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Oxidative Stress in Patients with Hepatitis, Cirrhosis, and Hepatoma Evaluated by Plasma Antioxidants

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We have applied our method for the simultaneous detection of plasma ubiquinol-10 (reduced form) and ubiquinone-10 (oxidized form) (S. Yamashita and Y. Yamamoto, *Anal. Biochem.* 250, 66–73, 1997) to plasmas of normal subjects (n=16) and patients with chronic active hepatitis (n=28), liver cirrhosis (n=16), and hepatocellular carcinoma (n=20) to evaluate the pressure of oxidative stress in these patients. The average ubiquinone-10 percentages (± S.D.) in total ubiquinone-10 and ubiquinol-10 in the four groups were 6.4 ± 3.3, 12.9 ± 10.3, 10.6 ± 6.8, and 18.9 ± 11.1, respectively, indicating a significant increase in ubiquinone-10 percentage in patient groups in comparison to normal subjects. These results and a significant decrease in the plasma ascorbate level in patient groups indicate that oxidative stress is evident after the onset of hepatitis and the subsequent cirrhosis and liver cancer.

Key Words: **ubiquinol; ubiquinone; oxidative stress; hepatitis; cirrhosis; hepatoma.**

Human liver cancer often develops after the onset of chronic hepatitis and the subsequent cirrhosis. Measurement of oxidative stress at each stage is of interest since oxidative stress has been suggested to be a causative factor in cancer (1). However, only a limited number of papers have documented the occurrence of oxidative stress in these liver patients. Patients with acute and chronic hepatitis were shown to have increased level of serum thiobarbituric acid reactive substances (TBARS) (2, 3), a maker of oxidative damage of lipids, and reduced level of plasma glutathione (4) and erythrocyte glutathione (5). Reduced levels of plasma and erythrocyte glutathione were also reported in patients with cirrhosis (6). Patients with severe hepatitis has reduced plasma levels of vitamin E (7). Increase in serum protein carbonyl content, a marker of oxidative damage of proteins, was observed in patients with hepatitis but not in patients with cirrhosis (8). Obviously some data are inconsistent and the measurement of oxidative stress by a more sensitive method is desired.

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favor of the former (9). Therefore, the ratio of the oxidized to reduced forms of redox compounds should increase under conditions of oxidative stress. Amongst of many biological redox compounds we selected ubiquinol-10 (reduced form of coenzyme Q-10) and ubiquinone-10 (oxidized form) couple since ubiquinol-10 is very labile in the oxidation of low density lipoprotein or plasma (10-12). In fact, when human plasma was incubated under aerobic conditions at 37 °C, ubiquinol-10 decreased after the depletion of ascorbate without having any significant decrease in a-tocopherol level (12) and concomitant formation of ubiquinone-10 was observed (unpublished data). The ratio of ubiquinone to ubiquinol has been used as a sensitive marker of oxidative stress and a significant increase in this ratio was observed in hyperlipidemic Nagase analbuminemic rats (13) and in Long-Evans cinnamon (LEC) rats (14) as compared with control rats. The plasma ratio of ubiquinone to ubiquinol also increased in patients with coronary artery disease (15) and hyperlipidemia (16).

We recently developed a simple and reliable method for the simultaneous detection of plasma ubiquinol-10 and ubiquinone-10 (17) and applied this method to plasmas of normal subjects and patients with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma to evaluate the pressure of oxidative stress in these patients. We also measured other antioxidants such as vitamin C, uric acid, unconjugated bilirubin, vitamin E, lycopene, and β-carotene for comparison.

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Abbreviations: TBARS, thiobarbituric acid reactive substances; LEC rat, Long-Evans cinnamon rat; HPLC, high pressure liquid chromatography; ECD, electrochemical detector; FC, free cholesterol; CE, cholesteryl esters; TC, total cholesterol; %CoQ-10, ubiquinone-10 percentage in total ubiquinone-10 and ubiquinol-10.

