they function as a prooxidant. Polyphenols easily generate hydrogen peroxide by the reaction with oxygen as evidenced by the fact that hydrogen peroxide is industrially produced by the reaction of a naphthohydroquinone derivative with oxygen. Whether polyphenols function as a prooxidant or an antioxidant depends on the reaction conditions such as oxygen concentration and coexisting substances. Bowry and Stocker [175] have reported a model case where even vitamin E functions as a radical initiator. Therefore, the antioxidant effects of food factors evaluated in a simple *in vitro* reaction cannot always be expected *in vivo*.



**Fig. (12).** Polyphenols as an antioxidant as well as prooxidant. The reaction of hydroquinone is shown as an example of a polyphenol.

Recently, many studies have been made to identify whether ASC and polyphenol cause apoptosis in cultured cells. However, the effect of hydrogen peroxide, which is an apoptosis inducer as discussed above and is formed by these factors and oxygen in almost quantitative yield, must be evaluated carefully, since these experiments are made under air, in a considerably higher concentration of oxygen than in an animal tissue.

To evaluate the effects of food factors *in vivo*, an appropriate model system is necessary, because experiments using animals are not so easily made. In the following sections, two models are presented.

## 12-2. Inhibition of Radical Reaction of ApoB and $\alpha$ -Tocopherol in Human Plasma by Green Tea Catechins

As discussed in section 11, oxidative modification of LDL is involved in atherogenic plaque formation, which is the main cause of coronary artery disease [1, 146, 147]. An epidemiological study [176] showed that intakes of dietary antioxidant flavonoids including tea polyphenols [177] were inversely related to mortality due to coronary artery heart disease. Flavonoids are polyphenolic antioxidants naturally present in vegetables, fruits and beverages such as tea and wine. Among these dietary sources, green tea contains relatively large amounts of polyphenols named catechins. *In vitro* studies [178-181] have shown that tea flavonoids inhibit the oxidation of isolated LDL utilizing  $\text{Cu}^{2+}$  as a radical initiator.

To clarify the role of tea catechins in the prevention of atherosclerosis, it is necessary to evaluate the antioxidative effect in a more physiological condition rather than in a reaction utilizing isolated LDL. Based on the discussion in the previous section, the capacity of food factors to inhibit apoB fragmentation caused by radical reaction in plasma may be an effective indicator to evaluate their anti-atherogenic activity. The antioxidative activity of tea catechins was evaluated based on this method. At the same time the antioxidative effect of catechins was estimated based on their interaction with  $\alpha$ -tocopherol.

Human plasma was subjected to a radical reaction initiated by 400  $\mu\text{M}$  of  $\text{Cu}^{2+}$  at 37°C as described previously [169]. When epigallocatechin gallate (EGCg) was added, the decrease in apoB was inhibited in a dose-dependent manner. Other catechins also inhibited the fragmentation of apoB in a dose-dependent fashion. To compare the inhibitory activity among these antioxidants, the concentration of each antioxidant for 50% inhibition ( $\text{IC}_{50}$ ) of apoB fragmentation relative to the control was determined graphically. The  $\text{IC}_{50}$  value of EGCg for apoB fragmentation is  $21.3 \pm 3.8 \mu\text{M}$  (Table 7) [182].

Similar experiments were carried out, and  $\text{IC}_{50}$  values for epicatechin (EC), epigallocatechin (EGC), Trolox (water-soluble vitamin E derivative), and epicatechin gallate (ECg) were determined as  $39.1 \pm 7.1$ ,  $42.2 \pm 5.9$ ,  $36.2 \pm 9.4$ , and  $14.6 \pm 3.6 \mu\text{M}$ , respectively (Table 7). The  $\text{IC}_{50}$  values of ECg and EGCg are significantly lower than those of EC, EGC and Trolox. These results indicate that EC and EGC have a comparable activity with Trolox as an antioxidant to apoB in the radical reaction of plasma. ECg and EGCg show significantly higher activity than EC, EGC, and Trolox, suggesting that the gallate group functions as a strong antioxidant.

