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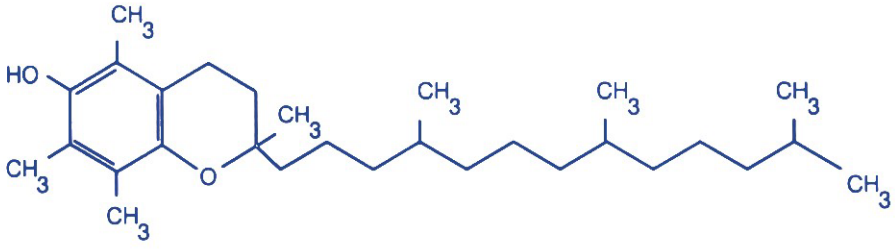
Molecular and Cell Biology Updates

Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications

Edited by

A.S.H. Ong

L. Packer



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Preface

Antioxidants inhibit the formation and spread of free radicals which can be damaging in biological systems. Free radicals form in biological systems through metabolism, but it is also realized that exogenous environmental sources, such as radiation, food, and drugs, contribute significantly to the generation of free radicals in biological systems. Being reactive species, free radicals are short-lived and do not travel far from cellular targets. Their concentration in biological systems is very low and is difficult to detect directly by electron spin resonance spectroscopy (ESR). Indirect methods of reactions of radicals with specific biomolecules are also sufficiently sensitive to detect quantitatively their presence. Thus the response of antioxidant defenses which react with radical species, can serve as an indirect measure that free radicals have been formed. Redox-based antioxidants change their oxidation state and antioxidants become free radicals themselves. Often, however, the antioxidants give rise to more persistent free radicals, sometimes owing to delocalization of the lone electron around ring structures (in vitamin E, ubiquinones, and certain carotenes). Persistent free radicals react only rarely and the precursors often can be regenerated in biological systems.

In recent years, it is becoming clearer from biochemical studies on how the major lipophilic antioxidants work. Particular attention has been given to vitamin E and quinones found in animal and plant membranes and in carotenoids, for the protection of membranes in lipoprotein systems. Flavonoids form another rich and varied source of natural antioxidants.

A distinction is also becoming clearer between the physiological and pharmacological usefulness of antioxidants for health benefits and for the treatment of disorders related to free radical generation in cells and tissues. Studies in this area are now reaching a point where many clinical interventions are being tested with antioxidants for the treatment of acute, degenerative and chronic diseases to verify evidence of their health benefits.

Since the major antioxidants are associated with dietary habits, some aspects of nutrition are closely linked to the presence and importance of antioxidants. In particular,

for lipophilic antioxidants, the nutritional effects of dietary fats and oils are very significant.

There have been many new developments occurring that make us confident that a volume highlighting some of the major lipophilic antioxidants, in terms of their biochemistry and applications for biomedical use, should prove of value for the biological community.

The stimulus for developing this volume came from a very successful conference sponsored by UNESCO's global Network on Molecular and Cell Biology (MCBN) and COSTAM, the Confederation of Scientific and Technological Associations in Malaysia, 19-22 September, 1991 in Penang, Malaysia, co-sponsored by ASTA Medica, the Council for Tobacco Research U.S.A., Eisai Company, the Federation of Asian Oceanic Biochemists (FAOB), Federation of Asian Scientific Societies and Academies (FASAS), Forschungarat Rauchen und Gesundheit (Research Council on Smoking and Health), Henkel Corporation, Hoffman La Roche, International Society of Free Radical Research (SFRR), International Union of Biochemistry (IUB), International Union of Pure and Applied Biophysics (IUPAB), Malaysian Palm Oil Promotion Council (MPOPC), Palm Oil Research Institute of Malaysia (PORIM), Pharma Stroschein GmbH, and University of Science Malaysia (USM), to whom we are grateful.

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Augustine S.H. Ong

Lester Packer

Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

NEW HORIZONS IN VITAMIN E RESEARCH - THE VITAMIN E CYCLE, BIOCHEMISTRY, AND CLINICAL APPLICATIONS

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Summary.

Probably most diseases at some point during their course involve free radical reactions in tissue injury. In some cases, free radical reactions may be involved in multiple sites and at different stages of a chronic disease. So, both acute and degenerative diseases are thought to involve free radical reactions in tissue injury. An overview will be given of the evidence for the occurrence of free radicals and the importance of antioxidant interventions with particular reference to the lipophilic antioxidant vitamin E (tocopherols and tocotrienols).

Introduction

Vitamin E is the name given to a group of naturally occurring lipid soluble antioxidants, the tocopherols and the tocotrienols, that are found naturally in certain plant oils. Since the discovery of vitamin E in Berkeley by H.M. Evans in 1922, when it was first described as an anti-sterility agent, many scientists and physicians have sought to elucidate its biochemistry, health benefits and clinical applications.

The Vitamin E Paradoxes

Vitamin E is the major, if not the only, chain-breaking antioxidant in membranes, but its membrane concentration is very low, usually equal or less than 0.05 - 0.1 nmoles/mg of protein (less than 1 per 1000 - 2000 membrane phospholipids). Yet the rate of lipid radical generation in membranes can be very high, about 1 - 5 nmoles/mg protein/min. Nevertheless, under normal conditions "rancidification," that is oxidation of membrane lipids and proteins, does not occur. Moreover, it is very difficult to render animals deficient in vitamin E, and vitamin E deficiency is seldom found in adult humans. Hence, there must exist a remarkably efficient mechanism for permitting low concentrations of vitamin E to have such high efficiency in protecting membranes against damage and in supporting normal biological activity (1).

The Vitamin E Cycle

We hypothesize that vitamin E acts catalytically, being efficiently reduced from its free radical form, its form after quenching radicals, back to its native state (2).

This catalysis occurs through the interactions between water and lipid soluble substances by both non-enzymatic and enzymatic mechanisms, which regenerate vitamin E from its radical (tocotrienoxyl and tocopheroxyl radical back to tocotrienol and tocopherol, respectively) (3). Under conditions where these auxiliary systems act synergistically to keep the steady state concentration of vitamin E radicals low, the loss or consumption of vitamin E is prevented (4-10).

The thioctic acid/dihydrolipoic acid couple (TA/DHLA) is a unique antioxidant system (4). Normally covalently-bound lipoamide exists in small amounts as the cofactor of α-ketodehydrogenases in animals. However, larger amounts of fed TA confers protection in tissues and membranes against oxidative damage. After absorption, TA may be reduced enzymatically or nonenzymatically to form an active antioxidant. It acts as a "double-edged sword," in that it appears to interact directly with the membrane to reduce tocopheroxyl radicals (weak effect) or to reduce ascorbate, which in turn acts at membranes to reduce tocopheroxyl radicals (stronger effect). Thus, the TA/DHLA couple

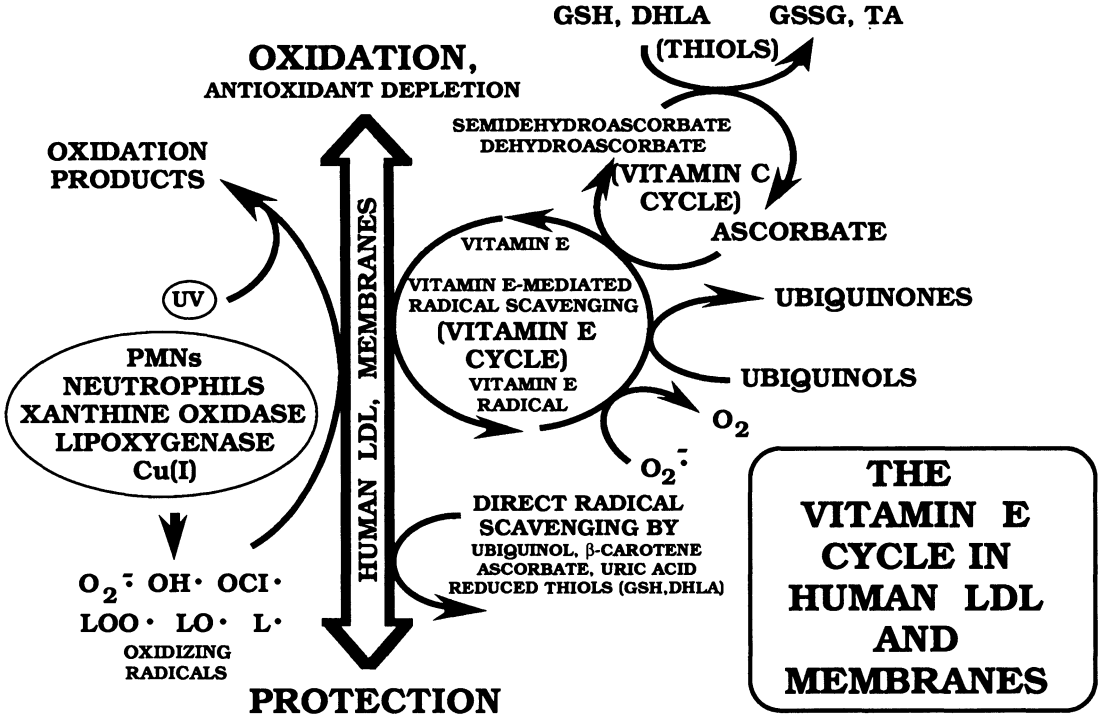

Figure 1: The vitamin E cycle-synergistic action of water- and lipid-soluble antioxidants. Non-enzymatic and enzymatic mechanisms for the regeneration of chromanoxyl radicals.

works in recycling vitamin E both in membranes and in low density lipoproteins (LDL), where it acts to stabilize them. The interactions between these antioxidants in membranes and LDL are shown in Figure 1. Thus, vitamin E will only be lost when these backup systems, either in the aqueous or membrane domains, become rate-limiting. At this point, the loss of vitamin E will be accompanied by increased rates of lipid and protein oxidation, destruction of membrane function, and inactivation of membrane enzymes and receptors. Thus, vitamin E not only has an antioxidant action, but also acts as a biological response modifier. By modulating membrane-associated enzyme systems, vitamin E markedly affects the production of small molecular weight substances, like secondary messengers and products of the arachidonic acid cascade, which have profound effects at low concentrations in cell regulation and proliferation (1).

Oxidative damage will be minimized while vitamin E is still present. The concentrations of other antioxidants that act in the cytosol to quench radical reactions or that donate their reducing power to vitamin E may also fluctuate up and down. Membrane associated redox couples, such as ubiquinol/ubiquinones and oxidized/reduced cytochrome c, can also lower the steady state concentrations of vitamin E radicals. Fluctuations in their concentrations, however, are not as important as changes in the concentration of vitamin E. The index of potential damage is high only when vitamin E is gone.

Amplifying the Vitamin E Message--Regulatory Effects of Vitamin E

Intracellular Signaling and Secondary Messenger Formation.

Protein kinase C (PKC), which is very important in intracellular signalling, may be down-regulated by vitamin E, but not by its esters (11,12). This would have the effect of inhibiting cell proliferation. These results may be relevant to the anti-cancer effects of vitamin E.

Production of Cell Mediators by the Arachidonic Acid Cascade.

Phospholipase A2 preferentially hydrolyzes peroxidized fatty acid esters of phospholipid membranes. Since lipid peroxides activate phospholipase A2, a decrease in

lipid peroxides by vitamin E will decrease phospholipase A2 activity (13,14). Vitamin E thereby modulates arachidonic acid release from membrane phospholipids and therefore arachidonic acid metabolism.

Vitamin E also inhibits plant and mammalian 5- and 15-lipoxygenases (15,16). Fatty acid hydroperoxides necessary for activation of lipoxygenase can overcome the inhibition. Finally, prostaglandin and HETE (hydroxyeicosatetraenoic acid) production from arachidonic acid in bovine seminal vesicles and kidney was inhibited by α-tocopherol; HETE production was inhibited less than that of prostaglandin. Thus, vitamin E seems to influence both the cyclooxygenase and lipoxygenase pathways; this modulation of arachidonic acid oxidation may have important in vivo implications.

Hypocholesterolemic Effects.

Tocotrienols, the form of vitamin E with an unsaturated isoprenoid side chain, can serve as powerful hypocholesterolemic agents. The γ-form is most active. These effects are not exhibited by the corresponding tocopherols. Tocotrienol may well act by inhibiting HMG CoA reductase activity (18), the rate limiting step in the biosynthesis of cholesterol.

Anticancer Effects.

Mevalonate-derived intermediates of sterol biosynthesis, including tocotrienols from red palm oil, suppress neoplastic growth (19). Increased linoleic acid content in fat diets also suppresses tumor growth. The growth of several types of transplantable tumors is also inhibited by tocotrienols, and particularly by the gamma isomer (20).

Thus, modulation of tumor growth rate by substances with polyunsaturated chains have potential anticancer activity. The extent to which these effects are mediated through actions on prostanoid formation or on HMG CoA reductase activity is still unclear.

Role of Vitamin E in Degenerative and Coronary Artery Disease

Several epidemiological investigations in recent years have provided strong circumstantial evidence for beneficial effects of vitamin E in degenerative and coronary heart disease.

Ischemic Heart Disease (IHD) Mortality.

The World Health Organization/Monica core study (27), carried out with 20 populations in Europe, is the most recent, extensive, and best controlled epidemiological investigation thus far conducted on risk factors involved in death from ischemic heart disease. In this study, a combination of classical risk factors accounted for only 20% of IHD mortality. A good correlation was found between elevated plasma cholesterol and IHD mortality in 12 populations, but not in 8 others. Of various measured parameters, the best correlation was found between low plasma concentrations of vitamins E and A and increased IHD mortality (27).

IHD mortality was highest in the extreme northern countries, e.g. Scandinavia, moderate in middle European countries, and lowest in the countries of southern Europe bordering the Mediterranean region. A seven-fold higher IHD was found in populations in the north as compared to the south. IHD mortality was inversely related to the amount of yellow and green vegetables in the diet of these European populations. When the data from the 12 cholesterol sensitive populations cited above were adjusted for cholesterol and lipid standardized factors, a lower risk to IHD mortality was still significantly correlated with higher plasma levels of vitamins E and A.

Angina Pectoris.

In a recent epidemiological study (28), the hypothesis was tested that plasma concentrations of antioxidant vitamins might be related to the risk of angina. Classic risk factors for coronary heart disease that were evaluated included age, habitual smoking, blood pressure, lipid composition, relative body weight, and also seasonal trends in vitamin and antioxidant concentrations in plasma samples from 6000 men aged 35 to 54. Without adjustment for the various risk factors, plasma concentrations of vitamin E, vitamin C and carotenoids were inversely related to an increased risk of angina pectoris. However, after adjustment for the various risk parameters, only plasma vitamin E remained as a significant factor. The adjusted odds ratio for angina between the lowest and highest quantities for vitamin E was 2.68 ($P = 0.02$). Thus, populations with a high incidence of coronary heart disease might well benefit from diets rich in vitamin E.

Coronary Artery Disease (CAD)--Atherosclerosis.

Cholesterol-rich low density lipoproteins are clearly involved in the multistep process of CAD. Many years ago, atherosclerotic lesions were shown to occur in rabbits fed diets high in cholesterol. Feeding with antioxidants, such as vitamin E, reduced the occurrence of these lesions. Polyunsaturated fatty acids, which are susceptible to lipid peroxidation, can form free radicals that can injure the endothelium, damage heart muscle cells, and provoke proliferation of smooth muscle. These processes are inhibited by vitamins C and E.

When polyunsaturated fatty acids, cholesterol and/or apoprotein B in low density lipoproteins (LDL) are oxidatively modified, the damaged LDL are phagocytized by macrophages. Extensive uptake of damaged LDL by macrophages results in the formation of foam cells. Foam cells accumulate under the unbroken layer of endothelial cells where they aggregate to form a fatty streak. The fatty streak serves as a nucleus for the development of the atherosclerotic plaque. Higher vitamin E concentrations would be expected to retard all of these processes. Thus, the multifactorial actions of vitamin E, not only as an antioxidant but also as a biological response modifier, account for the high correlation between low plasma levels of vitamin E and the high risk of CAD.

The Benefits of Vitamin E in Other Chronic Diseases and in Aging

Aging.

Deposits resulting from free radical reactions accumulate in tissue during the aging process. Lipid peroxidation may be an important factor in provoking premature aging.

Cancer.

Reactive oxygen species have been implicated in the process of cancer initiation and promotion. Vitamin E and the other antioxidants can function as anticarcinogens by quenching free radicals or reacting with their products. Epidemiological studies indicate

that vitamin E, alone or in combination with other antioxidants, is associated with a decreased incidence of certain forms of cancer.

Skin cancer is both the most common form of human cancer and the most common malignant cancer. Populations exposed to high levels of ultraviolet irradiation show an increased incidence of malignant and non-malignant melanoma. Alarmingly, the incidence of this disease has doubled in the United States in the past decade. Because antioxidants retard the multi-step process of carcinogenesis, molecular damage due to ultraviolet irradiation may well be reduced by vitamin E.

Anticancer agents that generate reactive oxygen species, such as adriamycin, damage normal tissue. Thus in patients treated with adriamycin (doxorubicin), vitamin E may be beneficial in protecting the skin, the heart, and other tissues.

Arthritis.

Increased free radical production has been observed in animal and human studies on arthritis. Vitamin E therapy is effective in relieving pain and in improving mobility in patients with osteoarthritis.

Circulatory Conditions.

Excessive platelet aggregation speeds the development of atherosclerosis. Vitamin E supplementation significantly decreases platelet aggregation in healthy adults and reduces elevated platelet aggregation rates in patients with high blood lipid levels and in oral contraceptive users. In patients with coronary artery disease who undergo bypass surgery, pretreatment with vitamin E reduces the elevated free radical concentrations in the plasma that usually occur.

Cataracts.

Photooxidative mechanisms and reactive oxygen species are important in cataractogenesis. Vitamin E delays or minimizes cataract development in isolated animal lenses. High plasma antioxidant concentrations correlate with reduced cataract risk in adults.

Exercise.

Strenuous physical exercise is associated with an increased rate of lipid and protein oxidation and of vitamin E consumption. Mountain climbers who were given oral doses of 400 IU/day vitamin E showed improved physical performance and decreased breath pentane output during prolonged exposure to high altitudes.

Air Pollution.

Vitamin E is an important component of the lung's defense against the injurious effects of smog, smoke, and smoking.

Relative Activities of Tocopherols and Tocotrienols

d-α-Tocopherol and d-α-tocotrienol, two forms of vitamin E, are natural membrane antioxidants. Both have the same aromatic chromanol "head," but d-α-tocopherol has a saturated whereas d-α-tocotrienol has an unsaturated polyisoprenoid "tail."

In membranes, d-α-tocotrienol shows 40- to 60-times higher antioxidant potency than d-α-tocopherol due to: (i) higher recycling efficiency from chromanoxyl radicals, (ii) more uniform membrane bilayer distribution, and (iii) better interaction of chromanols with lipid radicals (29). Thus, d-α-tocotrienol may have higher physiological activity than d-α-tocopherol under conditions of oxidative stress.

Both d-α-tocopherol and d-α-tocotrienol protect human LDL against oxidative modification induced by lipoxygenase, UV-irradiation, or peroxyl radicals initiated by azo dyes. As revealed by electron spin resonance spectroscopy, chromanoxyl radicals of both d-α-tocopherol and d-α-tocotrienol are formed in oxidatively stressed LDL. In the presence of ascorbate, the recycling efficiency for d-α-tocotrienol was higher than for d-α-tocopherol.

In myocardial ischemia-reperfusion studies (30) palm oil vitamin E (POE), (a gift of A. Gapor, PORIM) which contains 45% d-α-tocopherol and 55% d-α-tocotrienol, conferred protection to rat hearts against ischemia-reperfusion injury. After 40 min. of

ischemia, the hearts from animals fed POE for 45 days recovered 90% of the control value of their mechanical activity (contractility), whereas hearts from the control group recovered only 17% of the initial level.

Lipid and protein oxidation in the reperfused heart was greatly reduced by POE treatment. During the reperfusion period, more d-α-tocotrienol (79%) than d-α-tocopherol (59%) was consumed. d-α-Tocotrienol also demonstrated higher in vitro NADH-, NADPH-, succinate-, and ascorbate-dependent recycling efficiency from chromanoxyl radicals in heart membranes (mitochondria and microsomes) than d-α-tocopherol.

Thus, d-α-tocotrienol clearly is highly efficacious in protecting myocardial membranes against oxidation in the course of ischemia-reperfusion injury.

New Horizons in Vitamin E Research: Requirements for Vitamin E

The Vitamin E Paradox.

How do we reconcile the great efficiency of minute quantities of vitamin E in membranes with the beneficial effects of vitamin E supplementation on human health seen in chronic diseases and acute clinical conditions?

A local deficiency of vitamin E can arise rapidly in membranes under conditions of intense oxidative stress. Under these conditions, molecular damage to lipids, proteins, and nucleic acids can be expected. Examples of such acute situations are ischemia-reperfusion injury, e.g. myocardial infarction or stroke and subsequent reoxygenation, hemorrhagic and other forms of shock, exposure to extremes of environmental pollution or irradiation, or treatment with antineoplastic drugs.

The replenishment of vitamin E in depleted membranes often requires days to weeks, even in tissues in which vitamin E turnover is rapid. Thus, dietary supplementation with vitamin E during or after an episode of acute stress may prevent tissue injury.

Because water-soluble antioxidants that recycle vitamin E can be introduced in a matter of minutes, the effective concentration of the residual vitamin E in membranes or

lipoproteins, thereby may be rapidly increased. Similarly, water-soluble recyclers or water-soluble forms of vitamin E such as vitamin E phosphate or vitamin E succinate, may also be more effective than vitamin E itself. Thus, the concept of vitamin E recycling may prove to be crucial in the development of new strategies for the treatment of acute conditions involving oxidative damage.

Biokinetics and Tissue Absorption of Vitamin E.

The biokinetics and tissue absorption of different forms of vitamin E in humans has not been well studied. On the basis of animal studies, however, fast and slow turnover tissues have been identified. Furthermore, certain tissues, such as adipose tissue, the liver and the adrenal gland, accumulate vitamin E.

Dietary vitamin E is transported on chylomicra from the intestine to the liver and other tissues. In rat liver, an α-tocopherol binding protein has been identified, however its specificity for the different tocopherols, i.e. alpha, beta, gamma, and delta, and for the corresponding tocotrienols is unknown. Whether this binding protein is involved in the incorporation of vitamin E into lipoproteins is also unclear. In human plasma, tocopherols tend to be associated with phospholipid-rich lipoproteins, whereas tocotrienols are primarily found in triglyceride-rich lipoproteins (K.C. Hayes, personal communication). Thus, different pathways must exist for the incorporation of these two forms of vitamin E into lipoproteins. In regard to tissue uptake, adipose tissue becomes enriched in tocotrienols, while most other tissues contain more α-tocopherol than α-tocotrienol. Thus specificity is also expressed at the interface between plasma lipoproteins, probably including chylomicra, and various tissues.

In our laboratory, rats fed with large supplements of POE with roughly equivalent concentrations of tocopherol and tocotrienol accumulate both tocopherols and tocotrienols in many tissues, different skeletal muscle fibers and liver. Future biokinetic and tissue absorption studies in both humans and animals should allow us to define better the biological functions and actions of tocopherol and tocotrienol isomers.

Vitamin E Requirement.

Health status, lifestyle, diet, and environment markedly influence the requirements for vitamin E. Although demonstrable vitamin E inadequacy in apparently healthy adults is rare, vitamin E requirements may vary fivefold in individuals, depending on the dietary intake of polyunsaturated fat, tissue composition, the steady state concentrations of other interactive antioxidants in tissues, and genetic factors.

Free radical-mediated damage has been implicated in cellular and extracellular changes that occur over time in the aging process and in development of chronic diseases. Vitamin E and other antioxidants prevent or minimize oxidative damage in biological systems. How adequate the antioxidant defense should be to protect the body from excessive free radical concentrations is one of the many new horizons for vitamin E research.

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Table 1. Health Benefits and Clinical Applications of Vitamin E

Clearly Defined Need	
Malabsorption	Cholestatic liver disease, cystic fibrosis, abetalipoproteinemia, celiac disease, sprue, pancreatitis
Familial deficiency	Genetic factors
Prolonged total parenteral nutrition	Environmental factors
Some Evidence, Not Completely Accepted	
Premature infants	Reduced risk of intraventricular hemorrhage and reduced severity of retrolental fibroplasia
Intermittent claudication	Increased mobility and stronger exercise endurance
Peyronie's disease	Decreased fibrosis, pain
Hemolytic anemias	Sickle cell, G-6-P-D deficiency, Thalassemia
Other Indications	
Adult respiratory distress syndrome (ARDS)	Low plasma E levels in ARDS suggest a need for parenteral E administration, which may delay onset of acute respiratory failure (21)
Epilepsy	Vitamin E supplementation in addition to antiepileptic drug therapy resulted in reduction in seizures in a majority of epileptic children refractory to antiepileptic drugs (22)
Tardive dyskinesia	After vitamin E administration, scores on Abnormal Involuntary Movement Scale were lower in subjects with persistent tardive dyskinesia (23)

Table 1 (continuation). Health Benefits and Clinical Applications of Vitamin E

Cancer therapy	Radiotherapy. Plasma E and β-carotene levels were decreased in patients during radiotherapy prior to bone marrow transplantation. Antioxidant loss must be considered a possible cause of early post-transplant organ toxicity (24)
	Chemotherapy. Heart damage due to adriamycin therapy was decreased by vitamins A and E (25)
Burns	Vitamin E supplementation stimulated T-helper cells to near normal levels in patients after bum injury (20-64% of body surface area) (26)

Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

THE REACTIVITY OF TOCOTRIENOLS AND OTHER LIPID-SOLUBLE ANTIOXIDANTS TOWARDS PEROXYL RADICALS

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Summary

The relative reactivities of α- and γ-tocotrienol toward peroxyl radicals have been compared with those of α-T and other natural lipid soluble antioxidants by measuring their temporal disappearance when present in either homogeneous solutions or within human low density lipoprotein (LDL) previously enriched with α-tocotrienol. In homogeneous systems, the relative reactivities of the various antioxidants decreased in the following order: ubiquinol-10 > α-tocopherol ≈ α-tocotrienol > β-carotene ≈ lycopene > γ-tocopherol ≈ γ-tocotrienol. A similar sequence of consumption of antioxidants was also obtained when α-tocotrienol-enriched human LDL was exposed to peroxyl radicals. Under both conditions, α-tocotrienol reacted with peroxyl radicals as rapidly as α-tocopherol, though both forms of vitamin E were consumed after ubiquinol-10.

Introduction

The term vitamin E is a generic description for all tocols and tocotrienol derivatives which qualitatively exhibit the biological activity of α-tocopherol. The structure of tocotrienols differs from that of tocopherols only by the three unsaturated double bonds on the side chain of chromanol ring (Table I). While tocopherols and tocotrienols in general are present in vegetable oils, animal fats, grains, vegetables and

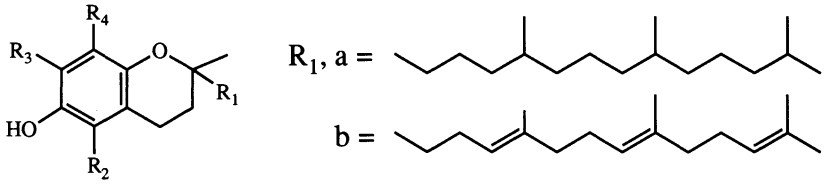
fruits (Slover, 1971), the main occurrence of tocotrienols is in palm, wheat germ, coconut and corn oil (Syvaoja et al., 1986).

Since its discovery in the 1920's the physiological effects of vitamin E have long been known and reported. The first notice of clinical use of vitamin E was as an antisterility agent as rats became fertile when wheat germ was added to the rancid lard in their diet (Evans, 1962) . Vitamin E deficiency has been associated with specific changes in neurotransmitters of the central nervous system, with decrements in cortical alpha-adrenergic binding sites and choline acetyl transferase (Chan et al., 1983). In both animals and humans, vitamin E deficiency has also been shown to be associated with an increase in aggregation of and prostaglandin synthesis by platelets *in vivo* (Stuart and Oski, 1979). Indeed, several reports have demonstrated a role for vitamin E as inhibitor of platelet aggregation (reviewed in Machlin, 1991). Perhaps more importantly, vitamin E, and α-tocopherol (α-T) in particular, possesses high antioxidant activities and is generally regarded as the most important lipid-soluble antioxidant in human blood plasma (Burton et al., 1983).

Whereas there are numerous reports on the biological activities of tocopherols there are only few such studies available for tocotrienols. Recent reports suggested that tocotrienol may have antitumor activity in mice (Kato et al., 1985), inhibit biosynthesis of cholesterol in chicken (Qureshi et al., 1986) and increase the concentration of high density lipoprotein (HDL)-cholesterol in rats (Imaizumi et al., 1990).

The antioxidant activities of α-tocotrienol (α-T-3) and α-T have been compared by Nakano et al. (1980). They observed that both antioxidants had the same protective effect on iron-induced formation of cholesterol-5α-hydroperoxide in liposomal or microsomal systems. Similar protective activities were observed by others using dilinoleyl phosphatidyl choline liposomes exposed to peroxyl radicals generated by the thermolabile initiator 2,2'-azobis(2-amidino propane)dihydrochloride (AAPH) (Yamaoka and Komiyama, 1989). Interestingly, these authors noted that α-T-3 had slightly higher activity than α-T when added after liposome formation. Serbinova et al (1991) made the remarkable claim that compared to α-T, α-T-3 possesses 40-60 fold higher antioxidant

Table I. Structure of tocotrienols and tocopherols



R_1	R_2	R_3	R_4	Vitamin E	Abbreviation
a	CH_3	CH_3	CH_3	α-tocopherol	α-T
a	CH_3	H	CH_3	β-tocopherol	β-T
a	H	CH_3	CH_3	γ-tocopherol	γ-T
a	H	H	CH_3	δ-tocopherol	δ-T
b	CH_3	CH_3	CH_3	α-tocotrienol	α-T-3
b	CH_3	H	CH_3	β-tocotrienol	β-T-3
b	H	CH_3	CH_3	γ-tocotrienol	γ-T-3
b	H	H	CH_3	δ-tocotrienol	δ-T-3

activity against (Fe^{2+} + ascorbate) and (Fe^{2+} + NADPH)-induced lipid peroxidation in rat liver microsomes.

In light of these conflicting reports we decided to further investigate the relative antioxidants reactivities of α-T and α-T-3, and to compare them with those of other natural lipid-soluble antioxidants. To do this we measured the temporal consumption of antioxidants within either homogeneous solution or human low density lipoprotein (LDL) when exposed to a steady flux of aqueous peroxyl radicals. The latter system was used since LDL is the major vehicle for transport of lipid-soluble antioxidants within the blood circulation and oxidative damage to LDL lipids and protein has been implicated as an early and important event in atherogenesis (Steinberg et al., 1989) . We conclude that, in these systems, the activity of α-T-3 is similar to that of α-T.

Materials and Method

Materials

Organic solvents of HPLC quality were purchased from Mallinckrodt, Hipersolv HPLC water from BDH, d-α-tocopherol and d-γ-tocopherol from KODAK, β-carotene (synthetic) from Sigma, and (all-E)-lycopene were gifts from H. Keller (Hoffmann-La Roche, Basel) . Ubiquinol-10 was prepared from ubiquinone-10 by reduction with sodium dithionite (Lang et al., 1986). 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN) and 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH) were obtained from Polyscience. α-T-3 and α-T-3 were isolated from VITEE capsules supplied by the Palm Oil Research Institute Malaysia (PORIM) using reversed (C18) and normal phase (silica) HPLC columns (SUPELCO; 10 x 250 mm; 5 μm particle size) and identified by their infra red, ultraviolet, mass and proton nuclear magnetic resonance spectra.

Oxidation of α-T-3 and Other Antioxidants in Homogeneous System.

A mixture of two antioxidants (100 μM each final concentration) in ethanol was incubated at 37°C for 3 min before AMVN was added (10 mM final concentration). AMVN, like AAPH, thermally decomposes to yield peroxyl radicals at a constant rate. Aliquots (20 μL) of the reaction mixture were removed at various times and analyzed for the antioxidants. Tocotrienols and tocopherols were detected simultaneously at 280 nm following separation on a C18 column equilibrated with 4% 50 mM sodium perchlorate in methanol (1.0 ml/min). Ubiquinol-10 was analyzed as described previously (Stocker et al., 1991).

Oxidation of α-T-3 Enriched Human LDL.

Enrichment of LDL with α-T-3 was carried out in principle as described for the enrichment of LDL with α-T (Esterbauer et al., 1991). LDL was isolated as described (Stocker et al., 1991). The isolated, α-T-3 enriched LDL (1.0-2.0 mg/ml) was incubated for 3 min at 37°C before AAPH (25 mM final concentration) was added to

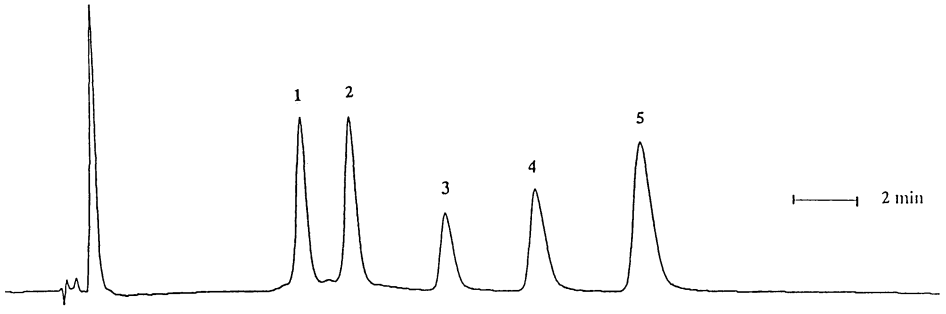


Figure 1. HPLC of Tocotrienols and Tocopherols Standards. HPLC Conditions LC-18 (25 x 0.46 cm) with guard column; electrochemical detection (+ 0.6 V); mobile phase, 4% 50 mm $NaClO_4$ in methanol at a flow rate 1.2 ml/min. Standard injected (10 μl) contained 100 pmol of each compound, γ-tocotrienol [1], α-tocotrienol [2], β-tocopherol [3], γ-tocopherol [4], and α-tocopherol [5].

initiate oxidation. At various time points aliquots of the solution were removed, extracted with aqueous methanol and hexane and the antioxidants present in the hexane phase analyzed as described (see above and Stocker et al., 1991). To obtain sufficient sensitivity for the detection of α-T-3, electrochemical (Fig. 1) rather than UV detection was used.

Results and Discussion

Homogenous System,

Since α-T has been reported to be the most important lipid-soluble antioxidant associated with human LDL (Esterbauer et al., 1991) we decided to use this form of vitamin E as a "reference" and to compare its reactivity with that of tocotrienols. Exposure of a mixture of α-T and α-T-3 to a steady flux of peroxyl radicals resulted in immediate and concomitant virtually linear disappearance of both forms of vitamin E with similar initial rates (Table II). When either α-T or α-T-3 was present alone at the

Table II. Reactivity of α-T and α-T-3 towards peroxyl radicals generated by AMVN in ethanol

Antioxidant	Rate of Consumption (μM/min)
α-T	0.41
α-T-3	0.39
α-T-3 alone	0.78

same concentration the rate of disappearance doubled (Table II) The obtained rates of antioxidant consumption are in good agreement with the expected rates of radical generation under our experimental conditions (Yamamoto et al., 1984) indicating that the antioxidants did not affect each other. In contrast to α-T-3, γ-T-3 failed to effectively compete with α-T for peroxyl radicals. Thus, under identical conditions as in Table II, but in the presence of γ-T-3 instead of α-T-3, initiation of the oxidation resulted in very rapid disappearance of α-T whereas γ-T-3 was initially "spared" from oxidation (Table III). Following complete consumption of α-T, the rate of γ-T-3 consumption increased significantly, to 0.18 μM/min. This value is low compared to α-T-3 alone (Table II), suggesting that compound(s) produced by the radical-mediated oxidation of γ-T-3 can themselves scavenge peroxyl radicals. A comparison of the relative reactivities of peroxyl radicals with different forms of vitamin E and-lycopene or β-carotene revealed that while α-T and α-T-3 were more reactive, α-T and γ-T-3 were at least partly spared from oxidation in the presence of either of the two carotenoids (data not shown).

From these results it may be concluded that in homogeneous systems the double bonds of the side chain of α-T-3 do not contribute to the antioxidant activity. In agreement, 2,2,5,7,8-pentamethyl-6-chromanol (PMHC), an analogue of α-T that totally lacks the phytyl side chain, has antioxidant activity similar to α-T against autoxidizing methyl linoleate at 40°C and safflower oil at 60°C (Suarna and Southwell-Keely, 1991). Similarly, a study by Niki (1985) with tocopherol analogous showed that the phytyl side chain of chromanol has little effect on the inhibition of peroxidation of liposomes.

Table III. Reactivity of α-T and γ-T-3 towards peroxyl radicals generated by AMVN in ethanol

Antioxidant	Initial Rate of Consumption (μM/min)
α-T	0.77
γ-T-3	0.065

Trolox, in which the side chain is replaced by carboxylic acid, is more soluble in water and has higher protective activity than either α-T or several synthetic food antioxidants (butylated hydroxytoluene, BHT; butylated hydroxyanisole, BHA; Nordihydroguaiaretic acid, NDGA and tertiary butylhydroquinone, TBHQ) when added to various vegetable oils, lard or chicken fat (Scott and Cort, 1976) Our observation that α-T-3 was more reactive than γ-T-3 towards peroxyl radicals is not surprising in light of the above and earlier reports by Burton and Ingold (1981) that under similar conditions the relative antioxidant activities of tocopherols decreased in the order of $\alpha > \beta > \gamma > \delta$. These results however do not rule out the possibility that different side chains of α-T-3 and α-T influence the accessibility of the antioxidant active chromanol structure when located within a membrane bilayer or lipoprotein monolayer.

Work from this laboratory has shown that ubiquinol-10 ($CoQH_2$, the reduced form of coenzyme Q, CoQ) is associated with human LDL and is the first lipid soluble antioxidant that disappears following exposure of the lipoprotein to a variety of different oxidizing conditions (Stocker et al., 1991) . We therefore tested whether α-T-3 could efficiently compete with $CoQH_2$ for peroxyl radicals. As shown in Table IV, when α-T-3 was exposed to peroxyl radicals in the presence of $CoQH_2$ the rate of consumption of the former was low as long as $CoQH_2$ was present but increased following $CoQH_2$ consumption to 0.39 μM/min. This finding is in agreement with a report by Frei et al. (1990) showing that $CoQH_2$ has a sparing effect on α-T within liposomes during peroxyl radical-mediated oxidation. The fact that the rate of $CoQH_2$ consumption in the absence of α-T-3 was higher than the sum of the rates of consumption of $CoQH_2$ and α-T-3 when present together (Table IV) suggests that in the former situation part of the $CoQH_2$ was

lost due to "wasting" as a result of autoxidation (see Frei et al., 1990). Taken together our results suggest that in homogeneous solution α-T-3 is more reactive towards peroxyl radicals than γ-T-3 and equally reactive as α-T though all forms of vitamin E appear to be less reactive than $CoQH_2$.

Table IV. Reactivity of α-T-3 and $CoQH_2$ towards peroxyl radicals generated by AMVN in ethanol

Antioxidant	Initial Rate of Consumption (μM/min)
α-T-3	0.22
$CoQH_2$	1.36
Control:	
$CoQH_2$ alone (-AMVN)	0.39
$CoQH_2$ + AMVN	2.19

Oxidation of Human LDL Enriched with α-T-3.

The relative importance of different antioxidants is likely to be influenced by a number of factors, including their relative reactivity towards the oxidant and their relative accessibility. To address the latter issue we enriched human LDL with α-T-3. Previous work by Esterbauer et al. (1991) has shown that enrichment of this lipoprotein with lipid-soluble antioxidants can be achieved by incubating human plasma with the antioxidant before isolation of the LDL. We adapted this procedure for α-T-3. Preliminary experiments revealed that upon incubation with plasma, α-T-3 incorporated into HDL and LDL to a similar extent. In order to compare the reactivities of α-T and α-T-3 we chose conditions that resulted in ≈ equimolar ratios of these two antioxidants within the isolated LDL. When such supplemented LDL was exposed to a steady flux of

aqueous peroxyl radicals $CoQH_2$ was the first antioxidant to be consumed (Table V). LDL-associated α-T-3 disappeared together with endogenous α-T, but before lycopene, β-carotene, and γ-T. These results suggest that as in the homogeneous system both α-isomers of vitamin E possess similar reactivity towards aqueous or lipid-soluble peroxyl radicals.

