Effect of Ingestion of Excess Methionine Diet on Aging of Erythrocytes in Mice

Puming He and Kyoden Yasumoto

Research Institute for Food Science, Kyoto University, Gokasho, Uji, Kyoto 611, Japan

(Received September 17, 1991)

Summary Accelerated senescence prone mice and resistant mice (SAM-P/1 and SAM-R/1) were fed 10% casein diet or that supplemented with 2.5% methionine (Met) diet for 4 weeks. Erythrocytes withdrawn from animals of each group were separated by density gradient centrifugation into four fractions of different cell density, that is, different age of erythrocytes, and were analyzed for the activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and the level of oxidized protein. Excess Met decreased the specific activity of GSH-Px in each fraction and the whole population of erythrocytes in SAM-P/1 but not in SAM-R/1. The changes in SOD activities were least significant among the erythrocyte fractions of every dietary group. The oxidized protein level in cell lysate increased with the increase of cell density in both strains of animals. These results were interpreted as indicating that excess Met had a positive effect on the erythrocytes senescence, and suggested that the activity of GSH-Px may be a useful enzymatic marker for the age of erythrocytes.

Key Words aging (senescence), glutathione peroxidase, oxidized protein, excess methionine, oxidative reaction, erythrocytes, senescence accelerated mice

Many studies of rodents have revealed the effects of excess methionine (Met) on the control of feed intake (1, 2), the tissues alternations (3, 4), and methyl group metabolism (5, 6). The most notable adverse effects of excess Met in low-protein diets (10% casein) are marked suppression in voluntary food intake and near-cessation of growth (1, 2). In addition excess Met exhibits morphological alterations of the kidney and liver and most notably hypertrophy of spleen, caused by an extensive deposition of hemosiderin (2). These changes have been postulated to be closely associated with the metabolism of the methyl group of Met (4).

Excess dietary Met has been shown to accelerate the turnover of hemoglobin by increasing the turnover and destruction of erythrocytes (7). This increase is
likely to explain the splenic enlargement. Histological examination of rats fed excess Met for 2 years shows an advanced stage of vascular aging (8); aortas from these rats exhibited thicker intima and media due to hypertrophy of smooth muscular cells, and some smooth muscular cells presented degenerative aspects and necrosis. Excess dietary Met, therefore, may accelerate aging process in cell and tissues.

Our previous studies showed that the accelerated aging of mice erythrocytes was partly associated with a defect in cellular oxidant defense mechanism, including glutathione peroxidase (GSH-Px, EC 1.11.1.9) (9). In the present study, we investigated the effects of ingestion of excess Met diet on aging of erythrocytes from senescence accelerated mice.

**MATERIALS AND METHODS**

**Animal and diets.** The senescence accelerated mice (SAM), a murine model of accelerated senescence, was developed by Takeda and his coworkers at the Chest Disease Research Institute, Kyoto University (10). Mating pairs of SAM-P/1 (prone to accelerated senescence) and SAM-R/1 (resistant) were obtained as a generous gift from Takeda and mated to establish the breeding colonies. Mice were maintained in an animal room with a 12-h light-dark cycle at 23±1°C. The breeders and postweanling offsprings were fed a commercial diet (CE-2, Nihon CLEA), which contained 0.25 ppm selenium on fluorometric analysis by the method of Watkinson (11). At 7 weeks of age, mice were housed in groups of 4 to 6 per cage and given the experimental diets for 4 wk: 10% casein diet (10C) and 10% casein diet supplemented with 2.5% Met (10C2.5M) (Table 1). These diets were shown by analysis to contain 0.13 µg Se/kg. The diets and deionized water were freely accessible throughout the experiment.

**Collection of blood and tissues.** At the end of experimental period, diet was removed at 0900 h, and the animals were anesthetized with ether and killed by decapitation at 1400–1600 h. Trunk blood was collected in a heparinized tube. The collected whole blood was centrifuged at 1,100 × g for 10 min at 4°C. Plasma was withdrawn and stored in a refrigerator maintained at −25°C. The remaining blood cells were washed twice with an equal volume of saline. Following blood collection, liver and heart were removed, washed, weighed and stored, together with plasma, at −25°C until analysis. The hematocrit value (13) was determined for the blood immediately after removed from heart.