The antioxidative activity of tea catechins and Trolox to  $\alpha$ -tocopherol was also evaluated in the radical reaction of plasma initiated by  $\text{Cu}^{2+}$  at 37°C based on  $\text{IC}_{50}$  (Table 7). The  $\text{IC}_{50}$  of each antioxidant for  $\alpha$ -tocopherol resembles that for apoB. These  $\text{IC}_{50}$  values are also divided into two groups and again gallate groups have a stronger antioxidant activity than other constituents of catechins. This result indicates that catechins have a protective effect on  $\alpha$ -tocopherol and apoB in a similar dose-dependent manner. The activity sequence of catechins is similar to that of the reaction of isolated LDL using metmyoglobin [183].

Yamanaka *et al.* [180] reported that EC and EGC had both prooxidant and antioxidant effects on the oxidation of isolated LDL by 5  $\mu\text{M}$  of  $\text{Cu}^{2+}$  based on conjugated diene formation and apoB fragmentation. The prooxidant effect of catechins was not observed in the concentration range of this study using plasma [182].

The reaction condition for isolated LDL is considerably different from that for plasma. Firstly, a similar rate profile of apoB fragmentation to the reaction of isolated LDL is observed when the concentration of  $\text{Cu}^{2+}$  is two orders of magnitude higher in the oxidation of plasma, probably because of antioxidants and metal binding proteins contained in the plasma. This indicates that apoB in plasma is protected more from oxidants than that in isolated LDL and that a similar fragmentation takes place once the reaction is initiated by a sufficient amount of  $\text{Cu}^{2+}$ . Secondly, a comparable concentration of catechins to  $\text{Cu}^{2+}$  is necessary for their antioxidant effect in the reaction of isolated LDL [180]. However, a much smaller amount of catechins compared to the  $\text{Cu}^{2+}$  concentration is needed to exhibit the antioxidant effect in the reaction of plasma [182]. This result supports the view that the antioxidative effect of catechins does not arise from only a chelating effect to  $\text{Cu}^{2+}$  [184], but also a radical quenching effect by hydrogen donation is essential. This is also supported by a study showing that tea catechins do not inhibit but rather promote DNA cleavage in the presence of  $\text{Cu}^{2+}$  [185].

Finally, the physiological implications of catechin intake must be evaluated. Piskula and Terao [186] reported that the rat plasma concentrations of catechins including their metabolites such as glucuronide and sulfate conjugates amount to 1-12  $\mu\text{M}$  after oral administration of EC at 172  $\mu\text{mol/kg}$ . Therefore, it is probable that the total concentration of antioxidative catechins including their metabolites in plasma is in the range that displays antioxidative effect on apoB as well as on  $\alpha$ -tocopherol [182].

This method was applied further to examine the antioxidant effect of fluvastatin, a synthetic inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase used for patients with hypercholesterolemia, and its metabolites [187].

In the last section, one method to evaluate the antioxidative effect of food in a whole animal is proposed. As a food source, propolis was examined.

**Table 7.  $\text{IC}_{50}$  Values ( $\mu\text{M}$ ) of Catechins and Trolox for apoB and  $\alpha$ -Tocopherol in the Oxidation of Plasma with  $\text{Cu}^{2+}$  [179]**

	ApoB	$\alpha$ -Tocopherol
EC	$39.1 \pm 4.1^a$	$41.8 \pm 13.2^a$
EGC	$42.2 \pm 3.4^a$	$42.7 \pm 3.0^a$
Trolox	$36.2 \pm 5.4^a$	$60.8 \pm 5.4^a$
ECg	$14.6 \pm 2.1^b$	$11.5 \pm 1.1^b$
EGCg	$21.3 \pm 2.2^b$	$17.3 \pm 2.2^b$

Each value is mean S.E. of three plasma samples from three individuals. Different letters indicate significant differences in each column among antioxidants by Bonferroni/Dunn protected least significant difference test ( $P < 0.05$ ).

### 12-3. *In Vivo* Antioxidative Activity of Propolis Evaluated by the Interaction with Vitamins C and E, and the LOOH Level in Rats

The propolis of honeybee hives has long been used in folk medicine going back as far as the ancient Greeks. Recent studies have shown that propolis exhibits a broad spectrum of activities such as antibiotic, anti-inflammatory, antifungal and antitumorigenic properties [188]. Along with these activities, the antioxidative effects of propolis and its components have also been studied *in vitro* [189-193]. Concerning the *in vivo* antioxidative activity of propolis, protective effects of intraperitoneally administered propolis extract against doxorubicin-induced cardiomyopathy [194], CCl<sub>4</sub>-induced liver damage [195], galactosamine-induced hepatitis [196], and gamma irradiation [197] have been reported.