Table V. Oxidation of α-T-3-enriched human LDL by peroxyl radicals: Comparison of the behavior of α-T-3 with endogenous antioxidants

Antioxidant	Rate of Antioxidant Consumption*	
	%/min	μM/min
$CoQH_2$	6.08	0.149
α-T	0.36 followed by 1.23	0.065 followed by 0.218
α-T-3	0.31 followed by 1.27	0.049 followed by 0.204
Lycopene	0.13 followed by 0.80	0.006 followed by 0.033
β-Carotene	0.29 followed by 0.64	0.007 followed by 0.015
γ-T	0.09 followed by 0.73	0.003 followed by 0.017

*Rates are given as initial rates (i.e., when all antioxidants were present in LDL) followed by the rate of consumption obtained immediately after complete disappearance of $CoQH_2$.

Recent work by Stocker et al. (1991) showed that in human LDL $CoQH_2$ was the most efficient antioxidant against peroxyl radicals. This was contrary to the report by Kagan et al. (1990) on the inhibition of lipid peroxidation induced by (Fe^{2+} + NADPH) or by (Fe^{2+} + tert-butyl hydroperoxide) in rat microsomes in which α-T was more effective than ubiquinols-9 or -10. The reasons for these apparently contradicting reports are likely related to differences in the two experimental models used, such as the nature of "lipid models" (i.e., lipoprotein vs. membrane bilayer), the type of the oxidant, and

the form of antioxidant administration (i.e., endogenous vs. exogenously added in ethanolic solution). We are presently investigating these possibilities in order to obtain further insight into the nature of $CoQH_2$'s antioxidant activity within LDL and other lipoproteins in more detail.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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PHYSICAL/CHEMICAL STUDIES OF VITAMIN E IN MEMBRANES

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Summary

Interaction of α-tocopherol and its analogue vitamin E acetate in model membranes has been studied using ^{1}H, ^{31}P and ^{13}C NMR. α-tocopherol binds strongly with the lipid molecule through intermolecular hydrogen bond. The possibility of such a hydrogen bond is excluded in Vitamin E acetate which binds loosely to the membrane through normal hydrophobic interactions. ESR spin labeling experiments indicate that whereas α-tocopherol broadens gel to liquid crystalline phase transition, vitamin E acetate lowers the phase transition temperature. Profile of permeation of small molecules through liposomes has been found to alter on incorporation of these drugs. However, no alteration in the mode of packing has been revealed by ^{31}P NMR and Electron Microscopy. The membrane retains normal lamellar bilayer arrangement. A model for α-tocopherol-lipid interaction has been constructed using computer aided graphics. Protection offered to intact cells (spermatozoa) by α-tocopherol against peroxidation caused by UV treatment has been studied using biochemical methods and spin labeling ESR technique.

Introduction

α-tocopherol is a lipid soluble antioxidant which plays a major role in maintenance of health of nervous and cardiovascular systems (Boland, 1990). Even immune systems require α-tocopherol for their unimpaired functioning. It is important to understand the mode of interaction and the process involved in prevention of oxidation of lipids at molecular levels. Such knowledge is likely to help evolve new, stronger and less toxic antioxidant compounds.

We have studied physicochemical aspects of lipid-α-tocopherol interactions using a variety of techniques. The mode of interaction and extent of penetration of α-tocopherol in lipid matrix has been investigated using ^{1}H, ^{13}C, ^{31}P NMR spectroscopy (Srivastava, et al., 1983, 1986). The alterations induced in the lipid matrix as a result of α-tocopherol incorporation have been estimated using ESR spin labeling and electron microscopy. More recently, the potency of α-tocopherol in preventing lipid-peroxidation has been assayed by monitoring colorimetrically malonaldehyde formed as a result of primary and secondary lipid peroxidation products (Wills, 1987). We have studied model membranes (dispersions of Dipalmitoylphosphatidyl choline (DPPC), Dioleoylphosphatidyl choline (DOPC) and egg phosphocholine (EPC) and goat spermatozoa.

We are trying to develop a new method based on spin labeling ESR to evaluate total oxidation. The preliminary results are encouraging.

Materials and Method

Materials

α-tocopherol was a gift from Malati Chemicals India. Spin labels 2,2,6,6-tetramethylpiperidine-N oxyl (TEMPO) and 2',2'-dimethyl-N-oxyloxazolidine derivative of 5-ketostearic acid (5-SASL) were purchased from SYVA Research Chemicals, USA. L-α-Dipalmitoylphosphatidyl choline (DPPC) and (DOPC) were obtained from SIGMA Chemical Company, USA. Egg PC was extracted were following standard method (Singleton, et al., 1965). Other reagents used were of analytical grade.

Model Membranes Preparations

Liposomes were prepared by following standard method. Aliquots of chloroform solutions of lipids were subjected to a stream of nitrogen so as to form uniform thin films of the lipid on the walls of container. Residual solvent was removed by subjecting the films to vacuum drying for at least 2 hours. Appropriate amount of buffer was added and

allowed to equilibrate for 30 minutes or more. Multilamellar liposomes were procured by vortexing. Unilamellar dispersions were obtained by subjecting the aqueous dispersions to sonication until they obtained optical clarity. Sonication was done with B-30 Branson sonicator fitted with microtip.

Spermatozoa: Sample Preparation

Goat testes were procured from the slaughter house. The epididymis was segmentally dissected into caput and cauda regions. Sperm cells were obtained by gentle mincing and tweezing in buffer. Tissue was removed by settling. The cells were washed by centrifugation and resuspended in buffer in desired concentrations. Sperms exhibiting 60-70% or more motility were used for experimentation.

Methods

NMR experiments were carried out on a Bruker AM-500 FT-NMR spectrometer. ESR experiments were done using X-band ESR spectrometer with a Varian 12 inch magnet and associated accessories. The sample temperature was measured by placing a copper constantan thermocouple placed in close proximity of the sample. Electron Microscopic (EM) studies were conducted with the help of Joel JEM 100 S electron microscope at a high voltage of 60 KV. The sample was prepared by placing a drop of suspension on a thin film of formvar coated copper grid and allowing it to dry before placing it on the microscope. The optical measurements were done on SPECTRONIC 1201 UV visible spectrophotometer.

Incorporation of α-tocopherol was achieved by addition of ethanol solution of α-tocopherol into cell samples or by dissolving lipids and α-tocopherol in chloroform solution, evaporating to dryness and subsequently dispersing into buffer. The system was allowed to equilibrate for 15 minutes. Samples were exposed to UV irradiation for 20 minutes for ESR experiment and 1 hour for biochemical assay. Lipid peroxidation was induced using Fe^{+2} + Ascorbate as oxidizing agent. Samples were incubated with 0.5 mM ascorbate and 10 μM $FeSO_4$ for 1 mg lipid/ml or 1 x 10^6 cells/ml. for 1 hour

duration. Lipid peroxidation was assessed by the formation of thiobarbituric acid reactive substances (TBARS) (Beuge and Aust, 1978). 2 ml of TBA reagent (0.375% W/V thiobarbituric acid, 15% W/V trichloroacetic acid in 0.25 N-HCL) was added to the samples, incubated for 10 minutes at 1OO°C, cooled, centrifuged and absorbance of supernatant read at 532 nm. The TBARS formation was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Mode of Interaction

NMR

^{13}C NMR (Chemical shift and relaxation times) provides information about the extent of immobilization and segmental motion of the interacting molecules (Levine et al., 1972). We have used for purpose of comparison, vitamin E acetate which is analogous to α-tocopherol except that the hydroxyl group is replaced by acetate moiety. Proton decoupled ^{13}C NMR spectra of both the compounds in $CDCl_3$ are almost similar. (Fig. 1(d)) Assignments of resonance lines have been reported earlier (Johnson & Jankawski, 1972). The resonances arising from sonicated DPPC vesicles without and with α-tocopherol or vitamin E acetate are depicted in a,b,c, (Fig. 1) respectively. One observes that whereas resonances belonging to vitamin E acetate remain sharp even after incorporation in lipid vesicles, those from α-tocopherol become very broad and are not detectable. This indicates that α-tocopherol gets strongly immobilized in the lipid matrix but its analogue vitamin E acetate is mobile. Measurement of spin-lattice relaxation times indicates that the hydrocarbon chain part of the vitamin E acetate molecule experiences same degree of motional freedom as that of lipid hydrocarbon chains (Srivastava, et al., 1983).

It is envisaged that the presence of hydroxyl group in α-tocopherol is responsible for tight binding to the lipid molecules through the formation of the hydrogen bond. The formation of hydrogen bond A-H ... B modifies the electron density around the proton of

the A-H group resulting in its shielding (Govil and Hosur, 1982). The proton chemical shift of the hydroxyl proton of α-tocopherol has been monitored (Fig. 2a). There is significant shift in the hydroxyl proton position on increasing the lipid content of the sample (Fig. 2(b-g)). At the highest lipid concentration (lipid:α-tocopherol = 1:1) the hydroxyl proton exhibits downfield shift of about 1.2 ppm. Moreover, the line becomes

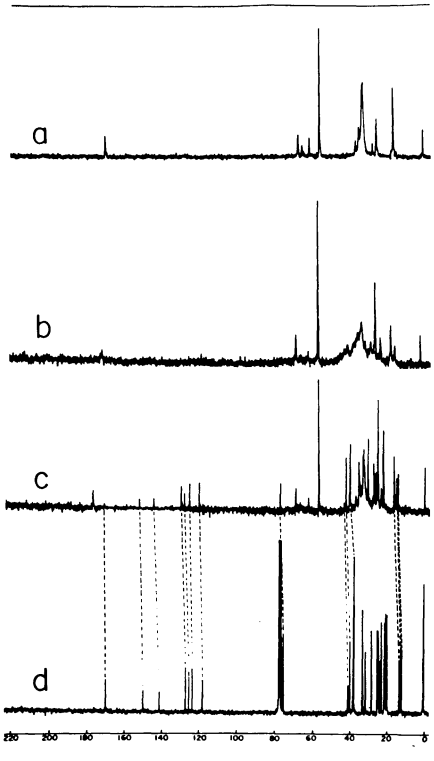

Fig.1 Proton decoupled ^{13}C-NMR spectra of
(a) DPPC unilamellar dispersion at 323K
(b) α-tocopherol + DPPC unilamellar dispersion (1:2 molar ratio) at 323K
(c) Vitamin E acetate + DPPC, unilamellar dispersion (1:2 molar ratio) at 323K
(d) Vitamin E acetate in $CDCl_3$.

considerably broad. The downfield shift of the hydroxyl proton can be explained only by assuming formation of a hydrogen bond between hydroxyl proton and one of the oxygen atoms of the lipid molecule. The electronic structure of tocopherol O-H group is distorted by the proton acceptor oxygen. The electronic field of this acceptor oxygen tends to draw the hydrogen away from the bonding electron of O-H bond and thereby reduces the electron density in the immediate vicinity of hydrogen. The contribution of this effect to the hydrogen bonding shift is negative since the deshielding of the hydrogen will cause its resonance signal to occur at lower magnetic fields than in the absence of proton acceptor oxygen. Thus, the shift in the position of O-H resonance clearly indicates involvement of this group in lipid- α-tocopherol interaction.

Further support has been provided by ^{31}P NMR results. The intermolecular interaction can manifest itself through alterations in the chemical shift positions or through line-broadening (Govil and Hosur, 1982). Fig. 3(a) shows a characteristic ^{31}P NMR spectrum of unilamellar vesicles in liquid crystalline state. Incorporation of α-tocopherol in the vesicles results into an upfield shift of the resonance line by about 1 ppm (Fig. 3 (b-d)). Moreover one observes a considerable increase in the line width. An increase in line width corresponds to decrease in the spin-spin relaxation time. Thus, ^{31}P NMR experiments indicate involvement of phosphate moiety in binding of α-tocopherol with lipid matrix.

Quantum chemical calculations based on PCILO method also indicate that the hydroxyl group of α-tocopherol is involved in binding with the phosphate group of phospholipid through intermolecular hydrogen bonding (Srivastava, et al., 1986).

Model of Interaction

X-ray diffraction data (Abrahamsson and Pascher, 1966) indicates that two hydrocarbon chains of the lipid molecule in liquid crystalline phase are separated by a distance of about 4.7A. Our experiments with magnetic resonance techniques suggest that the hydrocarbon chain of vitamin E acetate is packed in a manner similar to the loose packing observed for lipid-chains in liquid crystalline state. A computer model of

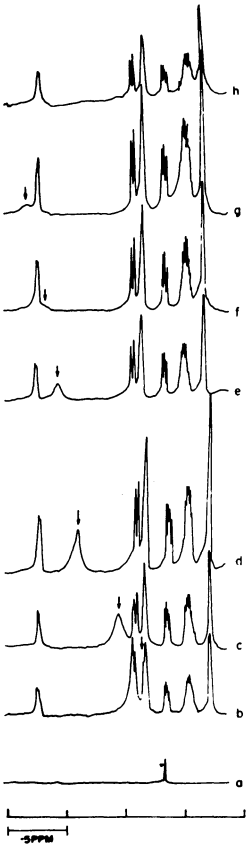

Fig. 2 Proton NMR spectra of (a) α-tocopherol, 100, mM (single hydroxyl peak at 4.18 ppm) in $CDCl_3$ (b)-(g) with increasing lipid to α-tocopherol molar ratio (h) DPPC, 100 mM in $CDCl_3$.

interaction has been generated using computer aided graphics. The structure and conformation of vitamin E acetate and lipid molecules are based on reported X-ray diffraction data. It is observed that vitamin E acetate can be easily incorporated in lipid bilayers with a distance of about 4.70A between the hydrocarbon chains of lipid and vitamin E acetate. The aromatic moiety comes near the lipid water interface such that the acetate group remains in the water phase. The interaction between vitamin E acetate is therefore hydrophobic in nature.

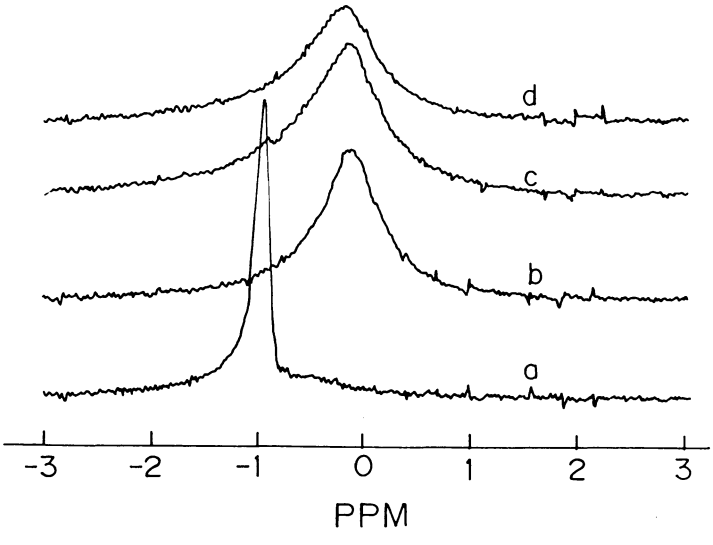

Fig. 3 Proton decoupled ^{31}P NMR spectra (a) Unilamellar DPPC vesicles (80 mM) in D_2O at 323K, (b) - (d) same as in (a), incorporated with 10, 20 and 40 mM α-tocopherol, respectively.

For modelling of binding of α-tocopherol to lipid bilayer, we have additional inputs from magnetic resonance and quantum chemical calculations. The hydroxyl group of α-tocopherol forms a hydrogen bond with the lipid molecule (Fig. 4). The exact location of hydrogen bond is not unequivocally defined on account of want of clear experimental indications. ^{31}P NMR results indicate definite involvement of phosphate group. This is suggestive of possible hydrogen bond formation between phosphate oxygen of lipid and hydroxyl hydrogen of α-tocopherol. However, some reports indicate towards formation of hydrogen bond between oxygen of glycerol moiety of lipid and hydroxyl oxygen of α-tocopherol (Kagan, et al., 1987; Shiro, et al., 1990). It is necessary to design an appropriate experiment which will decisively determine localization of hydrogen bond. However, our investigations indicate occurrence of hydrogen bond formation and its importance in prevention of peroxidation.

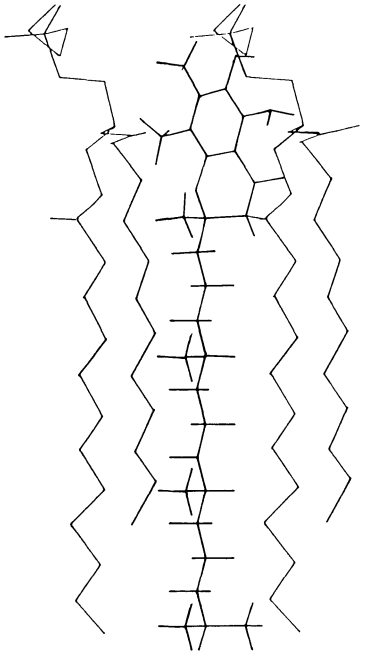

Fig. 4 Model for the interaction of α-tocopherol with DPPC. The hydroxyl group of the α-tocopherol is capable of forming hydrogen bonds with oxygen atoms of phosphate moiety and/or with water molecule at the lipid-water interface.

Alterations Induced in Lipid Matrix

Lipid Polymorphism

Biological membranes and model membranes (lipids dispersed in excess water) display bilayer arrangements. However, departures from normal bilayer structures e.g. hexagonal or cubic are probable due to local heterogeneities induced by external agents such as drugs, vitamins, etc (Srivastava, et al., 1989). Structural alterations in lipid matrix can be identified using a number of techniques including ^{31}P NMR (Seelig, 1978) and electron microscopy (Verkleiji, et al., 1978). As each phospholipid molecule contains only one phosphorous atom, ^{31}P NMR spectrum is relatively simple. The resonance pattern is

governed by the chemical shift anisotropy (CSA) and molecular motions. In a randomly oriented sample such as lipid dispersions in gel phase, the overall rotational rate is too slow to average CSA. One therefore observes a broad shoulder around -28 ppm and a relatively sharp peak around 18 ppm corresponding to $\Delta\sigma$ of $\approx$ 46 ppm (Fig. 5(a)). As

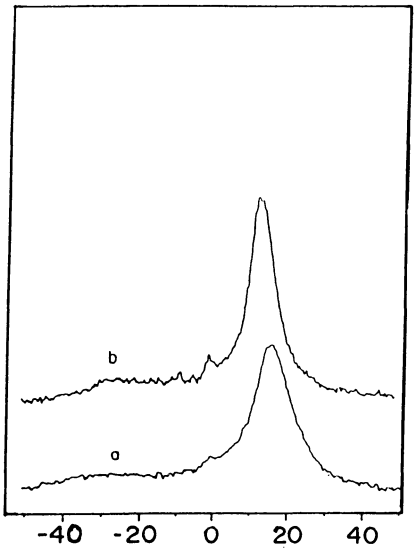

Fig. 5 ^{31}P Chemical shift anisotropy patterns with broad band decoupling for lipid dispersions in D 2 0 at 323K (a) DPPC, (b) DPPC + α-tocopherol (10:1 M ratio)

the temperature increases, averaging of CSA increases leading to reduction in $\Delta\sigma$ value. However, the characteristics of the pattern remain unchanged unless a change in structural organization occurs. We observed that incorporation of α-tocopherol or its analogue, vitamin E acetate leaves ^{31}P CSA pattern unaltered (Fig. 5(b)). This indicates that the bilayer arrangement of lipid matrix is unhampered by the presence of these molecules. Supporting evidence has been obtained from electron microscopic pictures. It has been observed that the spherical bilayer arrangement remains intact although the size of vesicles is larger in the presence of α-tocopherol as compared to normal vesicles.

Incorporation of α-tocopherol is likely to alter gel to liquid crystal transition characteristics, fluidity and permeability of model membranes. We have used ESR spin labeling techniques to study these characteristics.

Phase transition characteristics

The phase transition behavior has been studied using TEMPO partitioning (Shimshick and McConnell, 1973). TEMPO is known to partition in the lipid and aqueous phase. The extent of partitioning depends upon the degree of fluidity of lipid phase. The ESR spectrum consists of three line pattern (Fig. 6(a)) with high field line exhibiting two phase character, due to spin label dissolved in aqueous and lipid phase (peaks marked P & H respectively). The relative areas under these peaks correspond to the number of spin labels dissolved. The partition coefficient can be measured by the parameter

$$f = \frac{H}{(H+P)}$$

if the widths of the resonance lines remain constant over the range of temperatures (288-333K). Use of this dimensionless parameter is advantageous as it is not affected by factors such as decomposition or reduction of spin labels, settling of sample or any other factors which are likely to affect spectrometer sensitivity. Parameter 'f' increases initially gradually with increase in temperature and abruptly when the system undergoes gel to liquid crystal phase change. In the case of DPPC, the main transition occurs around 314K corresponding to melting of hydrocarbon chains and pre-transition at 308K which is associated with tilting of hydrocarbon chains with respect to the plane of the bilayer (Ruocco and Shipley, 1982) (Fig. 6). One observes that the membranes incorporated with α-tocopherol exhibit continuous gradual increase in 'f' with increasing temperature. The sigmoidal nature of phase However, membranes incorporated with vitamin E acetate show characteristics similar to DPPC vesicles namely, sigmoidal nature and appearance of main and pre-transitions. The main transition occurs at 301K, a temperature much lower than that of DPPC. Moreover, the pre-transition is less prominent (Fig. 6). Noteworthy is the difference in the behavior of the vesicles incorporated with α-tocopherol and its analogue.

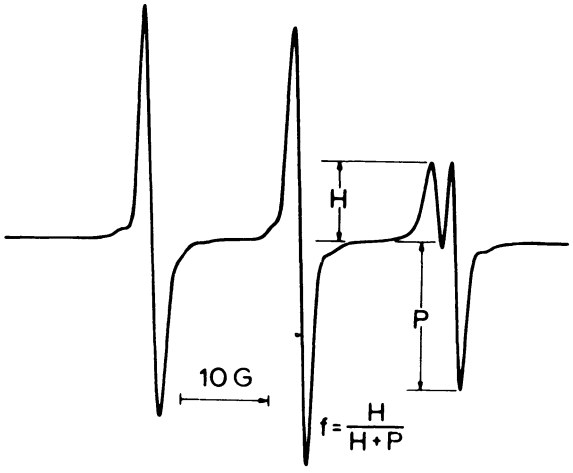

Fig. 6(a) X-band ESR spectrum of TEMPO in PPC dispersions, H and P correspond to signal heights due to TEMPO in lipid and water phase, respectively.

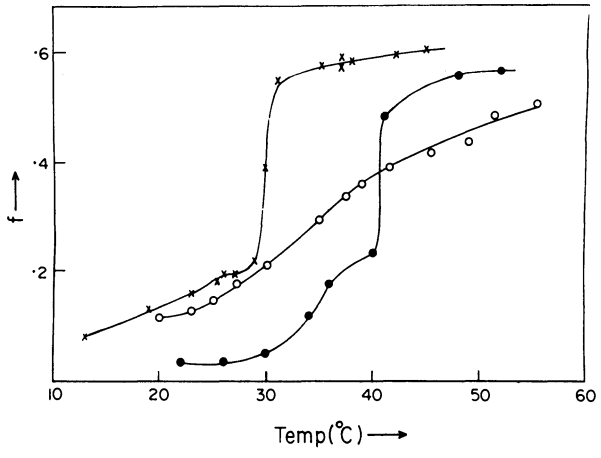

Fig. 6(b) Spectral parameter f = H/(H+P) as a function of temperature.(●) DPPC dispersion (○) DPPC + α-tocopherol (5:1 M ratio) (x) DPPC + vitamin E acetate (5:1 M ratio).

Permeability Changes

Lipid vesicles in the liquid crystalline state are able to transport small molecules and ions (passive transport) from the inner to the outer aqueous phase and vice versa (Benting and DePont, 1981). The transport is critically dependent upon the local organization and fluidity (Papahadjopouls, et al., 1972).

We have used 5-SASL for permeability measurements.It dissolves entirely in the lipid phase. It gets dispersed both on inner and outer monolayers of the lipid vesicles (Fig. 7). If we introduce a reducing agent such as sodium ascorbate in the aqueous phase, it permeates through the bilayer and reduces the spin labels (Schreier-Muccillo, et al., 1972; Aracava, et al., 1981). The spin labels residing in the outer monolayer undergo reduction at a faster rate than those in the inner monolayer. This can be monitored by measuring ESR signals (e.g. low field line) with time. The reduction follows an equation of type (Srivastava, et al., 1983)

$$S(t) = S_o(0)e^{-k_o t} + S_i(0)e^{-k_i t}$$

where S(t), $S_o(0)$, $S_i(0)$ correspond to number of total spin labels at time t, and initial concentrations of spin labels in outer and inner monolayers respectively. k_o and k_i are the rate constants for reduction of spin labels residing in outer and inner monolayers, respectively. We further note that

$$S(0) = S(0) + S_i(0) \text{ and } S_o(0)/S_i(0) = r_o^2/r_i^2$$

where r_0 and r_i are radii of the outer and inner monolayers, respectively. The outer diameter of sonicated vesicles is about 250 A and the thickness of bilayer is 50 A (Marsh, et al., 1972). The unknowns S_o, k_o and k_i can be estimated using least square fitting (Guest, 1961). The results of such analysis are listed in Table I. One observes that half lives for reduction of spin labels residing in outer and inner monolayers are significantly larger for DPPC vesicles than those for vesicles incorporated with α-tocopherol or its analogue. This clearly indicates that incorporation of α-tocopherol has resulted into increase in permeability of the lipid vesicles to small molecules.

Table I

Half life time (Minutes) for the reduction of spin label incorporated in lipid bilayer in different systems at 318K

	DPPC	DPPC + α-Tocopherol	DPPC + Vitamin E acetate
Outer Layer	7.7	4.5	5.7
Inner Layer	22	7.5	5.9

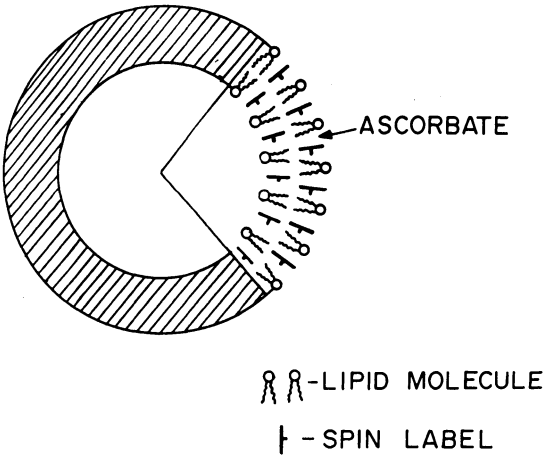

Fig. 7 Diagrammatic representation of permeation of ascorbate through unilamellar lipid vesicle.

Potency to Prevent Peroxidation

The ability of α-tocopherol to prevent lipid peroxidation has been judged by

monitoring formation of thiobarbituric acid-reactive substances (TBARS). We have studied three systems:

(i) Vesicles of DOPC - a synthetic lipid

(ii) Vesicles of egg PC - lipids extracted from biological membranes

(iii) Spermatozoa (Goat) - intact cells

Both DOPC and egg PC show some natural peroxidation which is nullified on incorporation of small amounts of α-tocopherol (about one molecule of α-tocopherol per 100 lipid molecules). It is not clear if it is the artifacts of the chemical method of estimation of peroxidation or otherwise. Noteworthy is the fact that addition of vitamin E acetate does not prevent peroxidation.

Aliquots of 1 mg/ml lipids in phosphate buffer at pH = 7 have been subjected to continuous irradiation of UV light for different duration of periods. Estimated peroxidation in terms of TBARS is plotted against time in figure 8. Untreated sample aliquots subjected to identical conditions served as controls while estimating peroxidation products. The peroxidation increases continuously for egg PC (Fig. 8(b)).

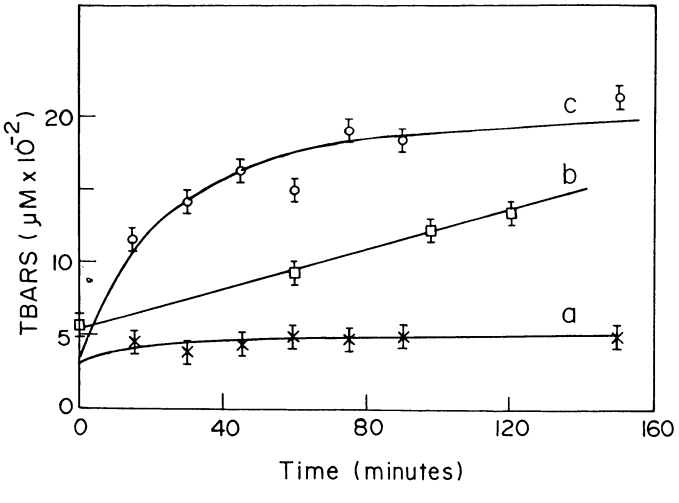

Fig. 8 Time-course of the lipid peroxidation during ultraviolet (UV) treatment, (a) Dioleoyl phosphatidyl choline (DOPC) without UV irradiation, (c) DOPC after UV irradiation, (b) egg PC after UV irradiation. TBARS have been estimated as discussed in the text.

DOPC vesicles on the other hand undergo peroxidation initially (till 40 min. or so) and then remains more or less constant. The discrepancy in the two cases is due to the differences in double bond character of lipid chains which is more complex in egg PC leading to a continuous increase in peroxidation product for a longer duration.

The behavior of spermatozoa procured from goat epididymis (Fig. 9) subjected to different periods of UV irradiation is depicted in figure 10. A sudden initial increase in peroxidation product (TBARS) is observed during first 10 minutes (in curve a) which goes down later. This decrease is probably due to the ability of cells to metabolize malonaldehyde (major peroxidation product) which is produced as a result of peroxidation. The two processes take place simultaneously resulting in a steady state concentration which is maintained at some level for some time. However, on UV exposure, the metabolic rate of the sperm cells is likely to go down as the cells die but the membrane continues to undergo peroxidation causing a gradual increase. On the other hand, presence of 10 μM of α-tocopherol (curve b) prevents the peroxidation due to UV irradiation almost completely, except when irradiation is continued for prolonged periods.

A detailed study has been undertaken to optimize tocopherol concentration required to effect total inhibition of peroxidation. Tocopherol concentrations have been varied from 0.1 to 1000 μM. Cauda and caput region spermatozoa (Fig. 11 (a,b)) display almost identical behavior. Peroxidation inhibition caused by the presence of α-tocopherol increases more or less linearly and attains total inhibition for about 1 μM concentration in the case of chemically induced (Fe^{++}- ascorbate) peroxidation. UV induced peroxidation is prevented when larger amounts of (α-tocopherol has been introduced (Fig. 11(c)). In general, peroxidation induction by UV is a complex phenomenon. It gives rise to cell death. Estimation of peroxidation products is also a difficult proposition as effects of chemical additives can give rise to alterations in cell metabolisms. With this view in mind we are trying to evolve a new method of estimation of peroxidation which is based on spin labeling ESR experiments.

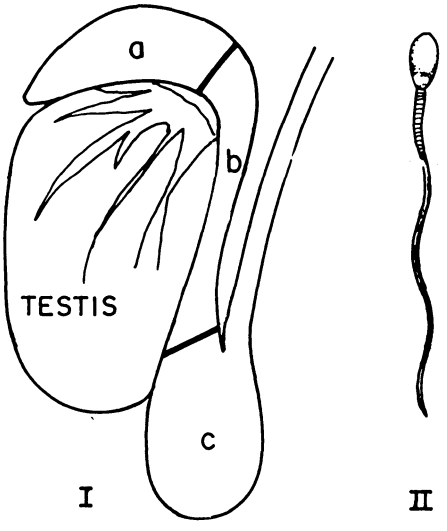

Fig. 9 (I) Schematic picture of goat testis showing different regions of epididymis, a-caput, b-corpus, c-cauda, (II) A typical sperm cell.

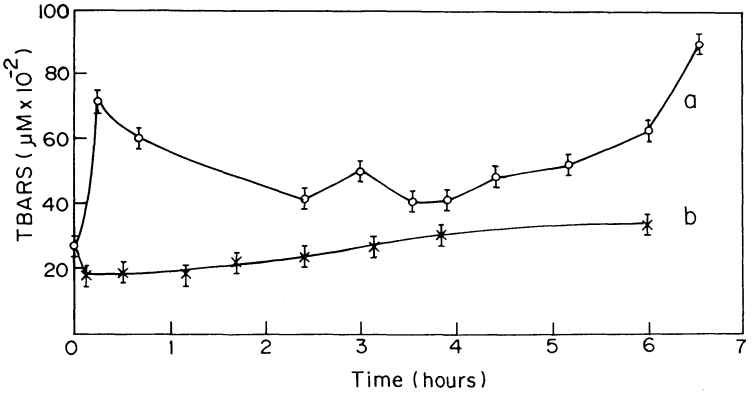

Fig. 10 Time-course of lipid peroxidation during UV treatment of goat spermatozoa (cauda cells). (a) cells without, antioxidant and (b) cells in the presence of 10 μM α-tocopherol. 1 x 10^6 cells/ml have been used for each experiment.

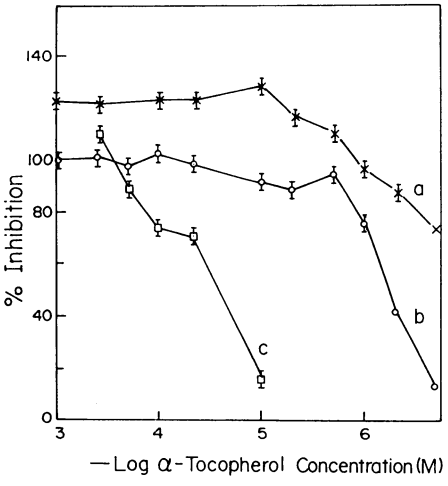

Fig. 11. Inhibition of lipid peroxidation in goat spermatozoa by α-tocopherol. Peroxidation was induced using Fe^{++}-Ascorbate and UV treatment. Cells were pre-incubated with α-tocopherol for 20 minutes before peroxidation. Fe^{++}-ascorbate peroxidation in (a) caput cells (b) cauda cells; UV treatment in (c) cauda cells.

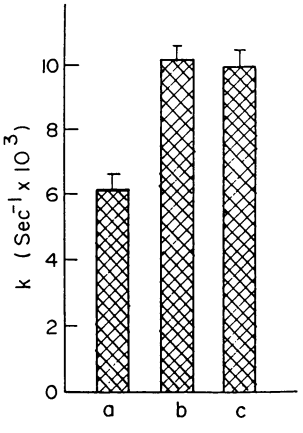

Fig. 12 Rate constant for spin label reduction by (a) goat sperm cells (b) goat sperm cell after UV treatment (c) goat sperm cells after peroxidation using Fe^{++}-Ascorbate. Results are expressed for 2 x 10^9, cells/ml from cauda epididymis.

ESR Method

Intact spermatozoa are known to cause spin label reduction (Chapman, et al., 1985; Bahl, et al., 1988; Phadke, et al., 1990). The rate of reduction follows a first order kinetics and follows the equation of type $h(t) = h(o)\ e^{-kt}$ where h(t) and h(o) correspond to unreduced spin label species at time t = t and t = o respectively and k is a parameter which is dependant on concentration of cells and initial spin label quantities. It has been observed that normalized k with respect to cell numbers and spin label concentrations is sensitive to cell type and cell environment. We have seen that k values for UV irradiated, chemically induced oxidation and normal spermatozoa lie in two distinctly different groups (Fig. 12). Preliminary results indicate that the presence of α-tocopherol assigns a k value which is intermediate to these two groups. However, it is too early to make definite conclusions as live cell is a complex entity. One needs to do extensive experiments with appropriate controls before arriving at definite conclusions.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

SYNERGISTIC EFFECT OF LIPID HYDROPEROXYL RADICAL SCAVENGING AND LIPID HYDROPEROXIDE REDUCTION IN THE INHIBITION OF LIPID PEROXIDATION IN BIOMEMBRANES

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Phospholipid hydroperoxides (PLOOH) can be generated in membranes by different mechanisms: lipid peroxidation, lipoxygenase reaction and addition of singlet oxygen. Whilst singlet oxygen addition is a non radical reaction, leading directly to the hydroperoxide, the lipid peroxidation initiator (usually an iron-oxygen complex) and lipoxygenases produce first a carbon centered radical, which than reacts with oxygen. The reduction of lipid hydroperoxyl radicals to lipid hydroperoxides is part of the lipoxygenase reaction, without a specific requirement for hydrogen donors. On the other hand, phospholipid hydroperoxyl radicals ($PLOO^{\bullet}$) produced during lipid peroxidation can start a chain reaction,getting the hydrogen atom from a polyunsaturated fatty acid, which eventually contains a new carbon centered radical (1).

From PLOOH new radicals are generated by electron transfer reactions or molecule assisted homolysis of the O-O bond. Peroxides, on the other hand, and presumably PLOOH, activate lipoxygenases. For this reason the control of PLOOH in membranes, plays a pivotal role among biological systems controlling the oxidative stability of cells.

The antioxidant cellular system protecting biomembranes from oxidative damage must deal with a control of initiation reactions and with the length of the peroxidative chain, once initiated.

The most commonly used model of a peroxidative damage of membranes is microsomal lipid peroxidation, induced by a suitable ferric iron chelate, and a source of reducing equipments,usually ascorbate or NADPH and cytochrome P450 reductase (2). Requirements for an iron chelate to induce lipid peroxidation are : i) reducibility of the ferric iron chelate; ii) interaction of the ferrous iron chelate with oxygen; formation of a relatively stable oxidizing intermediate (perferryl). It follows that primary protection is afforded by any agent perturbing one of those requirements. For instance, chelating agents hampering iron reduction (e.g. Desferrioxamine) or iron-oxygen interaction, (e.g. CN^- or orthophenantroline), as we chelates releasing superoxide (EDTA), prevent the initiation of microsomal lipid peroxidation (2). Another interesting mechanism of protection could be the reduction of the oxidizing initiator by a hydrogen donor in competition with lipids. Once the iron-oxygen complex succeeded to extract an hydrogen atom from a polyunsaturated fatty acid, the forthcoming reaction, i.e. the addition of oxygen, is so fast that the competition with an antioxidants stabilizing the carbon centered radical, would be just a theoretical claim.

Hydroperoxyl radicals are the peroxidation driving radicals, and against those radicals Vitamin E is specifically active. Vitamin E in fact, fits the basic requirements for being an antioxidant, since, following the hydrogen transfer a relatively stable radical is generated, and the reaction is kinetically favored. The rate constant for the reaction of $PLOO_\bullet$ with Vitamin E is, indeed, several order of magnitude faster than the reaction with a polyunsaturated lipid (3).

Accordingly, Vitamin E cannot prevent microsomal lipid peroxidation, unless a misleading condition is set up in which Vitamin E can interact with initiating species. Vitamin E, on the other hand, is effective in slowing down peroxidation rate, since propagation is minimized. This is in agreement with the experimental evidence that, during iron induced peroxidation, the consumption of Vitamin E parallels the peroxidative oxygen consumption (3,4).

During microsomal lipid peroxidation PLOOH are generated when $PLOO^{\bullet}$ receive an hydrogen atom either from a polyunsaturated fatty acid or from Vitamin E. From these hydroperoxides the ferrous iron chelate produces alkoxyl radicals ($PLO^{\bullet}$). Those radicals are very reactive and the possibility of their quenching by an antioxidant suffers the same kinetic limitation as for $HO^{\bullet}$. Since those radicals react with the antioxidant and the target molecule to be spared with approximately the same rate constant, to get a reasonable antioxidant effect we would load our membranes with a molar concentration of Vitamin E.

The consequences of the limited capacity of Vitamin E to play a significant antioxidants role when peroxidation is driven by $PLO^{\bullet}$, is the observed co-oxidation of Vitamin E without a significant antioxidant effect (4). These considerations, on the other hand, highlight the key role of PLOOH reduction, as a fundamental antioxidant mechanism. When PLOOH are reduced to PLOH, the $PLO^{\bullet}$ driven peroxidation is prevented, and Vitamin E can fully exert its chain breaking effect by quenching $PLOO^{\bullet}$.

