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Protection by vitamin E, selenium, trolox, ascorbic acid palmitate, acetylcysteine, coenzyme Q, β-carotene, and (+)-catechin against oxidative damage to rat liver and heart tissue slices measured by oxidized heme proteins

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Male rats were fed a basal diet containing vitamin E and Se which was supplemented with three different doses of trolox, ascorbic acid palmitate, acetylcysteine, β-carotene, coenzyme Q_{10}, coenzyme Q_0, and (+)-catechin. Liver and heart tissue slices were incubated at 37°C. The effect of antioxidants on oxidative damage to rat liver and heart tissue slices was studied by measurement of the production of oxidized heme proteins during spontaneous oxidative reactions. Supplementing the basal vitamin E and Se diet with the other seven antioxidants at levels that would be appropriate for humans resulted in considerable additional protection. Increasing further the dosage of the seven supplementary antioxidants did not result in a proportional increase in protection. Maximum protection against tissue oxidation was reached at a practical dietary level of antioxidants. (J. Nutr. 452–458, 1995.)

Keywords: heme proteins; vitamin E; selenium, coenzyme Q; acetylcysteine; β-carotene

Introduction

Free radicals and related oxidants have long been studied as agents of tissue damage. Lipid peroxidation is a well-established example of free radical toxicity, as is oxidative damage to RNA, DNA, and even carbohydrates. The reaction of proteins with various radicals/oxidants has not been studied extensively. Amino acids, peptides, and proteins are vulnerable to attack by a variety of free radicals and related oxidants.[1]

Among proteins the heme compounds hemoglobin and myoglobin are particularly prone to oxidation which occurs both spontaneously and through the action of extrinsic agents.[2] Oxidative damage of heme proteins involves a redox reaction of the heme group with a one- or two-electron(s) transfer and the denaturation of the globin structure.[3] Under conditions of oxidative stress, oxyhemoglobin is oxidized to methemoglobin due to the oxidation of the heme ferrous ion to ferric ion. When the sixth coordination site is occupied by either the distal histidine or an external ligand, methemoglobin is converted to hemichrome.[4,5]

During the investigation of oxidative processes, it is often desirable to quantify damage to proteins, lipids, and other cellular components. Existing methods of quantification usually measure secondary irreversible oxidative prod-

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ucts. A computer-aided heme protein spectra analysis program (HPSAP) was recently developed in our laboratory. HPSAP can measure both reversible and irreversible damage to hemeproteins using visible light spectroscopy. An example of a reversible reaction is the reduction of methemoglobin to hemoglobin. This program is based on the knowledge that the absorbance spectrum of a mixture of heme proteins is the sum of the spectra of the individual heme proteins plus the absorbance from turbidity of the biological samples.[3] Quantitation is achieved by matching the calculated spectrum with the experimental spectrum through regression analysis provided in the Lotus 123 program, followed by successive approximations taking into consideration known concentrations of heme compounds in tissues and the known redox reactions of heme components.

The scientific literature contains some evidence that oxidant damage to cells and the physiological mechanisms involved are the basic causes of many inflammatory and degenerative diseases.[5] Dietary antioxidants can play an important role in decreasing oxidant radical involvement in disease processes. It is the purpose of this study to evaluate the protection against spontaneous oxidative damage to liver and heart tissue slices as affected by the basal vitamin E and Se diet, compared with dietary supplementation of the basal diet with three different concentrations of trolox, ascorbic acid palmitate, acetylcysteine, coenzyme Q, β-carotene, and (+)-catechin. Visible light spectroscopy and HPSAP were used to measure oxidative damage to heme proteins. A comparison of resistance to oxidative damage in heart and liver tissue is discussed.

