*Free Rad. Res.*, Vol. 21, No. 2, pp. 109-118
Reprints available directly from the publisher
Photocopying permitted by license only



# EFFECT OF DIETARY VITAMIN C AND CATALASE INHIBITION ON ANTIOXIDANTS AND MOLECULAR MARKERS OF OXIDATIVE DAMAGE IN GUINEA PIGS

S. CADENAS, C. ROJAS, R. PÉREZ-CAMPO, M. LÓPEZ-TORRES and G. BARJA

*Department of Animal Biology II (Animal Physiology), Faculty of Biology, Complutense University, Madrid 28040, Spain*

*(Received January 4th 1994; in revised form February 18th, 1994)*

Guinea pigs were fed for five weeks with two diets with different levels of vitamin C, low (33 mg of Vit C/Kg diet) and high (13,200 mg of Vit C/Kg of diet). Catalase was inhibited with 3-amino-1,2,4-triazole (AT) in half of the animals from each dietary group. AT caused an almost complete depletion of liver catalase activity (90%) in both dietary groups. Vitamin C supplementation increased total glutathione peroxidase activity and tissue vitamin C level and decreased levels of protein carbonyls and malondialdehyde (MDA) in both treated and non-treated animals. This vitamin C supplementation did not change any of the other antioxidant defences studied. Our results show that dietary vitamin C supplementation increases global antioxidant capacity and decreases endogenous oxidative damage in the guinea pig liver under normal non-stressful conditions. This supports the protective value of dietary antioxidant supplementation.

KEY WORDS: Vitamin C, 3-amino-1,2,4-triazole, antioxidants, peroxidation, guinea pig, diet.

## INTRODUCTION

Cellular respiration leads in part to the generation of oxygen free radicals which are known to produce oxidative damage to biological macromolecules including proteins, lipids and DNA. Even at resting respiratory conditions some oxidative damage is always present in the cell, oxidative damage that will be increased under certain physiological or pathological situations. Several studies suggest the involvement of oxidative damage in the appearance of many degenerative diseases such as cancer[1], arteriosclerosis, cardiovascular problems[2] and the aging process[3-6]. Thus, enhancing tissue antioxidant capacity will reduce oxidative damage and may protect from the development of these degenerative diseases. In fact, there is increasing evidence that antioxidants like vitamin C decrease the incidence of cancer[7-8] and cardiovascular diseases[9].

In order to increase antioxidant capacity safely and easily, diet controlled antioxidants such as vitamin C or E may be the best choice. Supplementation with antioxidant enzymes or GSH (which is under feed-back cellular control) presents several problems and does not seem to be suitable.

Address for correspondence: Dr. G. Barja. Departamento de Biología Animal II (Fisiología Animal), Facultad de Biología, Universidad Complutense, Madrid 28040, Spain. TEL: 34-1-3944988. FAX: 34-1-3944935.

Taking all this into account we have studied the effects of two levels (high and low) of vitamin C supplementation in the diet on protein and lipid oxidation, and enzymatic and non-enzymatic antioxidants, in the guinea pig liver. We have performed this comprehensive study under basal aerobic conditions and we have also included animals exposed to an additional oxidative stress, using for that purpose 3-amino-1,2,4-triazole (AT) a very convenient catalase inhibitor since it can lead to an almost complete depletion of tissue catalase (CAT). In the guinea pig this enzyme activity is not only located inside the peroxisome but also in the cytosol and nuclear matrix[10-11]. This, together with the problems of using other type of antioxidant enzyme inhibitors because of the high toxicities they induce at the doses required for inhibition, prompted us to inhibit catalase activity with 3-amino-1,2,4-triazole. Besides, guinea pig is a useful model for these diet-controlled experiments since it cannot (like higher primates) endogenously synthesize ascorbate. On the other hand it has been shown in previous studies[4-6] that cellular endogenous antioxidants are subject to homeostatic control. Exposure to a strong oxidative stress *in vivo* can lead to compensatory inductions of endogenous antioxidants[4-6]. But the contrary could also be possible, addition of high amounts of exogenous antioxidants in the diet could promote a reactive depression of endogenous antioxidants, limiting then the usefulness of diet antioxidant supplementation. This is why in this work we have used animals, chronically feeding diets with low or high vitamin C levels.