Although the reduction of PLOOH can be obtained by the coordinate reaction of PLA_2 and GPX, the specific enzyme for this reaction is Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPX) (1).

The substrate specificity of PHGPX for the peroxidic substrate is broad, the enzyme indeed is active on all phospholipid hydroperoxides, as well as on cholesterol and cholesterol ester hydroperoxides in natural and artificial membranes as well as in oxidized low density lipoproteins (5,6)

Kinetic data indicate that an identical kinetic mechanisms, shared by GPX and PHGPX, has been adopted by nature to control hydroperoxide concentrations in different

environments, being one enzyme specific for soluble hydroperoxides and the other for membrane hydroperoxides (7)

PHGPX is a monomeric selenoenzyme (MW 18,000 d.). The structure of the enzyme purified from pig heart was partially elucidated by means of peptide and cDNA sequencing (8). Homologies between PHGPX and GPX are obvious and reveal a common molecular ancestor of these enzymes, but the percentage of identical amino acids in homologous position ranges below 40%. This poor relatedness suggests an early divergence in the molecular evolution of these proteins. From analogy considerations it was possible to assume that the use of selenoproteins for the reduction of hydroperoxides was a rather evolutionary achievement.

Although, at the present level of knowledge, the protection against lipid peroxidation is the only known function of PHGPX it is tempting to speculate that PHGPX is also involved in the physiological control of hydroperoxides that would cope with cellular functions.

Recent data, indeed, indicate that PLOOH can be formed directly by the action of 15 lipoxygenases (15-LO), a family of iron containing dioxygenases originally supposed to react only with free fatty acids (9). This interesting observation could link 15-LO catalyzed lipid peroxidation with iron induced lipid peroxidation, described above. In fact 15-LO might initiate lipid peroxidation by producing PLOOH from decomposition of which peroxidative chain reactions could start. This mechanism could cope with programmed membrane destruction during cell maturation, and possibly other biologically relevant processes. A lipoxygenase active on membrane-bound substrates has been described in reticulocytes, where the enzyme is expressed in the last steps of erythrocyte differentiation and seems to cope with the destruction of mitochondria and intracellular membranes (9). Moreover, there is suggestive evidence for this reaction to take place in other cell systems. A notable example is the generation in the aorta of hydroxy-derivatives of phospholipids apparently by sequential hydroperoxidation and reduction reactions (10).

Therefore, apart from peroxidative damage of membranes, phospholipid hydroperoxidation and reduction (as the "off" event) could be involved in other physiological processes. Lipid peroxides in membranes, indeed, have an ionophoric effect

on calcium, generate the "peroxide tone" of membranes and can control the activity of key enzymes such as phospholipases, cycloxygenase, and possibly protein kinase c(1).

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DETERMINATION OF RATE CONSTANTS FOR ANTIOXIDANT ACTIVITY AND USE OF THE CROCIN ASSAY

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Summary

Antioxidative activity of a given substance can be inferred from the protective effect under in vivo oxidative stress conditions. To quantitate such effects, however, the exact scavenging rate constants for the various oxygen radicals have to be determined.

In the following, two methods to obtain such data will be presented in detail: (1) pulse radiolysis in combination with kinetic spectroscopy to directly monitor build-up and decay of antioxidant radicals and (2) competition kinetics for indirect evaluation of scavenging rates. The most versatile substance for such competition studies, especially for alkoxyl and peroxyl radicals, has turned out to be the water-soluble carotenoid crocin.

Introduction

Any substance which is capable of inhibiting reactions occurring under oxidative stress conditions could be termed 'antioxidant'. However, a more limited definition would be more accurate, i.e. only those substances which are capable of scavenging oxygen radicals effectively without propagating oxidative chain reactions are true antioxidants.

Therefore, it is a prerequisite that for each substance with antioxidative potential rate constants for the scavenging of oxygen radicals as well as for the decay of the intermediate antioxidant radicals are determined. Already at this point we have a problem: shall we look only at inorganic oxygen radicals, especially hydroxyl radicals ($^{\bullet}OH$), or do we mainly consider organic oxygen radicals such as peroxyl or alkoxyl radicals, which

are more likely the dominant chain carriers during lipid peroxidation? Since freely diffusible hydroxyl radicals are rather unlikely to be formed under biological conditions and, furthermore, react quite indiscriminately with almost any organic substrate, studies of the reactivities of organic oxygen radicals would more closely relate to conditions of oxidative stress.

To determine kinetic parameters, i. e. rate constants, for prospective antioxidants, two predominant methods can be recommended:

(1) pulse radiolysis combined with kinetic spectroscopy and

(2) competition kinetics with steady-state radical sources.

Since pulse radiolysis is technically and financially rather extravagant, in most cases competition studies may be preferred.

Kinetic Spectroscopy

Methodology

Any determination of rate constants hinges on specific radical sources. Of all known systems, radiolysis of aqueous solutions offers the most versatile source of various types of radicals (Bors et al., 1984), while organic solvents have a much more limited applicability. Based on the long established chemistry of the radiolysis of water (Buxton, 1987, Jonah et al., 1989), the rapid radical generation by pulse radiolysis is certainly the most powerful method. In contrast, enzymatic systems, e.g. xanthine/xanthine oxidase should be applied with great caution, as they most likely generate a variety of radicals simultaneously.

Several types of accelerators and monitoring systems are known for pulse radiolysis experiments, but this is not the place for an extensive review (Sauer, 1982, Patterson, 1987). In our laboratory, we use a Febetron type accelerator and a spectrograph array (Saran et al., 1987) to monitor time-dependent absorption changes at 15 wavelengths simultaneously.

Another important feature of our set-up is the solvent delivery system, which is based on a syringe-driven mixing device. It allows both flexible, computer-controlled

concentration variations and, most important, the use of low sample volumes as small as 50 ml for concentration-dependent runs (alternatively, 50 μl aliquots of rare and/or expensive probes can be injected manually into the irradiation cuvette, containing 1.6 ml of buffer or sample solution).

Most modern pulse radiolysis facilities incorporate signal digitization to allow direct computer evaluation of kinetic traces. In the simplest case first or second order kinetics (logarithmic or reciprocal dependencies on substrate concentration) can be evaluated directly. In case of complex kinetics, e.g. chain reactions, 'kinetic modeling' must be applied. Both this term as well as 'numerical simulation' denote computational methods by which competing or sequential reactions are taken into consideration to arrive at rate constants for each individual reaction. This approach inherently also allows to test the veracity of such kinetic schemes, provided that the individual rate constants remain invariable when changing the substrate concentration(s) (Erben-Russ et al., 1987b).

Transient Spectra

Most hydroxy-aromatic compounds with antioxidative potential show absorption characteristics which can easily be monitored. Observation of transient spectra is commonly the first step. If hydroxyl radicals are present, however, the picture may be complicated, as they first add to dihydroxybenzene structures before water is eliminated to yield semiquinones (Adams & Michael, 1967). This could lead to different transient spectra even though the $^{\bullet}$OH-adduct radicals may be extremely short-lived (Neta &, Fessenden, 1974). A way to circumvent this problem is the univalent oxidation of aromatic hydroxy groups by azide radicals ($^{\bullet}N_3$). This yields directly peroxyl radicals by electron transfer from the dissociated phenolate group to $^{\bullet}N_3$ (Alfassi & Schuler, 1985) and, since quite a number of antioxidants contain aromatic hydroxy groups,, this reaction of $^{\bullet}N_3$ radicals is of particular relevance to our studies.

Some hindered phenols (6-hydroxy-2.5.7.8-tetramethylchromane-2-carboxylic acid, Trolox c, or 6-hydroxy-2.2.5.7.8-pentamethylchromane, PMC, as water-soluble analogs of α-tocopherol), α-keto phenols, and a number of flavonoid aglycones have been

investigated in our laboratory. One might expect that flavonoids, showing a clear structural dependency of the transient absorption of their respective aroxyl radicals (Bors & Saran, 1987), would lend themselves most easily to structure activity relationship (SAR) studies. Yet, as demonstrated below, this is only partially true for kinetic parameters. The transient spectra are also more strongly influenced by pH, i.e. the degree of dissociation of the various hydroxy groups, than by the type of attacking radicals.

There are exceptions to the latter statement, evidenced by the different transient spectra of kaempferol or quercetin after attack by $^{\bullet}OH$, $^{\bullet}N_3$ or (CH3)2C(OH)OO$^{\bullet}$, the isopropanol-derived peroxyl radical (Fig. 1).

Whether this suggests different sites of attack at the flavonoid molecule, is still a matter of debate. Incidentally, the strong absorption of the quercetin or kaempferol aroxyl radicals in the visible region - indicative of an extensive electron delocalization - has led to the use of these radicals in competition studies (Erben-Russ et al., 1987a).

Scavenging rate constants

Formation of transient spectra is a time- and concentration-dependent event which directly yields scavenging rate constants. Azide and $^{\bullet}OH$ radicals may form the antioxidant radical most efficiently, however, their rate constants may have only a tenuous relationship to oxidative stress conditions owing to the facts

(i) that these radicals are unlikely to be produced under biological conditions and

(ii) that these radicals, due to their high reactivities, are unlikely to show selective attack.

Hydroxyl radicals, in particular, react with almost any organic substance with diffusion-controlled rate constants. Nevertheless, the electrophilic nature of $^{\bullet}OH$ is apparent from a correlation of phenolic Hammett substituent coefficients with the respective rate constants (Bors et al., 1983). In the case of flavonoids reacting with $^{\bullet}OH$ or $^{\bullet}N_3$, there seems to be a not very significant but consistent preference for substances with a 4'-OH group as opposed to a catechol structure in the B-ring (e.g. kaempferol vs. quercetin, Bors et al., in press).

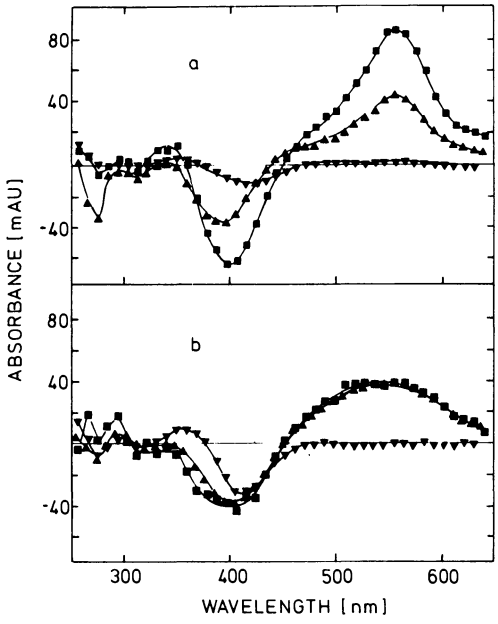

Figure 1: Transient spectra of kaempferol (a) and quercetin (b) after attack by $^{\bullet}OH$, $^{\bullet}N_3$ and $(CH_3)_2C(OH)OO^{\bullet}$ radicals in unbuffered aqueous solutions.

(a) kaempferol: (■) $^{\bullet}OH$ radical concentration 3.4μM, substrate concentration 43μM, pH 8.6; (▲) $^{\bullet}N_3$: 8.8 μM, 62μM, pH 8.0; (▼) $(CH_3)2C(OH)OO^{\bullet}$: 16.2μM, 58μM, pH 9.4
(b) quercetin: (■) $^{\bullet}OH$ radical concentration 3.3μM, 56μM, pH 9.2; (▲) $^{\bullet}N_3$: 8.3μM, 56μM, pH 8.0; (▼) $(CH_3)_2C(OH)OO^{\bullet}$: 9.0$\mu$M, 25$\mu$M, pH 8.5

While rate constants with t-$BuO^{\bullet}$ as a model alkoxyl radical will be discussed below (see competition studies), rate constants with peroxyl radicals as models of the predominant radicals in lipid peroxidation have recently been compiled in an extensive list (Neta et al., 1990). Yet the list contains only few examples for phenolic compounds (mainly Trolox c) and even less for fatty acid-derived peroxyl radicals. Studies with peroxyl radicals are mostly limited to organic solvents using lipophilic antioxidants with peroxyl radicals generated either by slow autoxidation (Porter et al., 1981) or by

thermolysis of azo-initiators (Barclay et al., 1984, Niki et al., 1984, 1985, Burton & Ingold, 1986).

Alternatively, pulse radiolysis studies of fatty acid peroxyl radicals in aqueous solution require strongly alkaline conditions to ensure sufficient solubility of the fatty acid monomers (Erben-Russ et al., 1987b). Micellar or liposomal systems above critical micellization concentrations (cmc) have typically shown reduced reactivities due to diffusion constraints (Barclay, 1989). We have recently explored alcohol-derived peroxyl radicals near neutral pH as possible models for fatty acid peroxyl radicals. Yet the difference of the kaempferol and quercetin transient spectra (see Fig. 1) as well as the fact that these α-hydroxyalkyl-peroxyl radicals, aside from decaying bimolecularly, may release O_2^- or decay unimolecularly in base-catalyzed reactions (Bothe et al.,1978, Petryaev et al., 1983), could make them inappropriate for antioxidant studies.

Decay rate constants and fate of antioxidant radicals: It is a prerequisite of antioxidants, that the radical formed after scavenging the more reactive oxygen intermediates, is sufficiently stable and does not propagate chain reactions on its own. Unfortunately, rate parameters for decay kinetics can obviously only be obtained from the observation of the transient spectra and are thus available only from pulse radiolysis studies. Consequently, these data are more limited to specialized equipment than scavenging rate constants, which can also be obtained from competition studies (see below).

The most intensively studied flavonoid aroxyl radicals, with few exceptions, decay by second order kinetics, i.e. they disproportionate (Bors & Saran, 1987). In strongly alkaline solution we found a highly significant correlation of the relative stability of aroxyl radicals with the presence of a catechol structure in the B-ring. Evidently, this moiety further promotes electron delocalization by participating in the dissociation equilibria (Bors et al., 1990). That the dissociation state of the parent flavonoid indeed governs this behavior, is apparent from the absence of such a stabilization effect of B-ring catechols near neutral pH (Bors et al., in press) and is subject of further studies. We are particularly interested in the influence of the 7-OH group, which owing to the biosynthetic pathways, exists in practically all known flavonoids (Heller & Forkmann, 1988) and evidently is the first hydroxy group which dissociates with increase in pH (Slabbert, 1977).

We have previously pointed out, that only scavenging of peroxyl radicals closely approaches biological conditions. Another important aspect of studying these radicals lies in the fact, that only for peroxyl radicals a 2:1-stoichiometry seems to exist: two peroxyl radicals are scavenged in sequence by phenolic or aromatic amine antioxidants (Boozer et al., 1955), ultimately leading to non-radical adducts. While we could not unequivocally verify this reaction for the flavonoids kaempferol and quercetin due to the high instability of the adducts preventing their HPLC separation, kinetic modeling clearly showed the importance of this reaction (Erben-Russ et al., 1987b).

In contrast to di- or polyhydroxylated aromatic compounds, which preferentially decay by disproportionation reactions, phenoxyl radicals derived from hindered monophenols (e.g. α-tocopherol and its model compounds), are sufficiently stable to be eventually reduced by intermolecular electron transfer - hence the synergistic effect of ascorbate and tocopherols in preventing lipid peroxidation (Niki et al., 1989).

Competition Studies

Methodology

Complementing pulse radiolysis experiments, these studies normally require only standard laboratory spectrophotometers and, for the generation of radicals, X- or gamma ray machines, or UV lamps. Basically a steady-state radical source is combined with a strongly absorbing reference substance and the diminished effect of radicals on this absorbance by increasing concentrations of a test substance is measured (Bors et al., 1985). This 'competition plot' is analog to the so-called Stern-Vollmer plot used in photochemical studies (Turro, 1967).

Aside from a high molar absorptivity, an optimal reference substance has to meet some additional criteria:

(a) it should allow to discriminate between different types of radicals;

(b) the radical produced from the reference substance should not react by itself with the test substance;

(c) the absolute rate constants of the reference substance with different types of radicals must be known if the relative rate constants, obtained from the competition plot, are to be converted into absolute values.

Properties of Reference Substances

The most obvious difference exists between reference substances, whose absorption is depleted after radical attack and those which form a new radical species. In the latter case, under optimal conditions, different absorption characteristics (λ_{max},ξ) might be attributed to selectively attacking radical species. In the first case, where usually the effective bleaching after a constant time lapse is measured, information on the type of radical can not be obtained directly. Nevertheless, as has been shown for the bleaching behavior of para-nitrosodimethylaniline (p-NDA or RNO) with different radical sources and competitors with known rate constants, one can easily distinguish between radiolytically produced $^{\bullet}OH$ radicals and '$^{\bullet}OH$ analogs' formed in enzymatic reactions (Bors et al., 1979).

Competition studies with substances, where formation of radicals is monitored, are far less frequent. Since the observed radicals in these cases are mostly short-lived, these substances only lend themselves to pulse radiolysis studies. The two most versatile compounds are 2.2'-azino-bis-(3-ethylbenzthiazoline-6- sulfonic acid), ABTS (Wolfenden & Willson, 1982) or quercetin (Erben-Russ et al., 1987a), of which ABTS was originally developed as a reference substance for peroxidase-catalyzed reactions (Childs & Bardsley, 1975, Gallati, 1979). Thiocyanate, SCN^-, where the observable radical, $(SCN)_2^{\bullet -}$, is exclusively formed by $^{\bullet}OH$ radicals ideally fulfills the first requirement. Yet it is a negative example for the second criterion, as the $(SCN)_2^{\bullet -}$ radical is reactive enough to form aroxyl radicals from phenols (Bors, 1985, Erben-Russ et al., 1987b). If used as a competitor for $^{\bullet}OH$ radicals, the secondary formation of aroxyl radicals would thus diminish the inhibitory effect due to scavenging of the primary $^{\bullet}OH$ radicals and consequently falsify the competition experiments.

The best-known substances from the third category are ABTS and the water-soluble carotenoid crocin, for which a number of rate constants with various types of radicals have been determined (Table I).

TABLE I.

Rate constants of the reference substances ABTS and crocin with different radicals in aqueous solution.

(all values $x10^{10}$ $M^{-1}s^{-1}$)

Radical	ABTS	Crocin
e^-_{aq}	0.5[a]	0.5[b]
$^{\bullet}OH$	1.2[a]	3.1[b]
$^{\bullet}N_3$	n.d.	1.2[c]
t-BuO$^{\bullet}$	0.2[d]	0.3[d]
SO_3^-	n.r.[e]	0.1[e]
O_2^-	n.d.	n.r.[b]

a - Wolfenden & Willson, 1982
b - Bors et al., 1982
c - unpublished value
d - Erben-Russ et al., 1987a
e - Erben-Russ et al., 1987c

n.d. - not determined
n.r. - no reaction

As mentioned before, formation of the $ABTS^{\bullet+}$ radical cation limits this substance to pulse-radiolytic competition studies. However, rate constants with a number of peroxyl and thiol radicals are known (Wolfenden & Willson, 1982, Mönig et al., 1983, Lal et al., 1988, Schuchmann & von Sonntag, 1988), and the substance might therefore be particularly useful for competition studies with biologically relevant radicals.

Crocin, on the other hand, is bleached both by reducing and oxidizing radicals, but not by O_2^- (as opposed to $HO_2^{\bullet}$) or $^{\bullet}CH_3$ radicals (Bors et al., 1982). As shown in the Table, absolute rate constants are known for a number of inorganic radicals and t-BuO$^{\bullet}$, others are still to be determined. The extremely high molar absorptivity of $1.35x10^5$ M^{-1} cm^{-1} at 440nm allows for very sensitive measurements especially with alkoxyl and peroxyl

radicals as these - in contrast to $^{\bullet}OH$ radicals - are not scavenged by the optically transparent gentiobiose moieties (Bors et al., 1984). Since t-BuO$^{\bullet}$ radicals can easily be generated by UV photolysis of t-BuOOH (or di-tert-butylperoxide, t-BuOOBu-t) - or peroxyl radicals after thermolysis of azo-initiators (Ursini, personal communication) - two readily available radical sources exist. Comparing the competition results for these two radical sources and a selected few phenolic and flavonoid antioxidants shows the sensitivity of the method (Fig. 2).

At the present, we have no explanation why Trolox c alone, in contrast even to its close analog PMC, shows such a strong preference for ROO$^{\bullet}$ radicals. Unfortunately, rate constants with pulse-radiolytically generated (haloalkane) peroxyl radicals in aqueous solution are only known for Trolox (Davies et al., 1988; Neta et al., 1989) and are thus of no help.

Since the original list of relative rate constants was published (Bors et al., 1984) and especially after the pulse-radiolytic determination of the absolute rate constant of crocin with t-BuO$^{\bullet}$ (Erben-Russ et al., 1987a), this reference substance has been used in a number of additional cases (Erben-Russ et al., 1987c, Bors et al., 1989, 1990b).

Crocin, together with its hydrolyzed dicarboxylic acid form, crocetin, and a similar carotenoid derivative, bixin (Hicks & Draper, 1981), are the only water-soluble carotenoids and could therefore be flexibly used in combination with all radicals generated in aqueous solution. However, as shown with canthaxanthin in n-hexane, similar competition experiments with lipophilic antioxidants and alkoxyl radicals (probably also with peroxyl radicals) can also be performed (Bors et al., 1984).

Conclusions

Kinetic methods to determine rate constants of prospective antioxidants with various types of radicals have been described. The technically demanding method of pulse radiolysis is the most useful one, yielding both scavenging rate constants as well as transient spectra and decay rate constants of the respective antioxidant radicals.

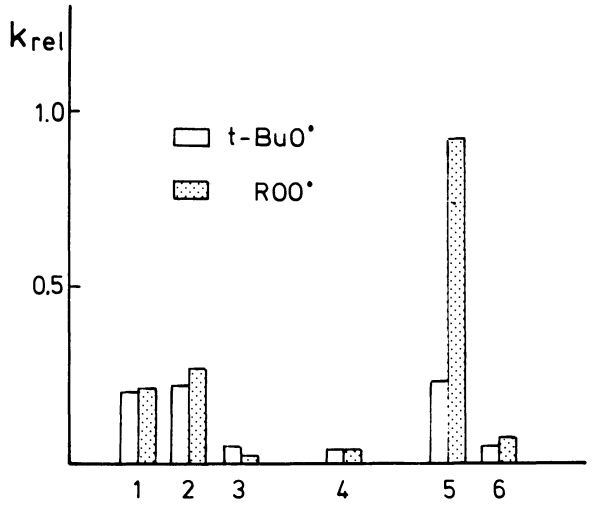

Figure. 2: Relative rate constants of phenolic antioxidants with t-BuO•, and ROO• determined by the 'crocin assay'.

Legend: t-BuO•, generated by UV photolysis of t-BuOOH in aqueous solution, containing 0.1 M t-BuOH; ROO•, from the thermolysis of 2.2'-azobis-(2-amidinopropane hydrochloride), ABAP.
1 - kaempferol; 2 - quercetin; 3 - epicatechin; 4 - 2.5-dihydroxyphenyl-acetic acid; 5 - Trolox c; 6 - pentamethylchromane.

Competition studies, which can be done both during pulse radiolysis experiments or with steady-state radical sources, yield only scavenging rate constants. However, as the latter method is more readily accessible and since an increasing number of suitable reference substances have been established, it is the preferred method to rapidly accumulate kinetic data of substances with antioxidative potential.

These data can then be used to interpret inhibitory effects of such substances in biological systems, exposed to oxidative stress conditions. However, a given substance can only be defined as a genuine antioxidant, when the relative stability of its radical derivative has been determined and it has been shown that this radical is incapable of promoting chain reactions due to its low reactivity.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

VITAMIN E IN PROTECTION OF OXIDATIVE IMPAIRMENT IN ENDOTHELIAL AND PLATELET FUNCTIONS.

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Summary

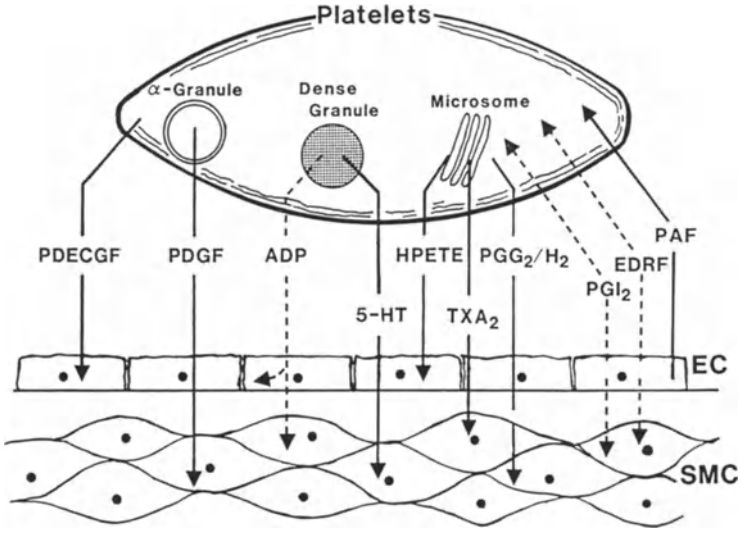
Effects of vitamin E-deficiency on endothelial and platelet functions were investigated in rats. The 7 week deficiency from weaning reduced serum α-Toc level to 1/5 and doubled serum thiobarbituric acid reactive substances. Acetylcholine-induced endothelium dependent relaxation in aortic rings precontracted with norepinephrine (10^{-7} M), was almost abolished at 7th week, but not at 4th week. Hyperaggregability of washed platelets was observed at 4th week and the magnitude became greater with the increasing period. Five day administration of d-tocopherol analogues (200 mg/kg/day) to the deficient rats completely recovered the endothelium-dependent relaxation with α-tocopherol, but partially with β-tocopherol. γ- and δ-Tocopherol were ineffective. Platelet aggregation was also inhibited by the supplementation in the order $\alpha > \beta > \gamma > \delta$. There was a strong inverse correlation between platelet aggregability and serum α-tocopherol levels of rats each given the analogues. These results suggest that α-tocopherol plays an important role in prevention of atherogenesis.

Introduction

The vessel wall and platelets interact with each other to regulate their functions and growth as shown in Scheme 1. Through this cooperative interplay, platelets and endothelial cells play regulatory functions in the circulation system. We have previously

reported that vitamin E (VE)-deficiency resulted in an increase of not only lipid peroxides, but also in various aliphatic aldehydes such as n-hexanal and 4-hydroxy-2-nonenal in rat plasma (Tomita et al., 1987; Yoshino et al., 1991). In addition, we found that low density lipoprotein rapidly and reversibly inhibited thrombin-induced endothelium--dependent relaxation in porcine coronary arteries (Tomita et al., 1990). Oxidatively modified LDL reportedly has more potent inhibitory effects on endothelium dependent relaxation (Kugiyama et al., 1990) and also proaggregatory effects than native LDL (Ardlie et al. 1989).

Some lipid peroxides and other related aldehydes are highly cytotoxic and lead to deterioration of cell membranes and impairment of their functions. VE as an antioxidant agent prevents peroxidation of unsaturated fatty acids by scavenging lipophilic radicals within membranes. Despite the numerous assumptions of its protective role in lipid peroxidation, few studies have shown functional damages due to VE deficiency in the



Scheme 1. Interaction of the endothelium with platelets

circulatory system. This study was therefore undertaken to investigate the effects of VE-deficiency on both endothelial and platelet functions, and their recovery by supplementation of various d-tocopherol (Toc) analogues.

VE deficiency resulted in a complete loss of acetylcholine-induced endothelium dependent relaxation in the aorta following an enhancement in platelet aggregation, and d-α-tocopherol supplementation recovered their impaired functions.

Materials and Methods

Animals

Male SD rats were divided into two groups at weaning, and had been maintained on either VE-deficient diet (dl-α-Toc. lmg/kg diet) or a supplemented diet (dl-α-Toc. acetate 20 mg/kg) for 4-8 weeks. In recovery experiments, d-Toc analogues (α, β, γ, δ), 200 mg/kg in 5 % Tween 80, were given orally for 5 days to 7 week deficient rats. Rats were killed 24 hrs after the last administration unless otherwise stated.

Measurement of Blood Pressure

Systolic blood pressure was measured by a tail-pulse pick-up method in unanesthetized rats using Ueda UR1000 (Ueda Co. Tokyo).

Endothelium-Dependent Relaxation

Ring segments (6 mm long, 3.5-4.0 mg wet weight) of rat thoracic aorta from each group were precontracted with norepinephrine (10^{-7}) or U46619 (10^{-8} M) , and relaxed with cumulative additions of acetylcholine (10^{-9}-10^{-4} M) in normal Tyrode's solution (pH 7.3, 370). The magnitude of relaxation was expressed as a percentage of norepinephrine- or U46619-contraction.

Preparation of Washed Platelets, and Measurement of Platelet Aggregation

Washed platelets from each rat were prepared as previously described (Tomita et al. 1984). Platelet aggregation was measured by a turbidimetric method using 4-channeled aggregometer (AGGRETEC TE-500, Elma, Tokyo). Aggregation was induced by thrombin or collagen.

Materials

SPF male SD rats were obtained from Clea Co. Tokyo. Reagents used here were purchased from the following sources: d-α, β, γ, δ-Toc (Eisai co. Tokyo) , norepinephrine bitartrate, and acetylcholine (Sigma Chemicals St. Louis, MO), U46619 (Funakoshi Pharmaceuticals, Tokyo), human thrombin (Midori Cross Co. Osaka), collagen (Collagen reagent Horm, Hormon-Chemie, MUnchen).

Results

Experimental Parameters

Two groups of rats were kept on VE deficient or supplemented diets 7 weeks from weaning. Body weights and blood pressure remained the same within the two groups during the entire experimental period. However, hemolysis and serum thiobarbituric acid reactive substances(TBARS) of the deficient group markedly increased; hemolysis was 98 % and TBARS was doubled at the 7 week deficiency. α-Toc concentration in serum from the deficient group decreased to 1/5 of that in the supplemented group.

Reduction of Endothelium Dependent Relaxation Due to the Deficiency

Furchgott (Furchgott et al. 1980) found that factors released from the endothelium relaxed the vascular smooth muscle. Fig. 1 shows the effects of the 7-week VE deficiency on acetylcholine-induced endothelium dependent relaxation in rat aortas. The ring segments of rat thoracic aortas were precontracted with either norepinephrine (10^{-7} M) or U46619 (10^{-8} M, a thromboxane agonist), and relaxed with cumulative additions

of acetylcholine. The magnitude of the relaxation response was expressed as a percentage of norepinephrine contraction. In the case of the aorta from control rats 10^{-8} to 10^{-4} M acetylcholine induced dose-dependent relaxation, whereas in a segment of the aorta from 7-week deficient rats the relaxation was almost abolished. Sodium nitroprusside induces vasodilation through the direct action on smooth muscle cells. An addition of 10^{-6} M sodium nitroprusside relaxed the ring from the deficient group to the basal line. Relaxation responses to various concentrations(10^{-9}-10^{-6} M) of sodium nitroprusside of endothelium denuded vessels were similar to each other in the two groups.

These results suggest that endothelial functions were impaired due to the 7 weeks VE deficiency. This dys-function not observed at the 4 week of the endothelium was deficiency.

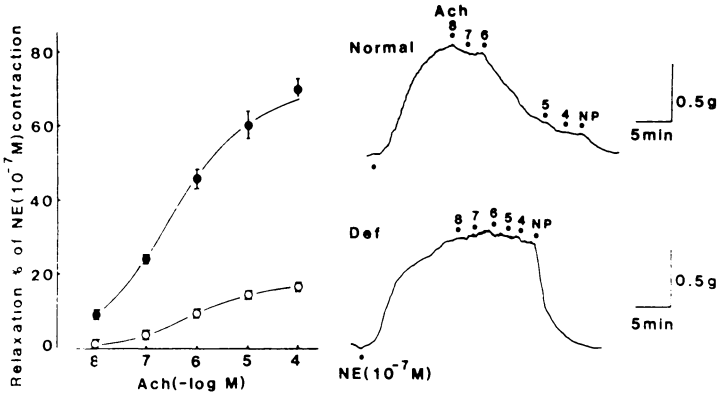


Fig.1 Reduction by VE deficiency of acetylcholine induced endothelium-dependent relaxation. Thoracic aorta were dissected from male SD rats maintained for 7 weeks on diets either deprived of vitamin E (dl-α-tocopherol less than 1 mg/kg) or supplemented (dl-α-tocopherol acetate 20 mg/kg) beginning at weaning. Rings were precontracted with norepinephrine (NE 10^{-7} M) and relaxed with cumulative addition of acetylcholine (10^{-8}-10^{-4} M) . The magnitude of relaxation responses was expressed as a percentage of NE contraction. - ○ -: deficient rats, - ● -:control rats. Each point and vertical bar indicate mean±S.E. for 5 preparations. Left: dose-response curves, Right: typical tracings. Ach: acetylcholine, NE: norepinephrine, NP: Sodium nitroprusside(10^{-8} M)

Recovery of Endothelium-Dependent Relaxation by Supplementation of d-tocopherol Analogues

d-α-Toc (200 mg/kg) was given orally for 5 days to the 7 week deficient rats. Endothelium-dependent relaxation once lost in the deficient group, reappeared after 5 day-supplementation. The magnitude of relaxation in the deficient rats was not statistically different from that in the control rats.

However, one-day supplementation resulted in only a partial recovery of the relaxation. Fig. 2 shows recovery from the impaired endothelium-dependent relaxation by the supplementation of various d-Toc analogues. Rats maintained on VE deficient diet for 7 weeks were each given orally either d-α, β, γ, δ-Toc or the vehicle for 5 days.

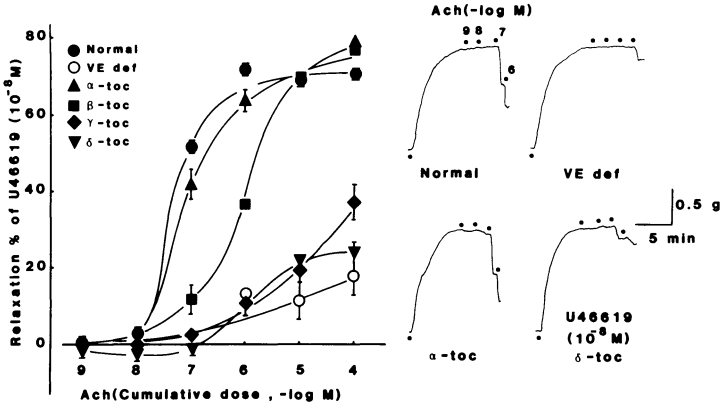


Fig. 2 Recovery of endothelium-dependent relaxation by supplementation of d-tocopherol analogues. Male SD rats maintained for 7 weeks on VE-deprived diet, received orally d-tocopherols(200 mg/kg) for 5 days. Endothelium dependent relaxation responses of aortic rings were measured as described in the legend to Fig. 1. Rings were precontracted with U46619 (10^{-8} M). - ○ -: deficient (n=7), - ● -: control (n=7), - ▲ -: α-, - ■ -: β-, - ◆ -: γ-, - ▼ -: δ-Toc supplemented (n=8). Each point and vertical bar indicate mean±S.E. for the indicated number. Left: dose-response curves, Right: typical tracings.

Significance : non-significant by ANOVA, control vs α-supplemented.

d-α-Toc supplementation brought a complete recovery of endothelium-dependent relaxation, while d-β-Toc showed only partial recovery at the Fig. 2 shows recovery from the impaired endothelium-dependent relaxation by the supplementation of various d-Toc analogues. Rats maintained on VE deficient diet for 7 weeks were each given orally either d-α, β, γ, δ-Toc or the vehicle for 5 days. d-α-Toc supplementation brought a complete recovery of endothelium-dependent relaxation, while d-β-Toc showed only partial recovery at the concentrations of 10^{-7}-10^{-6} M acetylcholine and a complete recovery at 10^{-5}-10^{-6} M acetylcholine. Relaxation of d-γ-Toc groups was slightly higher than the deficient group. d-δ-Toc was ineffective.

Enhancement in Platelet Aggregation Due to VE Deficiency

Within blood cells and plasma, platelets reflect most accurately the amount of digested Tocs (Ardlie et al. 1989). Thus, it is assumed that platelet functions are greatly influenced by Toc state. In order to exclude humoral factors, washed platelets were prepared. Special care was taken to minimize the exposure of platelet membranes to air during the preparation. Fig. 3 shows thrombin-induced aggregation of washed platelets from 4-7 week VE-deficient rats and the respective control. In contrast to the endothelium, higher aggregability of platelets was observed as early as at the 4-week deficiency. with prolonged periods of deficiency, a more significant difference was observed between the two groups.

Alleviation of Platelet Hyperaggregability by Supplementation of d-tocopherol Analogues

When d-α-tocopherol 25 mg/kg was given for 5 days to 7 week deficient rats, hyperaggregability due to the deficiency was partially alleviated. Administration of 50 and 100 mg/kg for 5 days brought a complete recovery. Aggregation of platelets from rats given the highest dose (200 mg/kg) was lower than that of the control group. Thus, higher doses of d-α-Toc inhibit platelet aggregation.

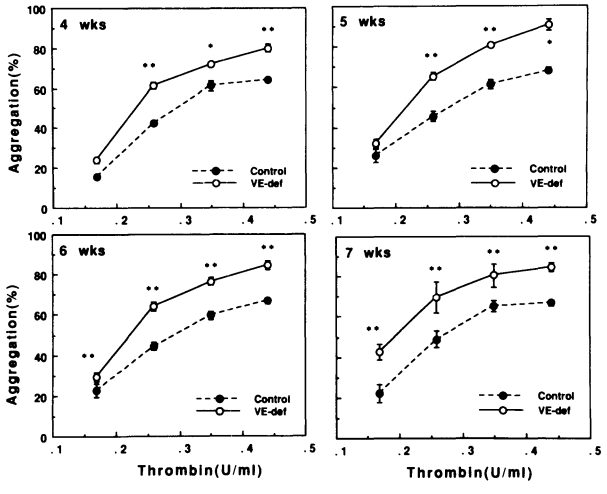


Fig. 3 Changes in platelet aggregability due to VE deficiency. Male SD rats were maintained for 4-7 weeks on diets either VE-deprived or supplemented from weaning, and washed platelets were prepared for each rats.

In another experiment, d-Toc analogues (200 mg/kg) were given for 5 days to the 7 week deficient rats and their effects on platelets were compared (Table I). α-Toc most greatly reduced thrombin-induced aggregation lower than that of the control group. β-Toc was the next most effective. Aggregation of platelets from rats given γ-Toc was also slightly lower than that in the control group. δ-Toc administration just normalized aggregation to control level.

Correlation Between Platelet Aggregation and d-Toc Contents in Platelets

The contents of tocopherol analogues in plasma from each group were determined by HPLC. There was a strong inverse correlation between the α-Toc levels in plasma from each group and the respective platelet aggregability (Fig 4).

Table I

Alleviation of platelet hyperaggregability by supplementation of d- tocopherol analogues. Male SD rats maintained for 7 weeks on VE deprived diets, were given d-tocopherol analogues(200 mg/kg orally for 5 days) or vehicle.

Groups	Aggregation(%) Thrombin(U/ml) 0.13	0.17	0.26
Control	38.1±4.1	57.1±5.4	67.6±2.4
Deficient	50.9±5.9	72.0±2.3	84.4±3.4
Supplemented			
α	30.1±4.1	41.5±2.3	49.0±2.2
β	33.3±5.4	50.0±5.6	54.2±2.7
γ	36.2±4.3	53.1±3.5	58.6±9.9
δ	42.8±3.0	58.4±4.6	63.5±5.3

mean±S.E. for 3 rats
Significance by ANOVA: deficient vs α, β, γ ($P<0.001$), deficient vs δ ($P<0.05$), control vs deficient α, β ($P<0.01$), control vs γ ($P<0.05$)

Discussion

There is now considerable evidence that oxidation of LDL takes place in vivo in contact with vascular endothelial cells, monocytes and so forth during the circulation, and oxidative modification of LDL has been implicated as an essential event in the pathogenesis of atherosclerosis. Various antioxidants such as VE, β-carotene, butyl hydroxy toluene, probucol etc. are reported to retard LDL oxidation. We have shown in

this study that VE deficiency abolished endothelium-dependent relaxation following the enhancement in platelet aggregation and an increase in serum TBARS. Platelet membranes seems to be more sensitive to oxidative damage than endothelial cell membranes. The potency of d-Toc analogues to recover their cell functions were in the order of $\alpha > \beta > \gamma > \delta$-Toc both in the endothelium and platelets.