Methods and materials

Chemicals

The antioxidants used in this study were α-tocopherol acid succinate (1210 I.U./g), coenzyme Q_{10}, (+)-catechin, trans-β-carotene, L-ascorbic acid 6-palmitate, N-acetyl-L-cysteine (all from Sigma Chemical Co., St. Louis, MO USA), sodium selenite (Difco Laboratories, Detroit, MI USA), trolox (97%), and coenzyme Q_0 (both from Aldrich Chemical Co., Milwaukee, WI USA). Trolox is a model compound of vitamin E which does not have a phytyl side chain but instead has a 2-carboxyl on the hydroxychromane ring.

Animals and diets

Male Sprague–Dawley rats (Bantin & Kingman, Fremont, CA USA) weighing 55–75 g were fed experimental diets. The diet containing the major components was vitamin E- and selenium-deficient with 10% tocopherol-stripped corn oil (Teklad test diet #TD 77068 with mineral mix #170911, Teklad Test Diets, Madison, WI USA). Animals were housed according to NIH guidelines. Animals had free access to deionized water, and food was changed daily in order to prevent oxidation. The rats were divided into four groups of six animals each. The four groups received the same amount of vitamin E and Se as would be considered suitable for humans. The concentrations of the other antioxidants (β-carotene, coenzyme Q_{10}, ascorbic acid palmitate, trolox, acetylcysteine, coenzyme Q_0, and catechin) included 0, ⅓, 1, and 3 times the amounts considered suitable for humans. In selecting antioxidant concentrations we used the following rationale which is similar to that used by others.[6] A person eats about 1 kg of dry diet per day. The RDA for the human nutrients (expressed as mg/day) is roughly similar to the nutritional requirements for a rat (expressed as mg/kg of rat diet). The RDA for vitamin E is 10 mg/day. The requirement for rats is 35 mg/kg of diet. We used 25 mg/kg of diet. The RDA for Se is 0.05 mg/day. The requirement for rats is 0.04 mg/kg of diet. We used 0.3 mg/kg of diet. There are no RDA values for the seven other antioxidants we have used. β-carotene is often used in a dose of 30 mg/day for humans. In dietary group 2, we used 15 mg/kg of diet. Coenzyme Q_{10} and trolox have properties similar to vitamin E. Therefore, we used them in doses similar to the dose of vitamin E: 10 and 16.7 mg/kg of diet for coenzyme Q_{10}, and trolox, respectively. The doses used in diet #4 (highest concentrations of antioxidants) did not exceed the maximum doses used by other investigators. An overview of the composition of the antioxidants in the diets is given in *Table 1*. The rats were on the experimental diets for a period of 6 weeks.

Preparation and oxidative reactions of tissue slices

Rats weighing 250–330 g were first anesthetized with chloroform (Fisher Scientific, Fair Lawn, NJ USA) and then decapitated. The liver and heart were immediately dissected and immersed in ice-cold Krebs-Ringer phosphate (KRP) buffer (pH 7.4). The tissues were cut into 0.5 cm^3 cubes with a surgical knife and sliced into 0.5 mm thick pieces (80–100 mg) by a Stadie-Riggs tissue slicer (Thomas Scientific, Philadelphia, PA).

To induce spontaneous oxidation of heme proteins in both heart and liver slices, approximately 200 mg tissue slices was added to a 10 mL glass serum bottle containing 5 mL of oxygenated Krebs–Ringer phosphate buffer (10 mmol/L of glucose). The bottles contained excess oxygen and were covered with Parafilm. The bottles were incubated in a gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ USA) at 37°C with continuous shaking (180 cycles/min) for 30 min, 1 hr, 2 hr, and 3 hr.

