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Inhibition of Oxidative Injury of Biological Membranes by Astaxanthin

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Abstract: The value of astaxanthin, a carotenoid pigment, in the treatment of oxidative injury is assessed. Astaxanthin protects the mitochondria of vitamin E-deficient rats from damage by Fe²⁺-catalyzed lipid peroxidation both *in vivo* and *in vitro*. The inhibitory effect of astaxanthin on mitochondrial lipid peroxidation is stronger than that of α -tocopherol. Thin layer chromatographic analysis shows that the change in phospholipid components of erythrocytes from vitamin E-deficient rats induced by Fe²⁺ and Fe³⁺-xanthine/xanthine oxidase system was significantly suppressed by astaxanthin. Carrageenan-induced inflammation of the paw is also significantly inhibited by administration of astaxanthin. These data indicate that astaxanthin functions as a potent antioxidant both *in vivo* and *in vitro*.

REACTIVE OXYGENS are produced by various enzymatic and nonenzymatic processes in living organisms. Under pathological conditions they may induce peroxidation of polyunsaturated fatty acids in biological membranes leading to functional impairment (1,2). Such oxygen toxicity has been postulated to underlie the pathogenesis of several diseases such as postischemic reflow injury (3, 4), retinopathy in premature infants (5), shock (6) and cerebral infarction (7). For example, paraquat (8) and adriamycin (9) also catalyze the formation of reactive oxygens and result in pulmonary fibrosis and cardiomyopathy, respectively. Furthermore, both activated neutrophils and macrophages produce active oxygen and significant fractions of these metabolites are released extracellularly, and induce various inflammatory responses (10, 11).

Superoxide has been assumed to be a primary source for other reactive oxygens, such as H₂O₂ and \cdot OH (1). Intracellular compartments are highly enriched with anti-oxidants such as superoxide dismutase, catalase, glutathion peroxidase, and glutathion. Thus, reactive oxygens are efficiently detoxicated (12). However, in extracellular space (13, 14) such as

plasma, concentrations of these enzymes and scavengers are low. High levels of extracellularly released superoxide cannot therefore be dismutated efficiently enough to protect cell membranes from oxygen toxicity.

Astaxanthin (AX) is a carotenoid pigment (Fig. 1) found in many animals and plants (15), and the compound can be purified in large quantity from yeast, *Phaffia rhodozyma* Miller (16). This compound may serve as an efficient and safe antioxidant. However, relatively little attention has been brought to the action of this compound. During the course of our studies on the protective action of various drugs against the impediment of biological membrane by reactive oxygens, we have found by using vitamin E deficient rats that astaxanthin protects biological membranes from hazardous oxygen species *in vivo* and *in vitro*.

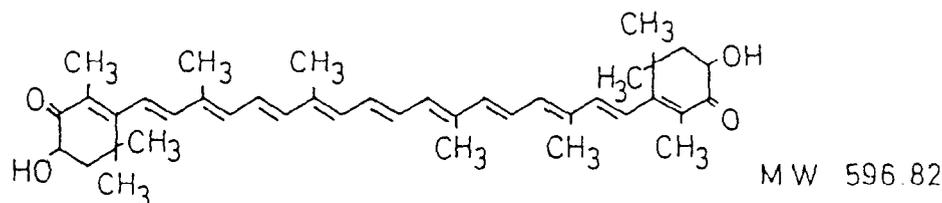


FIGURE 1. Chemical structure of Astaxanthin. Astaxanthin (AX) (3,3'-dihydroxy b,b-carotene-4,4'-dione), a carotenoid and a precursor of astacin.

Materials and Methods

Chemicals: Adenosine diphosphate (ADP), carrageenan (type IV), thiobarbituric acid (TBA), vitamin E (VE) and xanthine were purchased from Sigma Chemicals (St. Louis). Xanthine oxidase was obtained from Boehringer Mannheim Co. (West Germany). Astaxanthin was kindly donated by Suntory Co. (Osaka). Vitamin E deficient diet was obtained from Oriental Yeast Co. (Tokyo). Other chemicals were obtained from nacal tesque Co. (Kyoto). For *in vitro* experiments, AX was dissolved in dimethyl sulfoxide.