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TABLE 1

Plasma Antioxidants, Lipids, Free Fatty Acids in Normal Subjects and Patients with Hepatitis, Cirrhosis, and Hepatoma

				Hydrophilic antioxidants		
	Number	Male/female	Age (year)	Uric acid (μM)	Vitamin C (μM)	Unconjugated bilirubin (μM)
Normal:	16	12/4	57.7 ± 16.6	328 ± 65	52.7 ± 46.7	4.1 ± 2.6
Hepatitis:	28	18/10	59.5 ± 10.7	279 ± 79	**15.3 ± 15.0**	3.2 ± 2.0
			0.4832	*0.0560*	*0.0003*	*0.1426*
Cirrhosis:	16	10/6	60.4 ± 13.0	282 ± 86	**20.6 ± 15.8**	5.7 ± 4.1
			0.4552	*0.1331*	*0.0108*	*0.1851*
Hepatoma:	20	15/5	65.7 ± 70.	275 ± 118	**15.3 ± 21.6**	5.6 ± 3.6
			0.2414	*0.1425*	*0.0009*	*0.2499*

	Coenzyme Q-10				Other lipophilic antioxidants		
	$CoQH_2$-10 (nM)	CoQ-10 (nM)	Total Q-10 (nM)	%CoQ-10	Vitamin E (μM)	Lycopene (μM)	β-carotene (μM)
Normal:	759 ± 258	50 ± 26	809 ± 272	6.4 ± 3.3	26.9 ± 9.2	0.34 ± 0.27	1.30 ± 1.12
Hepatitis:	719 ± 270	**94 ± 64**	813 ± 278	**12.9 ± 10.3**	27.1 ± 9.2	**0.16 ± 0.13**	1.02 ± 0.91
	0.9417	*0.0015*	*0.6517*	*0.0011*	*0.8357*	*0.0427*	*0.4068*
Cirrhosis:	662 ± 217	79 ± 61	741 ± 234	**10.6 ± 6.8**	24.4 ± 8.0	**0.09 ± 0.08**	0.84 ± 1.06
	0.2913	*0.0703*	*0.4509*	*0.0070*	*0.5465*	*0.0044*	*0.0863*
Hepatoma:	652 ± 280	**149 ± 97**	801 ± 302	**18.9 ± 11.1**	21.7 ± 7.9	*0.12 ± 0.09*	**0.54 ± 0.37**
	0.1565	*0.0003*	*0.8735*	*<0.0001*	*0.0561*	*0.0108*	*0.0119*

	Coenzyme Q-10/total cholesterol			Lipophilic antioxidants/total cholesterol		
	10^6*$CoQH_2$-10/Tc	10^6*CoQ-10/TC	10^6*total Q-10/TC	10^3*vitamin E/TC	10^6*lycopene/TC	10^6*β-carotene/TC
Normal:	126 ± 41	11 ± 5	173 ± 42	5.82 ± 1.77	74 ± 58	285 ± 252
Hepatitis:	169 ± 51	**24 ± 16**	193 ± 50	6.63 ± 2.49	38 ± 29	240 ± 199
	0.6872	*0.0002*	*0.2318*	*0.1754*	*0.0570*	*0.6429*
Cirrhosis:	180 ± 51	**20 ± 10**	200 ± 53	6.53 ± 1.48	**24 ± 14**	227 ± 311
	0.4397	*0.0011*	*0.2066*	*0.1048*	*0.0122*	*0.2278*
Hepatoma:	197 ± 79	**44 ± 26**	**241 ± 80**	6.50 ± 2.01	34 ± 25	155 ± 86
	0.2582	*<0.0001*	*0.0025*	*0.1968*	*0.0502*	*0.1388*