Spectrophotometric measurement of heme proteins in tissue slices

After incubation of the tissue slices, the absorbance spectrum of each sample was obtained with a Beckman DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA USA). A 50 mg sample was blotted with filter paper and then transferred to a small cylindrical spectrophotometer cell of 5.5 mm i.d. and a light path of 2.0 mm. In the air-tight cell, the heme proteins in the tissue slices came to a redox equilibrium determined by the physiological conditions of the tissues. Spectra can be corrected for scattered light loss by using a reference material with similar scattering properties to blank the spectrophotometer. We have found Parafilm to be a useful reference material for tissues over the

Table 1 Addition of antioxidant nutrients to basal diet

	Amount of antioxidant (mg/kg diet) for each dietary group			
Antioxidant	1	2	3	4
Vitamin E	25	25	25	25
Selenium	0.3	0.3	0.3	0.3
β-carotene	0	15	45	135
Coenzyme Q_{10}	0	10	30	90
Ascorbic acid palmitate	0	33.3	100	300
Trolox	0	16.7	50	150
Acetyl cysteine	0	66.7	200	600
Coenzyme Q_0	0	33.3	100	300
Catechin	0	33.3	100	300

wavelength range 500–640 nm. Four layers of Parafilm representing turbidity were used as background to blank the spectrophotometer and to subtract some of the absorbance due to the turbidity of the tissues. The cell was sealed by a microscope cover glass and mounted on the center of the window of the spectrophotometer as close as possible to the photoreceptor to reduce light scattering caused by the tissue. The sample was scanned from 500–640 nm, and the absorbance versus wavelength at 5 nm intervals was automatically recorded by the scan program in the spectrophotometer.[7]

Analysis of absorbance spectra of heme proteins of liver and heart tissue slices with HPSAP

HPSAP is a spreadsheet program written with Lotus 123 (Lotus Development Corp., Cambridge, MA USA) that contains visible spectra of individual heme proteins from 500–640 nm. To determine concentrations of heme proteins, the absorbance versus wavelength (at 5 nm intervals) obtained from spectrophotometrically scanning (500–640 nm) the tissue was entered into the HPSAP. HPSAP calculates a theoretical spectrum based on the assumption that heme compounds closely obey the Lambert–Beer law. A quantitative determination of the different heme compounds in each sample was achieved by regression analysis, followed by successive approximations with least-squares criterion for minimizing the differences between the experimental and theoretical spectrum. The details of the development and application of HPSAP in tissue slice model systems have been described in previous studies.[2,3,8]

Statistical analysis

All the analyses were done in duplicate. Means of the duplicates were calculated, and results were expressed as means and standard deviations of the six animals in each group.

The statistical package SAS (SAS Institute Inc., Cary, NC USA) was used to analyze the experimental data. When significant F values were obtained using ANOVA, Duncan's LSD procedure was used to determine significant differences ($P < 0.05$) between treatment means.

Results

Spectra and oxidation of liver slices

Examples of absorbance spectra of liver and heart both experimental and calculated with HPSAP, are shown in *Figure 1*. Results of the HPSAP analysis of these spectra are presented in *Table 2*. *Figure 1L1* presents spectra of fresh and oxidized liver slices obtained from a rat fed diet 1 (smallest dosage of dietary antioxidants). The two spectra presented in *Figure 1L4* were obtained from a rat fed diet 4 (highest dosage of dietary antioxidants). In a fresh liver slice, the major heme proteins are hemoglobin, oxyhemoglobin, reduced mitochondrial cytochromes, and reduced microsomal cytochromes. As the tissues oxidize, the peaks in their visual spectra become more blunt, as some oxyhemoglobin and hemoglobin are oxidatively converted to methemoglobin and hemichrome while some reduced mitochondrial and microsomal cytochromes are converted to oxidized mitochondrial and microsomal cytochromes.

HPSAP analysis *(Table 2)* of the spectra recorded after 3 hr of incubation calculated 40% spontaneous oxidation in liver slices from a rat fed diet 1 *(Figure 1L1)* and only 25% spontaneous oxidation in liver slices obtained after 3 hr of incubation from a rat fed diet 4 *(Figure 1L4)*. This indicates a strong protective effect of diet 4 against oxidative damage.