Rats: Vitamin E deficient rats were prepared according to the method of Machilin *et al.* (17). Three groups (10 rats for each group) of male Wistar rats (6 to 7 weeks of age) were fed for 2–4 months. Each group had a different diet, normal (group 1), vitamin E-free (group 2) and vitamin E-free plus astaxanthin (containing 1 mg astaxanthin/100 g) (group 3).

Mitochondria and erythrocyte ghosts: Liver mitochondria were isolated from each group of animals according to a modification of Hogeboom and Schneider (18), and erythrocyte ghosts were prepared by the method of Dodge *et al.* (19).

Mitochondrial functions: Oxidative phosphorylation and respiratory control index (the ratio of phosphorylative accelerated respiration expressed by state 3 to the substrate level respiration expressed by state 4) were measured by a Clark-type oxygen electrode (20). Mitochondria were incubated in a medium containing 0.15 M KCl-10 mM Tris-HCl buffer (pH 7.4) to eliminate the inhibitory effect of sucrose on lipid peroxidation by Fe^{2+} and thiobarbituric acid reaction (21, 22).

Lipid peroxidation and lipid analysis: Erythrocyte ghosts were prepared according to the method of Blight and Dyer (22). Fe^{2+} -induced lipid peroxidation of the ghosts was assayed by thiobarbituric acid reaction (21). Lipid peroxidation *in vitro* was induced by Fe^{2+}

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and xanthine/xanthine oxidase system. Membrane phospholipids were analyzed by thin layer chromatography.

Protein content: Membrane protein was determined according to Lowry's method using bovine serum albumin as a standard (23).