Although there is considerable interest in the role of propolis as a dietary antioxidant, only limited studies are available on the antioxidative effect of orally administered propolis as well as the bioavailability of propolis [198]. El-Ghazaly and Khayyal [199] reported that orally administered aqueous propolis reduced the damage caused by gamma irradiation, and Basnet *et al.* [200] reported that orally administered propolis extract ameliorated liver damage induced by CCl<sub>4</sub>, D-galactosamine, and lipopolysaccharide. Although these reports suggest that propolis functions as an antioxidant, no effective method is yet available to evaluate directly the antioxidative activity of dietary propolis *in vivo*. We propose a method to evaluate the antioxidant activity of dietary propolis on the basis of ameliorative effects on the oxidative stress induced by vitamin E deficiency in rats [201].

#### Study Design

To evaluate the *in vivo* antioxidant activity of propolis, the rescuing effect of propolis on vitamins C and E was determined during the oxidative stress induced by vitamin E deficiency. Rats were divided into two groups. One group, designated as the control group, was fed a vitamin E-deficient diet and the other group, defined as the propolis group, received a vitamin E-deficient diet supplemented with 1% propolis. After 4 and 8 weeks, the tissue concentrations of vitamins C and E were compared between these two groups. At the same time, the tissue concentrations of LOOH as an indicator of oxidative stress were also compared.

#### Change in the Tissue Concentration of Vitamin E, ASC, and LOOH

No significant difference was observed in tissue vitamin E concentrations between these two groups after both 4 and 8 weeks. This result indicates that propolis does not rescue vitamin E, a hydrophobic antioxidant, during vitamin E deficiency. After 4 weeks, the plasma ASC concentration of the propolis group was significantly higher than that of the control group. This result suggests that propolis has a rescuing effect on ASC in plasma probably by an antioxidative mechanism. It is conceivable that some hydrophilic component(s) of propolis rescue(s) ASC, a water-soluble vitamin, different from the case of hydrophobic vitamin E as described above. No significant

difference was observed in the liver, heart, kidney, stomach, small intestine, and large intestine after 4 weeks.

After 8 weeks, the tissue concentrations of ASC in the kidney, stomach, small intestine, and large intestine of the propolis group were significantly higher than those of the control group.

These *in vivo* results indicate that one or more components of propolis are absorbed and circulate in the blood to affect the concentration of ASC in the kidney and plasma. These results also support the view that propolis rescues ASC in enhanced oxidative stress caused by prolonged vitamin E deficiency. It is conceivable that propolis exerts its antioxidative effect on tissues where it is assumed to be accumulated, as in kidney where it is excreted, and in the gastrointestinal tract where propolis affects these tissues even from the outside the cell.

Dietary propolis inhibits chemical carcinogenesis in the rat colon [202-203]. To investigate whether the antioxidative property of propolis contributes to the inhibition, the LOOH concentrations in the gastrointestinal tract of the control and the propolis groups were compared. A significant difference was observed after 8 weeks in the large intestine, where a significant difference in the ASC concentration was observed. These observations indicate that the oxidative stress in the large intestine is attenuated by propolis, although it is not clear whether the antioxidative effect is brought about intracellularly and/or from outside the cell.

These results show that the antioxidant effect of food factors *in vivo* can be evaluated on the basis of the effect of the factor on ASC, vitamin E and LOOH, namely key molecules as well as indices of oxidative stress. Among them, accurately determined ASC concentration in the tissue is the most sensitive indicator of oxidative stress [105].