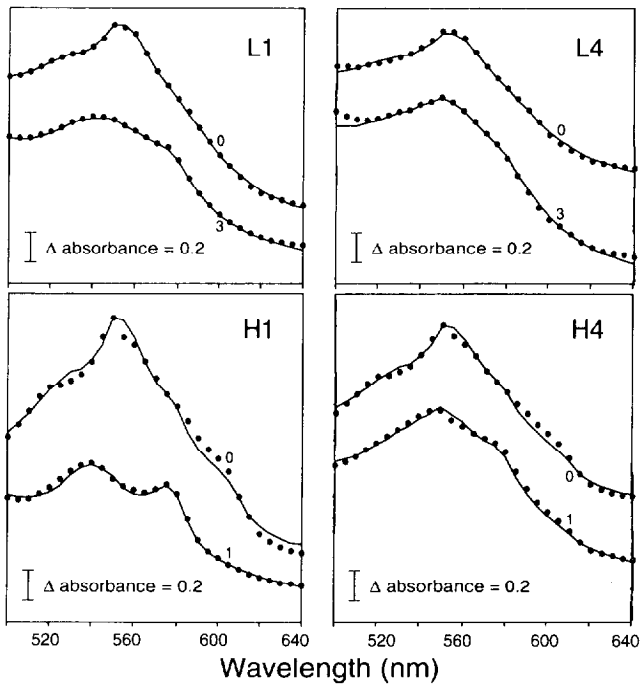

Figure 1 Visible spectra of heme proteins in liver (L) and heart (H) tissue slices incubated at 37°C; 0 hr (0); 1 hr (1); 3 hr (3); ···, experimental spectrum; ———, calculated spectrum. Liver and heart slices were from rats fed diet #1 (L1 and H1) or diet #4 (L4 and H4). The spectra are stacked, and the ordinate scale is given as Δ absorbance = 0.2.

In *Figure 2* the average percentage of oxidized heme proteins in liver tissue slices was plotted as a function of incubation time for all four groups of rats. Oxidation increased with incubation time. There was a distinct lag phase for up to 30 min of incubation. The dietary effect was highly significant ($P < 0.0001$). Percent oxidation decreased as the dosage of the antioxidants in the diets increased. Diet 1 provided the least protection, while diets 3 and 4 were the most effective in protecting against oxidative damage.

Figure 3 indicates that increasing the kinds and total amounts of antioxidants from diet 1 to diet 2 in liver markedly decreased the formation of oxidized heme proteins. The higher amounts of antioxidants in diet 3 further increased protection against oxidative damage, but the difference between diets 2 and 3 was not as pronounced as that between diets 1 and 2. The difference in protection provided by diet 4 compared with diet 3 was not significant at the 5% confidence level.

It is noteworthy that in liver slices diets containing all nine antioxidants (diets 2, 3, and 4) resulted in 0% hemeprotein oxidation levels after incubation for 30 min, while diet 1 (containing only vitamin E and Se) resulted in 6% hemeprotein oxidation levels after incubation for 30 min.

Table 2 Analysis with HPSAP of spectra obtained from oxidation of tissue slices incubated at 37°C

	Diet #1		Diet #4	
	0 hr	3 hr	0 hr	3 hr
Heme proteins of liver (mol%)				
Oxyhemoglobin	4	16	2	7
Hemoglobin	66	39	72	58
Methemoglobin	0	4	0	5
Hemichrome	0	18	0	4
Reduced mitochondrial cytochromes	18	3	15	7
Oxidized mitochondrial cytochromes	0	10	0	9
Reduced microsomal cytochromes	12	2	11	3
Oxidized microsomal cytochromes	0	8	0	7
Total oxidized heme proteins (mol%)	**0**	**40**	**0**	**25**
Sum of squares	0.003	0.001	0.004	0.01
	0 hr	1 hr	0 hr	1 hr
Heme proteins of heart (mol%)				
Oxyhemoglobin	11	48	10	18
Hemoglobin	54	20	61	52
Reduced mitochondrial cytochromes	35	0	29	15
Oxidized mitochondrial cytochromes	0	32	0	15
Total oxidized proteins (mol%)	**0**	**32**	**0**	**15**
Sum of squares	0.04	0.005	0.02	0.01