**Fractionation of erythrocytes by age.** The washed blood cells were suspended in isotonic phosphate-buffer (KRP buffer; 5 mM KCl, 1 mM MgSO4·7H2O, 1 mM CaCl2·2H2O and 127 mM NaCl in 10 mM phosphate buffer, pH 7.4). Leucocytes and platelets were removed by passing the suspension of washed blood cells through a column composed of α-cellulose and microcrystalline cellulose according to the method of Beutler et al. (14). The erythrocytes were collected by centrifugation at 1,100 × g for 10 min, and fractionated by cell density, which correlates with cell age.
Table 1. Composition of diets (%).

| Ingredients         | 10C | 10C2.5M |
|---------------------|-----|---------|
| Casein              | 10  | 10      |
| DL-Methionine       | 0   | 2.5     |
| Sucrose             | 30.12 | 29.12  |
| α-Corn starch       | 45.18 | 43.68  |
| Corn oil            | 5   | 5       |
| Cellulose           | 5   | 5       |
| Mineral mixture*    | 3.5 | 3.5     |
| Vitamin mixture*    | 1   | 1       |
| Choline bitartrate  | 0.2 | 0.2     |

*The composition based on AIN-76™ (12).

(15), by the modified method of Vottore et al. (1980). Briefly, 0.4 ml of the packed erythrocytes was suspended in 8 ml of density gradient mixture, Percoll/Angio-grafin-65/water 2.8:1.6:3.6 (v/v/v), and the suspension was placed in a round-bottomed centrifuge tube and centrifuged at 30,000 × g for 15 min at 4°C in an angle-rotor (Hitachi SCR20B centrifuge with RPR18-342 rotor). The generated density gradient was monitored using a mixture of colored density marker beads (Pharmacia Fine Chemicals). After centrifugation, the separated erythrocytes were recovered into 4 fractions of an equal volume by aspiration from the bottom to the top of tube using a density gradient fractionator (Hitachi DGF). The first fraction contained the erythrocytes with the density range of 1.108 to 1.121 g/ml. This fraction was referred to as F-1, and likewise, the remaining 3 fractions with the density ranges of 1.095 to 1.108, 1.073 to 1.095, and 1.062 to 1.073 as F-2, F-3, and F-4, respectively. Each fraction was washed with 5 volumes of saline to remove the gradient materials. This manipulation was repeated 3 times. The final packed cells from each fraction were resuspended in EGTA buffer (137.0 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄, 0.6 mM MgSO₄, 1.1 mM EGTA and 1.0 mM HEPES, pH 7.4) containing the protease inhibitors, leupeptin (500 ng/ml), phenylmethylsulfonyl fluoride (PMSF, 40,000 ng/ml), and pepstatin (700 ng/ml) to produce 1.0 ml final suspension. The erythrocytes were lysed by sonication in an internal ultrasonic cytosredder (UCD-200T, COSMO BIO Co., Ltd.). The lysates were kept at 4°C until analysis.

Determination of hemoglobin concentration and plasma protein. Hemoglobin concentration of the cell lysates was measured by conversion to cyanmethemoglobin using commercial kit (Wako Pure Chemical Ind.). Amount of cyanmethemoglobin was calculated from its absorption maximum at 540 nm and used in reporting the erythrocytes subpopulations, the level of oxidized proteins, and the enzyme activities. The plasma protein was determined as described by Lowry et al. (16).

Assay of enzyme activities. GSH-Px was assayed at 37°C by the coupled enzyme procedure of Paglia and Valentine (17) with the modification of using...
tert-butyl hydroperoxide as a substrate. One unit of GSH-Px was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH per min under the assay condition.

Superoxide dismutase (SOD) activity was determined in terms of its ability to prevent the reduction of cytochrome c by an enzymatic source of O₂⁻ (18). Results are reported in units, where 1 unit represents the amount of SOD required to inhibit by half the rate of cytochrome c reduction under the conditions described above.

**Determination of oxidized protein.** The oxidized protein concentration was determined according to the method of Starke et al. (19). The cell lysate containing about 1 mg hemoglobin was treated with 0.2% (wt/vol) dinitrophenylhydrazine (DNPH) in 2 M HCl. The concentration of DNPH incorporated into oxidized protein was calculated from the absorbance difference at 370 nm using an average value of 21.0 mM⁻¹ cm⁻¹ for the millimolar absorbance of aliphatic DNPH derivatives (20). The results were expressed as nanomols of DNPH incorporated per mg of hemoglobin.