## MATERIALS AND METHODS

### *Animals, Diets and Treatments*

Dunkin-Hartley male guinea pigs weighing 200–250 g were obtained from IFFA-CREDDO (Lyon, France). A basal vitamin C-deficient laboratory diet (U.A.R., France) containing 19.5% protein, 3.1% fat, 68% carbohydrates, and 4.4% non-nutritive bulk was used. The content of minerals and vitamins per Kg of diet was: phosphorus, 6,791 mg; calcium, 9,450 mg; potassium, 6,700 mg; sodium, 1,900 mg; magnesium, 2,003 mg; manganese, 90.7 mg; iron, 252 mg; copper, 30.6 mg; zinc, 113.4 mg; cobalt, 1.61 mg; iodine, 0.9 mg; vitamin A, 19,000 I.U.; vitamin $D_3$, 1,500 I.U.; vitamin $D_2$, 1,150 I.U.; thiamine, 18.5 mg; riboflavin, 18 mg; pantothenic acid, 16.5 mg; pyriodoxine, 3.15 mg; dl-$\alpha$-tocopherol, 15 mg; menadione, 2.5 mg; niacin, 190 mg; folic acid, 0.5 mg; biotin, 0.04 mg; choline, 1,600 mg; and vitamin $B_{12}$, 0.02 mg.

The diets with different vitamin C levels were obtained by adding 33 or 13,200 mg of L-ascorbic acid/Kg of basal diet. The animals were fed for five weeks the two experimental diets and they were maintained inside aseptic air positive-pressure animal cabinets (A 130 SP, Flfrance, Cachan, France) equipped with a HEPA air filter (99.999% for particles $>0.3\,\mu$m) at the inlet.

At the end of the dietary regimen the animals were sacrificed by decapitation and liver samples were immediately excised and stored at $-25°C$. Three hours before the sacrifice half of the animals in each dietary group were intraperitoneally injected with 3-amino-1,2,4-triazole (AT, 1 mg/g of total body weight) prepared in physiological saline (0.95% NaCl).

*Enzyme Activities*

Liver samples were homogenized in 50 mM phosphate buffer (pH 7.4). Superoxide dismutase (SOD) was measured after 24 hours of dialysis, quantifying the inhibition of pyrogallol autooxidation at 420 nm[12]. Catalase was assayed following $H_2O_2$ disappearance at 240 nm[13]. Total glutathione peroxidases (GPx), selenium and non-selenium, were determined following NADPH oxidation at 340 nm in the presence of excess glutathione reductase (GR), GSH and cumene hydroperoxide[14]. Glutathione reductase (GR) was assayed following NADPH oxidation at 340 nm in the presence of GSSG[15]. Cytochrome oxidase (COX) was measured following the rate of cytochrome c oxidation at 550 nm[16]. All the enzymatic reactions were performed at 25° C. Protein concentration was determined as described by Lowry *et al.*[17]

*Non-enzymatic Antioxidants*

Liver samples were homogenized in 5% trichloroacetic acid with 0.01 N HCl and total glutathione was measured by the spectrophotometric recycling assay[18] in the presence of 5,5'-dithiobis (2-nitrobenzoic acid), NADPH and GR at 412 nm. GSSG was assayed by the same method after derivatization of GSH with 12.5 mM N-ethylmaleimide (NEM) followed by alkaline hydrolysis of NEM[19]. GSH values were obtained after subtracting GSSG from total glutathione. Samples of liver tissue were homogenized in 50 mM perchloric acid for simultaneous analysis of ascorbic and uric acid by ion pair HPLC with UV detection at 280 nm[20]. $\alpha$-Tocopherol was measured by HPLC[21] with UV detection at 292 nm.