HPLC analysis of Toc analogues in serum after their administration, disclosed that α-Toc was detected even in the serum from rats given β-, γ- or δ-Toc, suggesting biotransformation among these analogues. Furthermore, platelet aggregation of each group given respective analogue, inversely correlated with α-Toc levels in their serum. Therefore, the potency of tocopherol analogues might depend on the biotransformation to α-tocopherol from the other analogues.

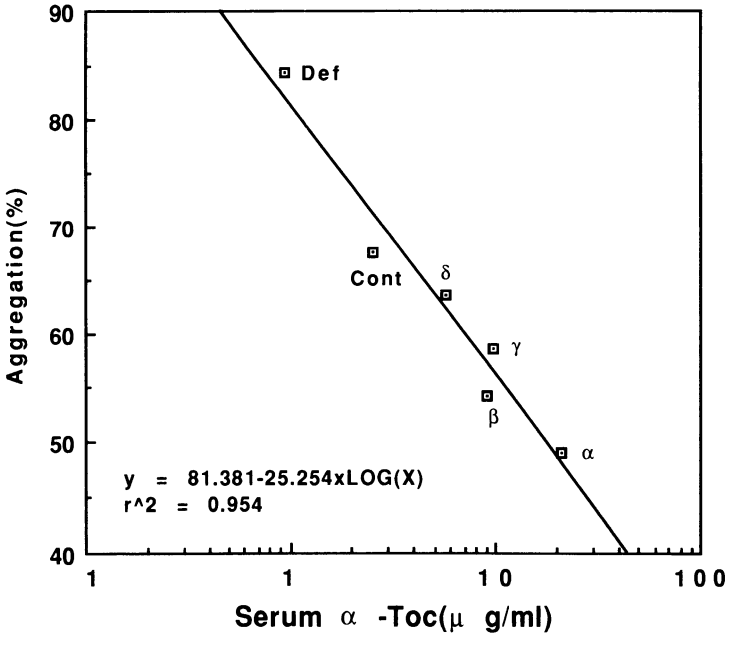


Fig. 4 Inverse correlation between serum α-tocopherol levels and platelet aggregation from each group. Male SD rats kept on VE-deprived diet for 7 weeks, were given orally for 5 days either d-tocopherol analogues (200 mg/kg) or vehicle. Def: deficient group, Cont: control, α, β, γ, δ: α-, β-, γ-, δ-groups

Conclusion

VE deficiency causes the deterioration of membranes in platelets and endothelial cells, and an impairment of their functions. The supplementation of α-Toc completely recovered these damages. The results suggest that α-Toc plays a critical role in maintaining normal functions of the endothelium and platelets, whose abnormalities are deeply involved in atherogenesis.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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ANTIOXIDANT EFFECTIVENESS OF TOCOPHEROL ISOMERS

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Summary

The antioxidant properties of several tocopherol isomers and of "Palm Vitee" were assessed by measurement of their ability to inhibit NADPH-induced peroxidation of hepatic microsomes prepared from vitamin E-deficient rats. The microsomal concentrations of d γ-, d α- and dl α-tocopherol required to inhibit peroxidation by 50% were 160, 750 and 1850 μg/mg protein respectively; d γ-tocopherol was also the most effective in prolonging the lag phase prior to the initiation of peroxidation. "Palm Vitee" was 3 times more potent than d γ-tocopherol in preventing TBARS formation; this may be due to the tocotrienol components of the preparation. Electron spin resonance (ESR) spectroscopy and spin trapping suggested that the tocopherols were preventing the formation of a carbon-centered radical adduct derived from linoleic acid. Signal height correlated with TBARS formation suggesting that the TBA assay is an accurate measure of lipid peroxidation in this study.

Introduction

The pathogenesis of many diseases can involve free radical-mediated lipid peroxidation of biological membranes. Adequate dietary intake of vitamin E, a major lipid soluble inhibitor of peroxidation, may therefore be important in inhibiting the development of conditions such as heart disease, cancer, cataracts, neuropathies and

myopathies (Packer and Landvik, 1989). Since vitamin E exists as several natural and synthetic tocopherol and tocotrienol isomers, it is important to establish which of these forms are the most effective antioxidants under defined conditions. Therefore, the present study has assessed the antioxidant properties of various tocopherols and tocotrienols by measurement of their ability to inhibit NADPH-induced lipid peroxidation of hepatic microsomes from vitamin E-deficient rats. Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS). As this index of peroxidation is relatively non-specific (Duthie, 1991), peroxidation was also monitored by electron spin resonance (ESR) spectroscopy and by assay of hexanal production; the latter probably arises from peroxidation of omega-6 fatty acids (Frankel et al.,1989).

Materials and Methods

Weaning male rats of the Rowett Hooded Lister strain were offered, ad libitum, a semisynthetic diet (Abdel-Rahim et al., 1986) containing 0.1 mg selenium/kg (as Na_2SeO_3) and less than 0.5 mg vitamin E/kg. After 6 weeks, the rats were anesthetized with ether. Following in situ perfusion with 0.15 M KC1, the livers were removed for the preparation of microsomes (Lim et al., 1980) which were adjusted to a protein content of 10 mg/ml in 0. 05 M potassium phosphate buffer (pH 7.4) .

The effects of added dl α-tocopherol, d α-tocopherol, d γ-tocopherol and "Palm Vitee" (composition:α-tocopherol 32%; α-tocotrienol 23%; γ-tocotrienol 28%; δ-tocotrienol 13%) were assessed as follows:- 40 μl of an ethanolic solution of isomer or "Palm Vitee" (final concentrations 0 mM, 0.004 mM, 0.04 mM and 0.4 mM) were incubated at 37°C with stirring for 30 min in a diluted microsomal suspension (100 μl in 5.6 ml of potassium phosphate buffer, 0.05 M, pH 7.4) . Peroxidation was then initiated by the addition of NADPH (final concentration 0.05 mM) and 1 ml aliquots were removed at intervals for the determination of TBARS (Yagi, 1987). Incorporation of the isomers into the microsomes was assessed by HPLC (Bieri et al., 1979), using a washed pellet

prepared by centrifugation of aliquots of the incubation mixture at 105,000g. The lag phase prior to the initiation of peroxidation was calculated as described in Hill & Burk (1984).

Microsomes were also prepared for ESR spectroscopy as previously described (Duthie et al., 1990) . At the start of the incubations, the spin trap α-(4-pyridyl-l-oxide) -N-tert-butylnitrone (4-POBN) was added to a final concentration of 100 mM. ESR spectra were recorded in a flat quartz cell with a Varian E104 X-band spectrometer, operating at 9.5 GHZ with 100 KHZ modulation frequency, l0 mW microwave power and 0.2 mT modulation amplitude. In addition to TBARS production, hexanal release from the incubations was measured by the method of Frankel et al.(1989).

Results

Tocopherol isomers

Preincubation of microsomes from vitamin E deficient rats with increasing concentrations of dl α-, d α- and d γ-tocopherol progressively decreased the rate and extent of lipid peroxidation. The length of the lag phase was significantly correlated with microsomal tocopherol content (Fig. 1) . The antioxidant effectiveness of the isomers was d γ- > d α- > dl α-tocopherol. For example, the microsomal concentrations of d γ-, d α- and dl α- required to inhibit peroxidation by 50% were 160, 750 and 1850 μg/mg protein respectively.

"*Palm Vitee*"

Addition of increasing concentrations of "Palm Vitee" also progressively inhibited TBARS formation which was completely suppressed when the preparation was present in the incubation at a final concentration of 0.4 mM. "Palm Vitee" was 3 times more effective in reducing the extent of peroxidation compared with the equivalent concentration of γ-tocopherol; the latter was the most potent of the tocopherol isomers (Fig 2).

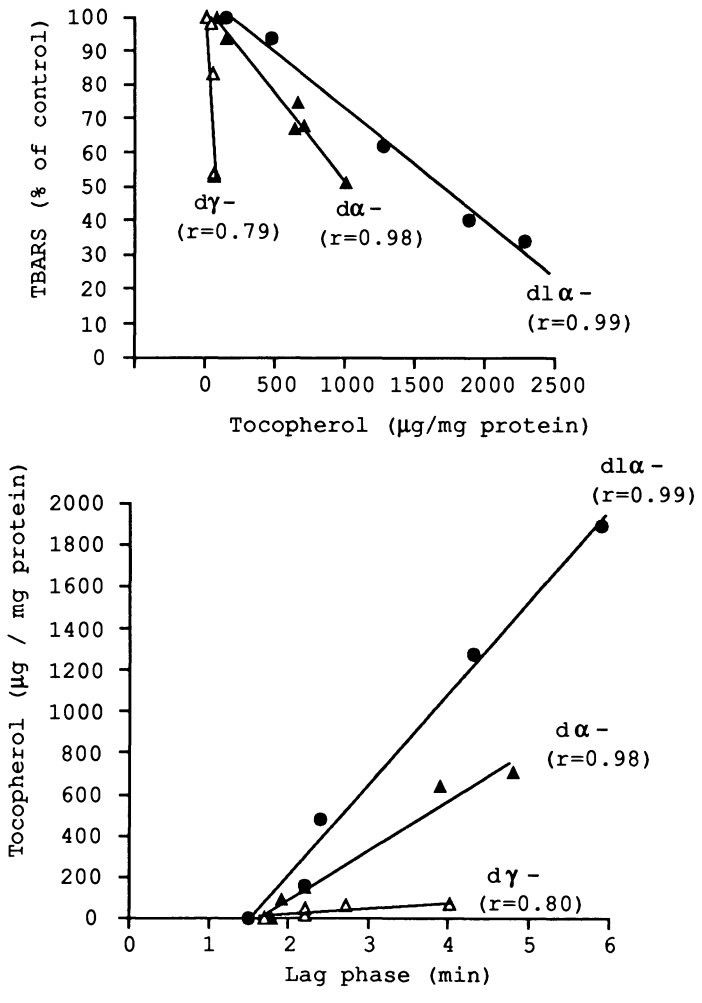

Fig. 1. Effects of microsomal dl α-, d α- and d γ-tocopherol content on (a) the magnitude and (b) the lag phase of lipid peroxidation

Electron Spin Resonance

The ESR spectra obtained from microsomal incubations containing 4-POBN consisted of a triplet of doublets representing interactions of the unpaired electron with 14N nucleus further split by interaction with a single hydrogen. The isotropic hyper-fine

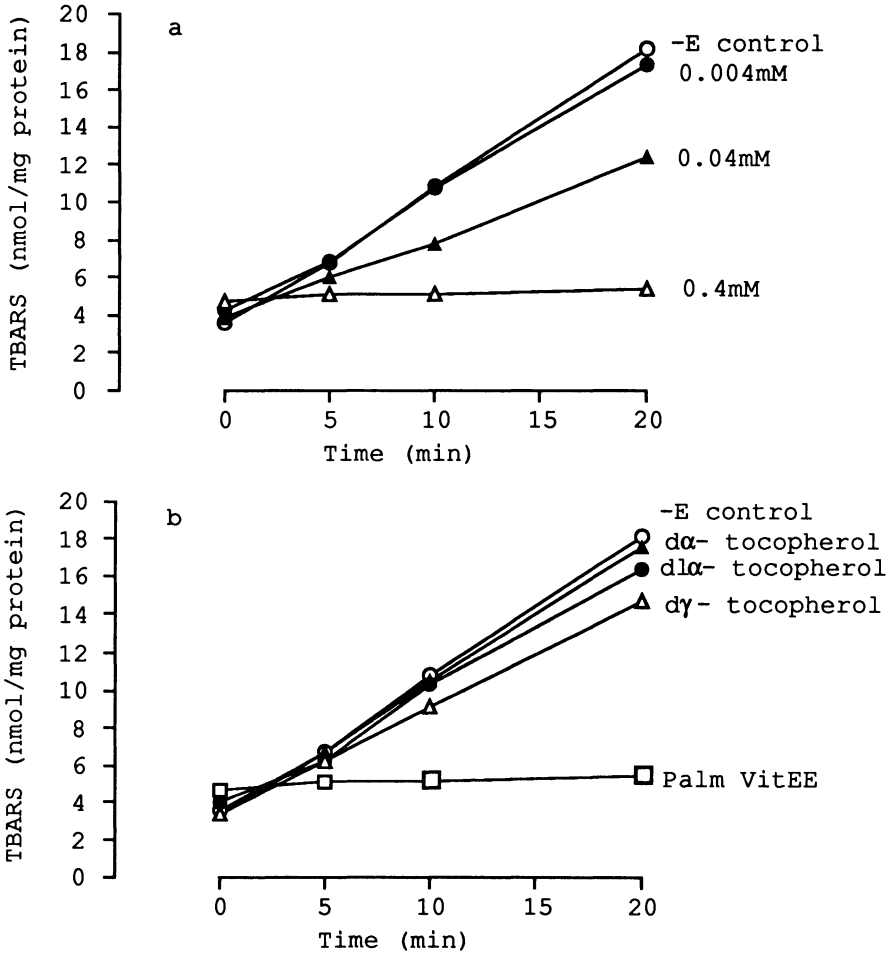

Fig. 2. Effects of the addition of various concentrations of "Palm Vitee" on the lipid peroxidation of microsomes from vitamin E-deficient rats. Also shown (b) is a comparison of the effect of equimolar concentrations (0.4 mM) of "Palm Vitee" and individual tocopherol isomers.

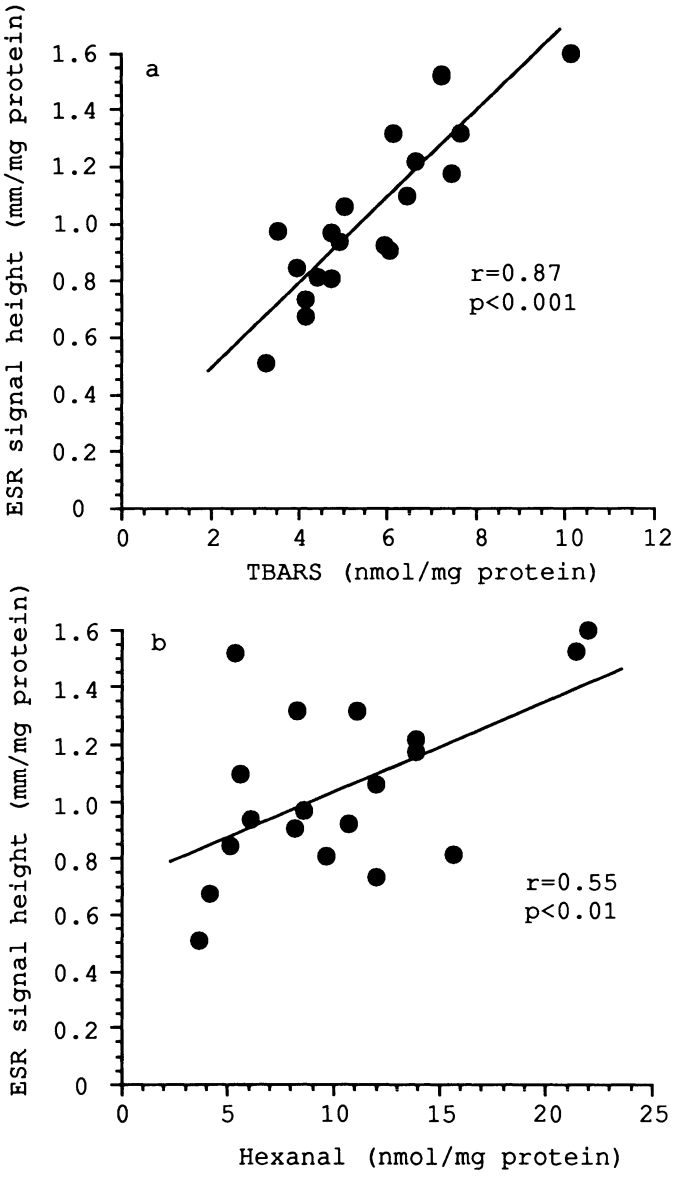

Fig. 3. The relationship between ESR signal intensity of the adduct trapped with 4-POBN and (A) TBARS (B) hexanal.

splitting constants A(14N) and A(1H) were 15.8 G and 2.6 G respectively. Signal intensity was significantly correlated with the production of TBARS ($P<0.001$, $r=0.87$) and hexanal ($P<0.05$, $r=0.55$) (Fig. 3).

Discussion

All eight naturally occurring isomers of vitamin E are derivatives of 6-chromanol and differ in the number and position of the methyl groups on the ring structure. The four tocopherol isomers (d α-, d β-, d γ-, d δ-) have a saturated 16-carbon phytol side chain whereas the tocotrienols (d α-, d β-, d γ-, d δ-) have three double bonds on the side chain. The widely available synthetic form, dl α-tocopherol, consists of a mixture of eight stereoisomers in approximately equal amounts; these isomers are differentiated by rotation around optical (chiral) centers at each methyl branch point of the phytol chain.

In the hepatic microsomal model used in the present study, the d γ-tocopherol was the most effective antioxidant. Compared with dl α- and d α-tocopherol, less was required to reduce the magnitude of peroxidation and the lag phase was longer. As intercalation of vitamin E within lipid bilayers may be affected by the position of the methyl groups on the chromanol ring and by the configuration of the carbons of the phytol side chain (Kagan et al., 1990), the differences in antioxidant effectiveness between the isomers may reflect variation in their orientation within the microsomal membranes.

"Palm Vitee" was much more effective than the tocopherol isomers in inhibiting microsomal TBARS formation. Such antioxidant potency may be ascribed to the tocotrienol components of the preparation. It is unclear at present which of the tocotrienol isomers (α -, γ -, or δ -) is the most active. However, recently Serbinova et al. (1991) have hypothesized that α-tocotrienol may have a higher antioxidant potency than α-tocopherol due to: (a) a higher recycling efficiency from chromanoxyl radicals, (b) a more uniform distribution in the membrane bilayer (c) a membrane disrupting effect which increases the efficiency of the interaction of chromanols with lipid radicals.

The rat foetal-resorption test indicates that the biological activity of γ-tocopherol is only 10% of that of the α-form (Horwitt, 1960) Therefore there is a considerable discrepancy between their in vitro antioxidant potencies and their biologically-relevant activity. In vivo, the tocopherol-transporting chylomicron remnants are taken up by the liver which preferentially secretes the α-form into the plasma in newly formed very low density lipoproteins (VLDL) but excretes most of the γ-tocopherol into the bile (Traber and Kaden, 1989) . In addition, plasma and cytosolic transporters and membrane receptors which are specific for the α- isomer (Behrens & Madere, 1982; Kaplowitz et al., 1989) may be required to achieve the correct alignment of the molecule in the membrane. Whether such receptors are needed to position tocotrienols in the membrane so that the functional hydroxy group of the chromanol ring is in the appropriate position to donate a hydrogen to the adjacent peroxidizing phospholipid is not known. Dietary repletion studies and clinical trials need to be undertaken before the nutritional and pharmacological relevance of the tocotrienols can be established.

The fluorescent determination of TBARS in biological samples can be subject to interference from iron, sucrose, and biliverdin (Duthie, 1991). However, in the present study, TBARS production correlated significantly with ESR signal intensity. Lack of spectral anisotropy in the ESR signal indicated that the radical was of low molecular weight with little motional restriction. Adduct parameters obtained using 5,5-dimethyl-l-pyrroline-N-oxide (DMPO) A(N) = 15.6 G, A(H) = 23.4 G, data not shown) which may be more sensitive to the trapped radical than 4-POBN were far removed from those parameters expected for $OH^{\bullet}$, O_2^{-}, thiyl and alkoxyl. This provides further evidence for the detection of a carbon-centered species, which may be derived from linoleic acid. Moreover, the significant correlation between ESR signal and hexanal also suggests that microsomal oxidation involved the decomposition of omega-6 fatty acids. Consequently, in the hepatic microsomal model used in this study, TBARS provided a reliable method of determining lipid peroxidation.

Acknowledgements

Our thanks to BASF, Germany for supplying the tocopherol isomers and to PORIM for the "Palm Vitee" preparation. We are grateful to Neil Gibson, University of Aberdeen, for providing DMPO.

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EFFECT OF VITAMIN E ON METABOLISM OF UREMIC LOW DENSITY LIPOPROTEINS IN HUMAN MONOCYTE-DERIVED MACROPHAGE

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Summary

In vivo malondialdehyde (MDA) rich low density lipoproteins (LDL) obtained from hemodialysis (HD)-patients may undergo more degradation than native LDL (n-LDL) obtained from healthy controls in human monocyte-derived macrophage. Vitamin E treatment reduced MDA in LDL and suppressed degradation in macrophage.

Introduction

It is well known that LDL modified in vitro are mainly metabolized in scavenger cells such as macrophage without being mediated by LDL receptors. The macrophage could develop into form cells, which have a close relation to the early stage of atherosclerosis (1). MDA is known to be a factor which could form modified LDL (2). However, all the studies on MDA-rich LDL (MDA-LDL) previously reported used artificially produced MDL-LDL and there have been no reports concerning the metabolism of MDA-LDL formed in vivo.

The present study was undertaken to investigate the metabolic profile of LDL in human macrophage, using MDA-LDL obtained from HD-patients who have usually higher MDA concentrations (3). Furthermore, alpha-tocopherol (TOC) was administered to the patients in order to examine possible metabolic changes of the LDL in macrophage due to reduction of MDA.

Materials and Methods

Subjects

Five age- and sex-matched healthy controls (controls) and HD-patients were enrolled in this study.

Methods

1) Preparation of LDL and ^{125}I-LDL. Fasting sera from control and from HD-patients at two weeks before and after administration of TOC of 600 mg a day were ultracentrifuged to obtain LDL at a density of 1.019 to 1.063 g/ml. The LDL obtained was dialyzed with 0.15 M NaCl, lmM EDTA, pH 7.4, and most part was pooled and then labelled with ^{125}I using the method of Mac Farlane et al. 2) Preparation of macrophage. A 20 ml sample of early morning fasting peripheral venous blood was obtained from healthy males, and macrophage were separated from monocytes using conventional method, cultured , supplemented with 10% LPDS, and used for experiments 48 hours later. 3) Metabolic profiles of ^{125}I-LDL in macrophage. After macrophages and ^{125}I-LDL were co-incubated for three hours, the binding, uptake and degradation of LDL was examined by the method of Goldstein et al (4). 4) Plasma lipids and composition of LDL. Proteins in LDL were determined by the Lowry method, lipids in plasma and LDL by enzyme method, and MDA in plasma and LDL by Yagi's thiobarbituric acid method, respectively (5).

Results

In plasma lipids, triacylglycerols (TG) and MDA levels were significantly higher in HD-patients than in controls, while high-density lipoprotein (HDL) cholesterol (HDL-C) showed lower values in HD-patients than in controls. MDA in HD-patients treated with TOC tended to be lower (Table 1).

Cholesterol level of LDL in HD-patients untreated with TOC was significantly lower than in controls and somewhat recovered after TOC treatment; TG level of LDL before and after TOC treatment in HD-patients were significantly higher than in controls and was slightly lowered after TOC treatment (Fig.1).

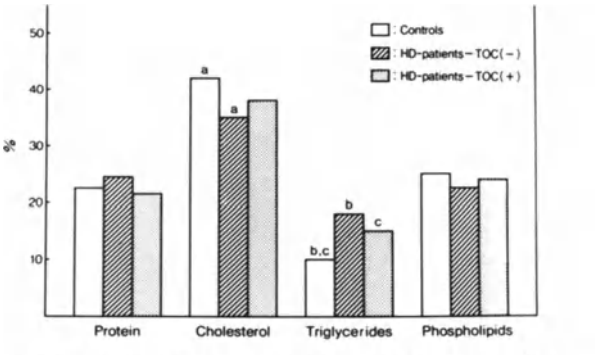
Apolipoproteins in LDL by 10% SDS-PAGE showed no noticeable differences between controls and HD-patients (data not shown). MDA concentration in LDL were significantly higher in HD-patients untreated with TOC than in controls and tended to decrease after TOC treatment (Fig. 2). Degradation of ^{125}I-LDL in cultured macrophage was significantly increased in HD-patients compared to controls, as shown in the left panel of Fig. 3. On the other hand, the right panel shows a comparison between HD-patients with and without TOC treatment.

TABLE 1.

Plasma lipid composition in control and HD-patients with (VE (+)) and without (VE (-)). TOC treatment

	n	TG	TC	HDL-C	MDA
		mg/dl			nmol/ml
Controls	5	88±25	195±34	51±10	2.5±0.5
HD-patients VE (+)	5	143±26*	191±52	34±11**	4.0±0.6*
VE (-)	5	136±46*	188±51	34±10**	3.3±0.6

Mean±SD. Significance vs controls; *: $p < 0.01$, **: $p < 0.05$.



Value represents as mean. No.of cases : Controls, 5, HD-patients －TOC(－), 4 and HD-patients－TOC(＋), 3. a,b : $p<0.01$, c : $p<0.05$

Fig. 1. Chemical composition of LDL.

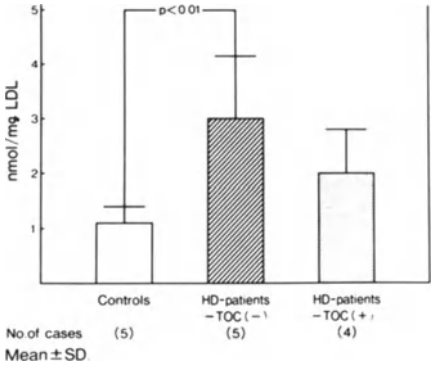


Fig. 2. Malondialdehyde concentration in LDL.

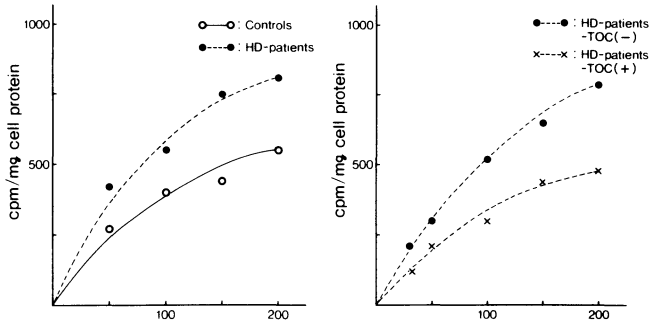


Fig. 3. Degradation of ^{125}I-LDL in cultured macrophage.

HD-patients treated with TOC showed a suppression of ^{125}I-LDL degradation compared to TOC-untreated HD-patients towards value obtained in control. There were no significant differences in binding and uptake of ^{125}I-LDL between HD-patients and controls (data not shown).

Discussion

It is well known that LDL artificially modified by acetylation or MDA is metabolized mostly in scavenger cells without mediation by LDL receptors (1, 2). There have been numerous reports on modified LDL which was metabolized in vitro, but there have been few (6) reports on modified LDL which was formed in vivo. In the present study, we investigated the metabolic profile of a modified LDL, MDA-LDL, which seems to be actually present in vivo, in human monocyte-derived macrophage. Samples of modified LDL were obtained from HD-patients who have higher MDA concentrations (3).

To reduce MDA in blood, TOC was administered to the patients, and the metabolism of MDA-LDL was examined before and after TOC treatment. MDA levels in total LDL were significantly higher in HD-patients than in controls. TOC treatment tended to reduce LDL-MDA levels in HD-patients. In parallel with this finding, degradation of ^{125}I-LDL in human macrophage was accelerated in HD-patients compared to controls, and such acceleration of degradation was suppressed after TOC treatment. This indicates that LDL from HD-patients is oxidized by MDA at higher concentrations and is developed to MDA-LDL, whose degradation is accelerated via scavenger pathway of macrophage (2); TOC treatment reduces MDA in blood, resulting in suppression of degradation of LDL in macrophage. The results of this study suggest a possibility that MDA-LDL from HD-patients may undergo degradation similar to artificial MDA-LDL in vitro (1, 2). This seems to be in agreement with the fact that HD-patients on long-term dialysis develop atherosclerosis at an earlier stage than controls with the same age (7).

Conclusion

In the present study, we investigated the metabolism of in vivo modified LDL obtained from HD-patients by macrophage and the effect of TOC treatment. The results were as follows: 1) It was found that in vivo MDA-LDL may undergo more degradation than N-LDL in macrophage. 2) TOC treatment reduced MDA in LDL and suppressed degradation in macrophage.

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DIFFERENCE OF ANTIOXIDATIVE EFFECT BETWEEN VITAMIN E AND SELENIUM

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Summary

To study antioxidative effects of vitamin E and selenium, two experiments were conducted. Experiment I: To study effects of vitamin E and/or selenium deficiency on malondialdehyde (MDA) levels in tissues and on oxidation of erythrocyte of rat fed with natural deficient diet. Experiment II: To study effects of vitamin E and/or selenium deficiency on MDA levels and free radicals levels in tissues, and on fluidity of erythrocyte membrane of rat fed with semisynthetic diet. It was found that MDA levels in tissues of vitamin E deficient groups were higher than that of selenium deficient groups in both experiments. In contrast to MDA level, free radicals levels detected by ESR in tissues of vitamin E deficient groups were lower than that of selenium deficient groups. Results of oxidation of erythrocyte with oxidants in vitro showed that vitamin E protected against hemolysis of erythrocyte induced by ascorbic acid, whilst selenium protected against oxidation of haemoglobin by superoxide anion. Effects of vitamin E and selenium deficiency on fluidity of erythrocyte membrane estimated by ESR and expressed as rotational correlation time (r_c) were also different: vitamin E deficiency increased r_c, when fluidity was estimated by labelling with 5-doxy stearic acid or 16-doxy stearic acid. Selenium deficiency increased r_c, when fluidity was estimated by labelling with 3-maleimido proxy and 3-(3-maleimidopropyl-carbamyl) proxy. It means that vitamin E deficiency caused decrease of fluidity of erythrocyte membrane due to damage to lipid of membrane and selenium deficiency caused decrease of fluidity of erythrocyte membrane due to damage to -SH group in membrane protein. These results demonstrated that antioxidant action of vitamin E and selenium are different: (1) vitamin E acts on the biomembrane, and selenium acts in the form of Se-dependent glutathione peroxidase (GPX) in cytoplasm; (2) vitamin E prevents lipid peroxidation more effectively than selenium, whereas selenium prevents free radicals production more effectively than vitamin E; (3) vitamin E protects lipid of membrane and selenium protects -SH group in membrane protein against oxidation.

Introduction

It is well known that both vitamin E and selenium are important bioantioxidants that prevent peroxidation in cells. It is generally considered that these two micronutrients perform antioxidation synergistically, or they may substitute for each other in antioxidation. However, our previous work (Zhu et al. 1981) showed that effects of vitamin E deficiency and selenium deficiency on swelling of pig liver mitochondria were different: vitamin E deficiency caused increasing swelling rate and selenium deficiency caused decreasing swelling rate (Fig. 1). And it was found that effects of these two micronutrients deficiency on fluidity of mitochondria membrane were different too: Vitamin E deficiency decreased fluidity, whereas selenium deficiency increased fluidity (Fig. 2). So we conducted two experiments to study the antioxidation effects of vitamin E and selenium.

Experiment I: To study effects of vitamin E and/or selenium deficiency on MDA level in tissues and on oxidation of erythrocyte of rat fed with natural deficient diet.

Experiment II: To study effects of vitamin E and/or selenium deficiency on MDA level and free radicals level in tissues and on fluidity of erythrocyte membrane of rat fed with semisynthetic deficient diet.

Materials and Methods

Experiment I:

Animal experiment and analytical methods have been described in detail by He and Zhu (1987) and are only briefly noted below:

Animal and diet: 48 male weaning Wistar rats were divided into four groups with 12 each, and were raised with vitamin E and/or selenium deficient diet composed with natural selenium deficient crops (corn meal, soybean meal and wheat flour) removing vitamin E by extracting with ethanol. Vitamin E and selenium contents of diets of these four groups were shown in Table 1.

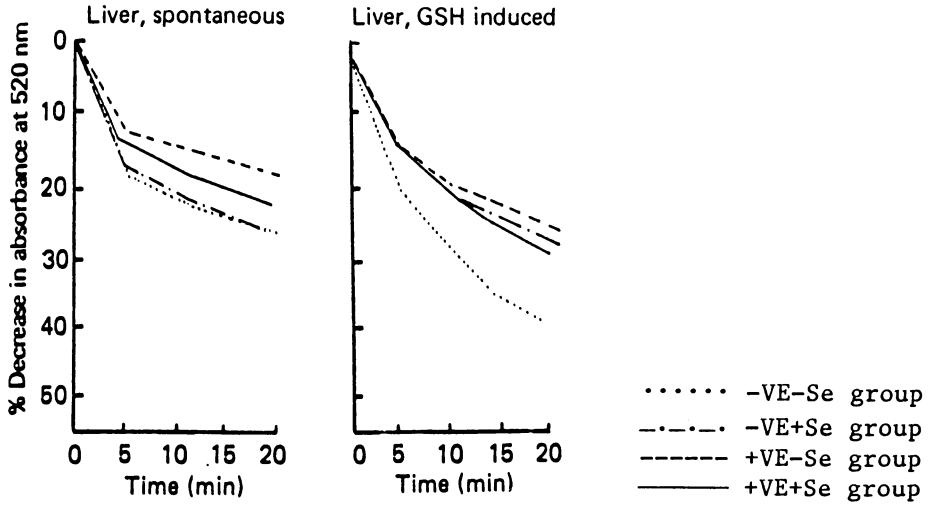

Fig. 1 Mitochondrial swelling of pigs.

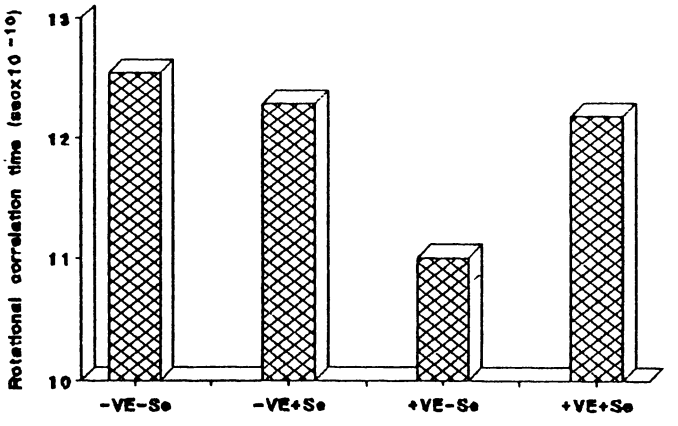

Fig. 2 Effects of supplementation of VE and/or Se to deficient diet on fluidity of liver mitochondrial membrane.

Table 1 Vitamin E and Selenium Contents of Diets

Group	Vitamin E(mg/kg)	Selenium(mg/kg)
-VE-Se	0.96	0.0067
-VE+Se	0.96	0.175
+VE-Se	25	0.0067
+VE+Se	25	0.175

Methods

(1) Vitamin E contents of diets was estimated by flourometric method (Hou and Zhu, 1982).

(2) Selenium content of diets was determined by flourometric method (Liu et al., 1985)

(3) MDA level in tissues was determined with TBA method (Wang, et al., 1980).

(4) Oxidation of erythrocyte in vitro:

a. According to Rotruck's (1972) method, erythrocytes was oxidized with ascorbic acid, but the concentration of ascorbic acid was adjusted to causing hemolysis of deficient erythrocyte only, and percentage of hemolysis was calculated.

b. According to Goldberg's (1977) method, erythrocyte was oxidized by superoxide anion generated with dihydroxy fumaric acid (DHF), concentration of which was adjusted to that only haemoglobin in deficient erythrocyte was oxidized, and percentage of haemoglobin oxidized was calculated. At this concentration of DHF, superoxide anion did not cause hemolysis.

Experiment II

Animal experiment

Design of animal experiment has been described in detail by Cai et al. (1992). 40 non-Se-deficient weaning rats were divided into two for -VE+Se groups

with 10 male and 10 female each. 40 Se-deficient weaning rats were divided into two for +VE-Se and -VE-Se groups with 10 male and 10 female each. Principal food of basal diets were torula yeast and corn meal in which vitamin E was removed by extracting with ethanol. α-Tocopherol and selenium were supplemented to the basal diet for non-deficient groups. α-Tocopherol and selenium contents of diets of these four groups were listed in Table 2.

Table 2 α-tocopherol and selenium contents of diets

Group	Selenium(ppm)	α-tocopherol(ppm)
-VE-Se	0.009	non
-VE+Se	0.274	non
+VE-Se	0.009	25.2
+VE+Se	0.274	25.2

Analytical methods

(1) Vitamin E content of diets was detected HPLC.

(2) Selenium content of diets was determined with fluorometric method (Liu et al., 1985).

(3) MDA levels in liver and lens were determined with TBA by calorimetric (Buege and Aust, 1978) and fluorometric methods (Wang et al., 1980) respectively.

(4) Free radicals levels and fluidity of membrane were detected by electronic spin resonance (ESR).

RESULTS AND DISCUSSION

Experiment I:

MDA levels in tissues: MDA levels both in heart and in liver of -VE+Se group was higher than +VE-Se group. However, it was significant in liver (Fig.3 and Fig.4). It

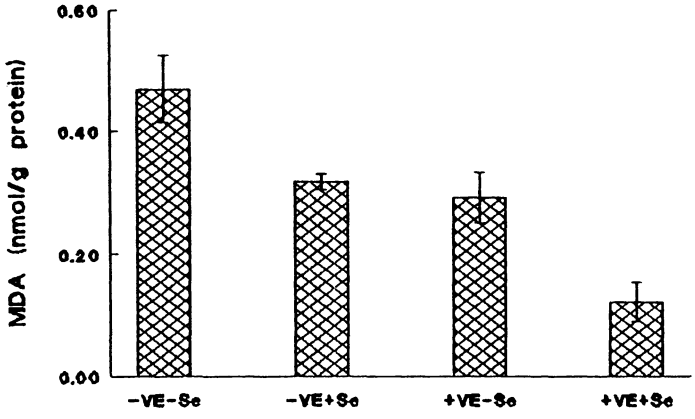

Fig. 3 MDA level in hearts of VE and/or Se deficient rats

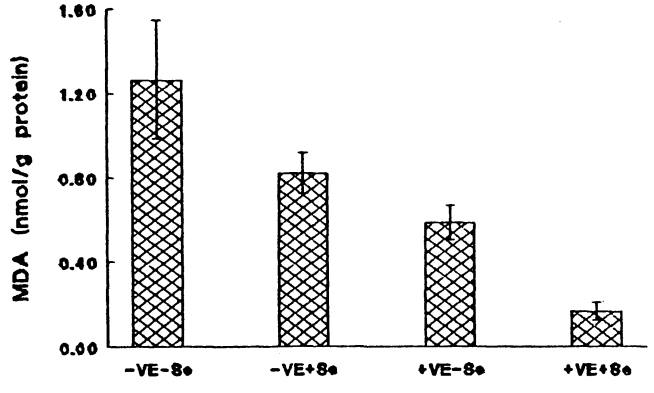

Fig. 4 MDA level in livers of VE and/or Se deficient rats

means that both vitamin E and selenium can prevent lipid peroxidation, but vitamin E is more effective than selenium.

Oxidation of erythrocyte

When erythrocyte was oxidized with proper concentration of ascorbic acid in vitro, hemolysis were serious in two vitamin E deficient groups. Supplementation of vitamin E prevented hemolysis no matter selenium deficient or no (Fig. 5). It showed that vitamin E can protect membrane broken caused by oxidation due to that vitamin E locates in membrane (Diplock and Lucy 1973).

When erythrocyte was oxidized by proper concentration of superoxide anion generate from DHF, hemolysis did not occur, but hemoglobin was oxidized. Percentages of oxidized haemoglobin in two Se-deficient groups were higher than that of two Se-supplement groups no matter vitamin E deficient or not (Fig.6). Protection of vitamin E against oxidation of haemoglobin by superoxide anion was not observed. Selenium prevented oxidation of hemoglobin by Se-dependent GPX in cytoplasm. This is the difference of antioxidation effect between vitamin E and selenium in acting sites.