**Chemicals.** All chemicals used were of reagent grade or better unless otherwise noted and were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Nacalai Tesque Co., Ltd. (Kyoto, Japan), or Wako Pure Chemical Ind. (Osaka, Japan).

**Statistics.** Data were reported as mean ± SEM (standard error of mean) and statistical analysis was performed by analysis of variance (ANOVA) and by Duncan's multiple range test. Data were considered significantly different when p < 0.05.

**RESULTS**

**Distribution of erythrocytes in density gradient**

The erythrocytes were separated over the density range of 1.062 to 1.121 after centrifugation. This distribution of the cells was divided into 4 fractions of decreasing density.

Distribution of erythrocytes in the density fractions varied with the strains and dietary regimens (Table 2). The excess methionine affected the distribution of density fractions in a similar, but not identical, way for both strains of mice: the erythrocytes from both strains fed 10C2.5M diet contained less F-1 (the oldest fraction) and more F-4 (the youngest fraction) than those from the mice fed 10C diet. When the mice were fed 10C diet, the distribution of density fractions was indistinguishable between the two strains, but when they were fed 10C2.5M diet, SAM-P/1 mice contained more of F-3 and less of F-1 than SAM-R/1 mice did. Additionally, the methionine excess feeding significantly lowered the hematocrit value in SAM-P/1 (Table 2).

**Antioxidative enzyme activities of erythrocytes**

The specific activity of GSH-Px significantly decreased, but that of SOD
Table 2. Percentage of hemoglobin concentration with erythrocyte obtained from each group by density and hematocrit values.* **

| Fractions of decreasing density | SAM-R/1  | SAM-P/1  |
|---------------------------------|----------|----------|
|                                 | 10C      | 10C2.5M  |
| F-1                             | 22.5±2.77<sup>ab</sup> | 10.5±2.54<sup>c</sup> | 25.1±1.58<sup>a</sup> | 16.0±2.27<sup>bc</sup> |
| F-2                             | 16.5±2.19<sup>a</sup> | 6.1±1.33<sup>b</sup> | 13.4±1.03<sup>a</sup> | 14.4±1.81<sup>a</sup> |
| F-3                             | 43.9±1.81 | 45.2±1.2 | 41.2±2.21 | 45.2±4.54 |
| F-4                             | 17.2±3.27<sup>b</sup> | 38.2±4.69<sup>a</sup> | 20.3±2.42<sup>b</sup> | 24.5±3.43<sup>b</sup> |

Hemoglobin concentrations (%)

| Hematocrit values (%)          | 37.0±0.82<sup>ab</sup> | 38.3±0.33<sup>a</sup> | 36.5±1.07<sup>ab</sup> | 35.2±0.58<sup>b</sup> |

* Mice erythrocytes were fractionated by density. Hemoglobin concentration in the separated fractions and hematocrit values were determined as described under Materials and Methods. ** The values are means±SEM from 5 mice in each group. Values within a row not sharing the same superscript are significantly different at p<0.05.

Fig. 1. Changes in specific activity of glutathione peroxidase in the erythrocytes of SAM-P/1 (circles) and SAM-R/1 (squares) fed 10% casein diet (10C, open symbols) and 10% casein diet supplemented with 2.5% methionine (10C2.5M, filled symbols). The erythrocytes were fractionated by density gradient centrifugation, and the enzyme activity was determined as described in Materials and Methods. The numbers 1, 2, 3 and 4 of abscissa represent the fractions F-1, F-2, F-3 and F-4 respectively. Each symbol and its associated vertical bar represent mean±SEM (n=5 and 6 for SAM-P/1 and SAM-R/1, respectively). The asterisk indicates significant difference from SAM-R/1 within the same density fractions and under the same dietary condition, and the star indicates significant difference from 10C group within the same density fractions and from the same strain of mice.
Fig. 2. Changes in specific activity of superoxide dismutase in the erythrocytes of SAM-P/1 (circles) and SAM-R/1 (squares) fed 10% casein diet (10C, open symbols) and 10% casein diet supplemented with 2.5% methionine (10C2.5M, filled symbols). The symbol denotes as described in Fig. 1. Number of mice were 5 in the group of 10C, and 7 in other groups.