*Protein Oxidation*

Protein carbonyl content was determined spectrophotometrically according to the 2,4-dinitrophenylhydrazine (DNPH) method[22]. Liver samples were homogenized in 100 mM potassium phosphate buffer (pH 7.4) containing 0.1% digitonin, 1 mM EDTA, phenylmethylsulfonyl fluoride (36 $\mu$g/ml), and the protease inhibitors leupeptin (0.5 $\mu$g/ml), pepstatin (0.7 $\mu$g/ml), and apoprotinin (0.5 $\mu$g/ml). After 15 min of incubation the samples were centrifuged (3,000 × g, 10 min) and the DNA was removed with 10% streptomycin (15 min incubation followed by centrifugation). The supernatant was divided into two 1 ml portions and 2.5 ml of 2 M HCl or 2.5 ml of 10 mM DNPH in 2 M HCl were added. Both portions were sequentially precipitated with 4 ml of 20% TCA and 4 ml of 10% TCA. Supernatants were discarded and the pellets were washed three times with 4 ml of ethyl acetate:ethanol (1:1, vol/vol). Pellets were redissolved in 2 ml of 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). Each DNPH sample was scanned from 320 to 410 nm in the spectrophotometer against the HCl corresponding sample and the peak absorbance was used to calculate protein carbonyls (molar extinction coefficient 22,000 $M^{-1}$ $cm^{-1}$). The protein concentration was read at 280 nm in the HCl sample using 6 M guanidine hydrochloride as a blank.

*Lipid Peroxidation*

Free malondialdehyde (MDA) was measured by ion-pair HPLC with UV detection at 267 nm[23] (Gilson115 UV detector) in supernatants of liver samples homogenized

in 0.1 M Tris buffer pH 7.4, immediately diluted (1:1) with acetonitrile to precipitate proteins, centrifuged at 3500 × g, filtered (0.4 μm) and injected (20 μl) in the chromatograph. The mobile phase was 10 mM phosphate (pH 7.4) containing 2.5 mM miristyltrimetylammonium bromide as counterion and 25% (v/v) acetonitrile, pumped (Waters 510) at 0.5 ml/min through a 100 × 4.6 mm Mechery-Nagel nucleosil (7 μm) reverse phase $c_{18}$ column. The MDA standard was prepared from malondialdehyde-bisdimethylacetal (Merck) in the presence of 0.16% (v/v) $H_2SO_4$.

*Statistical Analysis*

Data were analyzed by Two-Way Analysis of Variance (ANOVA). After the ANOVA, the Fisher's least significant difference (LSD) test was used to analyze significance between paired groups when necessary. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