Experiment II:

MDA levels in liver and lens

Results of this experiment was similar to that of experiment I. MDA levels both in liver and lens of -VE+Se group were higher than that of +VE-Se group (Fig. 7 and Fig. 8).

Free radicals levels in liver and lens

In contrast to MDA, free radicals levels both in liver and in lens of -VE+Se were lower than that of +VE-Se group, that of +VE+Se group were lowest (Fig. 9 and Fig. 10). This indicated that supplementation of vitamin E or selenium both could reduce production of free radicals, but selenium was more effective than vitamin E. It means that protect subject of these two nutrients were different too, vitamin E is more effective to prevent lipid peroxidation, whereas selenium is more effective to scavenge free radicals.

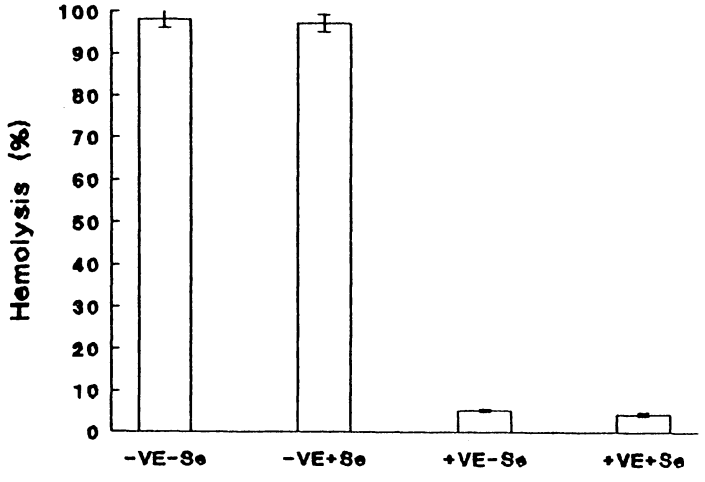

Fig. 5 The effects of dietary vitamin E and selenium on hemolysis of rats erythrocytes induced by vitamin C.

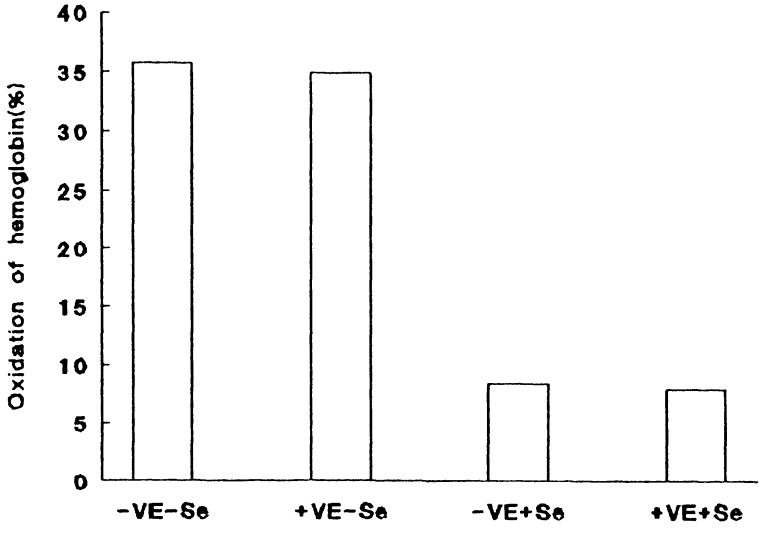

Fig. 6 The effects of dietary vitamin E and Se on oxidation of haemoglobin by superoxide anion.

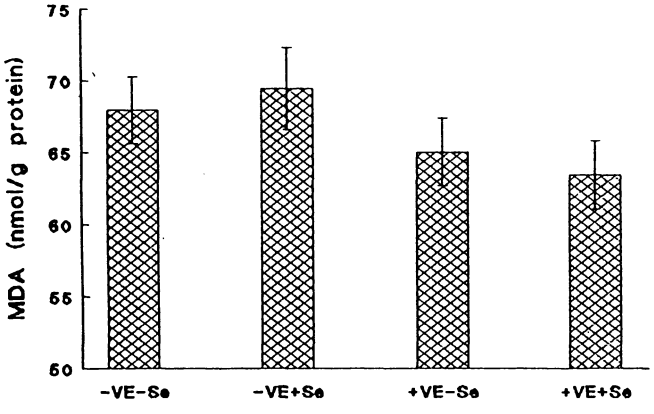

Fig. 7 MDA level in liver of VE and/or Se deficient rats.

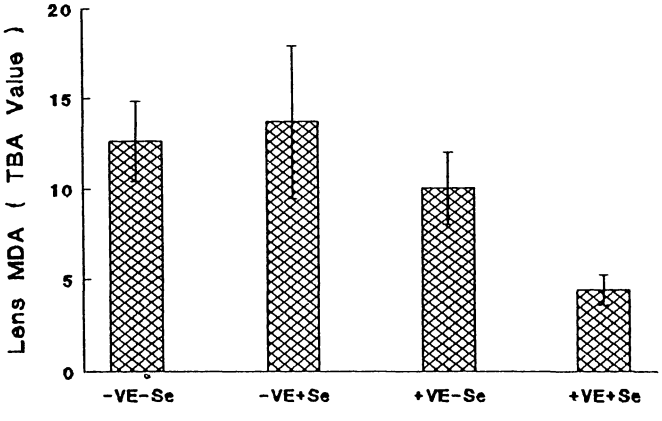

Fig. 8 MDA level in lens of VE and/or Se deficient rats.

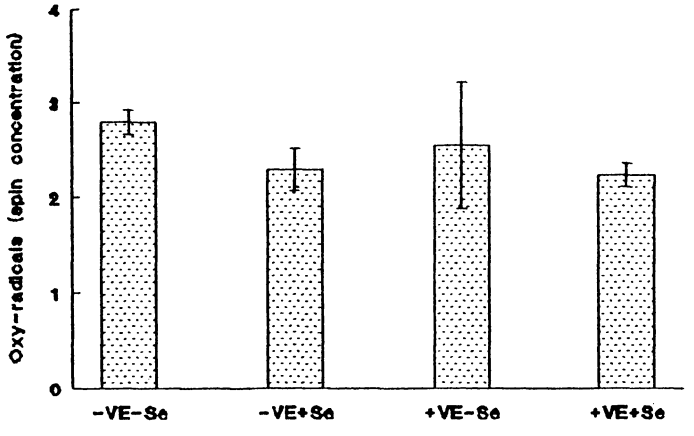

Fig. 9 Effect of supplementation of vitamin E and/or selenium to deficient diet on oxy-radicals in level in rat's liver.

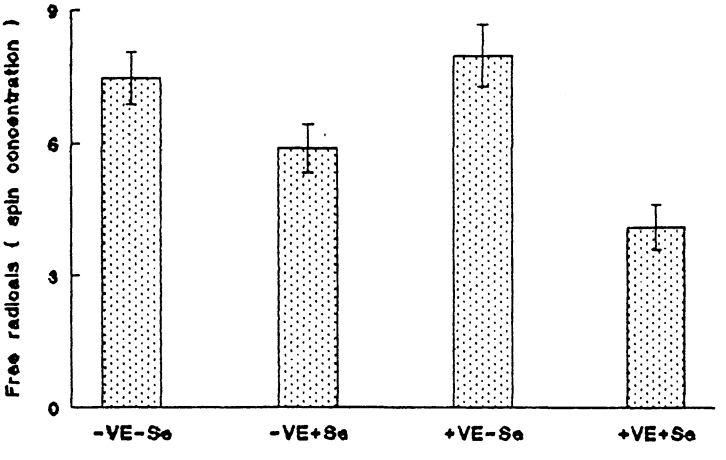

Fig. 10 Free radicals level in lens of VE and/or deficient rats.

Effect of fluidity of erythrocyte membrane

Fluidity of erythrocyte membrane detected by labelling with 5-doxy stearic acid and 16-doxy stearic acid decreased in vitamin E deficient group (expressed by increasing rotational correlation time (r_c) (Fig.11). Fluidity detected by labelling with 3-maleimido proxy and 3-(3- maleimidopropylcarbomyl proxy decreased in Se-deficient group (increasing r_c) (Fig.12). These results furthermore prove that vitamin E protects lipid and selenium protects -SH group of protein against oxidation.

CONCLUSION

Antioxidative effects of vitamin E and selenium are notably different.

1. They act on different sites

Vitamin E acts on the biomembrane, whereas selenium acts in the form Se-dependent GPx in cytoplasm.

2. They protect different targets

a) Vitamin E seems more effective in protecting against lipid peroxidation than selenium, whilst selenium seems more effective in preventing the production of free radicals than vitamin E.

b) Vitamin E protects fatty acid and selenium protects thiol group in erythrocyte membrane mainly.

It is suggested that antioxidant action of these two micronutrients is compensative, not substitutive for each other.

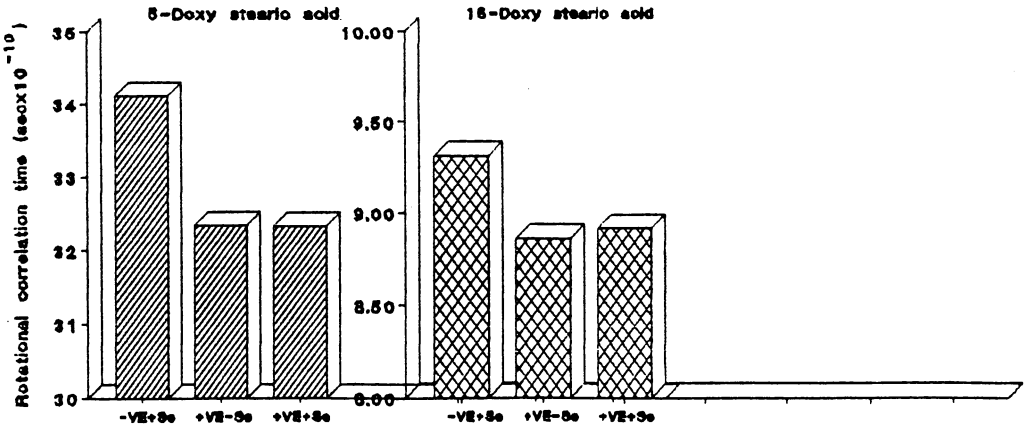

Fig. 11 Effect of VE or Se deficiency on fluidity of erythrocyte membrane labelled with 5-Doxy stearic acid and 16-Doxy stearic acid

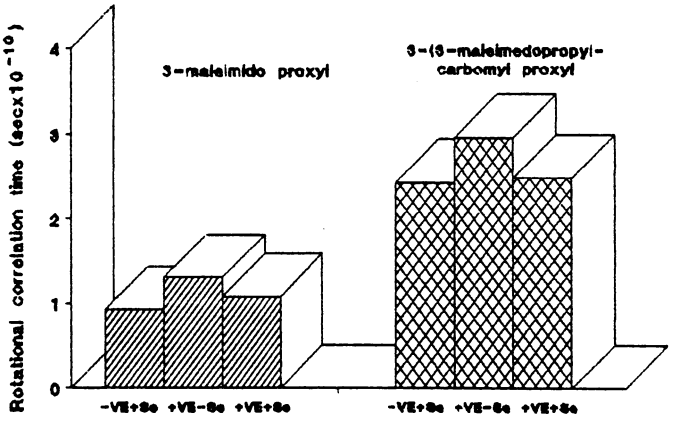

Fig. 12 Effects of VE or Se deficiency on fluidity of rat's erythrocyte membrane labelled with 3-maleimide proxyl and 3-(3-maleimidopropylcarbomyl) proxyl.

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TOCOPHEROL AND TOCOTRIENOL PLASMA TRANSPORT AND TISSUE CONCENTRATIONS: IMPLICATIONS FOR THEIR RELATIVE BIOLOGICAL FUNCTIONS

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Summary

The plasma transport and certain aspects of tocopherol and tocotrienol physiology were examined in humans and hamsters. First, their transport differed in that tocopherol was found primarily in LDL and HDL whereas tocotrienols disappeared from plasma with chylomicrons removal. Second, tocopherol concentration in LDL was inversely proportional to the "atherogenic potential" of LDL in an in vitro monocyte adhesion assay. Although vegetarian had more polyunsaturates and less total tocopherol in their plasma lipids, they maintained a higher tocopherol : cholesterol molar ratio. In essence, we find support for the concept that high intake of polyunsaturated fat is deleterious to the oxidation state of circulating LDL. Third, a high intake of tocotrienol did not appear to modulate plasma cholesterol levels in normolipidemic hamsters. We conclude that the transport, tissue concentration, and relative biological functions of tocopherol and tocotrienol are distinct and relatively unrelated.

Introduction

Tocopherol has long been recognized as the most important lipid antioxidant in mammalian systems. Recent attention has turned to the possible biological importance of tocotrienols and whether they might also function in an antioxidant capacity to modulate the pathway for cholesterol biosynthesis (Parker et al., 1991) and actually lower plasma cholesterol in certain hypercholesterolemic individuals (Tan et al., 1991; Qureshi et al., 1991).

It appears that tocotrienols may be involved in specialized microsomal systems (Komiyama et al., 1989; Serbinova et al., 1991), as opposed to the general membrane antioxidant function of tocopherol (Traber & Kayden, 1989). For example, in the original anti-fetal resorption assay in rats, α-tocotrienol revealed only 10-30% of the activity expressed by α-tocopherol, indicating its relative ineffectiveness in that system (Bunyan et al., 1961; Keth & Sondergaard, 1977). On the other hand, in subcellular hepatic microsomal redox systems tocotrienols may be 40x as effective as α-tocopherol (Serbinova eta], 1991).

It is in this context that we have re-examined certain aspects of lipoprotein transport and tissue storage of tocopherols and tocotrienols with a focus on their comparative biological function. For these experiments we utilized a variety of systems including humans, monkeys, hamsters and tissue culture.

Results and Discussion

Absorption and Lipoprotein Transport

Tocopherols:It is now well appreciated that the 4 isomers of tocopherol (α,β,γ,δ) (Figure 1) are not equally absorbed by the intestine or transported by lipoproteins. Nor are they equally sequestered by certain tissues (Biomeboe et al., 1990). By virtue of a specific α-tocopherol binding protein in hepatocytes (and possibly other cell types as well), the alpha isomer is preferentially retained and subsequently secreted in VLDL (Traber & Kayden, 1989). The α-tocopherol binding protein would appear to be present in the intestine, too, since even though all isomers of tocopherol are absorbed by the intestinal mucosa cell, α-tocopherol was preferentially secreted in hamster chylomicrons (Table 1). Once returned to the liver in chylomicron remnants, α-tocopherol is selectively bound to the cellular binding protein for resecretion in nascent hepatic lipoproteins while β, δ and γ-tocopherol rapidly disappear, except for a minor component of γ-tocopherol representing about 10-15% of the α-tocopherol mass (Traber & Kayden, 1989). The rapid disappearance of β- and δ-tocopherol may reflect the methylation of these two dimethyl isomers to the tri-methyl α-tocopherol by tissues (Baker et al., 1986). High α-tocopheryl acetate supplementation results in further displacement of plasma α-tocopherol (Pronczuk et al., 1991). The selective sequestration and transport of tocopherols is depicted in Figure 2.

TABLE 1

Absorption and Transport of Tocotrienols(T_3) and tocopherols(T) as evidenced by concentration in intestinal mucosa, lymph and plasma of hamsters fed a diet enriched with tocotrienols[a] and in plasma of humans supplemented with tocotrienols[b].

	N	Tocotrienol				Tocopherol				T_3:T
		α	γ	δ	Total	α	β+γ	δ	Total	
Hamster(μg/g or dl)										
Diet	4	80±02	113±4	30±1	233±6	12±1	13±0	2±0	80±1[c]	3:1
Intestinal Mucosa (postprd; 3hrs)	2	11±1	19±2	7±1	37±3	6±0	6±1	4±1	16±1	2:1
Lymph (postprd; 3 hrs)	2	250±60	400±7	360±20	1010±15	3160±530	220±80	70±20	3450±620	1:3
Plasma(overnight fast)[d]	4	<1	<1	0	<2	2520±470	60±10	30±10	2610±480	1:1300
Plasma (postprd; 3 hrs)	3	30±40	10±1	<1	40±10	870±110	70±20	40±10	980±100	1:25
Plasma (postprd; 3 hrs+Triton)[e]	3	220±40	110±20	20±2	350±40	1440±150	180±60	70±20	1690±130	1:5
Human Plasma (μg/dl)										
Baseline (fasted 6 hrs)	5	0	0	11±2	11±2	1070±190	120±40	4±4	1190±240	1:110
Supplemented (fast 6 hrs)	5	<1	<1	13±2	13±2	1594±330	50±20	11±1	1640±320	1:130
Supplemented (2 hrs postprd)	5	16±10	13±5	12±1	41±16	1940±210	54±8	18±4	2010±200	1:49

Values represented mean±S.D. (1 μmol of α-T_3 or α-T is equivalent to 425 μg and 431 μg respectively).

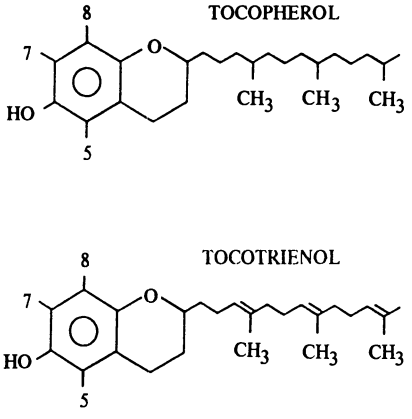
[a]Hamster were fed 4 wks a purified diet containing 223 ppm of tocotrienols and 70 ppm of tocopherols, of which 53 ppm was added with Vitamin mix in the form of dl-α-tocopherol acetate.

[b]Humans were supplemented with 2 Palm Vitee® capsules(80 mg of tocotrienols and 64 mg of tocopherols) per day for 10 days.

[c]Value includes tocopheryl acetate added to diet in vitamin mix at 53 ppm.

[d]Plasma of hamsters fed 11 wks a purified diet described on footnote a. Mean plasma cholesterol, 144±62 mg/dl; triglyceride 192±78 mg/dl.

[e]Plasma obtained from 3 hrs postprandial hamsters + 2 hrs post - Triton WR 1339 injection.



Position of Methyl Group	Tocopherol	Tocotrienols
5,7,8-Trimethyl	α-T	α-T_3
5,8-Dimethyl	β-T	β-T_3
7,8-Dimethyl	γ-T	γ-T_3
8-Monomethyl	δ-T	δ-T_4

Figure 1. Tocopherols and tocotrienols structure.

The preferential absorption and transport of tocopherols (T) over tocotrienols (T_3) suggests different lipid affiliations in plasma (see below). This was examined closely in the case of tocopherol by assessing its distribution among lipoproteins as well as its relation to the specific lipoprotein lipid constituents in humans (Pronczuk et al., 1991). The VLDL ($d < 1.006$ g/ml), IDL ($1.006 < d < 1.019$ g/ml), LDL ($1.019 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml) fractions were isolated from plasma of adult humans by means of sequential ultracentrifugation. For some adults and infants, HDL was also obtained by means of sodium phosphotungstate - Mg^{++} precipitation. Each lipoprotein was analyzed for its lipid composition and α-tocopherol concentration. Although generally agreed that lipoproteins transport tocopherol, reports vary in their assertion that most tocopherol is carried either by LDL (Davies et al., 1969) or by HDL (Takahashi et al., 1977). In terms of the mass distribution of tocopherol among lipoproteins in our population of adults, VLDL transported

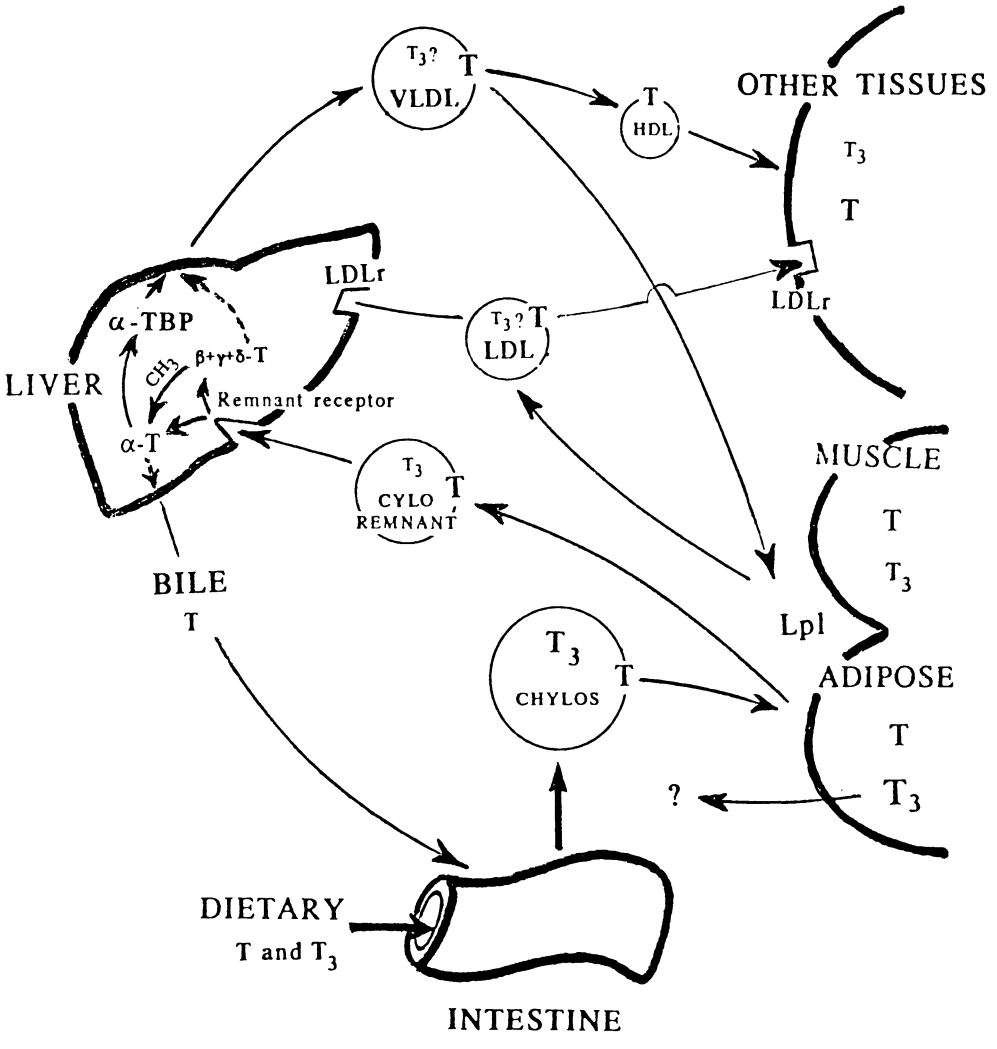


Figure 2. Transport of tocopherols(T) and tocotrienols(T_3) from intestine to other tissue. Both T and T_3 are absorbed from diet in chylomicrons and then T is transported mainly to the liver and with lipoproteins to other tissues, whereas T_3 is mainly transported and stored in adipose tissues.

TABLE 2.

Correlation coefficients between concentration of human plasma or lipoprotein lipid components and tocopherols.

	r	Significance
Plasma components		
cholesterol vs. γ-tocopherol	0.36	NS
cholesterol vs. α-tocopherol	0.73	$p < 0.005$
cholesterol vs α+γ-tocopherol	0.72	$p < 0.005$
triglyceride vs γ-tocopherol	0.58	$p < 0.05$
triglyceride vs α-tocopherol	0.34	NS
triglyceride vs α+γ-tocopherol	0.49	NS
phospholipids vs γ-tocopherol	0.51	NS
phospholipids vs α-tocopherol	0.74	$p < 0.005$
phospholipids vs α+γ-tocopherol	0.79	$p < 0.001$
Lipoproteins (VLDL, IDL, LDL, HDL) components		
cholesterol vs. γ-tocopherol	0.83	$p < 0.001$
cholesterol vs. α-tocopherol	0.85	$p < 0.001$
cholesterol vs. α+γ-tocopherol	0.86	$P < 0.001$
triglyceride vs γ-tocopherol	0.01	NS
triglyceride vs α-tocopherol	0.02	NS
triglyceride vs α+γ-tocopherol	0.02	NS
phospholipids vs γ-tocopherol	0.90	$p < 0.001$
phospholipids vs α-tocopherol	0.93	$P < 0.001$
phospholipids vs α+γ-tocopherol	0.94	$P < 0.001$

19%, IDL 3%, LDL 42%, and HDL 36%. Alpha -tocopherol represented 87-89% of the total tocopherol pool in each lipoprotein with the balance as γ-tocopherol (Table 1). In infants α-tocopherol represented 91% with γ-tocopherol the remaining 9% (Hayes et al., 1991).

Across all lipoprotein classes, α-tocopherol was highly correlated ($r=0.93$) with phospholipids (PLs) but not with core lipids such as triglycerides (Table 2). This suggests that tocopherol is associated with PLs on the surface of lipoproteins, presumably with polyunsaturated fatty acids in the PL molecule (Diplock et al., 1973). Also because HDL is relatively PL-rich, this lipoprotein has a higher concentration of tocopherol per mg of lipid than LDL or VLDL (Table 3) and transports the greatest mass of plasma tocopherol in infants where HDL is higher (infants HDL-C=28$\pm$7% of total plasma cholesterol, adults=23$\pm$7%). It is also apparent that for any given concentration of HDL cholesterol, infant HDL transports 10-20% more tocopherol than adult HDL for reasons that are unclear, but presumably related to greater concentration of HDL-PL in infant HDL (Figure 3). Because adults have a relatively expanded mass of LDL (adults LDL-C=124$\pm$7; infants=65$\pm$19), this lipoprotein fraction usually transports the largest mass of tocopherol in adults. This distinction between tocopherol concentration (depending on PLs) and bulk transport (depending on lipoprotein pool size) would appear to explain much of the discrepancy concerning which lipoprotein fraction carries the most tocopherol.

Tocotrienols: Since little is known about the transport and tissue concentration of the tocotrienols (vitamin E isomers of tocopherol with a triene-conjugated side chain, Figure 1), we explored these relationships in hamsters and humans (Davies et al., 1969; Liang et al., 1991). In neither species were tocotrienols readily detected in fasted plasma (Table 1). To increase the level in plasma we fed the tocotrienol-rich fraction isolated from palm oil to adult male Syrian hamsters in a purified diet enriched with 220 ppm tocotrienols and followed absorption and transport of the various tocotrienols into the postprandial mucosa (3 hour), mesenteric lymph (3 hour) and plasma lipoproteins at various time intervals. Plasma chylomicrons were also collected in the postprandial hamster following injection of Triton WR 1339. In addition a large number of tissues were examined for their relative tocopherol: tocotrienol ratio relative to the concentration of these two vitamin E molecules present in the

supplemented diet. In humans we compared the plasma level of tocotrienols and tocopherols in both supplemented and unsupplemented subjects. Supplementation was achieved with two capsules of Palm Vitee® providing 80 mg tocotrienol and 64 mg tocopherol per day for 10 days.

The results of these comparisons revealed that the tocotrienols behaved some what differently than the tocopherols, showing minimal preference among the isomers for absorption from the lumen into the mucosa and lymph (Table 4). Only in the adipose tissue of supplemented hamsters did the tocotrienols maintain their dietary concentration advantage over the tocopherols. This is corroborated by the fact that the T_3:T ratio was greater

TABLE 3.

Distribution of tocopherols (T) in lipoproteins from adult humans.

Lipoprotein fraction	N	α-T μg/dl	α-T:total lipids μg/mg	γ-T μg/dl	α+γ-T μg/dl	α+γ-T % distribution
VLDL	7	167±63 (88±4)†	1.58±0.17[a]	22±12	189±70	19±7
IDL	7	31±7 (89±5)	2.09±0.48	2±2	33±8	3±1
LDL	7	369±47 (87±1)	1.50±0.16[b]	57±11	426±56	42±5
HDL	7	323±62 (87±4)	1.91±0.18[a,b]	46±18	369±68	36±5
Total	7	891±88 (87±3)	1.74±0.15	127±34	1004±89	100

Values are expressed as means ±SD.
†Alpha-tocopherol as percent of total (α+γ-tocopherol) in each lipoprotein.
[a,b]Means with common superscript are significantly different by one factorial ANOVA and Fisher's protected LSD analysis ($p < 0.05$).

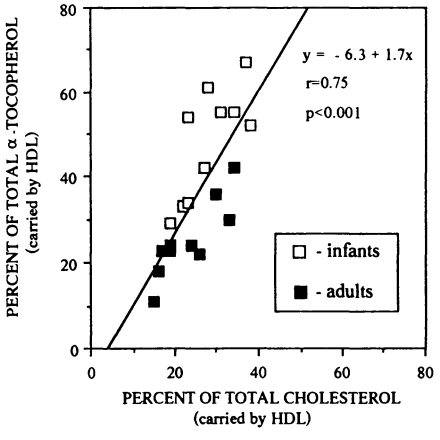


Figure 3. Relationship between cholesterol and tocopherol transported by HDL indicates that HDL carries an disproportionate amount of α-tocopherol when above 25-30% of the total cholesterol.

than 1.0 in adipose, which was the only tissue to accumulate more T_3 than T in supplemented hamsters. In contrast to tocopherols, the fasting hamster plasma contained almost no detectable tocotrienols. This was also true for the normal, fasting human. Even after supplementation with 80 mg T_3 per day, the fasting plasma level in humans averaged only 13 μg/dl of tocotrienols compared to 1655 μg/dl for tocopherols (Table 4). Tissue concentrations of T_3 in T_3-supplemented hamsters were typically much lower than tocopherol (1: 180 on average) and were undetectable (with exception of adipose tissue) in normal hamsters.

We interpret these observations to mean that the tocotrienols are initially transported like any lipid-soluble compound, most likely being incorporated along with triglyceride in the core of the triglyceride-rich chylomicron. Once transported to the adipose tissue, a modest level of tocotrienols appears to be deposited and stored with the triglyceride, presumably to be released during lipolysis. Unlike α-tocopherol, none of the tocotrienols appeared to be sequestered by a cytosolic binding protein in the liver to enhance their

TABLE 4

Content of tocotrienols(T_3) and tocopherols(T) in different tissues of hamsters fed a diet enriched with tocotrienols[a].

Diet	N	Tocotrienol(μg/g or dl)				Tocopherol(μg/g or dl)				T_3:T
		α	γ	δ	Total	α	β+γ	δ	Total	
Adipose[b]	7	3.8±0.4	5.3±0.7	2.1±0.3	11.2±1.3	4.9±0.7	1.4±0.3	1.0±0.4	7.3±0.5	2:1
Heart	7	2.0±0.4	0.5±0.1	<0.01	2.5±0.4	28.2±2.5	1.5±0.3	2.8±0.6	32.6±3.0	1:13
Testes	7	0.8±0.1	0.6±0.1	<0.01	1.4±0.1	14.4±2.9	1.2±0.2	1.3±0.1	17.6±3.0	1:13
Kidney	4	0.5±0.1	<0.01	<0.01	0.5±0.1	16.8±2.9	0.9±0.3	1.5±0.4	19.8±3.5	1:40
Adrenal glands	6	2.6±0.8	2.7±0.8	0	5.3±1.6	42.9±2.3	3.7±1.7	6.2±0.6	52.8±2.9	1:10
Liver	4	0.6±0.0	<0.01	<0.01	0.6±0.0	23.7±3.2	1.2±0.5	2.1±0.6	27.4±4.1	1:46
Cerebellum	5	<0.01	0	0	<0.01	18.9±2.1	0.9±0.1	0.4±0.2	20.2±1.9	1:>2000
Cerebrum	4	<0.01	0	0	<0.01	25.5±4.4	0.3±0.1	0.8±0.3	26.6±4.3	1:>2000
Spleen	3	0.2±0.1	0.1±0.0	0	0.3±0.1	35.8±7.0	0.5±0.2	0.4±0.1	36.7±6.7	1:120
Pancreas	3	1.6±0.7	1.2±0.1	0.2±0.1	3.0±0.7	78.4±2.8	1.3±0.5	0.9±0.3	80.6±3.2	1:27
Retina	4	0	0	0	0	79.0±20.6	1.1±0.6	1.0±0.4	81.1±21.2	-
Seminal vesicle	3	0.2±0.1	0.2±0.0	<0.1	0.5±0.4	9.1±1.4	0.3±0.2	0.2±0.0	9.6±1.2	1:19
Muscle(gastric)	3	0.9±0.3	0.3±0.2	0.3±0.1	1.4±0.5	35.2±7.9	1.4±0.4	0.7±0.1	37.3±7.5	1:27
Bile	4	0	0	0	0	64±17	0	0	64±17	-
Urine	4	0	0	0	0	0	0	0	0	-

Values represent mean±SD

[a]Hamster were fed 11 - 15 wks a purified diet containing 200 ppm of tocotrienols and 70 ppm of tocopherols.

[b]Only adipose tissue of control animals (3 ppm dietary T_3 revealed a detectable level of tocotrienol, γ-T_3 only (0.8±0.1 μg/g).

conservation and resecretion into lipoproteins. The essential lack of tocotrienols in LDL or HDL, in contrast to α-tocopherol, point to the striking difference in their transport and underscores the likelihood that T_3 and T associate with different lipid moieties during transport in plasma (Figure 2).

Antioxidants and atherosclerosis

α-Tocopherol: The concept that oxidized LDL (OX-LDL) contributes to atherogenesis arose with the observation that Probucol®, a lipid antioxidant drug, resulted on less experimentally induced atherosclerosis in rabbits (Carew et al., 1987; Kuzuya et al., 1991). One question is whether α-tocopherol, the natural biological antioxidant in LDL, functions similarly. Another is whether it behooves us to displace the polyunsaturated fatty acids (PUFA) in our LDL phospholipids and cholesteryl esters with less oxidizable fatty acids, namely monounsaturated or fully saturated fatty acids, to reduce the potential for developing oxidized LDL.

We addressed this issue in two ways. First, the natural experiment concerning the susceptibility of LDL to oxidation in humans was examined by comparing vegetarians with omnivores in terms of their plasma tocopherol carrying capacity relative to the cholesterol load and fatty acid profile of plasma lipoproteins (Pronczuk et al., 1991; Harding et al., 1962). In the first instance we found that even though vegetarians consume more polyunsaturated fat than non-vegetarians (Harding et al., 1962) and have more PUFA in their plasma fatty acids (Table 5), they actually maintain a lower plasma cholesterol concentration and relatively more plasma α-tocopherol per molecule of cholesterol (Table 6). This suggests that natural protection against oxidation is normally assured by an increase in the α-tocopherol concentration in plasma lipoproteins as they become more unsaturated. This is in keeping with the idea that tocopherol normally accompanies PUFA into the phospholipids of membranes (Diplock et al., 1973), including the modified PL-rich semi membrane surrounding lipoproteins. Thus, the vegetarian would appear to be at lower risk for atherogenesis on two counts. First, they have lower LDL and total plasma cholesterol concentrations due to their higher intake of polyunsaturated fat and minimal cholesterol consumption (Pronczuk et al., 1991). Second, even though LDL particles from vegetarians contain more PUFA among their phospholipids and cholesteryl esters than particles from non-vegetarians, more α-tocopherol is also present to protect against peroxidation.

As a second approach to the antioxidant issue we fed monkeys three fats that differed widely in their degree of saturation in an attempt to modify the LDL fatty acid saturation and tocopherol status in vivo as well as to test the atherogenicity of the LDL in vitro (Lindsey et al., 1990). Accordingly, in order to examine LDL unsaturation 10 cebus monkeys were rotated through cholesterol-free purified diets with 40% calories as palm oil (16:0 and 18:1 each at 16% kcals), hi-oleic safflower oil (18:1 at 30% kcals), or hi-linoleic safflower oil (18:2 at 30% kcals) for 6 weeks (Lindsey et al., 1991). LDL was isolated after each diet and split into two aliquots, one of which was oxidized with 5 μM copper for 24 hours (Quinn et al., 1987). Both native LDL and OX-LDL were then incubated in a monocyte adhesion assay (Alderson et al., 1986) to assess the atherogenicity of LDL as affected by dietary fat, their oxidative state measured as TBARS (thiobarbituric reactive substances) as well as their α-tocopherol status. TBARS increased in OX-LDL while α-tocopherol decreased (Table 7).

TABLE 5.
Plasma fatty acid profile of vegetarians and non-vegetarians.

Fatty acid	Non-vegetarian (n=10)[a]	Vegetarian (n=10)[a]
	% of total	
14:0	1.0 ± 0.4	1.0 ± 0.5
16:0	20.3 ± 1.8	21.3 ± 2.2
16:1n9	2.8 ± 0.7	2.2 ± 1.1
18:0	7.6 ± 0.7	7.9 ± 1.1
18:1n9	21.1 ± 2.2	18.3 ± 2.4*
18:2n6	31.5 ± 1.9	35.4 ± 4.6*
20:3n9	1.8 ± 0.4	1.8 ± 0.9
20:4n6	7.1 ± 1.2	6.1 ± 1.8
22:6n3	1.0 ± 0.5	0.8 ± 0.7
Others	5.9 ± 2.3	4.5 ± 3.5

Values are mean±SD.
[a]Each group represented 5 males and 5 females covering approximately the same age span (vegetarians 33±8 and non-vegetarians 31±8 years of age ±SD). *Significant difference in fatty acid of non-vegetarians vs. vegetarians ($p<0.05$).

TABLE 6.

α-tocopherol and total cholesterol in plasma of vegetarians and non-vegetarians.

Subjects	n	α-tocopherol μg/dl	cholesterol mg/dl	Molar ratio α-tocoph : chol
non-vegetarian	79[a]	899±178	194±31	1 : 246±45
vegetarian	79[a]	725±200*	132±25*	1 : 210±45*

Values are mean ± SD
[a]Group of 79 vegetarians represented 28 males and 51 females of 20-50 years of age. The control group included 79 age- and sex-matched non-vegetarians.
*Significant difference between values of non-vegetarians vs vegetarians ($p < 0.001$).

TABLE 7

Indices of lipid peroxidation and monocyte adhesion induced by native LDL and oxidized LDL from cebus monkeys

Adhesion	TRARS (nmol/mg protein)		α-Tocopherol (μg/mg protein)		Monocyte (% of PBS Control)	
Diet fat	Native	Oxidized	Native	Oxidized	Native	Oxidized
16:0	0.75±0.2	2.22±1*[a]	395±65	97±125*	124±4	158±11*
18:1n9	1.65±1	2.89±*	547±176	268±247*	128±4	139±3*
18:2n6	1.39±1	4.83±3*[a]	518±123	174±203*	114±4	167±12*

Values are ±SD, n=6-8
*Significantly different from native IDL by paired t-test ($p < 0.05$)
[a]Significantly different from each other by ANOVA and Fischer's protected LSD test ($p < 0.05$).

Although the more saturated LDL (16:0) maintained the lowest values for TBARS and α-tocopherol, monocyte adhesion did not differ between the native LDL isolated after the three dietary treatments, and although adhesion was increased 2-fold by OX-LDL, again no difference was noted between dietary fats. In fact, monocyte adhesion induced by native LDL tended to be lowest with the most unsaturated particles. However, these particles revealed the greatest increase in adhesion after oxidation, reflecting the negative, strong correlation (r=-0.71) between adhesion and LDL α-tocopherol content (Figure 4). These results suggest that the unsaturation of the dietary fat and LDL lipids is less important than the α-tocopherol status of the host (and the circulating LDL) in terms of dictating the atherogenic potential of the LDL particle. Furthermore, the LDL particle, though capable of being modified by altering the unsaturation of the dietary fat, is automatically compensated by the host's inclusion of extra tocopherol into PUFA-enriched particles. On the other hand, it seems likely that an individual with an elevated plasma cholesterol and highly unsaturated LDL fatty

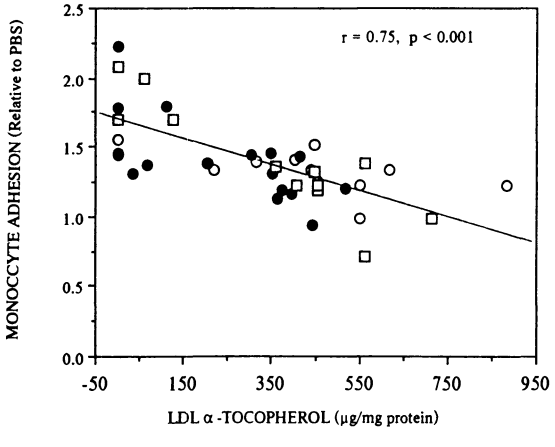


Figure 4. Stimulation of monocyte adhesion to endothelial cells by LDL in culture was measured using both native and oxidized LDL from cebus monkeys fed diets rich in 16:0 ( ), 18:1 ( ), or 18:2 ( ).(see text). After incubating cells with lipoprotein-enriched media for 4 hr, ^{51}Cr labeled U937 monocytes were added for adherence. Background adhesion was determined with PBS-treated controls and subtracted from each LDL preparation. Adhesion was then correlated with the LDL α-tocopherol content from both native and oxidized LDL.

acids without adequate tocopherol would be at increased risk for atherogenesis based on the potent promoting effect of OX-LDL on atherogenesis (Esterbauer et al., 1990; Weis et al., 1991; Dieber-Rotheneder et al 1991). However such a scenario seems unlikely since high PUFA intake is typically associated with decreased plasma and LDL cholesterol. Furthermore, dietary vegetable oils, the major source of PUFA, also contain the highest concentration of tocopherols and tocotrienols.