	Cholesteryl esters									
	CE (mM)	Ch20:4 (mM)	%Ch20:4	Ch18:2 (mM)	%Ch18:2	Ch18:1 (mM)	%Ch18:1	FC (mM)	TC (mM)	FC/CE
Normal:	3.22 ± 0.43	0.38 ± 0.09	12 ± 2	2.08 ± 0.28	65 ± 3	0.76 ± 0.10	24 ± 2	1.40 ± 0.39	4.62 ± 0.64	0.44 ± 0.12
Hepatitis:	**2.89 ± 0.77**	0.33 ± 0.11	12 ± 2	**1.77 ± 0.49**	**61 ± 4**	0.79 ± 0.21	**27 ± 3**	1.32 ± 0.28	4.21 ± 1.03	**0.47 ± 0.06**
	0.0256	*0.0583*	*0.9901*	*0.0168*	*0.0020*	*0.6604*	*0.0001*	*0.7977*	*0.0876*	*0.0324*
Cirrhosis:	**2.46 ± 0.90**	**0.27 ± 0.14**	10 ± 2	**1.47 ± 0.48**	**60 ± 3**	**0.72 ± 0.30**	**29 ± 2**	1.35 ± 0.38	**3.81 ± 1.24**	**0.59 ± 0.17**
	0.0010	*0.0098*	*0.1478*	*0.0003*	*0.0005*	*0.1087*	*<0.0001*	*0.7773*	*0.0059*	*0.0017*
Hepatoma:	**2.46 ± 0.90**	**0.27 ± 0.14**	10 ± 2	**1.47 ± 0.48**	**58 ± 4**	**0.63 ± 0.20**	**30 ± 4**	1.28 ± 0.33	**3.40 ± 1.04**	**0.62 ± 0.11**
	0.0001	*0.0039*	*0.8198*	*<0.0001*	*<0.0001*	*0.0247*	*<0.0001*	*0.3724*	*0.0006*	*0.0001*

Numbers in italic and bold show P values and significant differences compared to normal subjects, respectively, as determined by Mann–Whitney test. Abbreviations: $CoQH_2$-10, ubiquinol-10; CoQ-10, ubiquinone-10; total Q-10=$CoQH_2$-10+CoQ-10; %CoQ-10(%)=CoQ-10/total Q-10; Ch20:4, cholesteryl arachidonate; Ch18:2, cholesteryl linoleate; Ch18:1, cholesteryl oleate; CE=Ch20:4+Ch18:2+Ch18:1; FC, free cholesterol; TC, total cholesterol(=FC+CE); %Ch20:4 (%)=Ch20:4/CE; %Ch18:2(%)=Ch18:2/CE, %Ch18:1(%)=Ch18:1/CE.

Finally, we discussed the role of oxidative stress in carcinogenesis.

EXPERIMENTAL

Human plasmas. Patients examined in this study were hospitalized at First Department of Medicine in Kyoto Prefectural University of Medicine and were diagnosed with chronic active hepatitis (18 men and 10 women), liver cirrhosis (10 men and 6 women), and hepatocellular carcinoma (15 men and 5 women). All were infected with hepatitis C virus except for 3 patients with hepatitis, 1 with cirrhosis, and 2 with hepatoma which had contracted hepatitis B virus. The normal subjects consisted of 12 men and 4 women between the age of 40 to 83 years. Average ages (± S.D.) in the four groups were 59.5 ± 10.7, 60.4 ± 13.0, 65.7 ± 7.0, and 57.7 ± 16.6, respec-

tively, providing no statistical difference. Blood was drawn from the above patients and volunteers using sodium heparin as an anticoagulant. Plasma was separated by centrifugation at 1500 *g* for 10 min and was stored at −80°C until analysis.

Analytical procedure. Plasma levels of ascorbic acid, uric acid, and unconjugated bilirubin were determined by high pressure liquid chromatography (HPLC) equipped with an aminopropylsilyl column (Type Supelcosil LC-NH_2, 5 μm, 250 × 4.6 mm i.d., Supelco) and a UV detector (275 nm) as previously described (18). The mobile phase consisted of methanol/40 mM sodium monobasic phosphate (= 9/1, v/v) delivered at a flow rate of 1.0 ml/min.

Plasma ubiquinol, ubiquinone, vitamin E (mixture of a-tocopherol and g-tocopherol), lycopene, β-carotene, free cholesterol, and cholesteryl esters were measured as previously reported (17). Briefly, plasma (50 μl) was mixed vigorously with 250 μl of cold methanol and 500 μl of cold hexane in a 1.5 ml-polypropylene tube. After centrifugation at 10,000 *g* for 3 min at 4°C, 5 μl of hexane layer (corresponding to 0.5 μl of plasma) was injected immediately and directly onto HPLC equipped with two guard columns (Type Supelguard LC-ABZ, 5 μm, 20 × 4.6 mm i.d., Supelco, Tokyo), an analytical column (Type Supelcosil LC-8, 5 μm, 250 × 4.6 mm i.d., Supelco), a reduction column (Type RC-10-1, Irica, Kyoto), a UV detector (Model SPD-10A, Shimadzu, Kyoto), and an amperometric electrochemical detector (ECD, Model Σ985, Irica). The UV detector was monitored at 210 nm and the oxidation potential for ECD was 600 mV on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/*tert*-butyl alcohol (85/15, v/v) delivered at a flow rate of 0.8 ml/min.