As discussed above, specific and sensitive determinations of key molecules are prerequisite to investigate the nature of oxidative stress. In the analysis of macromolecules like DNA, mRNA, or protein, the specificity of their measurements is assured by using biological products such as cDNA or antibody. However the specificity in the quantitation of low molecular weight molecules such as ASC and LOOH is not biologically guaranteed. Therefore reliable method must be developed for the determination of each molecule. For this purpose, chemical conversion and HPLC are very effective. Recently LC-MS/MS has become a powerful tool for the determination of bioactive substances in an extremely low content like ceramides [204]. These chemical analyses with high specificity and sensitivity will allow us more deep insight into biological phenomena, *i.e.* networks of a great many molecules.

#### ABBREVIATIONS

AAPH	=	2,2'-Azobis(2-amidinopropane) dihydrochloride
Ac-DEVD-MCA	=	Acetyl-Asp-Glu-Val-Asp- $\alpha$ -(4-methylcoumaryl-7-amide)
apoB	=	Apolipoprotein B-100
ASC	=	L-Ascorbic acid

d	=	Day(s)	[10]	El-Saadani, M.; Esterbauer, H.; El-Sayed, M.; Goher, M.; A.Y. Nassar. <i>A.Y.</i> ; Juergens, G. <i>J. Lipid Res.</i> , <b>1989</b> , <i>30</i> , 627.
DCFH-DA	=	2',7'-Dichlorodihydrofluorescein diacetate	[11]	Cramer, G.L.; Miller, Jr. J.F.; Pendleton, R.B.; Lands, W.E.M. <i>Anal. Biochem.</i> , <b>1991</b> , <i>193</i> , 204.
DHA	=	L-Dehydroascorbic acid	[12]	Yamamoto, Y.; Brodsky, M.H.; Baker, J.C.; Ames, B.N. <i>Anal. Biochem.</i> , <b>1987</b> , <i>160</i> , 7.
2,3-DKG	=	2,3-Diketogulonic acid	[13]	Miyazawa, T.; Yasuda, K.; Fujimoto, K.; Kaneda, T. <i>J. Biochem. (Tokyo)</i> , <b>1988</b> , <i>103</i> , 744.
DMB	=	1,2-Diamino-4,5-methylenedioxy-benzene dihydrochloride	[14]	Yamamoto, Y.; Frei B.; Ames, B.N. <i>Methods Enzymol.</i> , <b>1990</b> , <i>186</i> , 371.
EC	=	(-)-Epicatechin	[15]	Meguro, H.; Akasaka, K.; Ohru, H. <i>Methods Enzymol.</i> , <b>1990</b> , <i>186</i> , 157.
EGC	=	(-)-Epigallocatechin	[16]	Akasaka, K.; Suzuki, T.; Ohru, H.; Meguro, H. <i>Anal. Lett.</i> , <b>1987</b> , <i>20</i> , 731.
ECg	=	(-)-Epicatechin gallate	[17]	Tokumaru, S.; Tsukamoto, I.; Iguchi, H.; Kojo, S. <i>Anal. Chim. Acta</i> , <b>1995</b> , <i>307</i> , 97.
EGCg	=	(-)-Epigallocatechin gallate	[18]	Mizushima, Y.; Harauchi, T.; Yoshizaki, T.; Makino, S. <i>Experientia</i> , <b>1984</b> , <i>40</i> , 359.
Fe-NTA	=	Ferric nitrilotriacetate	[19]	Kawai, T.; Nishikimi, M.; Ozawa, T.; Yagi, K. <i>J. Biol. Chem.</i> , <b>1992</b> , <i>267</i> , 21973.
GOT	=	Glutamate-oxaloacetate transaminase	[20]	Frei, B.; England, L.; Ames, B.N. <i>Proc. Natl. Acad. Sci. USA</i> , <b>1989</b> , <i>86</i> , 6377.
GSH	=	Glutathione	[21]	Tokumaru, S.; Takeshita, S.; Nakata, R.; Tsukamoto, I.; Kojo, S. <i>J. Agric. Food Chem.</i> , <b>1996</b> , <i>44</i> , 2748.