Thus, α-tocopherol as the principal antioxidant barrier to peroxidation of lipids at the cell boundary (plasma membrane) appears to be transported to cells by lipoprotein phospholipids, i.e. LDL and HDL, conferring antioxidant protection on these circulating particles in the process. Although the tocopherol-rich HDL would appear to be a particularly good donor of tocopherol and phospholipids to cells, this delivery system has not been reported. On the other hand, uptake of tocopherol by cells can occur via LDL and LDL particles (Thellman & Shireman, 1985). By contrast, tocotrienols do not appear to be transported quantitatively by LDL or HDL. The fact that only adipose tissue of nominal animals contains detectable tocotrienols suggests a more specific, limited role for these isomers in most cells.

Tocotrienols: Aside from the antioxidant potential of tocopherol, it has been reported (Tan et al., 1991; Qureshi et al., 1991) that tocotrienol supplementation of hypercholesterolemic humans can lower the plasma cholesterol 5 to 36%, especially the LDL fraction. A reduction in LDL cholesterol would be an obvious benefit to reducing the risk to atherosclerosis. Accordingly, the potential of tocotrienols to lower the plasma cholesterol was explored in normolipemic hamsters. The results (Table 8) indicate that tocotrienols supplementation was without effect on total lipids or distribution of the plasma cholesterol within lipoprotein fractions.

TABLE 8.

Effect of tocotrienols supplementation on hamster growth and plasma lipids[a]

	Dietary tocotrienols (ppm)		
	3	36	220
	N=7	N=8	N=8
Body weight (g)			
Initial	58±4	59±2	60±3
Final	118±9	121±13	116±10
Liver weight (g)	3.9±0.5	4.0±0.5	3.7±0.3
Plasma cholesterol (mg/dl)			
Total	120±28	125±18	124±33
HDL	63±13	65±15	66±17
Plasma triglyceride (mg/dl)	168±97	106±46	125±68

Values are mean±SD

[a]Male Syrian hamsters (Charles River), 4 weeks of age were fed for 4 weeks semi-purified diets containing 18% of dietary fat as either tocotrienols (T_3) stripped palm oil (diet with 3ppm of T_3), regular RBD palm oil (diet with 36ppm of T_3) or crude palm oil (diet with 220ppm of T_3).

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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MODULATION OF CELL PROLIFERATION BY TOCOPHEROLS AND TOCOTRIENOLS: ROLE IN ARTERIOSCLEROSIS

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Summary

α-tocopherol inhibits serum-induced proliferation of smooth muscle cells in culture. The antiproliferative effect of α-tocopherol is not correlated with its radical scavenging properties and is directly related to the inhibition of protein kinase C. Other derivatives of tocopherol are not effective in decreasing cell proliferation (trolox, α-tocopherol acetate) while others (tocotrienols, tocopherols with short phytol chains) inhibit cell growth by different mechanisms.

Introduction

Extracellular signals such as polypeptides, growth factors and hormones, can trigger in cells a transition from a quiescent (Go) to a proliferative state [1,2]. Positive signals (growth factors) and negative factors (growth inhibitors) [3] which cooperate in determining the rate of cell proliferation are subject to disorders leading to pathological conditions. Proliferation of smooth muscle cells, for example, plays a central role in arteriosclerosis [6-9]. Vitamin E has multiple cellular roles [8] such as antioxidant [9],

membrane stabilizer [10,11], cell growth inhibitor [12,13] and regulator of prostaglandin synthesis [14]. At physiological concentrations, (α-tocopherol, the most active form of vitamin E [15],is an inhibitor of smooth muscle cell growth [16,17]. In the following study tocopherol analogs[1], differing in their hydrocarbon chain or chromane ring substitutes and provided with different antioxidant activity [10,18,19], have been tested on the proliferation of synchronized smooth muscle cells and on their PKC activity. No correlation was found between the antioxidant potency of the different tocopherol isomers and homologues tested and their effect on cell proliferation and PKC activity.

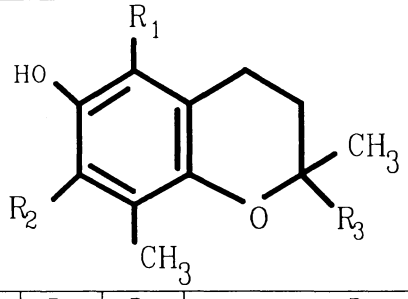
Materials and Methods

Materials

Tissue culture plastics were purchased from Falcon Labware (Becton Dickinson & Co.) and growth media and serum for cell culturing were obtained from Gibco Laboratories (Grand Island, NY). [Me-^{3}H]Thymidine (25 Ci/mmol) and [γ-32P]ATP (10 Ci/mmol) were from Amersham International. d-α-, d-β-, d-γ-, and d-δ-tocopherol were generous gifts from Hoffmann La Roche & Co (Basel, Switzerland) and Henkel Co (La Grange, IL, USA). d-α- and d-γ-tocotrienol were kindly provided by PORIM (Palm Oil Research Institute of Malaysia). (α-C6, α-C9 and α-C1 were generous gifts from Eisai Co, Tokyo, Japan. The structural formulae of the compounds are shown in Table I. A specific peptide (Bachem) Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, was used as substrate for the assay of PKC activity. Streptolysin-O (25,000 units) and PMA were from Calbiochem. A7r5 cells (rat aortic smooth muscle cell line, VSMC, clone DBLX from American Type Culture Collection) were grown in Dulbecco's modified Eagle medium (DMEM) containing 25 mM bicarbonate, 60 U/ml penicillin, 60 μg/ml streptomycin, and 10% fetal calf serum (FCS). Cells were seeded into 100-mm dishes and grown to confluence at 37°C in a humidified atmosphere of 5% CO_2-Culture media were changed every 3 days.

[1]Abbreviations used: PKC, protein kinase C; VSMC, vascular smooth muscle cells: FCS, fetal calf serum; PBS, phosphate-buffered saline;α-C1, α-C6 and α-C9 are the abbreviations for the (L-tocopherol analogues having side chain length of 1, 6 and 9 carbon atoms, respectively; α-C16 is equivalent to α-tocopherol.

Table I. Structural Formulas of tocopherols and analog compounds.



	R_1	R_2	R_3
α-tocopherol	$-CH_3$	$-CH_3$	$-(CH_2-CH_2-CH_2-CH)_3-CH_3$ CH_3
β-tocopherol	$-CH_3$	-H	$-(CH_2-CH_2-CH_2-CH)_3-CH_3$ CH_3
γ-tocopherol	-H	$-CH_3$	$-(CH_2-CH_2-CH_2-CH)_3-CH_3$ CH_3
δ-tocopherol	-H	-H	$-(CH_2-CH_2-CH_2-CH)_3-CH_3$ CH_3
PMC	$-CH_3$	$-CH_3$	$-CH_3$
Chromanol C-6	$-CH_3$	$-CH_3$	$-(CH_2)_3-CH-CH_3$ CH_3
Chromanol C-9	$-CH_3$	$-CH_3$	$-(CH_2)_3-CH-CH_2-CH_2-CH_2-CH_3$ CH_3
α-tocotrienol	$-CH_3$	$-CH_3$	$-(CH_2-CH_2-CH=C)_3-CH_3$ CH_3
γ-tocotrienol	-H	$-CH_3$	$-(CH_2-CH_2-CH=C)_3-CH_3$ CH_3

Synchronization at the G_1/S boundary was obtained by serum deprivation and hydroxyurea treatment [20]. Exponentially growing cells (in 6-well containing 1.5 ml of DMEM with 2% FCS), were made quiescent (Go) by treatment with 0.2% FCS for 48h. After re-stimulation by 2% FCS during 8h, 1.5 mM hydroxyurea was added to each plate. After 14h of treatment cells were washed with PBS and transferred to the fresh

complete medium (DMEM, 2% FCS). Onset and duration of the S-phase was determined by pulse labelling with [^{3}H]thymidine. A7r5 VSMC cell number remained unchanged during 22h from the stimulation and then cell divided, thus making a cycle in around 24h.

Effect of Tocopherol Homologues on Cell Growth

α-Tocopherol and tocopherol homologues were dissolved in absolute ethanol. The final ethanol concentration during cell growth never exceeded 0.5% and the same amount of ethanol was added to control cells. Compounds were added when restimulating the cells with serum following 48h deprivation. Cells were trypsinized and counted in a hemocytometer in triplicate after the completion of the cell cycle (approximately 24h). Viability was assessed by the trypan blue dye exclusion method.

Measurement of [^{3}H]thymidine Incorporation

Cells were pulsed with [^{3}H]thymidine (0.5-1 μCi/well) for 6h during S-phase following removal of hydroxyurea. Then, cells were washed twice with PBS supplemented with 10 mg/ml glucose and 1 mg/ml bovine serum albumin, fixed for 30 min with ice-cold 5% trichloroacetic acid, and solubilized in 0.1M NaOH/2% Na_2CO_3 (w/v). The radioactivity incorporated into acid insoluble material was determined in a liquid scintillation analyzer.

PKC Assay in Permeabilised Cells

Measurements of PKC activity in permeabilised smooth muscle cells were performed according to the procedure of Alexander et al. [21] with minor modifications. A7r5 cells in the late G_1 phase of the cycle, preincubated for 8 h in the presence of the indicated tocopherol homologue, were washed twice in PBS, resuspended in intracellular buffer (5.16 mM-$MgCl_2$, 94 MM-KC1, 12.5 mM-Hepes, 12.5 mM-EGTA, 8.17 mM-$CaCl_2$, pH 7.4) and aliquoted in 220 μl portions ($1.5x10^6$ cells/ml). Assays were started by adding [γ-^{32}P]ATP (40 cpm/pmol, final concentration 240 μM), peptide substrate (final

concentration 250 μM) and Streptolysin-O (0.6 I.U.). The reaction mixtures were incubated at 37°C for 5 min and the reaction was stopped by adding 100 ml of 25% (w/v) trichloroacetic acid in 2 M-acetic acid. After 10 min on ice, samples were centrifuged for 5 min and spotted on P81 ion-exchange chromatography paper (Whatman International) which were then washed several times with 30% (v/v) acetic acid containing 1% (v/v) H_3PO_4 and once with ethanol. The P81 papers were dried, and the bound radioactivity was counted in a liquid scintillation analyzer. To estimate the background phosphorylation of the peptide due to a kinase activity other than PKC, assays were performed in cells treated for 24 h with 1 μM PMA. Down-regulation of PKC was established to occur under these conditions by radioactive PDBu binding. The value of ^{32}P incorporated obtained in the latter conditions has been subtracted from the experimental data.

Determination of Uptake of Tocopherol Isomers in VSMC

Cells were incubated 24h in complete medium with 100 μM of the different tocopherol isomers (α-, β-, γ-, and δ-tocopherols). After washing, measurements of tocopherol content were performed by reverse phase HPLC using a C-18 column (Waters, Inc. with an in-line electrochemical detector essentially as described earlier [25]. The eluent was 20 mM-lithium perchlorate in methanol-ethanol (1:9) (v.v). Uptake is expressed as nmol of incorporated tocopherol isomer per $1x10^6$ cells. Control cells were incubated in the absence of any compound.

Results and Discussion

Effects of Tocopherol Analogs on VSMC Proliferation

Cell proliferation was studied by measuring [^{3}H]thymidine incorporation into DNA [19] or by the number of cells at the end of the cycle. The effect of (α-, β-, γ- and δ-tocopherols on the proliferation induced by fetal calf serum is shown in Table II.

Table II. Comparison of the effect of several tocopherols on smooth muscle cell growth an kinase C activity with their radical scavenging potency.

Compound	Inhibition of cell growth(%)	Uptake (nmoles/10^6 cells)	Protein kinase C (pmol/min/10^6 cells)	Radical Scavenging activity, %
Control	-	-	111	-
α-tocopherol	51	1.12	5	100
β-tocopherol	<1	0.95	125	60.6
γ-tocopherol	42	1.00	112	45.5
δ-tocopherol	41	1.81	110	27.3

The experiments were carried out using techniques described in the Methods. The correlation coefficient *"Scavenging/Antiproliferative activities"* was calculated to be 0.15

At a concentration of 100 μM, α-tocopherol, γ-tocopherol and δ-tocopherol, all inhibit VSMC FCS-induced proliferation; surprisingly, no effect of β-tocopherol was observed. α-Tocopherol was found to be slightly more potent than γ- or δ- tocopherols, the inhibition being 51%, 42% and 41% (compared to control), respectively. Moreover, α-, γ-, and δ-tocopherols have never been found to be toxic for smooth muscle cells even at high concentrations and cell viability was always greater than 95%. The content of added tocopherol isomers (α-, β-, γ-, and δ-tocopherols) was similar in A7r5 smooth muscle cells excluding that a different uptake was responsible for the diverse inhibition. To study the effect of tocopherol homologues on protein kinase C activity, streptolysin-O permeabilised VSMC and a PKC peptide substrate [21] were employed. As can be seen in Table II, α-tocopherol had a very strong effect on PKC activity (about 95% inhibition) whereas its analogs (β-, γ-, and δ-tocopherols) were ineffective.

The experiments summarized in Table II indicate that the antiproliferative activity of tocopherols cannot be correlated with their antioxidant potency. It is in particular striking that β-tocopherol, which possesses more than 60% of the radical scavenging capability of α-tocopherol inhibits cell proliferation less then 1%. α-tocopherol inhibition

is moreover associated to inhibition of protein kinase C. The inhibition of cell proliferation by γ- and δ-tocopherol is not linked to protein kinase C and it is presently under investigation.

Table III Effect of α-Cl, α-C6, α-C9 α-tocotrienol and γ-tocotrienol on cell growth and on PKC activity.

Compound	Concentration	Growth inhibition %	PKC activity (pol/min/10^6 cells)	Uptake (nmol/10^6 cells)
control		0	111	0.01
α-T	50 uM	55	5	1.12
C1	50 uM	82	106	0.014
C6	12 uM	99	89	0.29
C9	12 uM	29	84	0.25
α-T3	100 uM	51	85	5 . 4 9
γ-T3	25 uM	44	102	2.58

The maximal non-toxic concentration, inhibiting cell growth, was employed. α-T = α-tocopherol; C_1, C_6 and C_9 = α-tocopherol analogs with a 1,6 and 9 C phytol chain; T_3, tocotrienol

Synchronized smooth muscle cells were treated with different concentrations of α-Cl, α-C6, α-C9 αtocotrienol or γ-tocotrienol and cell number was determined after the completion of the cell cycle (24 h) . Results are expressed as percentage of control, (untreated cells) . 100% represents $93 \pm .6 \times 10^4$ cells /ml. PKC activity in permeabilised cells was measured as described in Materials and Methods. Cells were preincubated 8h with the tocopherol analogs as indicated. Results are the mean of three different experiments

Effects of the Tocotrienols and of Short Chain α-Tocopherol Homologues on VSMC Proliferation

In order to establish if the inhibitory action of α-tocopherol was related with the structure of the phytyl side chain, tocopherols with unsaturated phytyl chain (tocotrienols) and tocopherol homologues with different hydrocarbon chain lengths (α-Cl, α-C6 and α-C9) were tested. As shown in Table III, α- and γ-tocotrienols were both effective in inhibiting VSMC proliferation but at different concentration ranges; α-tocotrienol showed approximately 50% inhibition around 100 μM and γ-tocotrienol at a concentration of 25 μM. With the latter, toxicity was observed at concentrations above 45 μM .

The antiproliferative action of α-C6 and α-C9 occurred at very low concentrations. α-C6 was much more potent (IC_{50} = 1 μM) than α-C9 which never showed more than 30% inhibition in cell number compared to control. In contrast, α-Cl, a tocopherol homologue devoid of the phytyl chain, inhibited VSMC proliferation with an IC_{50} of 20 μM. In addition, α-C6 and α-C9 appeared to be toxic at concentrations greater than 10 μM whereas α-Cl showed toxicity around 100 μM. α-Cl was poorly incorporated into A7r5 smooth muscle cells (less than 10 pmoles/10^6 cells) possibly due to both its low hydrophobicity and to its ability to form fine micelles in the aqueous phase. It has been demonstrated earlier that the efficiency of membrane lipid peroxidation inhibition by α-tocopherol homologues with short side chain decreased monotonously in the order: α-Cl > α-C3 > α-C6 > α-C9 > α-Cll > α-Cl6 (α-tocopherol) [33]. Consistent with the afore mentioned, no correlation was found between the antioxidant properties of these α-tocopherol homologues and their antiproliferative activity which decreased in the order: α-C6 > α-Cl > α-Cl6. The results obtained by measuring PKC activity in permeabilised cells support our previous observations showing inhibition of the isolated rat brain protein kinase C and VSMC proliferation by α-tocopherol [17] . Inhibition of protein kinase C in permeabilised VSMC is specific to (-tocopherol, while the other tocopherol analogs and homologues have no effect. The inhibitory effect could be observed regardless of whether the cells were preincubated with α-tocopherol or the compound was added just before the kinase assay in the incubation mixture. This result is in favor of a direct

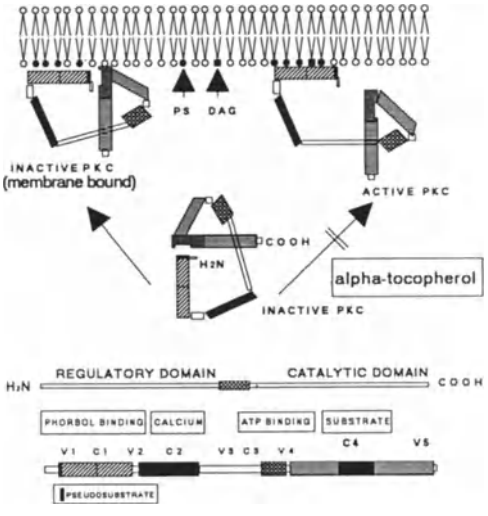


Fig. 1: A model of protein kinase C activation with the site of α-tocopherol action.

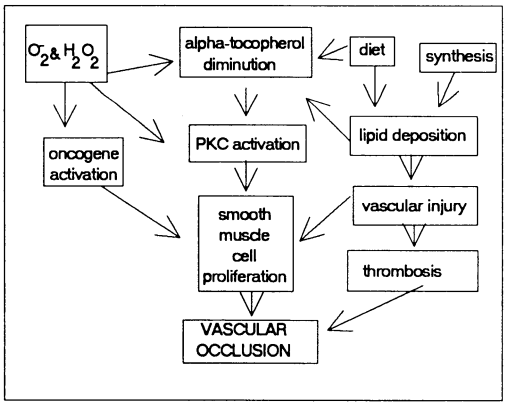


Fig. 2 A unifying pathogenetic model of arteriosclerosis.

protein-ligand interaction of PKC with α-tocopherol which could be in part responsible for the inhibitory effects on VSMC proliferation by α-tocopherol. However, the other tocopherol derivatives (γ- and δ-tocopherols, α- and γ-tocotrienol, short chain homologues) which were active in smooth muscle cell proliferation inhibition did not show significant PKC inhibition. This points out that the antiproliferative action of these tocopherol homologues might have more than one molecular target, besides PKC. The point of action of α-tocopherol based on the above and previously published results may be localized at the level of protein kinase C, since several reactions associated with the presence of an active protein kinase C (membrane translocation, 80 kD protein phosphorylation, transmodulation of the EGF receptor, and down regulation) have been all found to be sensitive to α-tocopherol (Figure 1)

A Unifying Pathogenetic Hypothesis of Arteriosclerosis

Proliferation of smooth muscle cells, has a central role in the arteriosclerosis process. We have described above and previously that proliferation of smooth muscle cells in vitro is under the control of α-tocopherol. This event will be verified in primary smooth muscle cell cultures and in vivo, to establish the degree of significance of our in vitro findings. It is however established that the extent of arteriosclerosis lesions responsible for a clinical manifestation (ischemic heart disease) inversely correlates with the blood α-tocopherol concentration (24). α-Tocopherol may be thus placed in a central position in the onset of this degenerative disease (Fig. 2). As a major regulator of smooth muscle cell proliferation all events leading to a decrease of this compound may result in smooth muscle cell growth. The possible causes of a low blood α-tocopherol may be dietetic in nature or may be caused by the destructive effect of radicals on the compound itself. Thus radicals may have a damaging role at a cellular level both in activating protein kinase C and diminishing α-tocopherol level, both events resulting in a stimulation of smooth muscle cell proliferation and occlusive arterial phenomena.

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VITAMIN E AND HEALTH IN THE MARMOSET MONKEY : A NON-HUMAN PRIMATE MODEL FOR NUTRITIONAL RESEARCH

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Summary

In association with various types of saturated and polyunsaturated fatty acid dietary supplements, the possible role of increased vitamin E intake on the health of adult marmoset monkeys (Callithrix Jacchus) was studied during several long-term feeding trials. Positive effects were observed on their growth rate, their reproductive capacity and possibly the mechanical performance of their heart muscles when under catecholamine induced stress. Further studies are urgently required to better define the extent of the effect of vitamin E upon the cardiac function of this small primate as positive findings could be of much benefit in reducing the incidence of cardiac arrhythmia under stress. Such findings would be of great relevance to a reduction in the occurrence of Sudden Cardiac Death in man.

Introduction

The relationship between vitamin E requirements and the absorption, transport and metabolism of dietary fat has recently been reviewed by Budowski and Sklan (1989). While common edible oils such as corn or safflower are good sources of this antioxidant vitamin, it is also known that the consumption of unsaturated fatty acids increases the need for protection against free-radical mediated damage to cell membrane structure and function (Packer 1991). However clinical symptoms of vitamin E deficiency are rare in

economically developed nations, and are usually confined to individuals with fat absorption problems. Nevertheless, when the dietary fat intake is greatly increased it is advisable to adjust the intake of vitamin E as suboptimal levels are frequently observed (Desai, 1968).

A major research interest in our laboratory has been the possible role of dietary lipids in modifying the age-dependent increase in the susceptibility of the heart to develop malignant arrhythmia when under ischemic stress. (Charnock 1985, 1991). As this condition is a precursor to Sudden Cardiac Death it is extremely difficult to study in free living populations. Consequently we have developed several animal models of ischemic arrhythmia, including that in the marmoset monkey (Callithrix Jacchus), a small non-human primate whose cardiac physiology and lipid metabolism are very similar to that of man. (McMurchie et al., 1987; Abbey et al., 1990; Charnock et al., 1991).

A series of relatively long-term feeding studies with a variety of different dietary lipid supplements, in conjunction with several different levels of additional α-tocopherol, have provided an opportunity for a retrospective examination of the role of vitamin E in the health of the marmoset. The results of this survey may also provide some information on the role of vitamin E in other primates including man.

Materials and Methods

The Experimental Design

In each of two consecutive experiments carried out during 1985/87 and 1987/90, breeding pairs of young adult marmosets about 1 year old, were housed together with any resulting progeny for periods of 24-36 months. During this time, both male and female marmosets shared the same diets, as did their progeny after weaning. Thus several generations of marmosets were fed and raised on lipid modified diets having predetermined levels of additional vitamin E along with the different lipid supplements. There were no restrictions on breeding, and all live progeny remained with their parents for at least six months after birth. At the completion of the experimental feeding

programs, the mature male marmosets were utilized for studies of their cardiac function, while all the females and their progeny were returned to the colony.

Over this 5 year period two separate experiments were carried out in which two similarly modified lipid supplemented diets were provided, but the second of these experiments contained considerably more additional vitamin E (α-tocopherol) than did the first. The major ingredients of these diets are summarized in Table I.

In addition to the vitamin E present in the basic primate meal (REF) which assayed at 27 or 42 mg/Kg in the first or second experiment respectively, the sheep fat (SF), sunflower seed oil (SSO) and fish oil (FO) lipid supplemented diets contained 50 mg/Kg additional α-tocopherol in "Diet 1" and 100 mg/Kg in the REF, SF and SSO, but 250 mg/Kg in the FO supplemented group of "Diet 2". Full details of the preparation and storage of these diets as well as details of the feeding regimes that were employed in these experiments have been published recently (Charnock et al., 1989; 1991).

Results and Discussion

The different vitamin E additions to the diet are reflected in the levels of vitamin E determined in food samples taken from the cold-stored fabricated diets on the day of presentation to the animals (Table II), as well as the plasma levels of vitamin E determined in the blood of the male marmosets at the end of the experiments (Table III). It is apparent that while the REF and SSO groups fed "Diet 1" had 'satisfactory' plasma levels of vitamin E, both the SF and the FO group possibly did not. However, the levels in these latter two dietary groups are far greater than those regarded as 'normal' in man (Desai 1968; Horwitt 1974), and are greater than those previously reported in apparently healthy marmosets (McIntosh et al., 1988). The increased levels of vitamin E added to "Diet 2", corrected these differences and all dietary groups now had similar plasma levels of this vitamin. In addition, determination of blood platelet levels of α-tocopherol (which were not carried out in the animals fed "Diet 1") revealed levels of vitamin E either equal to or greater than those previously reported by McIntosh et al (1987a) as being 'normal' for this species.

Table I Major Components of Marmoset Diets

Experiment 1985-87 *Diet 1		Components per Kg diet	Experiment 1987-90 *Diet 2	
		Primate Meal		
915g	20%	Protein	25%	935g
	65%	Carbohydrate	60%	
	4%	Fat	4%	
5g		Vitamin Mixture (A,B,C,D,E)		5g
80g	8%	Lipid Supplement (SF, SSO, FO)	6%	60g
		Additional Vitamin E		
50 I.U.		(REF, SF, SSO)		100 I.U.
50 I.U.		(FO)		250 I.U.
isoamyl acetate		Flavoring		black currant concentrate

* All animals received 30g fresh sliced banana per day, Monday-Friday inclusive. Lipid supplements (SF, SSO & FO) are defined in the text. REF refers to basic diet without lipid supplement.

Effect of Diet on Growth Rate

Both of our experiments were conducted under continuous veterinary care and all animals were weighed monthly. McIntosh and Looker (1982) have previously reported very little weight gain in healthy adult male marmosets after about 12 months of age. A similar result was obtained here, but comparison of the average live body weights of age matched marmosets fed either "Diet 1" or "Diet 2" consistently reveals greater weight gains for the animals fed on "Diet 2" irrespective of the absence (REF) or presence of the dietary lipid supplements (SF, SSO, FO). Although "Diet 1" and "Diet 2" were not identical in all respects other than their content of vitamin E (c.f. Table I), it is clear that

Table II Vitamin E Content of Fabricated Marmoset Diets

Lipid Supplement	Vitamin E (mg/Kg) * Diet 1	* Diet 2
None (REF)	14	17
Sheep Fat (SF)	37	31
Sunflower Seed Oil (SSO)	53	45
Fish Oil (FO)	64	83

* Diet 1 assayed by GLC (1985/87) : Diet 2 assayed by HPLC (1987/90). Note additional vitamin E added with lipid supplements as shown in Table 1.

the greater vitamin E enrichment of "Diet 2" was associated with a uniformly greater body weight in all dietary sub-groups (Table IV.) It is also clear that no matter what the type, lipid supplementation generally leads to greater body weight than does feeding the much lower fat REF diet which only contains about 4% by weight total fat. A similar study by McIntosh et al (1987a) also demonstrated an increased body weight of marmosets fed "adequate" levels of vitamin E, compared to those fed a lower level of this vitamin, irrespective of whether the diet contained additional sunflower seed oil or not. The differences in body weight reported in that study were reflected in altered skeletal muscle mass, but were not found in the wet weight of the hearts, livers or kidneys of the marmosets.

Effect of Diet on Reproduction

A complete record of the reproductive performance of all females in these experiments was maintained as a routine component of their health records. Consequently it is possible to compare the live birth rates, numbers of still births and spontaneous abortions which occurred for several years on "Diet 1" and at least one year on "Diet 2"

Table III Blood Level of Vitamin E in Marmosets

Lipid Supplement	Plasma (mg/dl)		Platelet ($\mu g/10^9$ cells)
	Diet 1	Diet 2	Diet 2
REF	5.8±0.2	6.2±0.2	0.34±.05
SF	*3.0±0.4	6.3±0.5	0.28±.03
SSO	6.2±0.3	5.0±0.4	0.43±.02
FO	2.4±0.2	6.6±0.7	0.61±.10

Value calculated from McIntosh et al., (1988). Lipid supplements described in text and Table 1. All values given as total α-tocopherol.

(after which the males were progressively utilized for other studies). A summary of these results is shown in Table V. When the overall birth rate during Diet 1 is calculated it is apparent that animals fed the fish oil (FO) dietary lipid supplement had considerably impaired performance (0.5 live births per female per year) compared to all the other dietary subgroups. The highest birth rate was seen in the group fed a saturated fat (SF) supplement where the average rate over two years was 2.1 live births per female per year compared to 1.4 in both the REF and the SSO groups.

However, in the second experiment, the addition of 250 mg/Kg of vitamin E to the FO diet ("Diet 2") compared to only 50 mg/Kg in "Diet 1" was associated with an increase in live births in this group to a level which was indistinguishable from that of any other dietary group in this experiment, or indeed from those rates seen in animals fed Diet 1 (other than the FO group).

As the birth rates in the low fat REF groups for "Diet 1" and "Diet 2" were virtually identical, it is quite reasonable to suppose that the differences seen in the FO

Table IV Effect of Diet on Body Weight of Male Marmosets

* Months	REF		SF		SSO		FO	
on Diet	Diet 1	Diet 2	Diet 1	Diet 2	Diet 1	Diet 2	Diet 1	Diet 2
0	316 ±13	315 ±7	304 ±9	346 ±5	308 ±11	348 ±22	304 ±5	345 ±8
6	321 ±11	342 ±14	333 ±7	347 ±3	333 ±7	344 ±15	326 ±10	316 ±7
12	337 ±7	362 ±16	349 ±9	360 ±4	349 ±9	357 ±16	318 ±10	331 ±6
18	349 ±10	357 ±24	360 ±9	375 ±8	363 ±9	366 ±17	336 ±11	362 ±7
24	334 ±28	355 2±4	336 ±14	358 ±12	334 ±18	362 ±17	337 ±13	363 ±7
30	321 ±12	351 ±21	356 ±8	369 ±9	349 ±13	361 ±13	339 ±14	365 ±8

* All lipid supplements introduced in 3 equal steps of one month each during first 6 month period. Values are mean weights ±SEM per animal for each dietary group: REF, SF, SSO, & FO are explained in the text and in Table I.

group of animals fed "Diet 1" and which were corrected in animals fed "Diet 2", were due to the considerably increased levels (x5) of vitamin E in the latter diet. Whether such increased levels of vitamin E should be regarded as necessary for optimal reproductive performance of New World monkeys in captivity is difficult to determine, as considerable biochemical and pathophysiological differences between species has been observed (Hayes, 1974; Hayes et al 1991), but certainly with our lipid supplemented marmosets it would seem to be desirable, although the lower level of vitamin E in "Diet 1" appeared to be 'adequate' in the absence of additional dietary polyunsaturated fat.

Table V Effect of Diet on Reproductive Performance of Female Marmosets

Lipid Supplement	Diet	Year	Births			*Annual Birth Rate
			Live	Still	Aborted	
REF	1	1	12	3	0	1.7
	1	2	7	11	0	1.0
	2	1	10	4	0	1.4
SF	1	1	11	4	0	1.8
	1	2	14	4	5	2.3
	2	1	10	5	3	1.7
SSO	1	1	10	3	2	1.7
	1	2	7	10	3	1.0
	2	1	10	3	0	1.7
FO	1	1	5	6	1	0.5
	1	2	5	4	2	0.5
	2	1	15	8	2	1.5

* Birth rate is calculated only from live births per female per year. The number of pairs were REF, n=7; SF, n=6; SSO, n=6 and FO, n=10.

The Effect of Diet Upon Blood Coagulation Indices

Several previous studies which were carried out in our laboratory (McIntosh et al., 1987a; 1987b) have suggested that the increase in clotting time that was seen when sunflower seed oil was added to the diet of marmosets, might at least in part be a result of a concurrent increase in vitamin E which normally occurs with the consumption of this edible oil (Budowski and Sklan, 1989). In this particular experiment ("Diet 2") there was an increase in bleeding (but not clotting) time when either PUFA enriched diets (i.e. SSO or FO) were consumed, with that found in the FO fed group being the most marked. A similar trend in plasma fibrinogen levels was also observed (Table VI). However as we had already established that the plasma levels of vitamin E were essentially identical in all dietary groups in this experiment (cf Table III) it is not obvious that vitamin E has a

role in these hemostatic effects. Nevertheless the increased level of platelet vitamin E in the FO fed group cannot be entirely excluded as the increased bleeding time in this group was markedly greater than that found in the SSO fed group. Of course, such changes in bleeding time are compatible with the numerous observations of change in thrombogenic tendency in human populations consuming an enhanced fish diet (Goodnight et al., 1982). However, in many of these human epidemiological surveys there is no compelling evidence for a direct involvement of vitamin E, and the effect is usually attributed to the role of the omega-3 PUFA's of fish oil on platelet aggregation (Leaf and Weber, 1988). Nevertheless an interaction between vitamin E and the balanced production of the prostanoids which so strongly influence thrombogenesis in the marmoset as well as man cannot be excluded (McIntosh 1987a; 1987b).

Effect of Diet on Blood Lipids

It is well known that the consumption of dietary lipids of different fatty acid composition has significant effects upon the levels of triglycerides and cholesterol in the blood of primates (Goodnight et al., 1982). Recent emphasis upon the inclusion of the omega-3 PUFA's of fatty fish to the human diet is dependent upon such observations (Leaf and Weber, 1988; Nordoy and Goodnight, 1990). However even in non-human primates studies under controlled conditions, the literature is not always consistent in this area, as for example, although there is general agreement that increased omega-3 PUFA consumption leads to reduction in plasma triglycerides, conflicting reports of their effect upon the levels of cholesterol are common (Ward and Clarkson, 1985; Abbey et al., 1990; Pronczuk et al., 1991).

This has not been the experience of our laboratory with marmosets, where significant reductions in both plasma triglycerides and cholesterol have consistently been observed after long-term feeding of a fish oil supplemented diet (Abeywardena et al., 1989). The results given here in Table VII were obtained from animals fed "Diet 2", and suggest that different effects of omega-3 and omega-6 PUFA's in the FO and SSO diets respectively is the most likely explanation for the decrease in cholesterol which was

Table VI Effects of Diet on Blood Coagulation Indices in Adult Male Marmosets

Dietary Group	n	Bleeding Time	Clotting Time	Fibrinogen
REF	5	2.4 ± 0.8	6.3 ± 1.1	199 ± 38
SF	6	2.6 ± 0.8	7.3 ± 1.6	207 ± 9
SSO	6	3.0 ± 0.8	6.8 ± 0.7	217 ± 21
FO	8	3.7 ± 0.8	7.9 ± 1.3	249 ± 49

* Diet was "Diet 2" with additional Vitamin E fed between 1987-90 c.f. Table I. Bleeding and clotting time are given in minutes. Fibrinogen is mg/dl. All values are means ± SEM.

observed in the FO fed group. It will be recalled that the plasma levels of vitamin E under these conditions were similar between the two dietary groups, and both levels were probably above the "normal" level for this species (cf Table III). No explanation can be offered for our observation that added saturated fat (SF) was as effective in lowering the total plasma cholesterol level of these marmosets as was the inclusion of additional omega-6 PUFA as sunflower seed oil (SSO) into their diet for a period of more than two years. Similar findings have sometimes been reported in studies in man where dietary conditions cannot be so well controlled, but it is possible that "fat loading" itself may effect the level of plasma cholesterol (Grundy, 1986; Bonanonme & Grundy, 1988).

Effect of Diet on Myocardial Lipidosis and Fibrosis

Considerable histopathological changes are known to occur in both Old and New World monkeys when they are exposed to reduced dietary levels of vitamin E (Hayes, 1974; 1979). However neither myocardial degeneration nor excessive "lipochrome pigments" were found in the cardiac muscle of the vitamin E deficient monkeys in these studies.

Because the ultimate objective of our experiments was to determine the extent of

Table VII Effect of Diet on Plasma Triglyceride and Cholesterol Levels in Adult Male Marmosets

Lipid Supplement	n	Triglyceride	Cholesterol
REF	6	74.8 ± 6.0	148.1 ± 12.8
SF	6	84.8 ± 5.9	117.9 ± 8.2
SSO	6	83.4 ± 9.1	118.5 ± 5.4
FO	6	41.7 ± 4.1	70.2 ± 6.4

* Diet was "Diet 2" fed between 1987-90 as in Table VI. All values are mg/dl (mean ± SEM). Values for FO group are significantly different (ANOVA; $p < .05$) from all other dietary groups. Source : Abeywardena et al (1989).

dietary induced change in the cardiac function of our marmosets (McLennan et al., 1987a; Charnock et al., 1987a), it was of interest to determine the extent to which our lipid supplemented experimental diets might influence the onset of either cardiac lipidosis or myocardial fibrosis, both of which have been implicated in severe cardiac myopathy. (Schiefer et al., 1978; Opstved et al., 1979; Svaar, 1982).

It can be seen from the summary of result given in Table VIII that there was an increase in the average number of cardiac muscle cells which contained extensive lipid after prolonged feeding of a fish oil enriched diet. Such an observation has also previously been reported in experimental rats exposed to prolonged fish oil dietary supplementation (Charnock et al., 1987b). This increase in lipidosis was accompanied by a slightly increased number of fibrotic lesions but it must be emphasized that these effects are very small indeed and almost certainly are of no biological significance particularly when compared to the lesions found after feeding erucic acid containing oils (Kramer et al., 1988; Rocquelin et al., 1989). This conclusion must also apply to our finding that the lowest levels of lipidosis and fibrosis were observed after the long term feeding of a saturated fat (SF) enriched diet. Furthermore, there is no evidence that these findings are associated with any long-term change in the plasma levels of vitamin E in these animals (c.f. Table III).

Table VIII Effect of Diet on Cardiac Lipidosis and Fibrosis in Adult Male Marmosets

Lipid Supplement	Lipidosis	Fibrosis/Lesions		
	Average % of cells with lipid droplets	Grade 1	2	3
REF	2.4	13	3	0
SF	1.3	5	1	1
SSO	1.8	33	2	0
FO	6.2	29	3	0

* Diet 2, (1987-90) : All values for lipidosis were from 3,000 fibers per ventricular section; 3 sections per animal (c.f Kramer et al., 1988). There were at least 6 marmosets per dietary supplemented group. Fibrosis : the grade of lesions is defined as the area of a lesion relative to that of a high power field (Opstvedt et al., 1979).

However very recent work by Chautan et al., (1990) has shown that in rats, decreased plasma vitamin E levels can be associated with markedly increased myocardial levels of this vitamin. This inverse relationship was particularly evident when dietary omega-3 PUFA's were increased. Consequently it will be necessary to make direct measurement of marmoset myocardial vitamin E levels after FO and other lipid supplemented diets before the possible role of vitamin E in cardiac lipidosis and fibrosis, or indeed many other aspects of cardiac muscle function, can be fully evaluated. This is particularly important as Janero & Burghart (1989) have recently reported greatly reduced levels of α-tocopherol in the heart muscles of spontaneously hypertensive rats compared to that found in normotensive animals.