Spectra and oxidation of heart slices

Figures 1H1 and *1H4* present spectra of fresh and oxidized heart tissue slices obtained from rats fed diet 1 and diet 4, respectively. Spectral changes were seen in the heart tissue slices, apparently due to the formation of oxidized heme proteins. HPSAP analysis *(Table 2)* revealed 32% spontaneous oxidation for heart slices from a rat fed diet 1 and incubated for 1 hr *(Figure 1H1)* and only 15% spontaneous oxidation for heart slices obtained from a rat fed diet 4 and incubated for 1 hr *(Figure 1H4)*. Thus, the addition of all nine antioxidants (diet 4) provided more protection than just vitamin E and Se (diet 1) in heart tissue slices.

A plot of the average percentage of oxidized heme proteins in heart tissue slices versus incubation time for all four groups of rats is shown in *Figure 4*. Hemeprotein oxidation levels rose with increasing incubation time; and the reaction was exponential, with oxidation rising markedly between 0 and 2 hr and plateauing after 2 hr. In contrast to the liver tissue slices, there was no visible lag phase for the heart tissue slices. For heart tissue slices, the dietary effect was also very significant ($P < 0.0001$), with maximum protection offered by diet 4 (highest dosage of antioxidants). Hemeprotein oxidation levels were always more elevated in heart tissue slices than in liver, probably due to the higher percentage of mitochondrial cytochromes in heart tissue. Apparently, mitochondrial cytochromes oxidize more readily than hemoglobin. The presence of more mitochondrial cytochromes in heart than liver may also explain the oxidation recorded in heart slices after 30 min of incubation for groups 2, 3, and 4. In the liver there was no oxidation at 30 min incubation for these same groups.

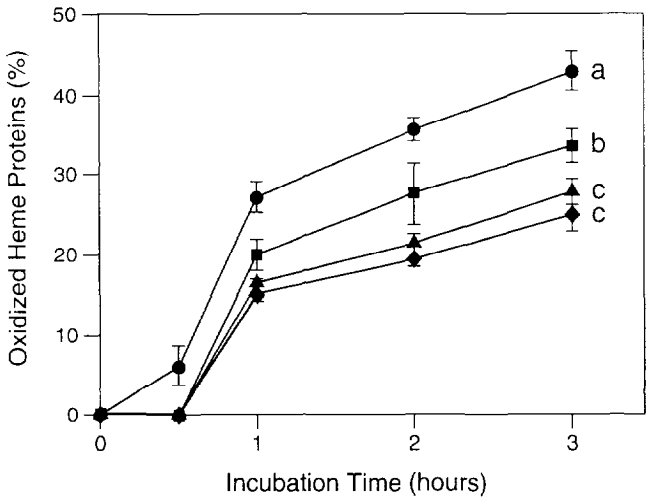

Figure 2 Production of oxidized heme proteins in mol% during oxidative reactions in liver tissue slices; ●, diet 1; ■, diet 2; ▲, diet 3; ◆, diet 4; for composition of each diet see *Table 1*. Tissue slices were incubated at 37°C. The values are expressed as mean ± SD for six rats. The curves marked with different lowercase letters are significantly different from each other at a 95% confidence level. The percent of oxidized heme proteins are denoted in alphabetical order with "a" being the largest and "c" being the smallest.

Figure 3 is a further analytical representation of the substantial protection against heme protein oxidation provided by diets 2, 3, and 4 containing all nine antioxidants over that of diet 1 containing only vitamin E and Se in heart tissue slices.