Fig. 3. Changes in oxidized protein levels in the erythrocytes of SAM-P/1 (circles) and SAM-R/1 (squares) fed 10% casein diet (10C, open symbols) and 10% casein diet supplemented with 2.5% methionine (10C2.5M, filled symbols). The symbol denotes as described in Fig. 1. Number of mice were 5, 6, and 7 in the groups of 10C, 10C2.5M, and the other 2 groups, respectively.

Tended to decrease, with the increase of cell density in both strains of mice on 10C diet (Figs. 1 and 2). Excess methionine decreased the specific activity of GSH-Px in each fraction from SAM-P/1 but not in the corresponding fractions from SAM-R/1 (Fig. 1). The changes in SOD activities were least significant among the erythrocyte fractions of every dietary group, except that SAM-P/1 fed 10C2.5M diet showed higher specific activity in F-3 than in F-1.

*J. Nutr. Sci. Vitaminol.*
ERYTHROCYTES AGING BY EXCESS METHIONINE

Table 3. The oxidized protein concentration in plasma and liver.* **

|                | SAM-R/1            | SAM-P/1            |
|----------------|--------------------|--------------------|
|                | 10C    | 10C2.5M | 10C    | 10C2.5M |
| Plasma         | 1.05±0.08 | 1.17±0.06 | 1.05±0.07 | 1.23±0.11 |
| Liver          | 2.30±0.21* | 3.14±0.15b | 2.67±0.21ab | 2.96±0.22b |
| ANOVA (p)      |         |          |         |          |
| Plasma         | NS**** | NS      | NS      | NS      |
| Liver          | NS     | <0.01   | NS      | NS      |

* Levels of oxidized protein in mice plasma and liver were determined by reactivity with 2,4-dinitrophenylhydrazine (DNPH) as described under MATERIALS AND METHODS. ** The values are means±SEM from 5 mice for the group of SAM-R/1 fed 10C diet and 7 mice for the other groups. Values within a row not sharing the same superscript are significantly different at p<0.05. *** NS = not significantly different at p<0.05.

Oxidized protein level

The oxidized protein level in cell lysates increased with the increase of cell density in both strains of animals (Fig. 3). When the oxidized protein levels were compared between the fractions of same cell density, excess methionine had little effect for SAM-R/1 (except for F-4) but produced a significant increase in all the fractions from SAM-P/1. When comparison was made in terms of the whole cell populations, feeding excess methionine resulted in a significant elevation of the oxidized protein level in SAM-P/1 but not in SAM-R/1.

The oxidized protein levels in plasma and liver are shown in Table 3. No significant difference was observed between the control and the excess methionine groups and between two mice strains.

DISCUSSION

In agreement with our previous finding (9), the results of this study showed that the distribution of erythrocytes in density gradient was hardly different between SAM-P/1 and SAM-R/1 when they were fed 10C diet (Table 2). Because the population of erythrocytes is made up by the cells of heterogeneous density, and as the cell density is demonstrated to correlate with the age of circulating erythrocytes (15), the distribution pattern observed here can be interpreted as indicating that the erythrocytes from SAM-P/1 and SAM-R/1 are composed in a similar ratio of the different physiological age cells.

The results presented in this study demonstrate that the excess methionine accelerated aging of the circulating erythrocytes in both strains of mice. Consumption of excess methionine significantly increased the subpopulation of young
erythrocytes and decreased the aged cells in SAM-P/1 and SAM-R/1 (Table 2). This is consistent with the results of earlier studies and indicates that feeding of excessive level of methionine increases the turnover of erythrocytes (7), and thus explains why the rodents fed excess methionine had enlarged spleen (2), accelerated hematopoiesis, and thus increased proportion of young erythrocytes in the circulation as observed here.

However, with SAM-P/1 mice, feeding excess methionine led to a mild condition compared with SAM-R/1, that is, SAM-P/1 fed excess methionine showed low hematocrit but insignificant increment of younger erythrocytes. It was supposed that the hematogenic function of SAM-P/1 mice was disordered and the adaptability to excess methionine dietary regimen decreased.