No evidence of abnormal or dietary induced lipofuscin pigmentation was observed in the myocardial fibers of any of our marmosets, even after 36 months of feeding the experimental diets (Turner & Charnock, unpublished observations). This is in marked contrast to the pigmentation of the vascular endothelium and smooth muscles of vitamin E deficient monkeys reported by Hayes (1974).

Table IX Effect of Diet on Heart Rate of Marmosets

Lipid Supplement	Diet 1	Diet 2
REF	242 ± 6	280 ± 25
SF	364 ± 13	268 ± 12
SSO	278 ± 9	247 ± 20
FO	n.m	226 ± 24

Values for heart rate are in beats/min (Means ± SEM) . There were at least 6 male marmosets per group. Dietary groups (REF, SF, SSO & FO) are as in text and previous tables. Diets 1 & 2 are as in Table 1. n.m is not measured in the experiment.

Effect of Diet on Heart Rate and Blood Pressure

The effect of diet on the heart rate of marmosets is shown in Table IX. Although there is insufficient information from these experiments to draw an unequivocal conclusion, it is possible that the additional vitamin E added to the lipid supplements of "Diet 2" may be associated with a decrease in heart rate. However, the inherent variability in collecting data of this type, even in lightly anaesthetized marmosets, certainly does not warrant too firm interpretation of the results. Perhaps the most interesting observation is the apparent decrease in heart rate of FO fed marmosets in the "Diet 2" experiment, where the vitamin E levels had been adjusted upward and the diets fed for more than two years. Certainly it would seem that in this particular experiment, the increased vitamin E content of the fish oil supplement did not limit the effect of omega-3 PUFA's in reducing the heart rate of marmosets by at least 15%. In addition it should be noted that the elevated heart rate seen in the SF fed group in the first experiment with "Diet 1" was not observed after feeding "Diet 2". It is possible that the increased level of Vitamin E in "Diet 2" may have contributed to this effect. However much further experimentation, with many more animals per dietary group will be required to resolve this question beyond doubt.

Table X Effect of Diet on Blood Pressure of Marmosets

Lipid	Diet 1		Diet 2	
Supplement	Systolic	Diastolic	Systolic	Diastolic
REF	96 ± 4	66 ± 2	90 ± 5.5	66.9 ± 5.7
SF	98 ± 3	71 ± 2	95 ± 4.1	66.5 ± 3.5
SSO	100 ± 5	69 ± 3	90 ± 5.0	59.1 ± 3.2
FO	n.m	n.m.	87 ± 3.7	60.3 ± 2.5

Diets and dietary groups are as in text and previous tables. All values are from adult male marmosets and are in mm Hg (means ± SEM). n.m. is not measured in experiment.

A similar equivocal conclusion must be drawn from our accumulated data on the blood pressure of these dietary manipulated marmosets (Table X) There is no evidence to suggest any difference between the blood pressures of animals fed either "Diet 1" or "Diet 2", nor is there any reason to believe that any form of lipid supplementation resulted in change in either systolic or diastolic pressures even after two or more years of dietary intervention. In fact, perhaps the most striking observation in either experiment is the complete lack of effect of any form of fat loading upon the blood pressure of these small non-human primates! It is interesting to speculate that this lack of effect may be influenced by the marmosets extremely active life-style which is markedly different from that of laboratory rodents or sedentary man.

Effect of Diet on Cardiac Muscle Dysrhythmia

One measurement of the cardiac function of animals which has been shown to be influenced by both age and dietary lipids, is the ability of their isolated papillary muscles to respond to a catecholamine induced stress in vitro (Charnock et al., 1985). Usually a high proportion of these isolated cardiac muscles develop marked dysrhythmia with increasing doses of isoprenaline. Those muscles from saturated fat fed animals are most severely effected (Charnock, 1985; Charnock et al., 1985; McLennan et al., 1987b).

The results given in Table XI were taken from two similar experiments with marmoset monkeys which were carried out after feeding either "Diet 1" or "Diet 2" for about 24 months (McLennan et al 1987;1988). Addition of isoprenaline to the incubation vessel containing the marmoset papillary muscles was again used to induce catecholamine mediated dysrhythmia, and the results which are expressed here (Table XI) in their simplest form are given only as the % of papillary muscles within a dietary group which developed pronounced dysrhythmia over a set period of time.

The most consistent observation is similar to that previously reported for laboratory rats fed a variety of lipid supplemented diets for about one year (McLennan et al., 1989). That is, inclusion of polyunsaturated fatty acids from either plant (omega-6 PUFA'S) or marine (omega-3 PUFA's) sources, significantly reduce the vulnerability of the heart to develop dysrhythmic contractions when subjected to isoprenaline induced stress (Charnock, 1985; Charnock et al., 1985).

Examination of the data from within and across these two experiments with marmosets cannot exclude a role for vitamin E, because like the effect on heart rate (c.f. Table IX) the proarrhythmic effect of the saturated fat (SF) found following the feeding of "Diet 1", may have been attenuated by the increased vitamin E content of "Diet 2". In this regard it is important to note some preliminary experiments in rats which we reported at the 1989 Palm Oil Conference in Kuala Lumpur, which suggested that an increased level of protection against "Reperfusion Arrhythmia" in ischaemically damaged hearts could be observed in the rats fed RBD palm oil compared to those which had received NBD palm oil dietary supplements (Charnock et al., 1991). Because the fatty acid composition of these two palm oil products is essentially identical, it is a definite possibility that their different levels of tocopherol and tocotrienols (that is their total levels of "vitamin E activity") may be responsible for this effect (Abeywardena et al., 1991).

Hence the experiments with marmoset papillary muscle reported here raise the interesting possibility that in primates as well as rodents, the stability of their papillary muscles to withstand catecholamine induced stress in vitro might be favorably influenced by the level of the animals cardiac membrane vitamin E. Certainly the level of membrane role for vitamin E in the protection of the heart from cardiac arrhythmia and hence

Table XI Effect of Diets on Cardiac Arrhythmia

Lipid Supplement	% Papillary Muscles Dysrhythmic	
	* Diet 1	# Diet 2
REF	44	50
SF	64	40
SSO	16	20
FO	n.d.	10

* Experiments with Diet 1 were with isoprenaline 10^{-5} μmols L^{-1}; # Diet 2 had isoprenaline 3.2 x 10^{-8} μmols L^{-1} : n.d. is not done. Source; adapted from McLennan et al., 1987a and McLennan et al., 1988.

vitamin E will be influenced by the type of dietary fat habitually consumed (Chauntan et al., 1991). If this suggestion should prove to be correct in marmosets, then an important Sudden Cardiac Death in primates would be established. Experiments to examine this possibility are currently in progress in our laboratory.

Conclusion

There is evidence that additional vitamin E added to several different types of lipid supplemented diets, is associated with increased growth and reproductive performance in adult marmoset monkeys (Callithrix Jacchus) . This was particularly evident when a fish oil lipid supplement rich in omega-3 PUFA's was added to the diet. While a dietary induced effect upon either the hemostatic parameters or the plasma lipids of the marmoset could not be directly attributed to different levels of vitamin E, neither could it be completely excluded. Similarly an effect of dietary vitamin E on the animals heart rate

was not unequivocally apparent. However when considered in conjunction with some recent evidence reported by ourselves and others from experiments in rodents, it is possible that an effect of vitamin E upon the mechanical stability of the marmosets heart muscle was observed when they were placed under catecholamine stress in vitro. Further experiments are urgently required to study this possibility in primates, as if vitamin E could be shown to have a direct effect upon the ability of the heart muscle to withstand catecholamine induced stress in vivo as well as in vitro, then a most important dietary link between vitamin E, fat consumption, cardiac arrhythmia and Sudden Cardiac Death would be established. As the incidence of this disease remains high in many economically well developed countries (Hetzel et al., 1989), and is currently increasing rapidly in such nations as Singapore and Mauritius (Beaglehole et al., 1988; Gareeboo et al., 1989) establishing such a link between vitamin E and this form of heart disease would be of immense clinical and economic importance.

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ANTITUMOR AND ANTIOXIDANT ACTIVITY OF TOCOTRIENOLS

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Summary

Antitumor activities of tocotrienols were tested on transplantable murine tumors. Intratumor injection of α- and γ-tocotrienols showed growth inhibition on sarcoma 180 inoculated subcutaneously into ICR mice. These agents also inhibited the pulmonary metastasis of Lewis lung carcinoma cells inoculated into footpads of BDF_1 mice. In in vitro studies, α-, γ- and δ-tocotrienols were found to possess direct cytotoxicity on various mammalian cells, and the IC_{50} values were 4-135 μg/ml when the cells were exposed to these agents for 72 hours. Tocotrienols suppressed the uptake of thymidine and uridine into the macromolecules of P388 leukemic cells. Malondialdehyde generation induced by $K_2Cr_2O_7$ in the, liver was suppressed when treated with γ-tocotrienol, but this suppression was lower than that of α-tocopherol.

Introduction

As part of our search for new physiological activities of tocotrienols from palm oil, we have already reported antitumor and antioxidant activities (Komiyama et al., 1989, Yamaoka Komiyama, 1989). In the present report, we demonstrate that tocotrienols exhibit antimetastatic activity and have direct cytotoxicity on various mammalian cells as well as inhibition of macromolecular synthesis and antioxidant activity.

Materials and Methods

Evaluation of cytotoxic activity

In vitro cytotoxic activity was measured by Giemsa staining assay as described by Mirabelli et al. (1985). Briefly, cells were cultured with tocotrienols in 0.2 ml of cultured medium at 37°C for 3 days. After cultivation, the cells were then fixed with methanol and stained with Giemsa solution for 30 min. Stained cells were solubilized with 0.2 ml of 0.1 N HCl, and absorbances were read by a microtiter plate reader at a wave length of 600 nm (reference wavelength at 405 nm).

Evaluation of Antitumor Activity

Sarcoma 180 tumor cells were maintained by sequential i.p. transplantation into ICR mice. For local tumor treatment, sarcoma 180 cells were transplanted into ICR mice on day 0. From day 5 to day 14, received intratumor injections (i.t.) of 0.5 mg/mouse/50 μl tocotrienols. The tumor size was measured by calipers every 5 days and calculated as follows;

$$\text{length (mm) x width(mm)} = \text{mm}^2$$

To determine antimetastatic activity of tocotrienols, Lewis lung tumor cells were inoculated into the footpads of BDF_1 mice on day 0. Mice received tocotrienols i.p. from day 1 to day 18. On day 17, footpads bearing tumors were amputated, and on day 23 mice were sacrificed to count pulmonary metastases.

Effect of Macromolecular Synthesis

P388 cells (2 x 10^4/ml) were plated on a 96-well microculture plate, and 24 hours later ^{3}H-labelled thymidine(TdR), uridine(UR) or leucine, was added to the culture. Tocotrienols were also added at the same time. After 2 or 4 hours of exposure, cells were collected on glass filters and washed three times with ice-cold water. The filters were dried and the radioactivity was counted by means of liquid scintillation.

Antioxidant activity

ICR mice were administered i.p. 20 mg/kg of $K_2Cr_20_7$ and 40 mg/kg of tocopherol or tocotrienols. Twenty four hours later, the mice were sacrificed and their livers were removed. Then, the livers were homogenized in 9 volumes of ice-cold 1.15 % KCl solution. The amount of malondialdehyde in the homogenate was determined according to the method of Uchiyama and Mihara(1978).

Results and Discussion

Cytotoxic and antimicrobial activity

Direct cytotoxicity of tocotrienols was examined using P388 leukemic cells. The IC_{50} values covered a wide range among the agents and the kind of cells. As shown in Table I, all tocotrienols showed direct cytotoxicity against various mammalian cells including tumor and normal cells, but the values were weaker than those of known antitumor agents. Tocotrienols did not show any antimicrobial activity on many micro-organism including Gram positive and negative bacteria, fungi and yeast, even at a concentration of 1,000 μg/ml (data not shown). Therefore, it is considered that the mode of action of tocotrienols differs from that of conventional nucleic acid synthesis inhibitors.

Antitumor Activity

The antitumor activity of a local injection of tocotrienol was examined using sarcoma 180 inoculated into ICR mice. Tocotrienol inhibited the tumor growth of the solid form of sarcoma 180 as shown in Table II, but tumor regrowth was observed after withdrawal of the agent. Antimetastatic activity of tocotrienols was examined using Lewis lung carcinoma cells which are well known as a highly metastatic experimental cell lines. As shown in Table III, the mean weight of amputated footpad bearing tumors was 677 mg, whereas with a dose of 40 mg/kg of tocotrienols, it was approximately 480 mg.

Therefore, it appeared that growth of original lung tumor was suppressed by tocotrienols. It was also clear that number of lung metastases in the control group was 13, but at a dose of 20 or 40 mg/kg of tocotrienols, the lung metastasis was suppressed.

Table I. Cytotoxicity of α-, γ- and δ-tocotrienols (IC_{50} value μg/ml)

Cell line		α-	γ-	δ-
HeLa	Human cervix ca.	116.2	13.1	77.4
B16	Mouse melanoma	11.2	3.8	20.0
P388	Mouse leukemia	16.2	11.2	4.7
L929	Mouse fibroblast	32.6	21.0	12.2
Chang	Human liver	25.6	19.8	12.2
MDBK	Bovine kidney	20.6	10.8	8.9
CHL	Hamster lung	46.9	23.1	135.4
Vero	Monkey kidney	64.7	34.7	35.4

Cells were exposed to α-, γ-, or δ-tocotrienols for 72 hours.

Table II. Antitumor activity of intratumor of tocotrienols on sarcoma 180

	Tumor size (mm^2)		
Agent	Day 7	Day 12	Day 17
Control(saline)	63 ± 16	165 ± 34	185 ± 50
α-Tocopherol	45 ± 22	121 ± 66	165 ± 84
α-Tocotrienol	43 ± 16	85 ± 23	141 ± 61
γ-Tocotrienol	49 ± 14	85 ± 15	129 ± 26

Mice were subcutaneously inoculated with sarcoma 180 on day 0, and received intratumor injections of 0.5 mg/mouse of tocotrienol on days 5-14. Tumor size were measured by calipers.

Table III. Effect of tocotrienols on Lewis lung carcinoma

Agent	Dose	Wt (mg) of footpad bearing tumor		No. of Metastases	
	(mg/kg)	Mean	Range	Mean	Range
Saline(control)	-	677	635-1151	13.0	10-19
α-Tocotrienol	20	608	474-850	6.0	2-10
	40	479	228-622	5.1	2-8
γ-Tocotrienol	20	536	435-831	5.7	0-11
	40	482	195-847	6.0	0-18
Mitomycin C	0.5	418	388-504	2.0	0-6

Lewis lung cells were inoculated i.p. into the footpads of mice on day 0. Mice were administered tocotrienols i.p. from day through day 18. On day 17, footpad bearing tumors were amputated, and on day 23 mice were sacrificed to count pulmonary metastases.

Effect on Macromolecular Synthesis

Since α-, γ-, δ-tocotrienols inhibited cell growth in vitro, macromolecular synthesis of P388 leukemia cells was examined using tritium labeled precursors of DNA, RNA and protein. As shown in Table IV, remarkable inhibition of the incorporation of thymidine was observed at a dose of 50 μg/ml of δ-tocotrienol. No remarkable difference was observed between the incorporation of TdR and UR. TdR incorporation was also inhibited in the group treated with α- or γ-tocotrienols. This inhibition did not increase with time.

Antioxidant activiy

We have previously reported that α- and γ-tocotrienols and α-tocopherol inhibited lipid peroxidation in mouse cardiac tissue due to the injection of doxorubicin (Komiyama and Yamaoka. 1991). Since we found that hexavalent chromium induces lipid peroxidation

Table IV. Effect of macromolecular synthesis by P388 leukemia cells

Agent	Conc (μg/ml)	Inhibition(%)					
		2 hr			4 hr		
		TdR	UR	Leu	TdR	UR	Leu
α-Tocopherol	50	13	3	0	0	0	1
α-Tocotrienol	6.3	27	22	0	12	30	0
	12.5	30	7	0	19	25	0
	50	42	7	0	61	36	19
γ-Tocotrienol	6.3	21	24	0	6	10	3
	12.5	30	19	0	26	11	8
	50	70	52	10	81	37	42
δ-Tocotrienol	6.3	21	35	0	9	22	0
	12.5	41	57	34	84	65	46
	50	96	83	62	95	85	72

Cells were exposed to the agents 4 or 2 or 4 hours.

in the mouse liver, the effect of tocotrienols on malondialdehyde generation in mouse liver was examined. As shown in Table V, α-tocopherol inhibited malondialdehyde generation by over 90 % but the same dose of γ-tocotrienol inhibited it by only 50 %. α-tocotrienol did not show any inhibition.

Table V Inhibitory effect on malondialdehyde generation in mouse liver

Agent	K_2Cr_2O7	MDA (nM)	Inhibition (%)
-	-	11.9	-
Saline	±	47.9	-
α-Tocopherol	±	15.0	91
α-Tocotrienol	±	48.5	0
γ-Tocotrienol	±	30.3	49

ICR mice were injected i.p. with 20 mg/kg of $K_2Cr_2O_7$ and tocotrienols. Twenty four hours later, mice were sacrificed and the amount of malondialdehyde in the liter was determined by the method of Uchiyama and Mihara.

Conclusion

Tocotrienols showed antitumor activity in the i.p. - i.p. system (i.p. inoculation of tumor cells and i.p. injection of the agents) in our previous experiment, but activity was weaker than that of known antitumor agents. One of the main reasons for this was the weak cytotoxic activity of tocotrienols in vitro compared with that of other antitumor agents. On the other hand tocotrienols showed antitumor activity against solid tumors grown in mouse footpads and inhibition of pulmonary metastasis. These findings suggested that the antitumor activity of tocotrienol is due not only to direct cytotoxicity but also to some other mechanisms. We previously reported that α- and γ-tocotrienol suppressed the generation of malondialdehyde (Komiyama Yamaoka, submitted) induced by doxorubicin in the heart tissue of mice. In the present experiment, only γ-tocotrienol inhibited malondialdehyde generation by hexavalent chromium. The antioxidant activity of tocotrienol seems to differ in accordance with the kind of radicals and/or the organs in mice.

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TOCOPHEROLS, CAROTENOIDS AND THE GLUTATHIONE SYSTEM

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Summary

Reactive oxygen species occur in tissues and can damage DNA, proteins, carbohydrates, and lipids. These potentially deleterious reactions are controlled by a system of enzymatic and nonenzymatic antioxidants which eliminate pro-oxidants and scavenge free radicals. Tocopherols are the most abundant and efficient scavengers of hydroxyl radicals in biological membranes. Water-soluble antioxidants include ascorbate and cellular thiols. The ability of lipid-soluble carotenoids to quench singlet molecular oxygen may explain anticancer properties of the carotenoids, independent of their provitamin A activity. Glutathione is an important substrate for enzymatic antioxidant functions and is capable of nonenzymatic radical scavenging. Thiols associated with membrane proteins may also be important to the antioxidant system. Interactions between the thiols, tocopherols, and other compounds enhance the effectiveness of cellular antioxidative defense.

Introduction

Oxidative stress is associated with a disturbance in the prooxidant/antioxidant balance in favor of the prooxidants (Sies, 1985). The occurrence of reactive oxygen species, known as pro-oxidants, is an attribute of normal aerobic life. The existence and development of cells in an oxygen-containing environment would not be possible without the presence of defense systems including powerful enzymes and nonenzymatic antioxidant components (Sies, 1986). Many of the pro-oxidants are free radicals, and the study of

these provides a new field of interest in biology and medicine (Halliwell & Gutteridge, 1990), including a number of physiological and pathophysiological phenomena and processes such as inflammation, ageing, carcinogenesis, drug action, drug toxicity, and defense against protozoa.

Many of the reactive oxygen species (Pryor et al., 1986; Kelm & Schrader, 1990) are oxygen-centered free radicals. Some, however, such as singlet molecular oxygen or hydrogen peroxide, are not radicals but are also involved in oxidative damage.

Antioxidant Defense

Detoxication of reactive oxygen species is one of the prerequisites of aerobic life, and many defenses have evolved, providing an important antioxidant defense system of prevention, interception, and repair of nonenzymatic scavengers and quenchers, known as antioxidants, as well as enzymatic systems.

The nonenzymatic systems include lipophilic and hydrophilic antioxidants. There are important interrelationships between these two systems, indicating the need for coupling to nonradical regenerative processes. The initial radical damage in a membrane needs to be repaired to allow for sustained free radical scavenging activity.

Tocopherols

Tocopherols (vitamin E) act as biological antioxidants (Tappel, 1962; Sies & Murphy, 1991). Vitamin E accounts for much of the lipid-soluble chain-breaking antioxidant capacity in the human blood plasma and erythrocyte membranes (Burton et al., 1983). Hence, a major biological function of tocopherol as an antioxidant lies in its reactivity as a free radical quencher. The rate constants with peroxyl radicals are in the range 10^6-10^8 $M^{-1}s^{-1}$, depending on the experimental conditions (Simic, 1981). The reaction involves a peroxyl radical and the phenolic hydroxyl group of tocopherol to generate the organic hydroperoxide and the tocopherol radical (Niki, 1987):

$$ROO^{\bullet} + \text{vit E-OH} \text{ -----> } ROOH + \text{vit E-O}^{\bullet} ,$$

transposing the radical function from the reactive organic radical (e.g., of a polyunsaturated fatty acid) into the less reactive chromanoxyl radical. Tocopherol breaks the chain reaction of peroxidation in that peroxyl radicals react with other lipids with rate constants of about 50 $M^{-1}s^{-1}$, but react with tocopherol 10^4 or 10^5 times faster. There is a consensus hypothesis regarding the reversibility of tocopheryl radical formation in which the hydrogen donor in the reaction

$$\text{vit E-O}^{\bullet} + \text{AH} \text{ --------> vit E-OH} + \text{A}^{\bullet}$$

is water-soluble, i.e., the radical challenge is transferred from the membrane to the aqueous compartment of the cell. A possibility is that vitamin C (ascorbate) interacts with vitamin E, as suggested by Tappel (1962), and consistent with this hypothesis is the observation (Reddy et al., 1982; Franco & Jenkinson, 1986; Wefers & Sies 1988) that tocopherol must be present in biological membranes for ascorbate to protect it from peroxidation. However, there remains some doubt as to whether this reaction occurs in vivo or even in the model systems (McCay, 1985). As evidence against a direct ascorbate-tocopheryl reaction in vivo, it has been reported that ascorbate deficiency has no effect on the rate of tocopherol turnover or loss in the guinea pig (Burton et al., 1990).

Carotenoids

Epidemiological investigations revealed an inverse relationship between serum carotenoid levels and the incidence of several types of cancer (Peto et al., 1981; Krinsky, 1988; Ziegler, 1989). Animal and cell culture studies demonstrated anticarcinogenic and antimutagenic properties of these compounds (Black & Mathews-Roth, 1991; Krinsky, 1989a). The underlying biochemical mechanisms are yet unknown, but there is evidence for a role of the antioxidative capacity of carotenoids, namely the quenching of singlet oxygen and reaction with oxygen-centered radicals (Foote & Denny, 1968; Burton & Ingold, 1984; Krinsky, 1989b). Research has focused on β-carotene, but other carotenoids may also be of importance.

We investigated the relative quenching ability of various naturally occurring carotenoids and compared them with α-tocopherol and bile pigments, using the thermodissociable endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate ($NDPO_2$) to

generate singlet oxygen (Di Mascio et al., 1989; Di Mascio & Sies, 1989). Lycopene, a biologically occurring carotenoid, exhibits the highest physical quenching rate constant with singlet oxygen, which is slightly higher than that of β-carotene.

This finding is of considerable general interest, since nutritional carotenoids, particularly β-carotene, and other antioxidants such as α-tocopherol have been implicated in the defense against pro-oxidant states. Also, albumin-bound bilirubin is a known singlet oxygen quencher. Interestingly, those compounds with low quenching values occur at higher plasma levels. When these differences are taken into account, the singlet oxygen quenching capacities of lycopene (0.7 μM in plasma), β-carotene (0.5 μM in plasma), albumin-bound bilirubin (15 μM in plasma), and α-tocopherol (22 μM in plasma) are of comparable magnitude.

Carotenoids may undergo trans- and cis- isomer interconversion by quenching singlet oxygen, and several cis-isomers have been detected in human serum and various fruits and vegetables. Little is known, however, on formation, distribution and biological relevance of the cis-isomers in human tissues (Zechmeister, 1962; Jensen et al., 1982; Chandler & Schwarz, 1987; Sowell et al., 1988; Kachik et al., 1989; Krinsky et al., 1990).

Since cis- or trans-isomers of carotenoids may have different biological reactivities, we recently measured the isomeric composition of lycopene and β-carotene in serum and seven human tissues (Stahl et al., 1991). In addition to all-trans lycopene, at least three cis-isomers (9-, 13-, and 15-cis) were present in serum, accounting for more than 50% of total lycopene. 15-cis β-Carotene, however, was present at only 5% of the all-trans isomer. In addition, 9-cis β-carotene was present in tissue samples but not in serum. There were inter-individual differences in carotenoid levels of the different tissue types, but liver, adrenal gland, and testes always contained significantly higher amounts of the carotenoids than kidney, ovary and fat; carotenoids in brain stem tissue were below detection limit. β-Carotene was the major carotenoid in liver, adrenal gland, kidney, ovary, and fat, whereas lycopene was the predominant carotenoid in testes (Kaplan et al., 1990; Stahl et al., 1991).

Glutathione

Thiols may also react with tocopheryl radicals to regenerate tocopherol and, conversely, tocopherols can repair thiyl radicals. In the cell, glutathione (GSH) is the major low molecular weight thiol present. An enzymatic mechanism, "glutathione free radical reductase"', has been proposed, evidence for which is indirect, however. Burk and coworkers (Burk, 1983; Hill & Burk, 1984) observed that lipid peroxidation in purified rat liver microsomes was inhibited by glutathione but protection was eliminated by heating the microsomes. The GSH-dependent "factor" has resisted further attempts at purification. Although there is no doubt that GSH has no overall sparing effect on tocopherol in rat liver microsomes, this mechanism in other tissues and other species is still uncertain (Murphy & Kehrer, 1989). Other thiols, such as dihydrolipoate, a lipid-soluble dithiol and powerful reductant, inhibit microsomal peroxidation (Haenen & Bast, 1983; Scholich et al., 1989) and spare tocopherol in a manner similar to glutathione, but this activity is not lost after heating or trypsinization.

It is possible that GSH enzymatically inhibits peroxidation in a way that requires but does not regenerate tocopherol, and GSH may act via the membrane-bound GSH peroxidase, which reduces phospholipid hydroperoxides (Ursini & Bindoli, 1987). In the absence of GSH, the hydroperoxides quickly accumulate by rapid and irreversible chain reactions. Tocopherol generates hydroperoxides without the initiation of further chain reactions and prevents the peroxidase from being depleted. Alternatively, the protection against autocatalytic peroxidation of lipids by tocopherol may be curtailed if tocopherol is used in reactions with cysteinyl or other radicals associated with proteins, which GSH may be able to intercept. Supporting this idea is the observation (Scheschonka et al., 1990) that the oxidation of protein thiols parallels the loss of tocopherol in microsomes subjected to a wide range of pro-oxidants.

Concluding Remarks

These brief comments make clear that there is a network of interlinked antioxidant systems to protect cells from oxidative damage. Interestingly, some vitamins and other micro-nutrients are important in this respect, linking nutrition physiology with biochemistry and toxicology.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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NUTRITION OF TOCOTRIENOLS AND LIPID METABOLISM

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Summary

Lymphatic absorption of α-tocotrienols (α-Toc3) given as a mixture of Toc 3 and α-tocopherol (α-Toc) was significantly higher than in γ-Toc3, δ-Toc3 and α-Toc in rats. This was confirmed when the rate of absorption of individual Toc3 was measured. Approximately 50 to 60% of the absorbed Toc3 and α-Toc was transported as chylomicrons in lymph. More α-Toc was excreted into feces than α-Toc3 when these were given to rats, but the content in the liver markedly higher in the former than in the latter. Either α-Toc or Toc3 concentrate contain α-, γ-, and δ-Toc3 and α-Toc (dietary level; 0.13% α-Toc or 0.07% Toc3 and 0.06% α-Toc) did not reduce systolic blood pressure of SHR when 0.5% salt solution was given as drinking water, whereas they suppressed an increase in blood pressure with age when the dietary was increased to 0.2% and no saline was given. The effect of Toc3 concentrate and α-Toc, in particular the former, on aortic production of $PGI_2$was diverse depending on their dietary level whereas no effect was observed on the degree of maximum platelet aggregation induced by ADP. Although the activity of liver microsomal Δ^5- and Δ^6- desaturates was not influenced by Toc3 concentrate and α-Toc, they repressed conversion of the linoleic acid to arachidonic acid. These results suggest that Toc3 have diverse nutritional and physiological impacts on various parameters, and their effects are not necessary the same as those of α-Toc.

Introduction

Tocotrienol (Toc3) have been known to exert a hypocholesterolemic effect (1,2) presumably through the reduction of hepatic cholesterol synthesis (3). A mixture of Toc3 improved the reduction of plasma HDL-cholesterol in rats due to cholesterol ingestion in a dose-dependent manner (4,5). These observations suggest that Toc3 are the potent regulator of cholesterol metabolism. Since α-tocopherol (α-toc) regulates the production of prostaglandins (6-9), it is noteworthy to know the function of Toc3 in those parameters under regulation of eicosanoids. In addition, since the absorbability defers among the Toc isomers (10), it is also important to know the rate of absorption of Toc3.

In the present study we compare the intestinal absorption of Toc3 with α-Toc in rats. The effects of Toc3 on blood pressure and other lipid indices were also examined.

Materials and Method

Animals and Diets

Male Sprague-Dawley rats were obtained from Seiwa Experimental Animals, Fukuoka, Japan, (Exps 1 and 2) or from Japan SLC Inc, Shizuoka, Japan (Exp. 3), and acclimated for 3-5 days in a room with controlled temperature (22-24°C) and lighting (alternating 12-hr periods of light and dark). In Exps. 1 and 2, rats weighing approximately 300g were given a commercial non-purified diet (Type NMF, Oriental Yeast Co., Tokyo, Japan) and subjected to cannulation of the left thoracic lymphatic channel, and an indwelling catheter was placed in the stomach (11). Each animal was administered 3ml of a test emulsion through the gastric tube and lymph was collected for 24 hrs. The composition was in exp.1: 200mg Na-taurocholate. 50mg bovine serum albumin (fatty acid free), 200mg high-oleic safflower oil and 100mg Toc concentrate prepared from palm oil (α-Toc 35%, α-Toc3 20%, γ-Toc3 30% and δ-Toc3 12%, Nissin Oil Mills Co., Tokyo, Japan); and in Exp. 2: 200mg Na-truocholate, 50mg bovine serum albumin, 200mg triolein (Sigma Chemical Co., St. Louis, MO) and 10mg individual Toc3 or α-Toc (Nissin Oil Mills Co., Tokyo, Japan). In Exp. 3, rats weighing approximately 77g were fed an AIN type purified diet (12) containing 20% partially hydrogenated sardine oil, and 0.2% of either α-Toc or α-Toc3 (purity 80%) (Eisai, Tokyo, Japan). In

Exps. 4 and 5, 6 or 8 week old male spontaneously hypertensive rats (SHR/NCrj, Japan Charles River Co., Kanagawa, Japan) were fed an AIN type purified diet (12) containing either α-Toc or Toc3 concentrate (α-Toc 36%, α-Toc3 24%, γ-Toc3 27 % and δ-Toc3 11%, Palm Oil Research Institute of Malaysia, Kuala Lumpur, Malaysia) at the 0.13% or 0.2 % for 6 or 5 weeks, respectively. The systolic blood pressure on the tail every week with a sphygmomanometer (MK100 Moromachi Kikai Co., Tokyo, Japan) in a chamber maintained at 33°C.

Analytical Procedures

Toc and Toc3 were extracted from the lymph by ethanol and hexane containing an internal standard, 2,2,5,7,8-pentamethyl-6-hydroxychromane (Eisai Co., Tokyo, Japan) (13) and applied for HPLC (Zorbax SIL column elution with hexane-diethylether, 9:1 v/v, detection at UV 282 nm). The activity of liver microsomal Δ^5- and Δ^6- desaturates (14) and fatty acid composition of tissue lipids (15) were analyzed as described elsewhere. The aortic production of PGI_2 was measured as 6-keto $PGF_{1\alpha}$ by a radioimmunoassay using a commercial kit (15), and the platelet aggregation by 5 μM ADP was measured with a platelet aggregation analyzer (AGGRECORDER II, Kyoto Daiichi Kagaku Co., Kyoto, Japan). Urinary L-ascorbic acid was by the dinitrophenylhydrazine method (16).

Statistical Analysis

One-way analysis of variance was followed by Duncan's new multiple range test to establish the exact nature of the difference among the group (17).

Results

Intestinal Absorption of Tocotrienols

When the mixture of α-Toc and Toc3 were introduced to the stomach by gavage (Exp. 1), lymphatic absorption of α-Toc3 was considerably greater than that of the γ- and δ-Toc3, and it was lowest in α-Toc (Fig. 1.). This was confirmed even when individual Toc3 were given separately (Exp. 2), and intestinal absorption of α-Toc3 was significantly

higher than other Toc3 or α-Toc throughout the lymph collection for 24 hr (Fig. 2.). There were no differences in the rate of absorption among γ-Toc3, δ-Toc3 and α-Toc. In addition, there was a slight but significant difference in the form by which Toc3 and α-Toc are transported in the lymph; the proportions as chylomicrons of α-Toc3, γ-Toc3, δ-Toc3 and α-Toc were 60.5 ± 1.6^{a}, 55.7 ± 2.6^{ab}, 50.2 ± 1.7^{b} and 55.9 ± 3.0^{ab}, respectively (values not sharing a common letter are significantly different at $p<0.05$).

The results of Exp. 3 in which either α-Toc or α-Toc3 were fed thr amount of Toc3 excreted into feces was approximately one-half that of α-Toc (Table 1). In contrast, the hepatic content was markedly higher in α-Toc than in α-Toc3.

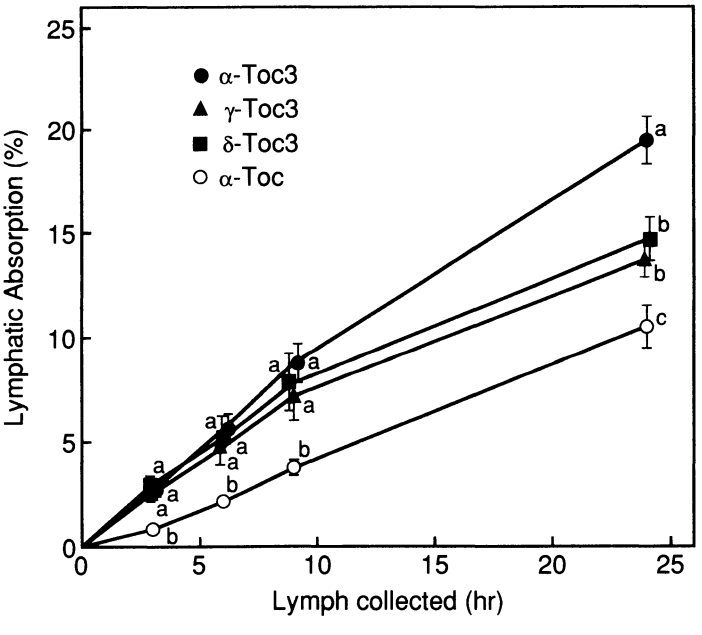

FIG 1. Lymphatic absorption of tocotrienols and α-tocopherols (Exp. 1.). Tocotrienol concentrate containing α-tocopherol (α-Toc) and α-, γ- and δ-tocotrienols (Toc3) were administered to lymph cannulated rats and lymph was collected periodically. Mean±SE of 6 rats. Values not sharing a common letter are significantly different at $p<0.05$.

Fatty Acid Compositions of Tissue Lipids and Desaturase Activity

In all feeding experiments (Exps. 3 to 5), there were no differences in food intake, body weight gain and liver weight among the control, Toc and Toc3 groups except for one occasion in which rats were fed diets containing hydrogenated sardine oil (Exp. 3), and liver weight was significantly lower in the α-Toc and α-Toc3 groups than in the control group.

Fatty acid compositions of liver and serum lipids were not significantly influenced by the α-Toc or Toc3 concentrate in all experiments in spite of the difference in the dietary fat source. However when the ratio of arachidonic acid to linoleic acid + dihomo--linolenic acid, an index of 6-desaturation, was compared, it was lowered by feeding α-Toc or Toc3 concentrate. An example of the polyunsaturated fatty acid composition of liver phosphatidylcholine is shown in Table 2.

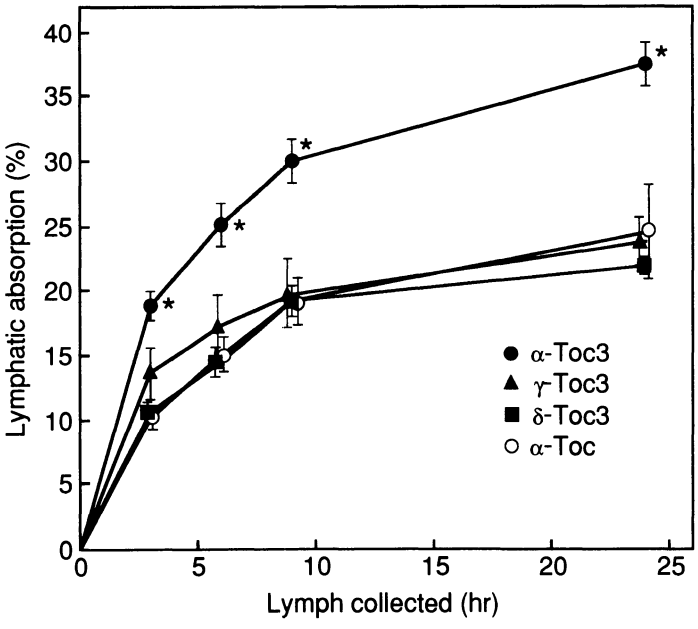

FIG 2. Lymphatic absorption of tocotrienols and α-tocopherol (Exp. 2). A mixture of α-, γ- and δ-tocotrienols (Toc3) or α-tocopherol (α-Toc) was administered to lymph cannulated rats and lymph was collected periodically. Mean±SE of 6 rats. Asterisk shows significant difference from other groups at $p<0.05$.

TABLE 1
Contents of α-tocopherol and α-tocotrienol in liver and feces of rats (Exp. 3)*

Group	Liver (μg/g)		Feces(μg/g/day)	
	α-Toc	α-T3	α-Toc	α-T3
Control	23±5.2	nd	198±44	nd
α-Toc	321±196	nd	9500±330	nd
α-T3	11.6±2.1	51.6±7.0	315±24	4050±160

*Mean±SE of 5 rats. nd:not detected. Feeding period, for 28 days.

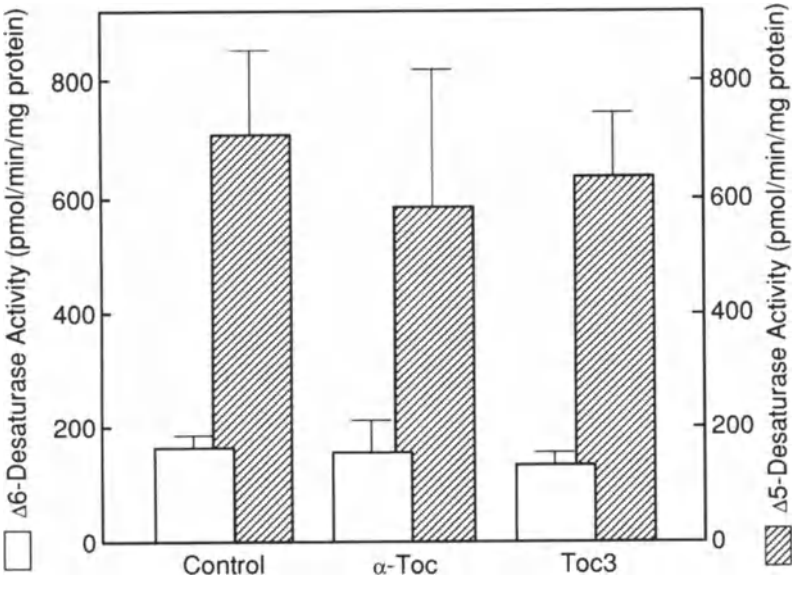

FIG 3. The activity of liver microsomal Δ^5- and Δ^6-desaturases in spontaneously hypertensive rats (SHR) given α-tocopherol (α-Toc) or tocotrienol (Toc3) concentrate (Exp. 5). Mean±SE of 4 to 6 rats.