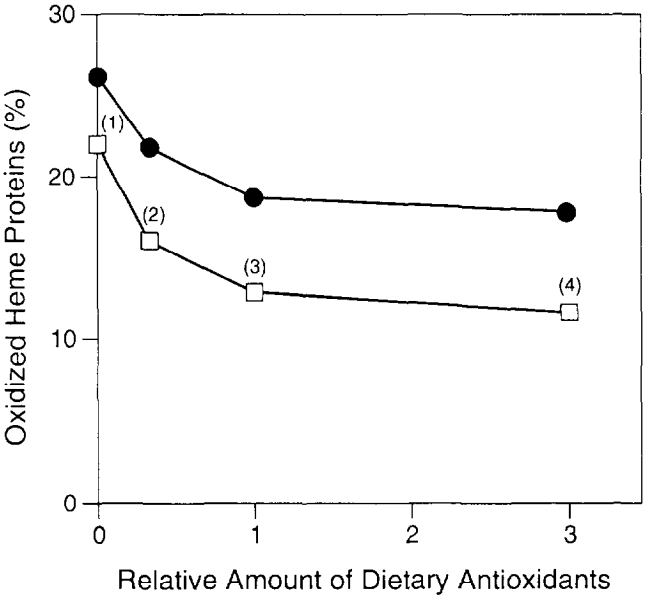

Figure 3 Average mol% oxidized heme proteins in liver tissue slices, □; and heart tissue slices, ●; versus relative amount of antioxidants in diets (1), (2), (3), and (4).

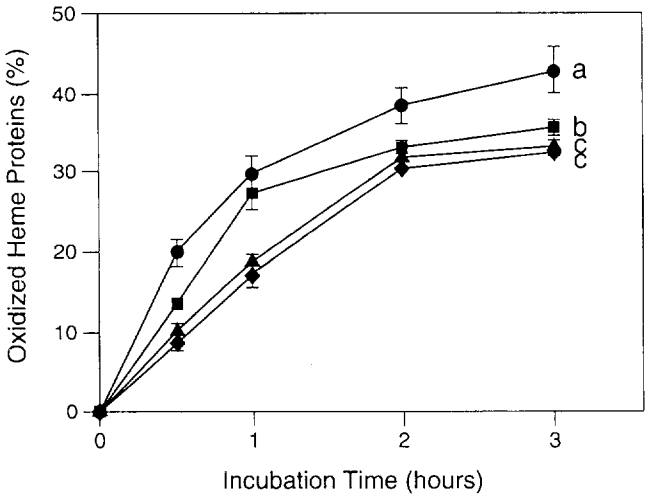

Figure 4 Production of oxidized heme proteins in mol% during oxidative reactions in heart tissue slices. Legend same as *Figure 2.*