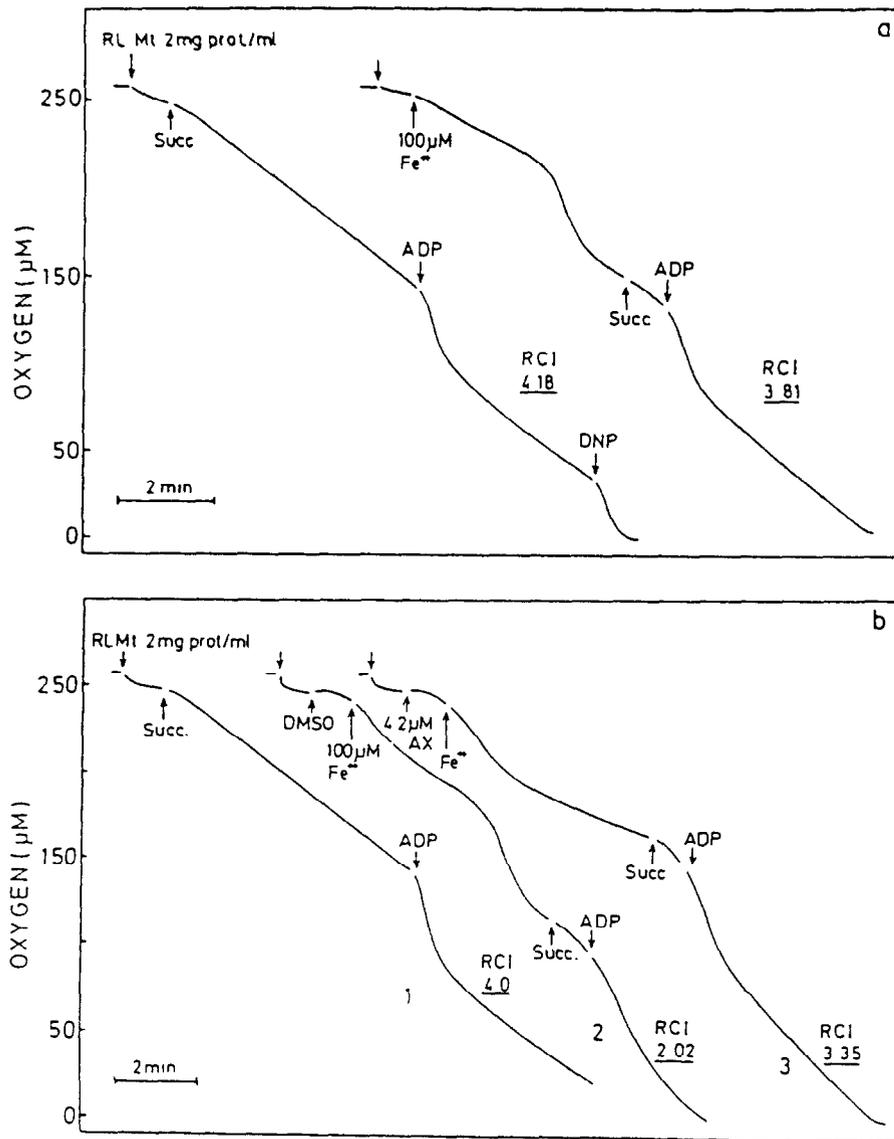


FIGURE 2. Protection by astaxanthin against Fe²⁺-induced dysfunction of rat liver mitochondria. Liver mitochondria were isolated from normal rats and animals fed a vitamin E-deficient diet for two months. The isolated samples were washed twice with a medium containing 0.15 M KCl and 10 mM Tris-HCl buffer (pH 7.4). Respiratory activity of mitochondria was assayed with a Clark type oxygen electrode in a medium containing 0.15 M KCl, 3 mM MgCl₂ and 5 mM potassium phosphate buffer (pH 7.4) at 25°C. Respiratory control index of untreated rat liver mitochondria was 4.18 (trace 1 in a), and that of vitamin E-deficient rat liver mitochondria was 4.0 under succinate as respiratory substrate (trace 1 in b). Dimethyl sulfoxide, a carrier for astaxanthin was added after adding 4.2 μM astaxanthin, and Fe²⁺-induced changes were observed (trace 3 in b). RL Mt, rat liver mitochondria; RCI, respiratory control index; DMSO, 5 μl/ml dimethyl sulfoxide; Succ, 1 mM sodium succinate; DNP, 25 μM dinitrophenol; ADP, 150 μM ADP; Fe²⁺, 100 μM Fe²⁺.

Carrageenan-induced inflammation: Saline solutions (1 ml) containing either vitamin E or astaxanthin were injected intraperitoneally 30 min before carrageenan treatment. Effect of astaxanthin on carrageenan-induced edema was examined by measuring the volume changes of rat paws after subcutaneous injection of carrageenan (24, 25). Anesthetized rats were given subcutaneous injections of 0.2 ml saline solution into the left paw and the same volume of saline solution containing 10 mg carrageenan into the right paw. Change in paw volume was measured hourly three times after the treatment.

Results and Discussion

Effect of astaxanthin on Fe^{2+} -induced changes in mitochondrial functions of vitamin E deficient rats. Figure 2a shows the effect of Fe^{2+} on the respiratory activity of mitochondria from intact rats. Addition of 10–100 μM Fe^{2+} to the reaction medium enhanced oxygen consumption of mitochondria and slightly decreased respiratory control index (RCI; state 3/ state 4). The mitochondria from vitamin E deficient rats (group 2) revealed RCI of 4.0 and ADP/O ratio 2.0; these values are within normal levels with succinate as a respiratory substrate (trace 1 in Fig. 2b). However, when Fe^{2+} was added to the medium, the rate of oxygen consumption increased and respiratory control index (RCI) decreased markedly (trace 2 in Fig. 2b). When astaxanthin was added prior to the treatment, the Fe^{2+} -induced increase in oxygen consumption and the decrease in respiratory

Table I
Effect of Fe^{2+} and astaxanthin on the oxidative phosphorylation of mitochondria from vitamin E deficient rats.

Fe^{++} (μM)	A (nM)	RCI	(% Decrease)	ADP/O Ratio	(%)
0	0	4.00	(0)	2.00	(100)
10	0	3.37	(21)	2.04	(102)
40	0	2.92	(36)	2.02	(101)
100	0	2.44	(52)	1.98	(99)
100	45	2.64	(45)	2.04	(102)
100	420	3.21	(26)	1.85	(93)
100	4200	3.35	(22)	1.77	(89)

Experimental conditions were the same as in Figure 2. Activities of mitochondrial oxidative-phosphorylation were expressed in two characteristics, respiratory control index (RCI) and ADP/O ratio. Numbers in parentheses represent % of the control values obtained from mitochondria which were not treated with Fe^{2+} and astaxanthin.