Statistical analyses. Data were analyzed by using StatView-J4.11 software (Abacus Concepts, Berkeley, California). Significant differences compared to normal subjects were determined by nonparametric Mann-Whitney test.

RESULTS AND DISCUSSION

Table 1 summarizes plasma antioxidants and cholesterols in normal subjects and patients with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma. A significant decrease in plasma ascorbic acid level was observed in patient groups as compared with normal subjects. This indicates that these patients were subject to oxidative stress since ascorbic acid is the most oxidatively vulnerable antioxidant present in human plasma (12, 19). A significant decrease in plasma ascorbic acid level was also observed (14) in LEC rat, an animal model of hepatitis and liver cancer. However, it is also possible that the lowered level of plasma ascorbic acid in these patients is caused by the shortage of ascorbic acid intake.

We, therefore, adopted a different approach and a newly developed method for the measurement of plasma ubiquinol-10 and ubiquinone-10 was applied. The ratio of ubiquinone-10 to ubiquinol-10 should be one of the most reliable marker of oxidative stress since it is a direct product of redox imbalance. Fig. 1 shows typical HPLC chromatograms of hexane extracts of plasmas from a normal subject and a patient with chronic active hepatitis. As demonstrated, coenzyme Q-10 exists mostly in its reduced form (ubiquinol-10) in both subjects, but a normal subject has higher level of plasma ubiquinol-10 and lower level

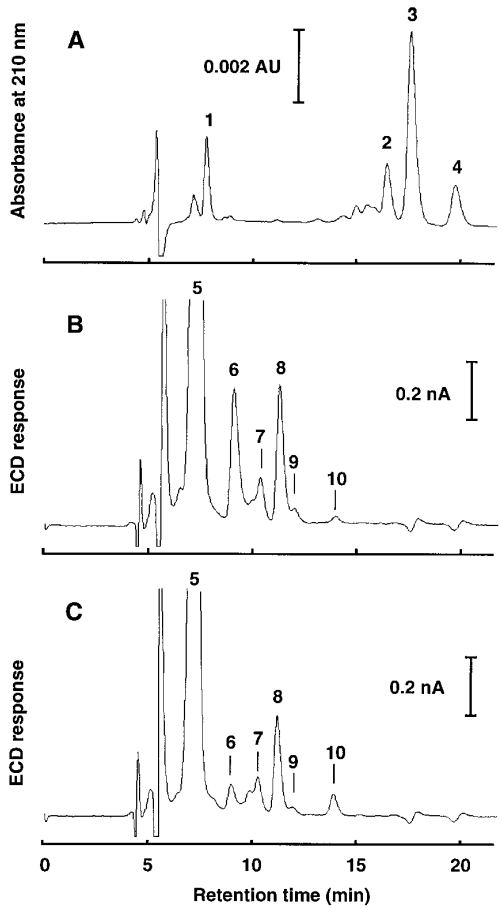

FIG. 1. Typical chromatograms of hexane extracts of plasmas from a normal subject (**A, B**) and a patient with hepatitis (**C**). Free cholesterol (**1**), cholesteryl arachidonate (**2**), cholesteryl linoleate (**3**), and cholesteryl oleate (**4**) are detected by UV (210 nm). Vitamin E (**5**), lycopene (**6**), β-carotene (**7**), ubiquinol-10 (**8**), unknown (**9**), and ubiquinone-10 (**10**) are detected by ECD (600 mV).

of ubiquinone-10 than does a patient with hepatitis. Fig. 2 shows that patients with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma had significantly higher content of plasma ubiquinone-10 expressed as %CoQ-10 = ubiquinone-10 / (ubiquinone-10 + ubiquinol-10) than normal subjects. These results indicate that oxidative stress is evident after the onset of hepatitis and the subsequent cirrhosis and liver cancer. A significant increase in the ratio of plasma ubiquinol-9 to ubiquinone-9 was also observed in LEC rat after the onset of hepatitis (14). It is noteworthy that there were no differences in total amounts of ubiquinone-10 and ubiquinol-10 among the four groups despite the impairment of liver function as observed in the reduction of lecithin:cholesterol acyltransferase activity (see below).