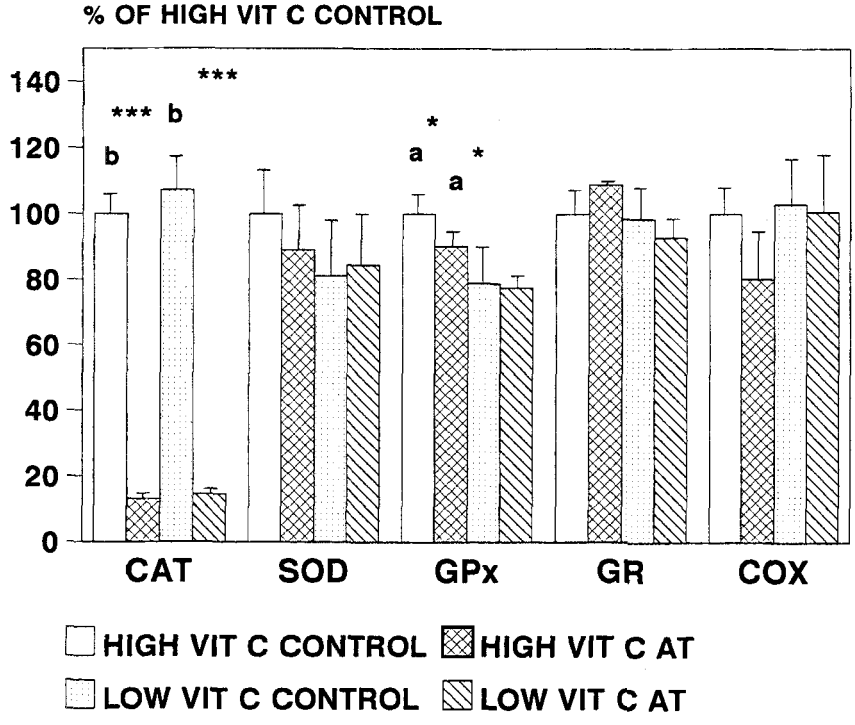


FIGURE 1 Antioxidant enzymes and cytochrome oxidase activity from the liver of guinea pig maintained for five weeks with two diets differing in the vitamin C level (33 mg VitC/Kg diet and 13,200 mg VitC/Kg diet) and treated or not with the CAT inhibitor 3-amino-1,2,4-triazole (AT). Values are represented as % of the activity found in the high Vit C control. Activities in high Vit C controls: 678.38 ± 38.48 (CAT); 11.12 ± 1.47 (SOD); 153.32 ± 9.57 (GPx); 29.09 ± 2.14 (GR); 8.09 ± 0.63 (COX). Data are means ± SEM from 5-7 animals (SOD: U/mg prot; μmol $H_2O_2$-CAT, μmoles cytochrome-COX or nmoles NADPH-GPx and GR-transformed per min per mg prot.). a: significant difference between groups with high and low vitamin C levels in the diet. b: significant difference between control and AT treated animals from the same dietary regimen. *$p < 0.05$; ***$p > 0.001$.

## RESULTS

### *Endogenous Antioxidants*

Aminotriazole treatment was very effective and resulted in almost total depletion (90%) of liver CAT in both dietary groups (Figure 1).

Vitamin C supplementation increased total GPx activity in control and AT treated animals (Figure 1). Liver ascorbate level was also significantly increased in the high vitamin C diet group (Table 1).

The dietary vitamin C level did not change any other antioxidant studied including SOD, CAT, GR and COX activities; vitamin E, GSH, GSSG and uric acid (Figure 1; Table 1).

### *Lipid and Protein Oxidation*

Vitamin C supplementation decreased endogenous MDA levels (measured by HPLC); they were smaller (66–78% decrease) in the group that ingested 13,200 mg vit C/Kg diet in both control and AT treated animals. Levels of protein carbonyls were also significantly decreased (57–22% decrease) in the group with a high vitamin C diet (Figure 2) in both cases (control and AT treated animals).

## DISCUSSION

In the present study we have used two different levels of vitamin C in the diet. Guinea pig must receive 0.5 mg of vitamin C/day to avoid the development of scurvy[24]. Animals in the low vitamin C group were receiving 33 mg of ascorbate/Kg diet; i.e. they were ingesting 0.54–1.1 mg of ascorbate per day. So, they can be considered non-scorbutic animals. The high vitamin C group was receiving 13,200 mg ascorbate/Kg diet. This group was designed to study if supplementing the diet with levels of vitamin C much higher (20 times) than those present in standard comercial diets has beneficial or detrimental effects on oxidation of macromolecules and levels of antioxidants in the liver.

Supplementation with vitamin C was very effective since ascorbic acid tissue levels were about 50–165 times greater in the high vitamin C group than in the low vitamin C group. On the other hand the treatment with AT was also very effective since it resulted in almost total depletion (90%) of liver catalase in both dietary groups. Similar degrees of CAT inactivation by analogous doses of AT *in vivo* to those used in this study have been previously described in guinea pig[10] and other species[25-30] and also in CAT-depleted rat hepatocytes cultured in the presence of AT[31]. Unlike the situation in the rat, between 56% and 87% of the hepatic catalase is localized in the cytosol in the male guinea pig[11]. And it has also been proved that this cytoplasmic catalase is provided with substrate under physiological conditions[10]. Thus, inhibiting CAT activity with AT treatment would mean increasing cell $H_2O_2$ levels and subsequentially a form of additional oxidative stress. In fact increases of tissue $H_2O_2$ concentration have been demonstrated in houseflies drinking water containing 2 mM AT[30].