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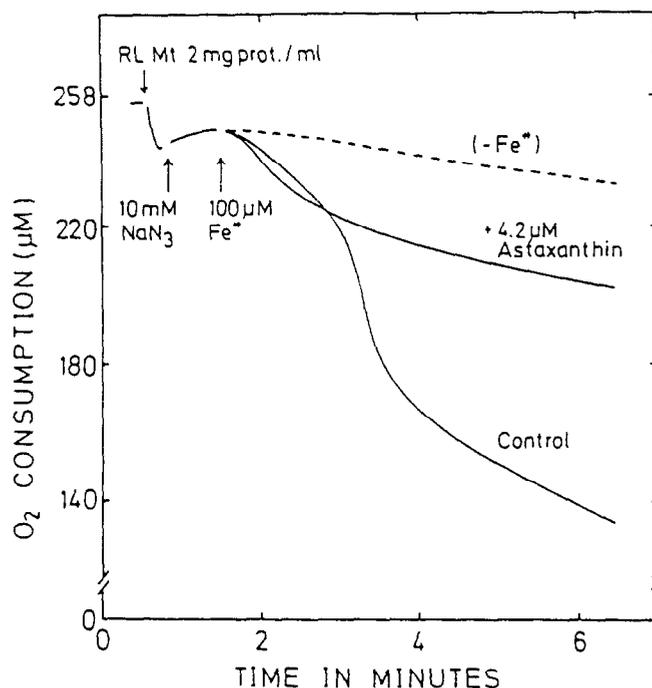


FIGURE 3. Enhancement of NaN_3 -insensitive oxygen consumption by Fe^{2+} and its suppression by astaxanthin. Mitochondria were isolated from normal rat liver (group 1). Astaxanthin ($4.2 \mu\text{M}$) was added prior to each experiment. Other conditions were the same as in Figure 2.

control index were inhibited significantly (Fig. 2b, trace 3). Both the hazardous effect of Fe^{2+} and the protective effect of astaxanthin depended on their concentrations (Table I).

Figure 3 represents the enhancement of the rate of NaN_3 -independent oxygen consumption by Fe^{2+} . Since NaN_3 inhibits electron transport of mitochondria, the Fe^{2+} -induced increase in oxygen consumption would have occurred independently from the mitochondrial electron transport system.

Since mitochondrial membranes are enriched with polyunsaturated fatty acids, the increased oxygen consumption might reflect the Fe^{2+} -induced lipid peroxidation. Consistent with this notion is the fact that the Fe^{2+} -induced increase in oxygen consumption was inhibited by astaxanthin that has a polyunsaturated carbon chain.

Mitochondrial function of the astaxanthin-administered vitamin E deficient rats. To test whether astaxanthin also protects mitochondrial function *in vivo*, the mitochondrial Fe^{2+} -induced lipid peroxidation was investigated in vitamin E deficient rats with or without astaxanthin administration. Figure 4 shows the effect of $100 \mu\text{M}$ Fe^{2+} on the RCI values of mitochondria isolated from different groups of rats.

The respiratory control index of mitochondria from group 1 rats was slightly inhibited by Fe^{2+} -induced lipid peroxidation; no remarkable disorders of mitochondrial function, such as oxygen consumption, were induced. Thus the defense mechanism against free radicals seems to function in control group (group 1). On the other hand, mitochondria from vitamin E deficient rats (group 2) showed a remarkable decrease in respiratory control index by Fe^{2+} . The deterioration in respiratory control index was inhibited significantly by treating animals with