GSSG	=	Oxidized GSH	[22]	Welch, R.W.; Bergsten, P.; Butler, J.D.; Levine, M. <i>Biochem. J.</i> , <b>1993</b> , <i>294</i> , 505.
GPX	=	Glutathione peroxidase	[23]	Bergsten, P.; Yu, R. Kehrl, J.; Levine, M. <i>Arch. Biochem. Biophys.</i> , <b>1995</b> , <i>317</i> , 208.
h	=	Hour(s)	[24]	Meister, A. <i>Biochem. Pharmacol.</i> , <b>1992</b> , <i>44</i> , 1905.
HDL	=	High density lipoprotein	[25]	Martensson, J.; Meister, A. <i>Proc. Natl. Acad. Sci. USA</i> , <b>1992</b> , <i>89</i> , 11566.
IMT	=	Intima-media thickness of the carotid artery	[26]	Harris, E.D. <i>FASEB J.</i> , <b>1992</b> , <i>6</i> , 2675.
i.p	=	Intraperitoneal	[27]	Cowan, D.B.; Weisel, R.D.; Williams, W.G., Mickle, D.A.G. <i>J. Biol. Chem.</i> , <b>1993</b> , <i>268</i> , 26904.
LDL	=	Low density lipoprotein	[28]	Ji, L.; Fu, R. <i>J. Appl. Physiol.</i> , <b>1992</b> , <i>72</i> , 549.
LOOH	=	Lipid hydroperoxide	[29]	Hurst, R.; Bao, Y.; Ridley, S.; Williamson, G. <i>Biochem. J.</i> , <b>1999</b> , <i>338</i> , 723.
NDPP	=	1-Naphthylidiphenylphosphine	[30]	Mashima, R.; Yamamoto, Y.; Yoshimura, S. <i>J. Lipid Res.</i> , <b>1998</b> , <i>39</i> , 1133.
NDPPO	=	1-Naphthylidiphenylphosphine oxide	[31]	Aviram, M.; Hardak, E.; Vaya, J.; Mahmood, S.; Milo, S.; Hoffman, A.; Billicke, S.; Draganov, D.; Rosenblat, M. <i>Circulation</i> , <b>2000</b> , <i>101</i> , 2510.
ODS	=	Osteogenic Disorder Shionogi	[32]	Mukai, K.; Nishimura, M.; Kikuchi, S. <i>J. Biol. Chem.</i> , <b>1991</b> , <i>266</i> , 274.
oxLDL	=	Oxidatively modified LDL	[33]	Packer, J.E.; Slater, T.F.; Willson, R.L. <i>Nature</i> , <b>1979</b> , <i>278</i> , 737.
PBS	=	Phosphate-buffered saline	[34]	Burton, G.W.; Wronska, U.; Stone, L.; Foster, D.O.; Ingold, K.U. <i>Lipids</i> , <b>1990</b> , <i>25</i> , 199.
ROS	=	Reactive oxygen species	[35]	Smith, D.; Shang, F.; Nowell, T.R.; Asmundsson, G.; Perrone, G.; Dallal, G.; Scott, L.; Kelliher, M.; Gindelsky, B.; Taylor, A. <i>J. Nutr.</i> , <b>1999</b> , <i>129</i> , 1229.
SAM	=	Senescence-accelerated mouse	[36]	Chen, L.H.; Lee, M.S.; Hsing, W.F.; Chen, S.H. <i>Int. J. Vit. Nutr. Res.</i> , <b>1980</b> , <i>50</i> , 156.
SAMP	=	Accelerated senescence-prone mice	[37]	Chen, L.H. <i>Am. J. Clin. Nutr.</i> , <b>1981</b> , <i>34</i> , 1036.
SAMR	=	Accelerated senescence-resistant mice	[38]	Chen, L.H.; Thacker, R.R. <i>Biotech. Appl. Biochem.</i> , <b>1986</b> , <i>8</i> , 40.
SOD	=	Superoxide dismutase	[39]	Chen, L.H.; Thacker, R.R. <i>Int. J. Vit. Nutr. Res.</i> , <b>1987</b> , <i>57</i> , 385.
TBARS	=	Thiobarbituric acid-reactive substances	[40]	Ginter, E.; Kosinova, A.; Hudecova, A.; Mlynarcikova, U. <i>J. Nutr.</i> , <b>1984</b> , <i>114</i> , 485.
Toc·	=	Tocopheryl radical	[41]	Hruba, F.; Novakova, V.; Ginter, E. <i>Experientia</i> , <b>1982</b> , <i>38</i> , 1454.
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