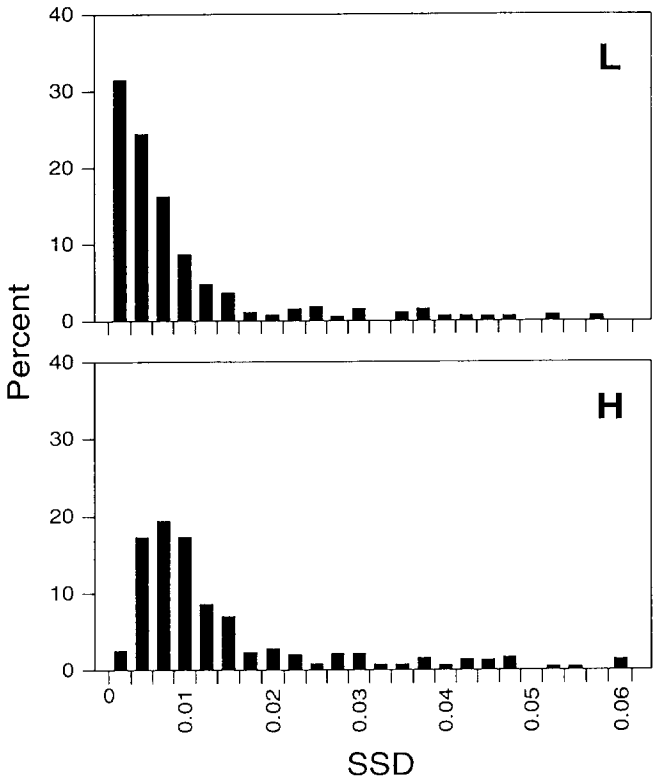

Figure 5 Frequency distribution of the sum of squared differences (SSD) between the experimental and calculated spectra for liver tissue slices (L); and heart tissue slices (H).

Evaluation of calculated spectra

The sum of squared differences (SSD) was used to evaluate the reliability of HPSAP in analyzing heme proteins in a tissue slice system by reconciling the experimental spectrum (recorded by the spectrophotometer) and the calculated spectrum (determined with HPSAP). The SSD is calculated as follows: SSD = Σ (calculated absorbance − experimental absorbance)^2. The best fit is indicated as the SSD approaches zero.

The SSD was calculated for all 480 spectra (240 liver tissue slice spectra and 240 heart tissue slice spectra). SSD values ranged from 0.00019 to 0.15. Frequency plots of the distribution of the liver SSD and the heart SSD are shown in *Figure 5*. Both SSD distributions were skewed to the left. Therefore, the median seemed more appropriate than the mean to describe the central tendency of the distributions.

For the liver SSD distribution the mode was 0.0025 and the median was 0.005. For liver, 80% of the SSD were less than 0.01 and 90% were less than 0.018. The fits of the liver spectra were generally excellent.

For the heart tissue spectra the mode was 0.0075, and the median was 0.01. For heart, 80% of the SSD were below 0.023, and 90% were below 0.043. The fits of the heart tissue spectra were generally very good. The accuracy of available information on the cytochrome composition of rat heart mitochondria may presently limit the accuracy of the heart tissue calculated spectra.

Discussion

To investigate biological oxidative processes, it is often desirable to quantify damage to proteins, lipids, and other cellular components. Existing methods of quantitation measure mostly secondary irreversible oxidative products. The HPSAP method developed in our laboratory can measure both reversible and irreversible damage to heme proteins using visible light spectroscopy and Lotus 123 spreadsheet programming. A combination of regression analysis and successive approximations shows particular strength in the quantitation of heme proteins in biological samples. The merits of applying HPSAP to tissue slices are discussed in our previous studies.[2,3,8] HPSAP has several other unique features. Using a tissue slice model to study the effects of oxidative stress has the advantage of being as close to the in vivo situation as possible. Hemoglobin and myoglobin, which have identical spectra and cannot be differentiated, are present in most major animal organs and tissues and are highly subject to spontaneous oxidation or extrinsic oxidants. Myoglobin, particularly in heart muscle, may be one of the best indicators of physiological oxidative reactions. Myoglobin is situated close to the mitochondria where various oxidants are produced. Thus, oxidative products of heme proteins can be considered as indexes for the quantification of oxidative damage, measuring both reversible and irreversible damage. The relative protective capabilities of nutrient and other antioxidants against oxidation can also be studied.

This research involving multiantioxidants is appropriate. These antioxidants were chosen for experimental study because there is a considerable amount of biochemical knowledge about each of them in the scientific literature: vitamin E, selenium, ascorbic acid palmitate, acetylcysteine, and coenzyme Q[9]; trolox[10]; β-carotene[11,12]; and (+)-catechin.[7,13] Previous studies have analyzed the deposition in various tissues following dietary supplementation by vitamin E and selenium,[14] coenzyme Q,[15,16] and β-caro-

tene.[17] Acetylcysteine may act as a reducing agent to regenerate vitamin E.[18,19]

We have previously shown that dietary supplements of vitamin E, β-carotene, coenzyme Q_{10}, and selenium protect against lipid peroxidation in rat tissue slices.[14] We have made quantitative simulation models of these protective effects.[20] Results of the present studies using multiantioxidants are necessarily complex with limited mechanistic interpretations, but they provide valuable empirical data.