Enhancing ascorbate content in the diet has decreased MDA levels (measured by HPLC) in the guinea pig liver (around 65–75%). These results show how ascorbate is able to decrease liver lipid peroxidation *in vivo* not only under normal conditions

TABLE 1
Hydrophilic and lipophilic antioxidants after vitamin C supplementation and AT (aminotriazole) treatment

| | High vit C | | Low vit C | |
|---|---|---|---|---|
| Antioxidants | Control | AT treated | Control | AT treated |
| GSH (μmol/mg prot) | 63.61 ± 9.91 (7) | 67.15 ± 9.63 (5) | 45.55 ± 10.09 (6) | 65.49 ± 7.53 (5) |
| GSSG (μmol/mg prot) | 4.49 ± 0.71 (7) | 3.02 ± 0.56 (5) | 4.12 ± 0.78 (6) | 4.73 ± 0.72 (5) |
| Uric acid (nmol/g) | 302.48 ± 44.62 (7) | 221.49 ± 47.34 (5) | 316.60 ± 42.41 (6) | 303.12 ± 45.19 (5) |
| Ascorbic acid (nmol/g) | 1988.20 ± 167.20 (7) | 2346.29 ± 393.22 (5) | 34.28 ± 21.02 (6)[a]*** | 13.96 ± 13.96 (5)[a]*** |
| α-tocopherol (μg/g) | 16.82 ± 2.04 (7) | 15.73 ± 2.76 (5) | 17.02 ± 5.49 (6) | 11.40 ± 0.93 (5) |

Values are means ± SEM. Number of animals in parentheses.
[a] represents significant differences between high and low vitamin C groups.
[b] represents significant differences between control and AT treated groups from the same dietary regimen.
***$p < 0.001$.

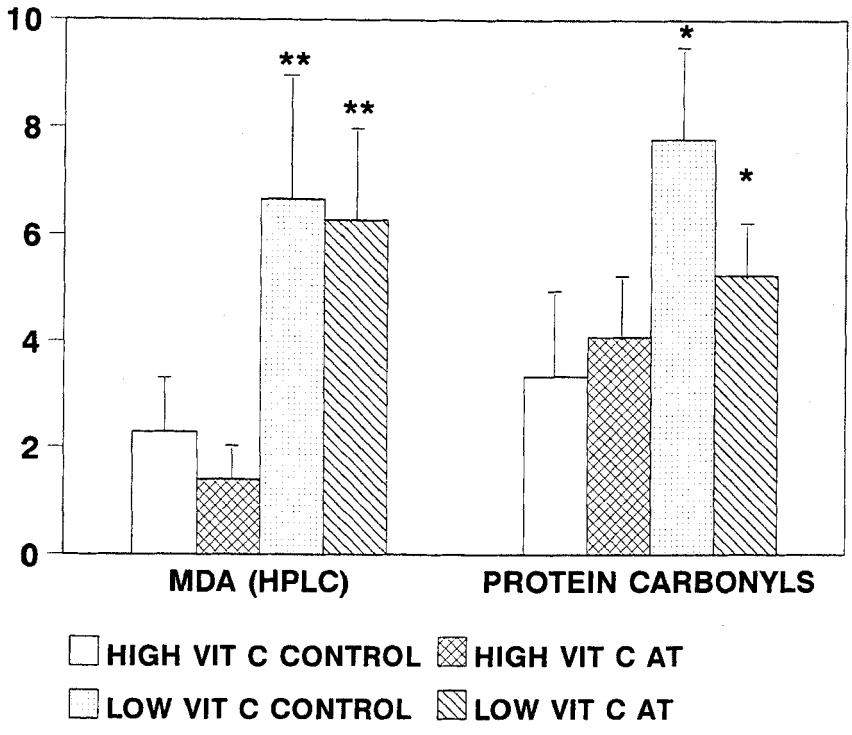


FIGURE 2 Endogenous MDA (nmoles/g tissue) and protein carbonyls (nmoles/mg protein) from liver of guinea pig fed for five weeks with two different amounts of vitamin C in the diet (33 mg VitC/Kg diet and 13,200 mg VitC/Kg diet) and treated or not with the CAT inhibitor 3-amino-1,2,4-triazole (AT). Data are means ± SEM from 5–7 animals. Asterisks represent significant differences in relation to the low vitamin C diet group. *$p < 0.05$; **$p > 0.01$.