Besides ubiquinol-10 and ubiquinone-10, levels of free cholesterol (FC), cholesteryl esters (CE = cholesteryl arachidonate + cholesteryl linoleate + cholesteryl

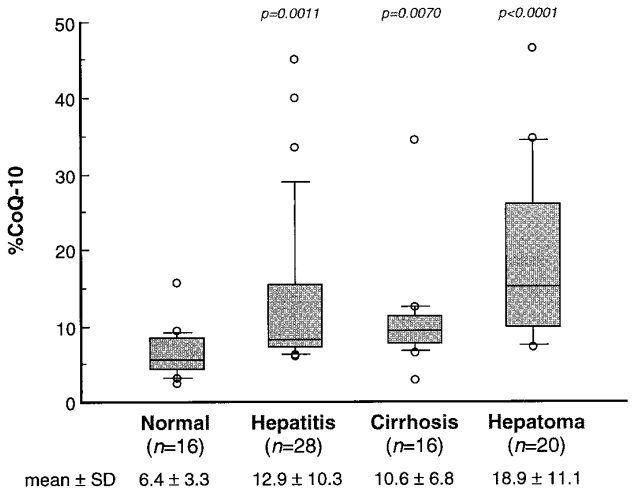

FIG. 2. Box and whisker plots of plasma CoQ-10 percentage (%CoQ-10) in total amounts of ubiquinone-10 and ubiquinol-10 among normal subjects and patients with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma (hepatoma). Significant differences compared to normal subjects were analyzed by non parametric Mann–Whitney test.

oleate), tocopherols (mixture of α-tocopherol and γ-tocopherol), lycopene, and β-carotene were determined by HPLC analysis (Fig. 1). We observed no differences in plasma levels of FC, but a significant decrease in plasma levels of CE was apparent in patient groups (Table 1), indicating impairment of lecithin:cholesterol acyltransferase. A reduction of this enzyme activities was observed in patients with liver diseases (20). It is well known that patients with hepatitis, cirrhosis, and hepatoma have increased level of plasma conjugated bilirubin but unconjugated bilirubin levels were unchanged (Table 1) as observed previously (21).

Although it is reported that patients with severe viral hepatitis had diminished plasma levels of vitamin E (7), plasma vitamin E levels in this study were unchanged in patients with hepatitis, cirrhosis, and hepatoma (Table 1). No significant change was observed when vitamin E levels were normalized to levels of total cholesterol (TC = FC + CE). These data are consistent with other results indicating that vitamin E levels do not decrease at an early stage in the oxidation of human plasma (10, 12, 19). A significant decrease in plasma levels of lycopene was observed in patient groups, but a significant decrease in the ratio of lycopene to TC was observed only in patients with cirrhosis (Table 1). A significant decrease of plasma levels of β-carotene was also observed in patients with hepatoma, but the ratios of β-carotene to TC were the same among the four groups (Table 1). These data suggest that plasma levels of vitamin E, lycopene, and β-carotene are unsuitable as markers of oxidative stress.

The role of oxygen radicals in the aetiology of carcinogenesis has not been fully investigated. However, it would be reasonable to expect that oxygen radicals would likely persist longer under conditions of oxidative stress presenting an increased risk of oxidative damage to DNA. If these oxidative modifications are unrepaired, they can cause errors in genetic replication having the potential to initiate carcinogenesis (22). Oxygen radicals have also been demonstrated to convert carcinogenic precursors to the ultimate active carcinogen (23) and, further to these roles in the initiation of cancer, oxygen radicals may also be involved in tumor promotion. We have recently demonstrated that oxidized 1,2-diacylglycerol which is formed from oxidized biomembranes by phospholipase C activates protein kinase C having the ability to act as an endogenous tumor promoter (24, 25). These data are thus consistent with chronic oxidative stress (redox imbalance) being an important cause of carcinogenesis. In this, and in a previous study (14), we have demonstrated that oxidative stress is evident after contracting hepatitis in humans and in LEC rats (14) which is a condition predisposed to the onset of liver cancer. In fact, chronic inflammation is recognized as one of the major contributing cause of cancer (26).