In the circulating erythrocytes, a progressive molecular aging of the nonrenewable enzyme proteins probably led to a decrease of erythrocytes life-span (21). For both strains of mice, the activity of GSH-Px, one of the important antioxidative enzymes, significantly decreased with the increase of cell density of erythrocytes, that is, with the increase in erythrocytes age (Fig. 1). This suggests that the aged erythrocytes are highly susceptible to the danger of oxidative damage. In agreement with the previous observation (9), the density (age)-associated decrease in the activity of GSH-Px was more significant in SAM-P/1 than in SAM-R/1. This decrease is consistent with the previous observation that the activities of age-marker enzymes of erythrocytes, hexokinase and pyruvate kinase, were higher in the cells of SAM-P/1 than those of SAM-R/1 (9).

A metabolic interaction between selenium and sulfur has been well established (22–24). Dietary supplementation of methionine increases GSH-Px activity in the erythrocytes of humans with low selenium status (25), and in liver, heart and plasma of rats (22). However, this concept of metabolic interaction between selenium and sulfur is not tenable with the results described in this paper for the effects of excess methionine on GSH-Px. The methionine level was far beyond the supplementation levels used in those studies. Besides, selenium contained in the casein-based diets used in the present study, 0.13 μg Se/kg, appears mostly to differ from selenomethionine. Previous work from our laboratory suggested that selenite and selenocysteine are the two major chemical forms of selenium contained in milk casein (26).

The present studies showed that feeding excess methionine impaired the antioxidant defense mechanism in the erythrocytes of SAM-P/1 but less significantly in the counterpart cells. A significant decrease in the specific activity of GSH-Px was observed in each density fraction (Fig. 1). On the other hand, we could not detect any statistically significant change in the specific activity of SOD, another important antioxidative defense enzyme (Fig. 2). The results can be interpreted as suggesting that the activity level of GSH-Px, unlike that of SOD, is a factor affecting the aging process and the toxicity of excess methionine in the diet.

The changes in these two enzyme activities were not parallel during the aging process of the mice erythrocytes. This disparity can be interpreted as suggesting...
that independent mechanisms are involved in the regulation of the two enzymes. Support is lent to this interpretation by the variations in age-related changes observed for those two enzymes in rat liver (27) and brain (28). In rat liver, expression of SOD decreased gradually with age while expression of GSH-Px changed insignificantly. In developing rat brain, SOD activity increases gradually with age while GSH-x activity is constant from birth.

The erythrocytes collected from the mice fed excess methionine appear to suffer from defect in the cellular antioxidation defense system. In order to assess much appropriately the extent of this defect, we measured the level of oxidized protein by the DNPH incorporation method of Oliver et al. (29). Feeding of excess methionine increased the level of oxidized protein not only in the erythrocytes (Fig. 3) but also in liver of the animals (Table 3). The oxidized protein is a group of proteins oxidatively modified by active oxygen species, such as $\text{O}_2^-$, $\text{H}_2\text{O}_2$, $\text{OH}^-$ and singlet oxygen, which the cells inevitably encounter throughout their life (30). These active forms of oxygen are generated in the mixed-function oxidation systems (31), through the oxygen metabolism, or during the course of detoxication of xenobiotics and excess nutrients. It thus appears, although there is no direct supporting evidence for this inference, that excess methionine increases the production of active oxygen species.

In harmony with our previous observation (9), the level of oxidized protein increased with the increase of cell density (i.e., the increase in age of erythrocytes), and was higher in the fractions from SAM-P/1 than in the corresponding fractions from SAM-R/1 (Fig. 3). The change paralleled the change in GSH-Px activity. The cellular enzymatic protective mechanism against oxidative damage may be low in the aged erythrocytes, so that their oxidative damage could be higher than that of young erythrocytes. The present results of oxidized protein levels support this inference.

It is noteworthy that excess methionine increased the oxidized protein levels in almost all the fractions of erythrocytes from both strains of mice. Similar increase was observed in the level of oxidized proteins of the aged erythrocytes fractions.