but also in CAT-depleted animals. Our results are in agreement with previous reports describing a 30% increase of *in vivo* TBARS in the liver from guinea pig receiving a vitamin C deficient diet for three weeks[32]. Genetically scorbutic rats also showed TBARS increases in plasma, LDL and liver in relation to control rats[33]. The acute effect of vitamin C on liver endogenous MDA levels observed in our work is probably due to the direct MDA measurement (by HPLC). The TBARS method suffers from interference problems with other substances when it is applied to *in vivo* measurements.

We have found an interesting parallel in the dietary ascorbate effect on lipid and protein oxidation *in vivo*. As has just been shown for lipid peroxidation, vitamin C supplementation strongly decreased (around 25–60%) protein carbonyls in the guinea pig liver. It has recently been described that protein carbonyls are increased in old animals[34] and at the same time caloric restriction, the only experimental manipulation capable of increasing maximum life span, decreases protein carbonyls in mice liver[35]. In our experiment extra ascorbate did not decrease food intake. On the contrary animals receiving high amounts of vitamin C are significantly more than animals receiving low levels of vitamin C. So we can rule out the possibility of caloric restriction being responsible for the decrease in carbonyls

observed. We have found in the literature how GSH, another hydrophilic antioxidant, was also able to inhibit both TBARS and protein carbonyl formation when it was added to an *in vitro* peroxidation system in rat liver microsomes[36]. Our data extends this inhibitory capacity for lipid and protein oxidation to vitamin C *in vivo* in the guinea pig liver. Recent reports also show that vitamin C inhibits 8-OH-deoxyguanosine formation both *in vitro* (calf-thymus DNA)[37] and *in vivo* (sperm from humans supplemented with vitamin C for 15 days)[38]. Thus, present information suggests that vitamin C supplementation protects the three principal types of biological macromolecules, lipids, proteins, and DNA, from oxidative damage.

In previous studies it has been demonstrated that endogenous antioxidants are subjected to homeostatic control. Exposure to an oxidative stress *in vivo* can lead to compensatory inductions of endogenous antioxidants[4-6]. Addition of high amounts of exogenous antioxidants in the diet could also generate a reactive depression of these endogenous antioxidants. The results obtained in our study reveal that neither antioxidant enzymes nor GSH, uric acid and vitamin E levels are depressed by supplementing the diet with high doses of vitamin C. This reactive depression of endogenous antioxidants did not take place and on the contrary total GPx activity from the tissue was increased. Decreases or absence of changes have been reported after AT treatment[28-29,31] for GPx activity. In the present study vitamin C supplementation resulted in an increased GPx activity even after AT treatment. Vitamin C supplementation is then capable of enhancing global antioxidant capacity in the guinea pig liver leading to a decreased oxidative damage not only under basal conditions but in both control and CAT-depleted animals. On the other hand, three hours after AT injection do not seem to be enough time to promote any significant change. In contrast, previous studies in our laboratory showed important antioxidant inductions after long term AT treatment[5] and increased oxidative stress indexes after 15 days of AT treatment[28-29]. Thus, the absence of negative effects after catalase depletion observed in the present study, could be due to the short time selected.

The capacity of vitamin C to decrease tissue lipid and protein oxidation *in vivo* supports the hypothesis that an adequate antioxidant protection is essential to maintain the integrity of proteins and lipids in the guinea pig liver. These results can possibly explain in part why in the majority of the epidemiological and longitudinal studies performed to date dietary vitamin C is protective and decreases the incidence of various degenerative diseases, including the main causes of death in industrialized countries today, cardiovascular diseases[9,39] and cancer[1,7-8].



## *Acknowledgments*

This work was financially supported by grant n°93/0145E from the National Research Foundation of the Spanish Ministry of Health (FISss). Predoctoral fellowships were received by C. Rojas (F.P.I., Ministry of Education), R. Pérez-Campo and S. Cadenas (Complutense University).