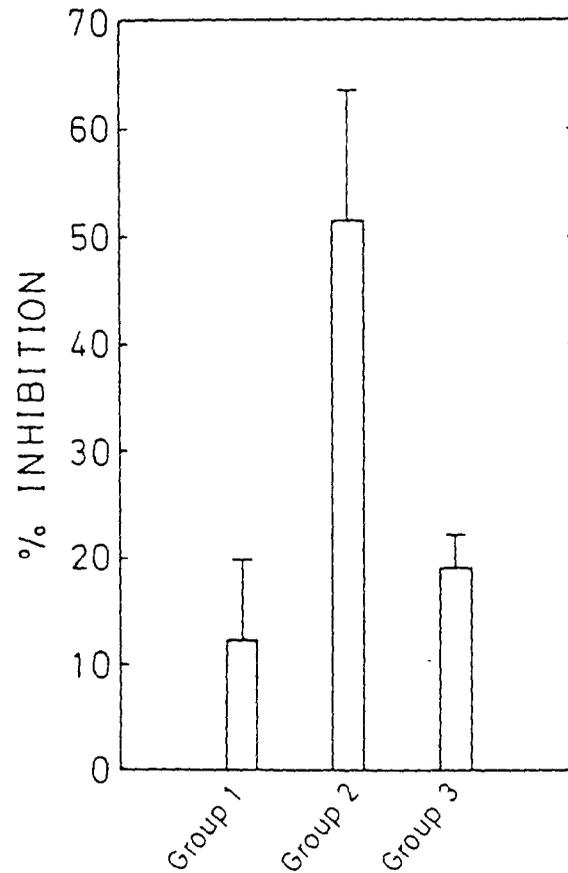


FIGURE 4. Effect of Fe^{2+} on the respiratory control index of rat liver mitochondria fed under different conditions. Animals were divided into three groups and fed different diets; group 1 fed normally, group 2 fed vitamin E-deficient (Oriental Yeast Co.) and molecular distilled corn oil, and group 3 fed vitamin E-deficient plus 1mg/100g astaxanthin. Rat liver mitochondria were prepared from each group of animals. Other conditions were the same as in Figure 2. Data show the effect of $100 \mu\text{M}$ Fe^{2+} on the respiratory control index of mitochondria from 3 groups. Each value is a mean + SD of five experiments.

astaxanthin (group 3). ADP/O ratio, another indicator for mitochondrial function, was not affected remarkably by Fe^{2+} -induced lipid peroxidation in any animal groups.

Lipid peroxidation by active oxygens of erythrocyte membranes from vitamin E deficient rats and its inhibition by astaxanthin administration. To confirm that the decrease in mitochondrial function of vitamin E deficient rats by Fe^{2+} might reflect the astaxanthin-inhibitable lipid peroxidation, changes in TBA-reactive metabolites were determined in erythrocyte ghosts from three animal groups. Figure 5 shows the formation of thiobarbituric acid (TBA)-reactants by Fe^{3+} and superoxide generated by xanthine/xan-