However, of the two mouse strains employed in the present investigation, SAM-P/1 can be considered to be much more vulnerable than the counterpart to the oxidative stress. As shown in Table 4, two-way ANOVA of effects of excess methionine on the activities of GSH-Px and SOD and on the levels of oxidized protein in the erythrocytes showed a significant interaction with the mouse strains. This interaction implies that the senescence process is dependent on the dietary conditions in SAM-P/1 much more than in SAM-R/1.

The oxidized protein level in plasma and liver tended to be higher in the mice fed excess methionine diet than in those animals fed the control casein diet, but the difference bore no statistical significance. It was supported that the erythrocytes were markedly susceptible to the oxidative damage rather than plasma and liver.

Excess methionine had positive effect on accelerating senescence of erythrocytes. This finding invites speculation that decrease in the cellular enzymatic
protection mechanisms against oxidative damages leads to an increase in the level of oxidized protein in the aged erythrocytes and in most fractions of erythrocytes from the mice fed excess methionine. The speculation is consistent with the commonly enjoyed hypothesis that oxidative damages promote the process of aging, and thus with the hypothesis presented by Harman that the aging proceeds with the destructive actions of free radicals (32).

Support is lent to the hypotheses raised regarding the observed changes in age subpopulations and the cellular impairment by the observed changes in GSH-Px activity and levels of oxidized proteins. It may be worth noting that the activity of GSH-Px is a marker enzyme for the age of erythrocytes, and that feeding excess methionine accelerates the senescence of the erythrocytes, that is, the senescence process is associated to a certain degree with the dietary levels of methionine.

This research was supported by a Grant-in-Aid 01480061 from the Ministry of Education, Science and Culture of Japan. The authors are grateful to Professor T. Takeda for his kindly supplying the senescence accelerated mice.

REFERENCES

1) Harper, A. E., Benevenga, N. J., and Wohlhueter, R. M. (1970): Effects of ingestion of disproportionate amounts of amino acids. *Physiol. Rev.*, **50**, 428–557.
2) Benevenga, N. J., Yeh, M.-H., and Lalich, J. J. (1976): Growth depression and tissue reaction to the consumption of excess dietary methionine and S-methyl-L-cysteine. *J. Nutr.*, **106**, 1714–1720.
3) Muramatsu, K., Odagiri, H., Morishita, S., and Takeuchi, H. (1971): Effect of excess levels of individual amino acid on growth of rats fed casein diets. *J. Nutr.*, **101**, 1117–1126.
4) Benevenga, N. J. (1974): Toxicities of methionine and other amino acids. *J. Agric. Food Chem.*, **22**, 2–9.
5) Krebs, H. A., Hems, R., and Tyler, B. (1976): The regulation of folate and methionine metabolism. *Biochem. J.*, **158**, 341–353.
6) Newberne, P. M., and Rogers, A. E. (1986): Labile methyl groups and the promotion of cancer. *Annu. Rev. Nutr.*, **6**, 407–432.
7) Cohen, H. P., and Berg, C. P. (1956): Erythrocyte turnover in rats fed diets high in

*NS = not significantly different at p < 0.05.