In summary, a significant increase in the plasma ratio of ubiquinone-10 (oxidized form) to ubiquinol-10 (reduced form) was observed in patients with with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma as compared to normal subjects. We believe this is one of the most reliable evidence of oxidative stress in these patients.

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REFERENCES

1. Ames, B. N., Gold, L. S. and Willett, W. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5258–5265.
2. Suematsu, T., Kamada, T., Abe, H., Kikuchi, S, and Yagi, K. (1977) *Clin. Chim. Acta* **79,** 267–270.
3. Higueras, V., Raya, A., Rodrigo, J. M., Serra, M. A., Roma, J., and Romero, F. J. (1994) *Free Radical Biol. Med.* **16,** 131–133.
4. Shigesawa, T., Sato, C. and Narumo, F. (1992) *J. Gastroenterol. Hepatol.* **7,** 7–11.
5. Swietek, K., and Juszczyk, J. (1997) *J. Viral Hepat.* **4,** 139–141.
6. Loguercio, C., Tarando, D., Vitale, L. M., Beneduce, F., and Del Vecchio Blanco, C. (1996) *Free Radical Biol. Med.* **20,** 483–488.
7. von Herbay, A., Stahl, W., Niederau, C., von Laar, J., Strohmeyer, G., and Sies, H. (1996) *Free Radical Res.* **25,** 461–466.
8. De Maria, N., Colantoni, A., Fagiuoli, S., Liu G. J., Rogers, B. K., Farinati, F., Van Thiel, D. H., and Floyd R. A. (1996) *Free Radical Biol. Med.* **21,** 291–295.
9. Sies, H. (1985) *in* Oxidative Stress (Sies, H., Ed.), pp. 1–8, Academic Press, London.

10. Cross, C. E., Forte, T., Stocker, R., Louie, S., Yamamoto, Y., Ames, B. N. and Frei, B. (1990) *J. Lab. Clin. Med.* **115,** 396–404.
11. Stocker, R., Bowry, V. W., and Frei, B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1646–1650.
12. Yamamoto, Y., Kawamura, M., Tatsuno, K., Yamashita, S., Niki, E., and Naito, C. (1991) *in* Oxidative Damage and Repair (Davies, K. J. A., Ed.), pp. 287–291, Pergamon Press, Oxford.
13. Yamamoto, Y., Wakabayashi, K., Niki, E., and Nagao, M. (1992) *Biochem. Biophys. Res. Commun.* **189,** 518–523.
14. Yamamoto, Y., Sone, H., Yamashita, S., Nagata, Y., Niikawa, H., Hara, K. and Nagao, M. (1997) *J. Trace Elem. Exp. Med.* **10,** 129–134.
15. Lagendijk, J., Ubbink, J. B., Delport, R., and Vermaak, W. J. H. (1997) *Res. Commun. Mol. Pathol. Pharmacol.* **95,** 11–20.
16. Kontush, A., Reich, A., Baum, K., Spranger, T., Finckh, B., Kohlschutter, A., and Beisiegel, U. (1997) *Atherosclerosis* **129,** 119–26.
17. Yamashita, S., and Yamamoto, Y. (1997) *Anal. Biochem.* **250,** 66–73.
18. Yamamoto, Y., and Ames, B. N. (1987) *Free Radical Biol. Med.* **3,** 359–361.
19. Frei, B., Stocker, R., and Ames, B. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9748–9752.
20. Cooper, R. A., Diloy-Puray, M., Lando, P., and Greenberg, M. S. (1972) *J. Clin. Invest.* **51,** 3182–3192.
21. Owens, D., Jones, E. A., and Carson, E. R. (1977) *Clin. Sci. Mol. Med.* **52,** 555–570.
22. Kuchino, T., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E., and Nishimura, S. (1987) *Nature* **327,** 77–79.
23. Ji, C., and Marnett, L. J. (1992) *J. Biol. Chem.* **267,** 17842–17848.
24. Takekoshi, S., Kambayashi, Y., Nagata, H., Takagi, T., Yamamoto, Y., and Watanabe, K. (1995) *Biochem. Biophys. Res. Commun.* **217,** 654–660.
25. Yamamoto, Y., Kambayashi, Y., Ito, T., Watanabe, K. and Nakano, M. (1997) *FEBS Lett.* **412,** 461–464.
26. Ames, B. N., Gold, L. S., and Willett, W. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5258–5265.