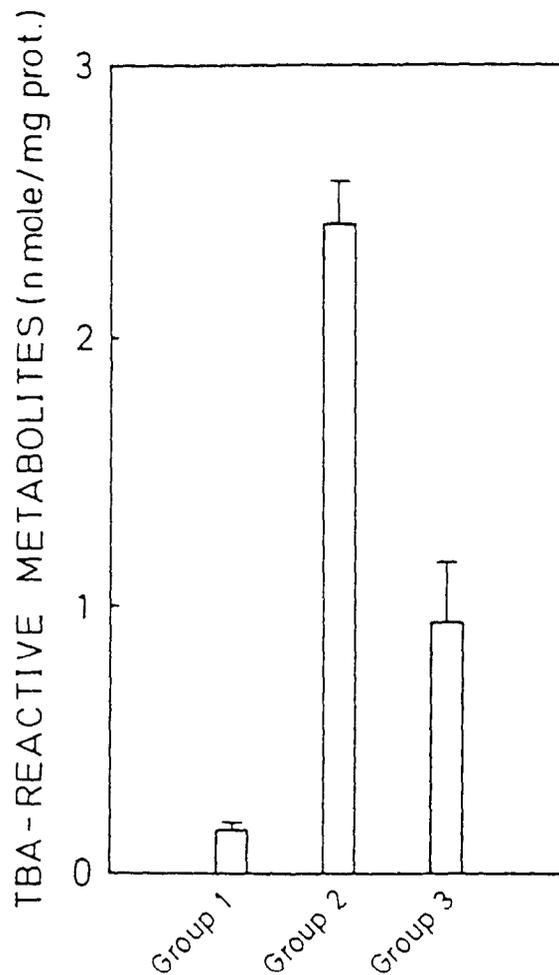


FIGURE 5. Lipid peroxidation of erythrocyte membranes from animals fed under different conditions. Erythrocyte ghosts were prepared from 3 animal groups following the method of Dodge *et al.*(19). Ghosts were suspended in a medium containing 20 mM Tris-HCl buffer (pH 7.5) 1 mg protein/ml. Lipid peroxidation of the membranes was induced by 50 μ M FeCl₃ and the xanthine (200 μ M)/xanthine oxidase (0.01 IU/ml) system for the production of \cdot OH. After incubation at 37°C, thiobarbituric acid (TBA)-reactive metabolites were determined by spectrophotometer. Each value is a mean + SD of five experiments.

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thine oxidase system. Control group represented a low level of thiobarbituric acid-reactants even after incubation for 210 min under oxidative stress. On the other hand, the amount of thiobarbituric acid-reactants increased markedly in group 2. In erythrocyte membranes from astaxanthin-administrated group the formation of thiobarbituric acid-reactants was inhibited significantly. Thus the administration of astaxanthin *in vivo* might inhibit the lipid peroxidation of erythrocyte membranes from VEDR under oxidative stress *in vitro*.

Effect of astaxanthin and vitamin E on the Fe^{2+} -induced lipid peroxidation of mitochondria obtained from vitamin E deficient rats. As described above, the administered astaxanthin protected the mitochondrial function of vitamin E deficient rats from being impaired by reactive oxygens. Since vitamin E has been used as a potent antioxidant, the effect of astaxanthin on lipid peroxidation of mitochondrial membrane was compared with that of vitamin E. Figure 6 represents the inhibitory action of astaxanthin and vitamin E on the formation of thiobarbituric acid-reactants. This inhibitory effect of astaxanthin being 100 to 500 times stronger than that of vitamin E.

To get further insight into the mechanism by which astaxanthin inhibited the Fe^{2+} catalyzed lipid peroxidation, the change in lipid components of erythrocyte membrane was determined in three groups. Figure 7 shows thin layer chromatographic profiles of erythrocyte membrane phospholipids. After incubation of erythrocyte membranes with Fe^{3+} and xanthine/xanthine oxidase system for 210 min, the formation of lipid peroxides markedly increased in the vitamin E-deficient group (group 2) but not in control group when detected when smears between each spot for the identified lipids in thin layer chromatography, suggesting the promotion of lipid peroxidation. Under identical conditions, addition

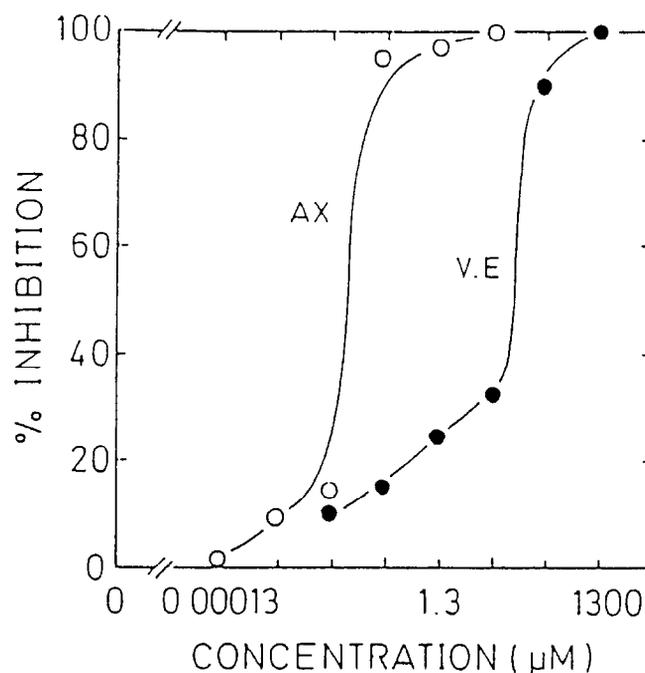


FIGURE 6. Effect of astaxanthin and vitamin E on Fe^{2+} -induced lipid peroxidation. Intact mitochondria (2 mg protein/ml) prepared under the same conditions as in Figure 2, and washed three times with 0.15 M KCl and 10 mM Tris-HCl buffer solution (pH 7.4) at 0°C . The washed mitochondria were preincubated for one min with various concentrations of astaxanthin or vitamin E at 37°C . After incubation with $100\ \mu\text{M}$ Fe^{2+} (Mohr's salt) at 37°C for one hour, level of thiobarbituric acid-reactants was measured. Data were expressed as % inhibition of thiobarbituric acid-reactant formation.