|                | GSH-Px   | SOD      | Oxidized protein |
|----------------|----------|----------|------------------|
| Strain         | NS*      | NS       | < 0.001          |
| Diet           | NS       | NS       | < 0.001          |
| Strain \times Diet | < 0.001 | < 0.02   | < 0.002          |
methionine. J. Biol. Chem., 222, 85–88.
8) Fau, D., Peter, J., and Hadjiisky, P. (1988): Effects of ingestion of high protein or excess methionine diets by rats for two years. J. Nutr., 118, 128–133.
9) He, P., and Yasumoto, K. (1990): Age-associated changes in glutathione peroxidase and oxidized protein in erythrocytes of senescence accelerated mice. J. Jpn. Soc. Nutr. Food Sci. (in Japanese), 43, 121–125.
10) Takeda, T., Hosokawa, M., Takeshita, S., Irino, M., Higuchi, K., Matsushita, T., Tomita, Y., Yasuhira, K., Hamamoto, N., Shimizu, K., Ishii, M., and Yamamura, T. (1981): A new murine model of accelerated senescence. Mech. Aging Dev., 17, 183–194.
11) Watkinson, J. H. (1966): Fluorometric determination of selenium in biological material with 2,3-diaminonaphthalene. Anal. Chem., 38, 92–97.
12) American Institute of Nutrition (1977): Ad Hoc committee on standards for nutritional studies. J. Nutr., 107, 1340–1348.
13) McGovern J. J., Jones, A. R., and Steinberg, A. G. (1955): The hematocrit of capillary blood. New Engl. J. Med., 253, 308–312.
14) Beutler, E., West, C., and Blume, K.-G. (1976): The removal of leukocytes and platelets from whole blood. J. Lab. Clin. Med., 88, 328–333.
15) Borun, E. R., Figueroa, W. G., and Perry, S. M. (1957): The distribution of Fe39 tagged human erythrocytes in centrifuged specimens as a function of cell age. J. Clin. Invest., 36, 676–679.
16) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265–275.
17) Paglia, D. E., and Valentine, W. N. (1967): Studies on the quantitative and qualitative of erythrocytes glutathione peroxidase. J. Lab. Clin. Med., 70, 158–169.
18) Mccord, J. M., and Fridovich, I. (1969): Superoxide dismutase: An enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem., 244, 6049–6055.
19) Starke, P. E., Oliver, C. N., and Stadtmann, E. R. (1987): Modification of hepatic proteins in rats exposed to high oxygen concentration. FASEB J., 1, 36–39.
20) Levine, R. L. (1984): Mixed-function oxidation of histidine residues. Methods Enzymol., 107, 370–376.
21) Seaman, C., Wyss, S., and Piomelli, S. (1980): The decline in energetic metabolism with aging of erythrocyte and its relationship to cell death. Am. J. Hematol., 8, 31–42.
22) Waschulewski, I. H., and Sunde, R. A. (1988): Effect of dietary methionine on tissue selenium and glutathione peroxidase (EC 1.11.1.9) activity in rats given selenomethionine. Br. J. Nutr., 60, 57–68.
23) Waschulewski, I. H., and Sunde, R. A. (1988): Effect of dietary methionine on utilization of tissue selenium from dietary selenomethionine for glutathione peroxidase in the rat. J. Nutr., 118, 367–374.
24) Butler, J. A., Beilstein, M. A., and Whanger, P. D. (1989): Influence of dietary methionine on the metabolism of selenomethionine in rats. J. Nutr., 119, 1001–1009.
25) Luo, X., Wei, H., Yang, C., Xing, J., Liu, X., Qiao, C., Feng, Y., Liu, J., Liu, Y., Wu, Q., Liu, X., Guo, J., Stoecker, B. J., Spallholz, J. E., and Yang, S. P. (1987): Bioavailability of selenium to residents in a low-selenium area of China, in Selenium in Biology and Medicine, ed. by Combs, G. J., Jr., Spallholz, J. E., Levander, O. A., and Oldfield, J. E., AVI books, New York, Part A, pp. 436–444.

Vol. 38, No. 1, 1992
26) Yasumoto, K., and Yoshida, M. (1988): Food chemistry of selenium. *Nippon Nōgeikagaku Kaishi*, 62, 1090–1093 (in Japanese).

27) Rao, G., Xia, E., Nadakavukaren, M. J., and Richardson, A. (1990): Effect of dietary restriction on the age-dependent changes in the expression of antioxidant enzymes in rat liver. *J. Nutr.*, 120, 602–609.

28) Mavelli, I., Rigo, A., Federico, R., Ciriolo, M. R., and Rotilio, G. (1982): Superoxide dismutase, glutathione peroxidase and catalase in developing rat brain. *Biochem. J.*, 204, 535–540.

29) Oliver, C. N., Levine, R. L., and Stadtman, E. R. (1987): A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging. *J. Am. Geriatr. Soc.*, 35, 947–956.

30) Fisher, A. B. (1988): Intracellular production of oxygen-derived free radicals, in Oxygen Radicals and Tissue Injury (Proceedings of an Upjohn Symposium), ed. by Halliwell, B., Upjohn, Bethesda, U.S.A., pp. 34–39.

31) Fucci, L., Oliver, C. N., Coon, M. J., and Stadtman, E. R. (1983): Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing. *Proc. Natl. Acad. Sci. USA*, 80, 1521–1525.

32) Harman, D. (1981): The aging process. *Proc. Natl. Acad. Sci. USA*, 78, 7124–7128.