Our results indicated that the diets supplemented with all nine antioxidants provided greater protection against oxidative damage to hemeproteins than an adequate diet supplemented with only vitamin E and Se (*Figures 2, 3,* and *4*). The enhanced protection can be attributed to three conditions present in the system. Both fat- and water-soluble antioxidants were supplemented in the diets. The fat-soluble antioxidants used were vitamin E, β-carotene, coenzyme Q_{10}, and ascorbic acid palmitate. The more hydrophilic antioxidants in the study included trolox, acetylcysteine, coenzyme Q_0, and catechin. Both lipophilic and aqueous oxygen reactive species are responsible for oxidative damage of heme proteins, even though the oxidative reaction is mainly carried out in the aqueous phase of the cell. Therefore, it is important to have both fat- and water-soluble antioxidants in the cellular antioxidant defense systems.[8]

The presence of different categories of antioxidants augmented the antioxidative protection provided by the system. Vitamin E, β-carotene, coenzymes Q_{10} and Q_0, and catechin are chain breakers or free radical scavengers. Ascorbic acid palmitate and acetylcysteine are reducing agents which can inactivate free radicals or regenerate oxidized free radical scavengers. Catechin, ascorbic acid palmitate, and trolox are sequestering agents that retard oxidation by binding reactive metal ions. A fourth category of antioxidants help in the catabolism of hydroperoxides which are strong initiators of free radical reactions. Se is an essential constituent of the enzyme glutathione peroxidase in its reduction of hydrogen peroxide.

The synergistic effect between vitamin E and vitamin C is well known. Ascorbic acid, glutathione and probably acetylcysteine can regenerate vitamin E by reducing the tocopheroxyl radical to tocopherol. Thus synergism among the various antioxidants can enhance the antioxidant protection of the diets supplemented with all nine antioxidants.

Diets 3 and 4 were not significantly different in providing protection to hemeproteins against oxidative damage, even though diet 4 contained three times the concentration of antioxidants in diet 3. Diet 3 may have reached the concentrations of antioxidants offering maximum protection to heme proteins against spontaneous oxidation.

It is appropriate to consider the differences between liver and heart tissue slice spectra. One of the most striking differences between heart and liver spectra was the presence of greater amounts of mitochondrial cytochromes in the heart compared with the liver. Heart tissue consists mostly of muscle, which needs larger amounts of mitochondria to supply the demand for energy.

Another major difference in the comparison of liver and heart spectra was the amount of microsomal cytochromes, which accounted for 10 to 12% of the heme proteins found in liver tissue slices.

Higher amounts of oxyhemoglobin were formed in the heart tissue slices, and heart tissue slices oxidized more rapidly than liver tissue slices. Reactions in the respiratory chain may explain the high levels of oxyhemoglobin and oxidized cytochromes in heart tissue. The respiratory chain in heart tissue includes substrates such as NADH, NADPH, dehydrogenases, cytochromes b, c, c1, and aa3, and coenzyme Q which are oxidized sequentially in order to ultimately reduce oxygen to water. If early irreversible oxidative damage occurred in the respiratory chain, (e.g., damage to the dehydrogenases), reduction of the cytochromes in the chain would be inhibited. Therefore, oxygen would accumulate and could combine with hemoglobin in the tissue-forming oxyhemoglobin. Perhaps the substrates and the intermediates involved in the respiratory chain are better protected in the liver than in the heart tissue.

In conclusion, multiantioxidant dietary supplements provided better protection against oxidative damage to both liver and heart tissue slices than diets supplemented with only vitamin E and Se. The presence of both fat- and water-soluble antioxidants, with different mechanisms of action and with synergistic effects between antioxidants contributed to this result.

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