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of 4.2 μM astaxanthin or 420 μM vitamin E to the incubation mixtures inhibited the peroxidative changes considerably; the thin layer chromatographic profiles of the astaxanthin- and vitamin E-treated groups were almost the same as those for the control membrane. In the experiment with astaxanthin-administered vitamin E deficient rats (group 3, lane 10), the changes in the thin layer chromatographic profile induced by Fe²⁺ was not remarkable when compared with that from group 2.

Effect of astaxanthin on carrageenan-induced paw edema in the rat. As described above, astaxanthin showed a protective effect against the membrane impediments by reac-

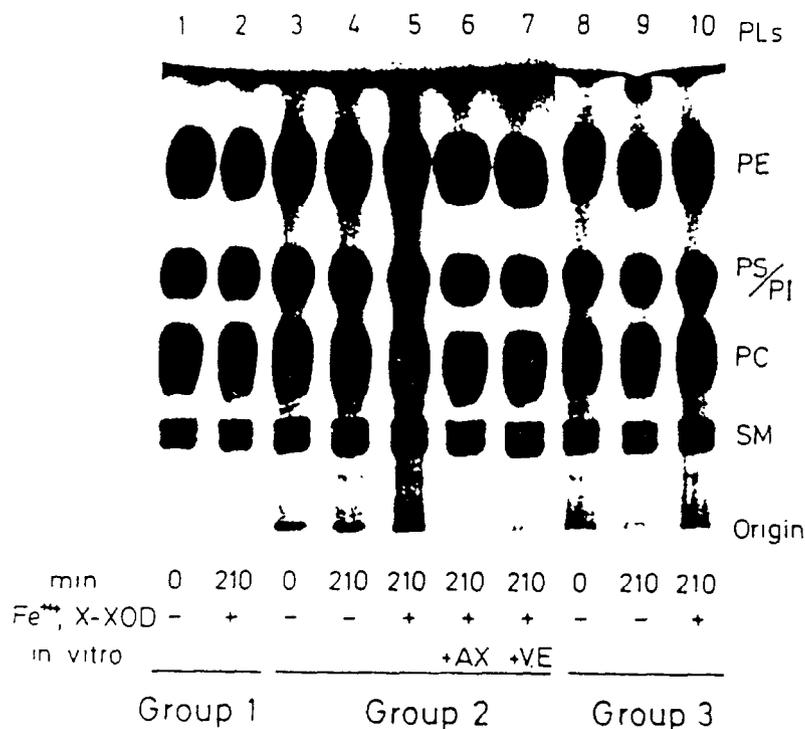


FIGURE 7. Effect of reactive oxygens on the phospholipid in erythrocyte membranes. After incubation for 210 minutes under identical conditions as in Figure 6, membrane lipids were extracted following the method of Blight and Dyer (22), and subjected to thin layer chromatographic analysis using a developer composed of chloroform/methanol/acetic acid/distilled water (100/60/16/8: v/v) and colored with 2% phosphorus molybdate. In the pattern of the vitamin E deficient ghosts (lanes 3, 4 and 5), some unknown lipid complexes appeared with the progress of peroxidation. The formation of these unknown lipid complexes was reduced in the ghost of group 3 (lanes 8, 9 and 10). Conversely, no such changes were seen with the membrane treated with 4.2 μM astaxanthin or 420 μM vitamin E *in vitro* (lanes 6 and 7). Added concentrations of Fe³⁺, xanthine, and xanthine oxidase were 50 μM , 0.2 mM, and 0.025 unit/ml, respectively.

PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PI, phosphatidylinositol; PC, phosphatidylcholine; X-XOD, xanthine-xanthine oxidase system; AX, astaxanthin; V.E, vitamin E; in vitro, added *in vitro*; Group 1, normal diet; Group 2, vitamin E-free diet; Group 3, vitamin E-free and astaxanthin containing diet.

tive oxygens and its effective concentration was significantly lower than that of vitamin E. To test whether such a potent protective action can also be seen in inflammatory tissues, we tested the effect of astaxanthin and vitamin E on carrageenan-induced paw edema in which reactive oxygens play a phlogogenic role (27). As shown in Figure 8, carrageenan markedly increased the paw volume by an astaxanthin-inhibitable mechanism. Under identical conditions, vitamin E failed to show such anti-inflammatory action. The mechanism by which astaxanthin inhibited the carrageenan-induced paw edema is not clear at present. In this context, the carrageenan-induced inflammation is highly sensitive

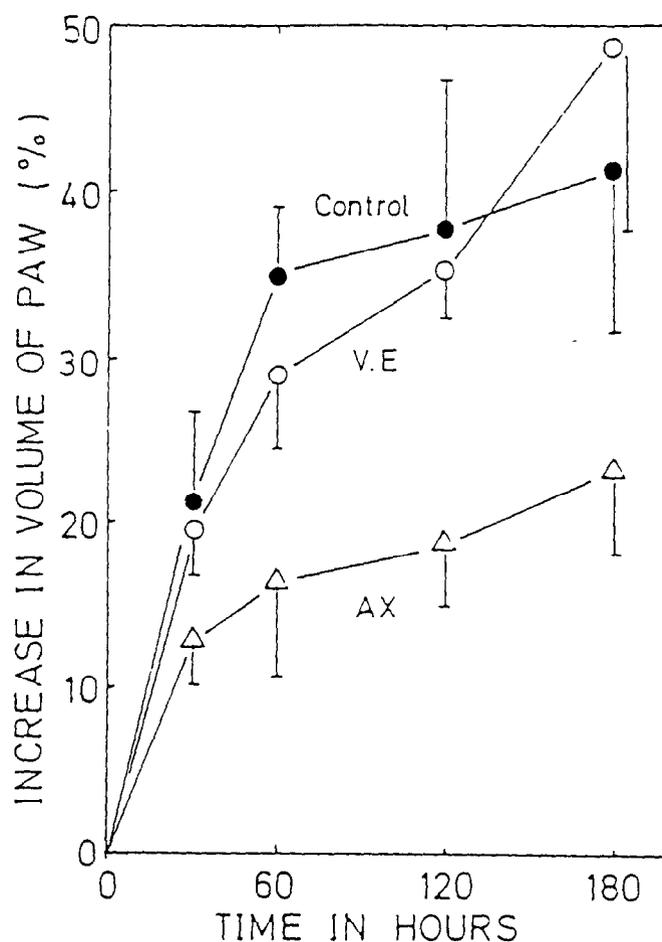


FIGURE 8. Effect of antioxidants on carrageenan-induced inflammation. Under light ether anesthesia, animals were subcutaneously injected with 0.2 ml saline solution in the left paw. The same volume of carrageenan solution (10 mg/0.2 ml) was administered in the right paw, and the change in paw volume was measured at the indicated times. The control group was treated with an intraperitoneal injection of 1 ml of saline solution (Control), and the experimental group was administered the same volume of saline solution containing 1 mg vitamin E (V. E) or astaxanthin (AX) 30 min prior to the experiment [26]. Each point is a mean \pm SD of five experiments.

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to inhibitors for phospholipase A₂ (25). Thus, the results do not rule out a possible contribution of the inhibitory activity of astaxanthin on phospholipase A₂. However, the pharmacological effects of astaxanthin make this antioxidant a valuable model for the development of novel anti-inflammatory agents for therapeutic use.

At present, the mechanism of protective action of astaxanthin against active oxygen induced membrane damage has not been clarified. It is known that carotene, having a polyunsaturated carbon chain like that of astaxanthin, has a higher reactivity with singlet oxygen (28–31) than does vitamin E (32). Therefore, it is possible that astaxanthin can also quench the active oxygen. However, further work is needed to fully understand the mechanism of antioxidant and anti-inflammatory actions of astaxanthin.

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