## *References*

1. B.N. Ames (1989) Endogenous oxidative DNA damage, aging, and cancer. *Free Radical Research Communications*, 7, 121-128.
2. B. Frei (1991) Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage. *American Journal of Clinical Nutrition*, 54, 1113S-1118S.

3. D. Harman (1956) Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology*, **11**, 298–300.
4. R.S. Sohal, K.J. Farmer, R.G. Allen and S.S. Ragland (1984) Effects of diethyldithiocarbamate on life span, metabolic rate, superoxide dismutase, catalase, inorganic peroxides and glutathione in the adult male housefly, *Musca domestica*. *Mechanisms of Ageing and Development*, **24**, 175–183.
5. M. López-Torres, R. Pérez-Campo, C. Rojas, S. Cadenas and G. Barja (1993) Simultaneous induction of SOD, glutathione reductase, GSH and ascorbate in liver and kidney correlates with survival during aging. *Free Radical Biology and Medicine*, **15**, 133–142.
6. M. López-Torres, R. Pérez-Campo, A. Fernández, C. Barba and G. Barja de Quiroga (1993) Brain glutathione reductase induction increases early survival and decreases lipofuscin accumulation in aging frogs. *Journal of Neuroscience Research*, **34**, 233–242.
7. G. Block (1991) Epidemiologic evidence regarding vitamin C and cancer. *American Journal of Clinical Nutrition*, **54**, 1310S–1314S.
8. T. Byers and G. Perry (1992) Dietary carotenes, vitamin C, and vitamin E as protective antioxidants in human cancers. *Annual Review of Nutrition*, **12**, 139–159.
9. J.M. Gaziano, J.E. Manson, J.E. Buring and C.H. Hennekens (1992) Dietary antioxidants and cardiovascular disease. *Annals of the New York Academy of Sciences USA*, **669**, 249–259.
10. A. Geerts and F. Roels (1982) *In vivo* cooperation between hepatic catalase and superoxide dismutase demonstrated by diethyldithiocarbamate. *FEBS Letters*, **140**, 245–247.
11. S. Yamamoto, A. Völkl, T. Hashimoto and D. Fahimi (1988) Catalase in guinea pig hepatocytes is localized in cytoplasm, nuclear matrix and peroxisomes. *European Journal of Cell Biology*, **46**, 129–135.
12. S. Marklund and G. Marklund (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, **47**, 469–474.
13. R.F. Beers and I.W. Sizer (1952) A spectrophotometric method for measuring the break down of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, **195**, 133–140.
14. R.A. Lawrence and R.F. Burk (1976) Glutathione peroxidase activity in selenium-deficient rat liver. *Biochemical and Biophysical Research Communications*, **71**, 952–958.
15. V. Massey and C.H. Williams (1965) On the reaction mechanism of yeast glutathione reductase. *Journal of Biological Chemistry*, **240**, 4470–4481.
16. L. Smith (1955) Spectrophotometric assay of cytocrome c oxidase. In *Methods of Biochemical Analysis* (ed. D. Gick), Wiley-Interscience, New York, pp. 427–434.
17. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
18. F. Tietze (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Analytical Biochemistry*, **27**, 502–522.
19. P. Sacchetta, D. Di Cola and G. Federici (1986) Alkaline hydrolysis of N-ethylmaleimide allows a rapid assay of glutathione disulfide in biological samples. *Analytical Biochemistry*, **154**, 205–208.
20. G. Barja de Quiroga, M. López-Torres, R. Pérez-Campo and C. Rojas (1991) Simultaneous determination of two antioxidants, uric and ascorbic acid, in animal tissue by high-performance liquid chromatography. *Analytical Biochemistry*, **199**, 81–85.
21. G.T. Vatassery, M.F. Brin, S. Fahn, H.J. Kayden and M.G. Traber (1988) Effect of high doses of dietary vitamin E on the concentrations of vitamin E in several brain regions, plasma, liver, and adipose tissue of rats. *Journal of Neurochemistry*, **51**, 621–623.
22. R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel and E.R. Stadtman (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology*, **186**, 464–478.
23. A.W. Bull and L.J. Marnett (1985) Determination of malondialdehyde by ion-pairing high-performance liquid chromatography. *Analytical Biochemistry*, **149**, 284–290.
24. National Research Council ed. (1978) Nutrient requirements of laboratory animals. National Academy of Sciences, Washington D.C., pp. 59–69.
25. G.L. Jones and A.R. Neill (1982) The effects of clofibrate and 3-amino-1,2,4-triazole on liver catalase and lipid metabolism in mice. *Biochimica et Biophysica Acta*, **712**, 420–426.
26. C.M.G. Aragon, F. Rogan and Z. Amit (1991) Dose- and time-dependent effect of an acute 3-amino-1,2,4-triazole injection on rat brain catalase activity. *Biochemical Pharmacology*, **42**, 699–702.
27. T. Yusa, J.S. Beckman, J.D. Crapo and B.A. Freeman (1987) Hyperoxia increases $H_2O_2$ production by brain *in vivo*. *Journal of Applied Physiology*, **63**, 353–358.

28. M. López-Torres, R. Pérez-Campo and G. Barja de Quiroga (1990) Aminotriazole effects on lung and heart $H_2O_2$ detoxifying enzymes and TBA-RS at two $pO_2$. *Pharmacology and Toxicology*, **66**, 27–31.
29. G. Barja de Quiroga, M. López-Torres and R. Pérez-Campo (1989) Catalase is needed to avoid tissue peroxidation in *Rana perezi* in normoxia. *Comparative Biochemistry and Physiology*, **94C**, 391–398.
30. R.G. Allen, K.J. Farmer and R.S. Sohal (1983) Effect of catalase inactivation on levels of inorganic peroxides, superoxide dismutase, glutathione, oxygen consumption and life span in adult houseflies (*Musca domestica*). *Biochemical Journal*, **216**, 503–506.
31. P.E. Starke and J.L. Farber (1985) Endogenous defenses against the cytotoxicity of hydrogen peroxide in cultured rat hepatocytes. *Journal of Biological Chemistry*, **260**, 86–92.
32. T. Mori, R. Kitamura, S. Imaoka, Y. Funae, M. Kitada and T. Kamataki (1992) Examination for lipid peroxidation in liver microsomes of guinea pigs as a casual factor in the decrease in the content of cytochrome P-450 due to ascorbic acid deficiency. *Research Communications in Chemical Pathology and Pharmacology*, **75**, 209–219.
33. H. Kimura, Y. Yamada, Y. Morita, H. Ikeda and T. Matsuo (1992) Dietary ascorbic acid depresses plasma and low density lipoprotein lipid peroxidation in genetically scorbutic rats. *Journal of Nutrition*, **122**, 1904–1909.
34. R.S. Sohal, S. Agarwal, A. Dubey and W.C. Orr (1993) Protein oxidative damage is associated with life expectancy of houseflies. *Proceedings of the National Academy of Sciences*, **90**, 7255–7259.
35. L.D. Youngman, J.-Y.K. Park and B.N. Ames (1992) Protein oxidation associated with aging is reduced by dietary restriction of protein or calories. *Proceedings of the National Academy of Sciences*, **89**, 9112–9116.
36. J.R. Palanda and J.P. Kehrer (1992).Inhibition of protein carbonyl formation and lipid peroxidation by glutathione in rat liver microsomes. *Archives of Biochemistry and Biophysics*, **293**, 103–109.
37. A. Fischer-Nielsen, H.E. Poulsen and S. Loft (1992) 8-hydroxydeoxyguanosine *in vitro*: effects of glutathione, ascorbate, and 5-aminosalicylic acid. *Free Radical Biology and Medicine*, **13**, 121–126.
38. C.G. Fraga, P.A. Motchnik, M.K. Shigenaga, H.J. Helbock, R.A. Jacob and B.N. Ames (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proceedings of the National Academy of Sciences*, **88**, 11003–11006.
39. K.F. Gey (1990) Lipids, lipoproteins and antioxidants in cardiovascular dysfunction. *Biochemical Society Transactions*, **18**, 1041-1045.

Accepted by Professor B. Halliwell