The activity of liver microsomal 5- and 6-desaturases was measured in Exp. 5, and the results are shown in Fig 3. The activity of both 5- and 6-desaturases was not influenced by α-Toc or Toc3 concentrate.

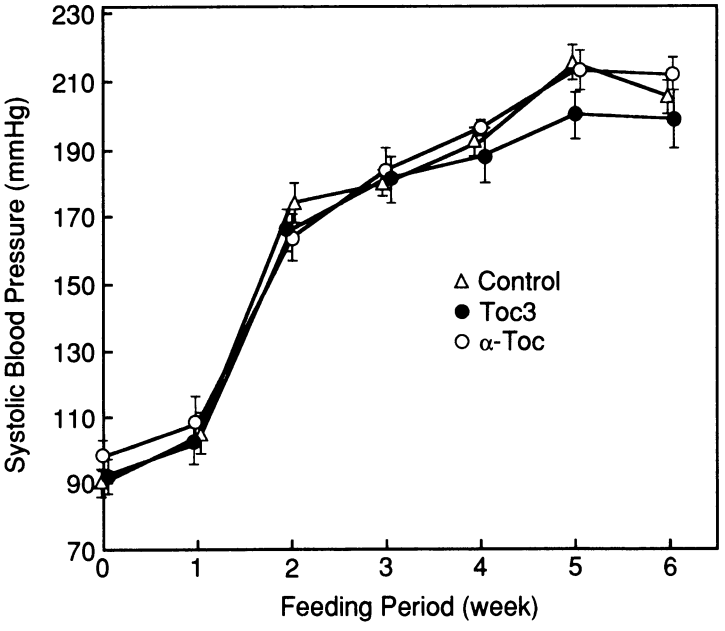

FIG 4. Systolic blood pressure of spontaneously hypertensive rats (SHR) given α-tocopherol (α-Toc) or tocotrienol (Toc3) concentrate together with a 0.5% salt solution as drinking water (Exp. 3). Mean±SE of 6 rats.

Blood Pressure

Fig 4 illustrates systolic blood pressure of SHR given α-Toc or Toc3 concentrate at a dietary level of 0.13% together with 0.5% salt solution as drinking water. There was no difference in the blood pressure among the three groups. In contrast, when the dietary level of α-Toc and Toc3 concentrate was increased to 0.2% and no salt solution was given, the rise of blood pressure with age was significantly suppressed by these compounds to a similar extent (Fig 5).

TABLE 2
Effect of α-tocopherol and tocotrienol concentrate on polyunsaturated fatty acid composition of liver phosphatidylcholine of SHR(Exp. 5)*

Group	Fatty acids (weight %)					(20:3+20.4) /18:2
	18:2n-6	20:3n-6	20:4n-6	22:5n-6	22:6n-3	
Control	9.1	0.4	35.2	1.3	3.3[a]	4.1[a]
α-Toc	11.5	0.5	32.3	0.9	2.3[b]	2.9[ab]
Toc3	11.2	1.0	33.2	0.8	3.0[a]	3.0[b]

* Mean±SE of 4 to 6 rats. α-Toc:α-tocopherol, Toc3:tocotrienol concentrate.
[ab]values not sharing a common letter are significantly different at $p<0.05$.

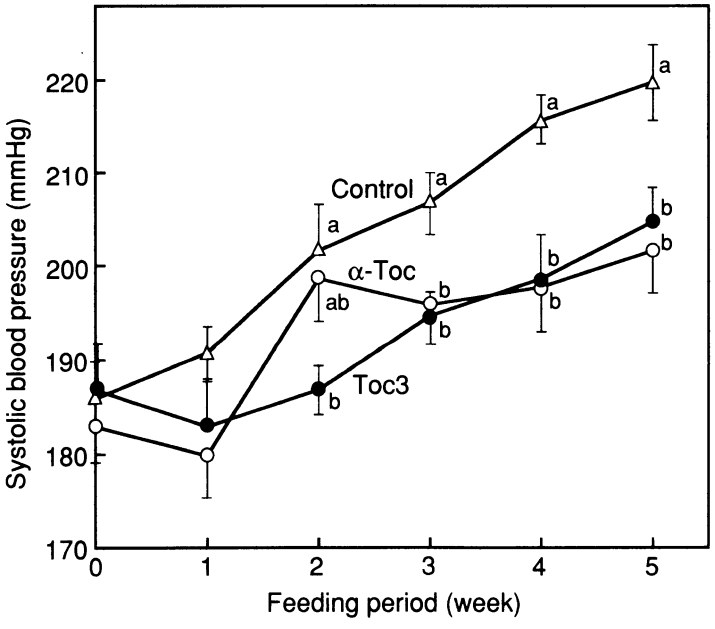

FIG 5. Systolic blood pressure of spontaneously hypertensive rats (SHR) given α-tocopherol (α-Toc) or tocotrienol (Toc3) concentrate (Exp. 4). No salt solution was given. Mean±SE of 4 to 6 rats.
Values not sharing a common letter are significantly different at $p<0.05$.

Eicosanoid Production and Platelet Aggregation

As shown in Fig 6, the aortic production of PGI_2 production was significantly low in the treated groups, in particular those given Toc3. In contrast, the production rather tended to increase in Exp. 5 and the difference between the control and Toc3 groups was significant. The platelet aggregation by ADP was not largely influenced by α-Toc or Toc3 concentrate in both experiments.

Urinary Ascorbic Acid Excretion

A temporal increase in urinary ascorbic acid excretion due to feeding partially hydrogenated fish oil at a relatively high level (18) was clearly ameliorated by the addition of α-Toc or α-Toc3 in the diet (Fig 7).

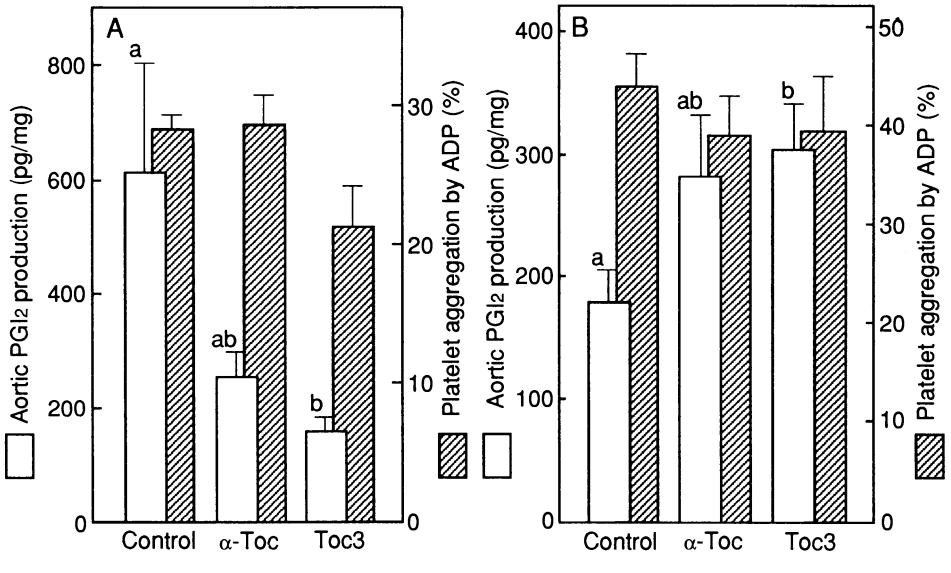

FIG 6. Aortic production of PGI_2 and platelet aggregation in spontaneously hypertensive rats (SHR) given α-tocopherol (α-Toc) or tocotrienol (Toc3) concentrate. A: Exp. 4, B: Exp. 5. Mean±SE of 6 and 4 to 6 rats for Exps. 4 and 5, respectively. Values in each experiment not sharing a common letter are significantly different at $p<0.05$.

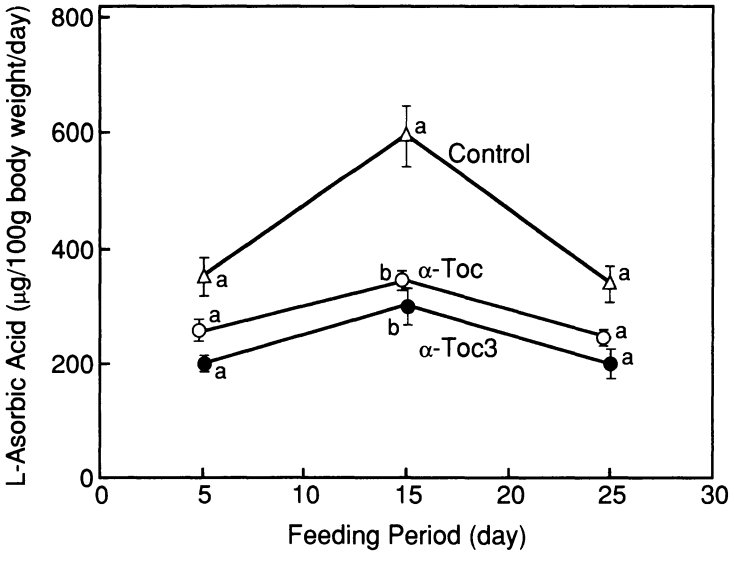

FIG 7. Urinary excretion of L-ascorbic acid in rats given α-tocopherol (α-Toc) or α-tocotrienol (α-Toc3) (Exp. 3). Mean±SE of 5 rats. Values not sharing a common letter are significantly different at $p<0.05$.

Discussion

A number of investigators reported intestinal absorption of α-Toc to be from 15 to 65% depending on the methods of administering α-Toc and the length of time the animals had to recover from surgery (10). No data is available regarding absorption of Toc3. The present study showed that lymphatic absorption of α-Toc is around 10 to 20%. Since absorption of fatty acids simultaneously dosed was quantitative in the present condition and since α-Toc absorption was reaching plateau at 24-hr, it seems reasonable that absorption of α-Toc is in the range of the lower borderline of the available data. In

addition, the proportion of α-Toc as lymph chylomicrons was considerably lower than previously reported (10).

In contrast, absorption of α-Toc3 was significantly higher than α-Toc, whereas that of γ- and δ-Toc3 was comparable with α-Toc. This was indirectly confirmed in the different approach in which fecal excretion of these compounds was measured. Although these observations suggest specific regulatory functions of α-Toc3 *in vivo*, the concentration of α-Toc3 in the liver was markedly lower than α-Toc.

Although α-Toc has been shown to influence prostaglandin production (6-8), this effect appears to be modified by the experimental condition as indicated in two sets of studies (Exps. 4 and 5). In rats fed a cholesterol-enriched diet, Toc3 concentrate tended to reduce aortic production of PGI_2 and plasma concentration TXA_2 (4,5). The reduction of PGI_2 production by α-Toc and Toc3 concentrate was confirmed in SHR given these compounds at a 0.13% level together with a salt solution as drinking water, but not in those receiving a 0.2% level of α-Toc or Toc3 (0.2%) and no salt solution. Under the latter situation, aortic production of PGI_2 increased significantly by Toc3 concentrate. It is thus likely that the effect of Toc3 on eicosanoid production is readily modified by the experimental conditions employed, and in this context, Toc3 appear to be more effective than of α-Toc.

The effect of α-Toc and Toc3 concentrate on blood pressure and urinary excretion of ascorbic acid was apparently indistinguishable, and they were at least effective to prevent these parameters.

A series of present studies showed a slight but significant difference in the metabolic effect between α-Toc and Toc3, and it seems likely that α-Toc3 may have peculiar functions since it was absorbed more efficiently than α-Toc.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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CAROTENOIDS AND VITAMIN A: AN OVERVIEW

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Summary

Carotenoids are primarily symmetrical, C-40, polyisoprenoid structures with an extensive conjugated double bond system. Although widely distributed in nature, they are synthesized in photosynthetic microorganisms and plants, but not in animals. Hydrocarbon carotenoids are metabolized to a host of products, including retinal, B-apocarotenals, hydroxylated derivatives (the xanthophylls), and keto and epoxy compounds. Some of these derivatives, such as retinal, abscisin and trisporic acid, show pronounced biological activity. Carotenoids function as accessory pigments in photosynthesis, as protective agents against light-induced cell death in microorganisms, and, in the absence of the dietary intake of preformed vitamin A, as an essential dietary source of the vitamin in mammalian growth and development. Carotenoids also have a variety of interesting actions, which may be of therapeutic value but not necessarily of basic physiological significance. Among such actions are their stimulation of the immune response, their inhibition of mutagenesis and cell transformation, and their prevention of leukoplakia and micronuclei formation in the buccal mucosa. Finally, carotenoid intake has been associated with a lower risk of some types of cancer and of cardiovascular incidents. Because of the many effects of carotenoids on biological systems, distinguishing among functions, actions and associations is important.

¿Vitamin A is a major product of approximately 50 of the roughly 600 characterized natural carotenoids. Dietary vitamin A is derived from both animal and plant sources. Whereas carotenoids are absorbed intact only by a few species, e.g. humans, cows, and birds, vitamin A is efficiently absorbed by all. Vitamin A is not synthesized de novo from small molecules, but is formed only by cleavage of carotenoids.¿ It exists in various biologically active forms, such as retinol, retinyl esters, retinal, retinoic acid, and the β-glucuronides of retinol and of retinoic acid. Vitamin A and its various active forms are inactivated by hydroxylation, epoxidation and chain cleavage. In vivo the most active forms of vitamin A are bound to a set of specific retinoid-binding proteins, some of which serve as nuclear transcription factors (the retinoic acid receptors). Forms of vitamin A function in vision, cellular differentiation and embryological development. Of various retinoids, retinoic acid and several of its chemically synthesized analogs show the most profound effects on cellular differentiation and on embryological development. Vitamin A is required for growth and development, but large amounts cause toxicity. Thus the separation of efficacy from toxicity is a major pharmacological concern. The only naturally occurring compounds with a high efficacy/toxicity ratio are retinoyl β-glucuronide and retinyl β-glucuronide.

Thus, the carotenoids and retinoids are a diverse set of compounds with a fascinating spectrum of biological effects. Thus far, their precise modes of action in various physiological processes and therapeutic applications have only partially been elucidated.

Introduction

Carotenoids are widespread in nature, but particularly among organisms that are exposed to light. Because of their extensive conjugated double bond system, carotenoids absorb light in the 400-500 nm region, i.e. show a yellow to orange coloration. They have attracted the attention of biologists and chemists for two centuries. In early attempts to separate and to purify them, adsorption chromatography was developed. Thus, a major powerful method of analytical biochemistry, now available in manifold and sophisticated forms, was stimulated by the carotenoids. More than 600 carotenoids have thus far been characterized, and each of them has a variety of cis isomers. Some commonly occurring carotenoids are depicted in figure 1. Many treatises have been devoted to carotenoids, of which the most comprehensive is that edited by Isler (1971).

Approximately 50 carotenoids serve as precursors of vitamin A in mammals. Indeed, the conversion of carotenoids into vitamin A is of crucial significance in human nutrition, in as much as most of the vitamin A obtained from the diet in less industrialized countries is derived from carotenoids. The so-called "parent" compound of the vitamin A class is all-trans retinol (Fig. 2) . Retinol is converted to a limited number of biologically active compounds in vivo. In addition to naturally occurring compounds, more than 2000 analogs of vitamin A, and particularly of retinoic acid (Fig. 2), have been synthesized. Some of these synthetic analogs are much more active than vitamin A in physiological systems. This whole group of chemically related compounds is termed the "retinoids". A valuable 2-volume treatise on the retinoids is that of Sporn, Roberts, and Goodman (1984).

Carotenoids

Structure

All-trans β-carotene (fig. 1) is generally considered as the reference compound of its class. Many variations of this structure occur in nature, including those shown in fig. 1, longer (e.g. C-50) and shorter (e.g. the β-apocarotenals, abscisin and trisporic acid)

compounds, and conjugated derivatives (e.g. acylated xanthophylls). The biological activity of these compounds resides in its long conjugated double bond system, which confers the properties of light absorption, singlet oxygen quenching, and antioxidant action. But these same properties make carotenoids unstable in an oxidative environment. Hence, they can protect other molecules from oxidation, but in turn, must also be protected. Being nonpolar, carotenoids are usually associated with lipid components of cells. The chemistry of carotenoids and polyterpenoids has recently been reviewed (Britton, 1989).

Biosynthesis

Carotenoids, which are polyisoprenoids, are synthesized in nature from acetyl-coenzyme A via β-hydroxy β-methyl glutaryl-coenzyme A, mevalonic acid, and geranyl pyrophosphate. Harrison (1990) has recently reviewed these pathways. Carotenoid formation is induced by light and inhibited by a variety of natural and synthetic compounds. Much attention is now being given to the cloning of carotenoid gene clusters and to elucidating the mechanisms of their control (Armstrong et al., 1990; Misawa et al., 1990).

Metabolism

Because of its nutritional importance, the cleavage of β-carotene into vitamin A has been extensively studied (Olson, 1988, 1989). β-Carotene is oxidatively cleaved at the 15,15' central double bond to yield two molecules of retinal by a cytosolic enzyme found in the intestinal mucosa, the liver, and other tissues. In plants and in some microorganisms, carotenoids are excentrically cleaved to give β-apocarotenals, β-ionone, and other products. In mammals, excentric cleavage has only recently been shown (Wang et al., 1991). The relative importance of central and excentric cleavage in the conversion of carotenoids into vitamin A in humans is not yet clear. The metabolism of other carotenoids in mammals is not well defined. Xanthophylls may be biologically acylated or may form sugar conjugates, however, and a variety of epoxides and other oxidation products may result from interactions with various oxidants in vivo and in vitro.

In a more physiological context, the intestinal absorption of dietary carotenoids depends on several factors; namely, its bioavailability from foods, its structure, the concomitant presence of fats and oils in the diet, and the total amount ingested. For example, α- and β-carotene from raw, sliced carrots are very poorly absorbed (5-10%). Upon pureeing and then cooking, however, its absorption increases to 40-50% in the presence of dietary fat. Carotenoids in oil are much better absorbed than those bound in other foods. As the intake of carotenoids increases, the absorption efficiency declines. Thus, at a dose of 0.1 mmole (54 mg) of β-carotene in humans, only 10-20% is absorbed.

After absorption, carotenoids are transported in chylomicra into the general circulation, and then, in the steady state, in lipoproteins of the plasma. The pattern of carotenoids in human plasma generally reflects that in the diet. Major plasma carotenoids are lutein, lycopene, cryptoxanthin, β-carotene and α-carotene (Parker, 1989). The patterns of carotenoids in plasma and in adipose tissue are closely related (Parker, 1989). Of various human organs, adipose tissue contains by far the most carotenoid, followed by liver. The highest concentration of carotenoids, however, is found in the corpus luteum. Thus, specificity exists in the way that carotenoids are distributed among tissues, although little is currently known of the mechanisms involved.

The absorption, metabolism, and plasma clearance of carotenoids in humans is very dependent on their structure. Thus ethyl β-apo-8'-carotenoate is absorbed very well, is not detectably metabolized, and shows slow plasma clearance, whereas β-apo-8'carotenal is absorbed less well, is rapidly metabolized, and its products are quickly removed from the circulation (Zeng et al., 1991). Thus, generalizations about carotenoid metabolism are constrained by our increasing knowledge about the specificity of many of these processes.

Functions

In plants and photosynthetic organisms, carotenoids serve as accessory pigments to absorb light of wavelengths not absorbed by the chlorophylls. Mutant photosynthetic organisms that lack carotenoids, when exposed to light, are killed, presumably by light-induced oxidative stress on chlorophylls and other essential molecules in cells

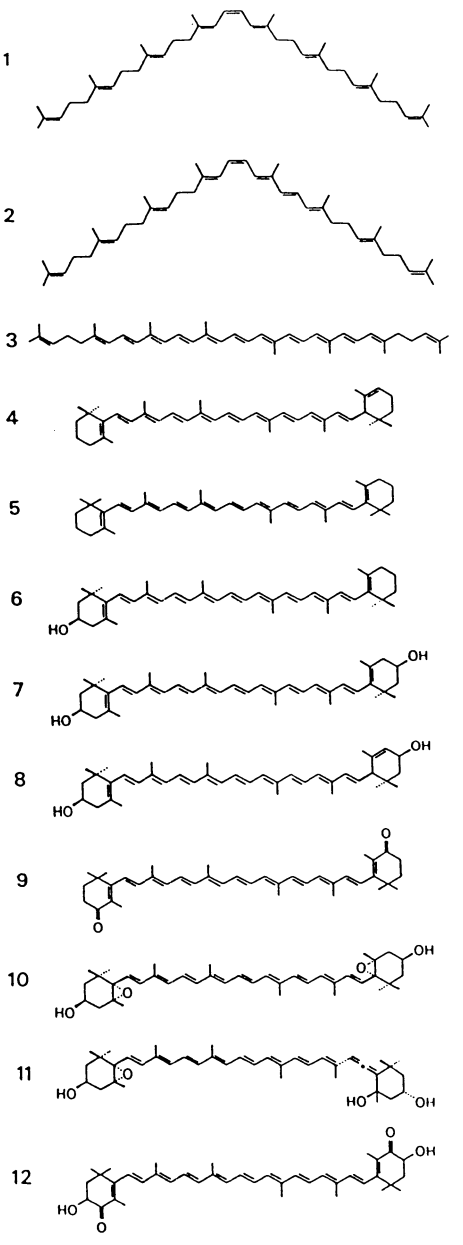

Figure 1. Polyenes and carotenoids in foods that may also be found in animal tissues. 1, phytoene; 2, phytofluene; 3, lycopene; 4, α-carotene; 5, β-carotene; 6, β-cryptoxanthin; 7, zeaxanthin; 8, lutein; 9, canthaxanthin; 10, violaxanthin; 11, neoxanthin; 12, astaxanthin. Reprinted with permission of the FASEB Journal (Bendich and Olson, 1989).

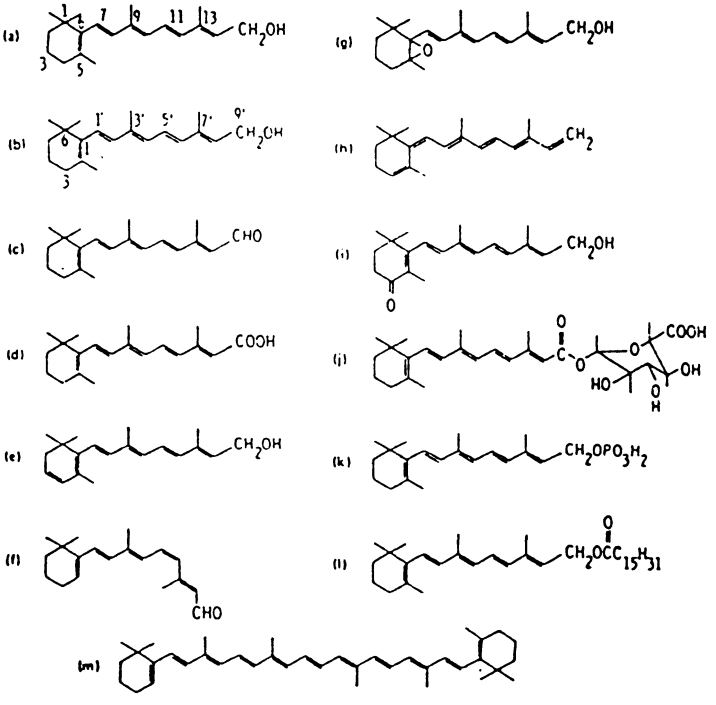

Figure 2. Formulas for retinol and its derivatives. a,b) all-trans retinol; c) all-trans retinal; d) all-trans retinoic acid; e) 3,4 didehydroretinol, f) 11-cis retinal; g) 5,6 epoxyretinol; h) anhydroretinol; i) 4 oxoretinol; j) retinoyl β-glucuronide; k) retinyl phosphate; l) retinyl palmitate; m) all-trans β-carotene. Reprinted with permission of Marcel Dekker, Inc. (Olson, 1990).

(Krinsky, 1989). Thus, light harvesting and photoprotection are two clear functions in plants and microorganisms. Phototropism, which may involve carotenoids as well as other light-absorbing molecules, such as flavins, also has survival value. Similarly, the plumage of birds and coloration of insects protect them in part from predators.

In mammals, carotenoids are only known to function as precursors of vitamin A. In the absence of dietary vitamin A, therefore, they are essential nutrients. Other effects of carotenoids might well be considered actions until more is known of their molecular mechanisms (Bendich and Olson, 1989).

Actions

Carotenoids serve as antioxidants at low Po_2, quench singlet oxygen, enhance the immune response, reduce photo-induced neoplasm, inhibit mutagenesis, reduce tumor development in vivo, cause tumor regression in vivo, lower cell transformation in vitro, prevent sister chromatid exchange, and reduce so-called "precancerous" changes, such as leukoplakia and micronuclei prevalence, in buccal epithelia (Bendich and Olson, 1989). Carotenoids also reduce lipid peroxidation in low density lipoproteins (Esterbauer et al., 1989), an observation of possible significance in the genesis of atheromas. Other effects might be added to this list. In most cases, carotenoids, both with and without provitamin A activity, are effective, whereas vitamin A itself usually is not. Thus, the cited actions seem due to the intact carotenoids and not to their conversion into vitamin A.

In some cases, the implications of these observations are clear. If individuals, who suffer from leukoplakia or cervical dysplasia, are benefitted by receiving large oral daily doses of β-carotene, such treatment is certainly a viable option. The chronic administration of β-carotene in large doses, although possibly causing the benign condition carotenosis, is not known to cause toxic manifestations. Similarly, the treatment of erythropoietic protoporphyria with β-carotene is known to reduce light-induced adverse reactions (Mathews-Roth, 1990). In other cases, however, the physiological implications of an observation are more difficult to interpret. For example, carotenoids clearly enhance the immune response in vitamin-A sufficient experimental animals under specified conditions (Bendich, 1989). That process may be important in protecting against

infection. On the other hand, the enhancement may be very small, or indeed not detectable, under normal dietary conditions. An enhanced immune response might be beneficial in reducing the severity of infection, or might be harmful in increasing autoimmune reactions. Thus, many actions of carotenoids are difficult to interpret physiologically.

Associations

In epidemiological studies relating dietary patterns with chronic diseases, and particularly with cancer, vitamin A and its precursor carotenoids were early identified as showing a protective effect, i.e. the higher the intake, the lower the risk of cancer (Ziegler, 1989, 1991). Thereafter, it became clear that carotenoid intake, rather than that of preformed vitamin A, was primarily, if not solely, responsible for the relationship. of various cancers investigated, the risk of lung cancer showed the best inverse relationship with carotenoid intake (Ziegler, 1989, 1991). Carotenoids other than β-carotene were often better correlated with reduced risk than β-carotene itself (Colditz et al., 1985). Most, but not all, studies showed a significant relationship between carotenoid intake and reduced risk of lung cancer (Ziegler, 1989, 1991).

Epidemiological studies can be confounded by uncontrolled variables, of which the most important for lung cancer is smoking. Most, but not all, such studies have been corrected for smoking habits. other nutrients and food components, such as fiber, vitamin C, and vitamin E, are also associated with foods rich in carotenoids. The possibility exists, therefore, that these other components, or a mixture of them with carotenoids, are serving as protective agents. Diets rich in vegetables and fruits also tend to be lower in animal meats, meat products, and saturated fat. Finally, fresh vegetables and fruits, which are more costly than many kinds of carotenoid free foods, are more commonly ingested by persons with a knowledgeable appreciation of the relationship of diet, exercise and life-style to health. Thoughtful epidemiologists are well aware of these factors (Bendich and Olson, 1989; Ziegler, 1989, 1991). Thus, caution must be exercised in interpreting such associations in a causal manner.

Intervention trials, i.e. providing supplements of given carotenoids to one group of subjects and a placebo to another matched group, provide more direct evidence of efficacy. In some cases, but not in all, carotenoids have shown chemopreventive actions against cancer (Santamaria and Bianchi, 1989).

In a mechanistic sense, carotenoids probably act as antioxidants and/or as singlet oxygen quenching agents, in all likelihood in consort with other cellular antioxidants. The relative contribution of carotenoids to the physiological protection system against adverse oxidative and free radical reactions within cells, however, has not yet been clarified.

Vitamin A

Structure

The carbon atoms in all-trans retinol (Fig. 2a,b) are conventionally numbered as shown in Fig la, although chemists often use the numbering shown in Fig. lb. Retinal (Fig. lc), retinoic acid (Fig. ld) and 3,4 didehydroretinol (fig. le) are naturally occurring analogs. The biologically active form in vision is 11-cis retinal (fig. lf). Physiologically inactive forms are 5,6-epoxy retinol (fig. lg) , anhydroretinol (fig. lh) , and 4-oxoretinol (fig. li) , although the 4-oxo derivative can be teratogenic in high doses. The β-glucuronides of retinoic acid (fig. ij) and of retinol are biologically active but show relatively little toxicity. Retinyl phosphate (fig. ik) may be formed in small amounts in cells, whereas retinyl palmitate (fig. 11) and other long chain fatty acyl esters are major storage forms. Because of its conjugated double bond system, retinol and its derivatives absorb maximally at 325 nm (in hexane), whereas retinoic acid and 3,4 didehydroretinol absorb at 350 nm (in ethanol). Uncharged vitamin A derivatives are all highly lipophilic, retinoic acid and retinyl phosphate are somewhat more polar, and the retinoid glucuronides are water-soluble. All are rapidly oxidized in the presence of oxygen, transition metals, and light. Thus, they require protection by antioxidants, of which BHT is one of the more effective. In vivo retinoids are primarily protected by specific associations with binding proteins.

Biosynthesis

Vitamin A is a required nutrient for vertebrates. It can be provided in the diet in the form of preformed vitamin A or of precursor carotenoids. As already mentioned, carotenoids can be cleaved both centrally and excentrically ultimately yielding Vitamin A. Thus, the only known pathway for the biosynthesis of vitamin A is via the carotenoids. Needless to say, however, elegant chemical procedures have been devised for the synthesis of vitamin A and its analogs.

Metabolism

Preformed vitamin A in foods is largely present as retinyl esters. These are hydrolyzed in the intestine to retinol, which is absorbed as a part of the mixed micelles derived from lipid digestion. Carotenoids are also absorbed in micellar form, albeit less efficiently than vitamin A. Retinal ultimately derived from carotenoid cleavage can either be oxidized to retinoic acid or reduced to retinol. Carotenoids that are excentrically cleaved may yield retinoic acid without passing through retinal.

Retinol of whatever origin is largely esterified in the intestinal mucosa and incorporated into chylomicra, which are transferred via the lymph to the blood. Chylomicron remnants are taken up by liver parenchymal cells, whereupon retinyl esters are hydrolyzed to retinol, transferred in large part to stellate cells, re-esterified, and stored in lipid globules. Upon demand, retinyl esters of stellate cells are hydrolyzed to retinol and released from the liver as a 1:1 complex with retinol binding protein. The dynamics of these events have been summarized (Olson, 1990; Blomhoff and Wake, 1991).

Retinol is reversibly oxidized to retinal, which in turn is irreversibly oxidized to retinoic acid. Retinoic acid, as well as retinol, can form water-soluble conjugates with glucuronic acid, can be oxidized via the 4-hydroxy derivative to the 4-oxometabolite, can be epoxidized at the 5:6 position, and can be oxidatively cleaved to chain shortened products. Most oxidized products of retinoic acid are biologically inactive (Olson, 1990).

Most retinoids bind to specific binding proteins in tissues; retinol to plasma (RBP) and cellular retinal-binding proteins (CRBP) , retinal to retinaldehyde binding protein

(CRALBP) and to opsin of the eye, and retinoic acid to cellular retinoic acid binding proteins (CRABP) of the cytosol and the nuclei. The latter, termed retinoic acid receptors (RAR), play roles in gene expression (Sporn et al., 1984; Darmon, 1990).

Functions

The best defined function of vitamin A is in vision. The visual pigment, rhodopsin, is formed in the rod cell by the binding of 11-cis retinal to opsin. Light induces the isomerization of the 11-cis to the all trans form, which destabilizes the conformation of the protein. Through a sequence of steps involving transducin, a specific G protein, and the ultimate hydrolysis of cGMP, the sodium pore in the membrane is closed. Thus a light impulse is transduced via amplifying chemical reactions into a membrane potential (Stryer, 1988; Olson, 1990).

Vitamin A is also involved in cellular differentiation (Sporn et al., 1984; Sherman, 1986; Sporn and Roberts, 1991). In vitamin A deficiency, mucus secreting cells of epithelia tend to disappear, and keratin producing cells take their place. A large variety of cells are stimulated to differentiate in vitro by retinoids, of which retinoic acid is one of the most active. Retinoic acid, having been transported into the nucleus by CRABP, interacts with RAR, which exists in three distinct forms: α, β and γ (Darmon, 1990). The complex, possibly as a dimer, interacts with the upstream response elements of genes to turn their expression on or off. For example, retinoic acid directly induces the formation of one of its nuclear receptors, RAR_{β}. During the process of differentiation of various cell types, of course, a large number of new proteins are produced. Thus, a major current challenge is to define the sequence of these changes. Furthermore, some retinoids, like retinoyl β-glucuronide, stimulate differentiation without binding to cytosolic or nuclear receptors (B. P. Sani, R. G. Mehta, A. B. Barua, and J. A. Olson, unpublished observations). The relationship of various retinoids to differentiation has been reviewed (Sporn et al., 1984; Sherman, 1986). Vitamin A-dependent processes, such as the immune response, in all likelihood are expressions of the role of vitamin A in cellular differentiation (West et al., 1991).

Vitamin A functions as well in embryological development. Both vitamin A deficiency and vitamin A excess are characterized by abortions, resorptions, and malformed fetuses. Vitamin A may well serve as a morphogen in development, either by stimulating the differentiation of clusters of special cells, such as the zone of polarizing activity (ZPA), or by serving as a gradient across one or more axes of the developing embryo (Summerbell, 1991). Of various retinoids tested, retinoic acid and some of its synthetic aromatic analogs are the most active. 3,4 Didehydroretinoic acid, which is formed from retinoic acid in the chick limb bud, may play a similar role (Thaller and Eichele, 1990). The mechanism by which retinoids play such roles in development is not well defined, although homeobox genes may be involved (Summerbell, 1991).

The growth-promoting function of vitamin A is probably a complex set of interactions among vitamin A and various hormones as well as an expression of programmed cellular differentiation.

Actions

Vitamin A and most retinoids are highly toxic when administered in large doses (Olson, 1990; Hathcock et al., 1990). Three types of toxicity exist: acute, caused by single huge oral doses; chronic, caused by repeated ingestion of large oral doses over time; and teratogenic, caused by large oral doses ingested early in pregnancy. Retinoic acid is more toxic than retinol, and the all-trans form is more toxic than the cis isomers. Interesting exceptions are retinoyl β-glucuronide and hydroxyphenylretinamide, which show biological activity with much less, if any, toxicity.

Retinoids have been used effectively in treating certain skin disorders, such as acne and psoriasis, as well as some types of recurrent cancer, such as that of the skin and breast (Meyskens and Prasad, 1986; Orfanos et al., 1987). Specific retinoids are preferentially used for a given clinical condition. Because large oral doses are most effective, a balance must be achieved in dosing between efficacy and adverse side effects. Interestingly, in the topical treatment of moderate acne, retinoyl β-glucuronide showed efficacy similar to that of all-trans retinoic acid without causing toxic manifestations (D.

B. Gunning, A. B. Barua, R. A. Lloyd, and J.A. Olson, unpublished observations). Retinoic acid has also been used to prevent and to repair photoaging of the skin (Kliqman, 1989). Vitamin A also influences wound healing and bone remodelling. Thus, a variety of actions, which may be either beneficial or noxious, are caused by retinoids.

Associations

Supplementation of preschool children in Indonesia with semi-annual large doses of vitamin A reduces mortality by approximately 30% (Sommer, 1989). Such children, although not showing clinical signs of vitamin A deficiency, grew faster, were less anemic, and died less frequently when supplemented with vitamin A than did their nonsupplemented peers (Sommer, 1989). When the vitamin A status of preschool children from the same region was determined by a new sensitive method, the modified relative dose response (MRDR), 60% of them were shown to be in a marginal status (Tanumihardjo et al., 1990). Similar effects of vitamin A supplementation on mortality were observed in some other third world countries but not in others. The most logical explanation of this effect is that a marginal vitamin A status adversely affects the immune system, thereby leading to a higher incidence of morbidity and ultimately of death. In several of these studies, however, morbidity was unaffected by vitamin A supplementation. Quantifying the nature and severity of morbidity, which are crucial considerations when assessing life-threatening situations, is difficult in field surveys. In various societies, where nutritional and environmental stresses differ, careful analysis of the overall situation may be an essential. step in predicting whether or not vitamin A supplementation will have a significant public health effect.

In initial epidemiological studies on the relation of nutrition to cancer, a dietary vitamin A index was calculated for each subject (Ziegler 1989, 1991). Only later, as already indicated, did it become clear that carotenoids , but not preformed vitamin A, in the diet were significantly associated with a lower risk of some forms of cancer. Thus far, supplementation with vitamin A does not seem to have a protective effect against cancer in well nourished subjects exposed to high risk environments. Similarly, plasma

retinol concentrations, although often reduced in cancer patients subjects to various forms of therapy, do not serve as useful predictors of cancer risk in carefully conducted prospective studies (Ziegler, 1989, 1991).

Conclusion

In an historical and nutritional sense, carotenoids have been inherently linked to vitamin A as its dietary precursors. They are now viewed, and correctly so, quite separately. This development has largely been due to the following considerations: 1) the close association of dietary carotenoids, but not of dietary preformed vitamin A, with a reduced risk of some types of cancer and possibly of atherosclerosis, 2) the realization by physicians and many biologists that the physicochemical properties of carotenoids are very different from those of vitamin A, facts well known to chemists working with these molecules, and 3) the demonstration that the nutritional benefits of vitamin A are only a small part of the biological spectrum of vitamin A activities, a realization enhanced by the synthesis and testing of a large number of retinoids. In contrast, carotenoids do not share, insofar as we are aware, in these other biological activities of retinoids. Thus, the two fields of carotenoids and vitamin A are diverging rather than converging, albeit linked by the important nutritional tie between them.

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METABOLISM OF CAROTENOIDS BY ENZYMES OF OXYGEN METABOLISM

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Summary

Carotenoids are metabolized by lipoxygenases and other oxygenases in bacteria, plants and animals; however, the mechanisms of these reactions are poorly understood. We have studied the oxidation of β-carotene by soybean lipoxygenase as a model system for understanding these reactions in vivo. β-carotene inhibits the rate of peroxidation of linoleic acid and the production of linoleic hydroperoxide product in a concentration-dependent fashion. In the presence of soybean lipoxygenase, carotenoid absorbance at 452 nm is rapidly diminished (bleached), however, we have not yet been able to identify the carotenoid metabolites. Although autoxidation products were identified, no enzyme-dependent carotenoid metabolite absorbing over the range of 300 to 600 nm was eluted from reverse phase HPLC columns. Experiments are in progress to identify the enzymatic metabolites. Hydroperoxide products of lipoxygenases are precursors to leukotrienes and other cytokines, the production of which are associated with a number of inflammatory and allergic states. As β-carotene is associated with enhancement of the immune response, the inhibition of lipoxygenase products in vivo may represent an important mechanism by which carotenoids serve in immunoregulation.

Introduction

Carotenoids are associated with protection against coronary heart disease, cataract and cancer (1-4,5-8). In a preliminary report, dietary carotenoids were inversely related to heart disease and stroke (9) and plasma β-carotene concentrations are inversely related

to risk of angina (10). It has been proposed that β-carotene, in combination with other antioxidants protects against lipoprotein oxidation (10-13) and thus potentially plays an important role in retarding the progression of atherosclerosis. It is of interest that smoking, which is correlated with lowered concentrations of plasma carotene (1) may result in LDL modification (14). The evidence implicating carotenoids in cancer prevention and enhancement of immune function has recently been reviewed (15).

The mechanisms by which carotenoids might exert these effects are not understood and likely involve a number of reactions. Possible mechanisms by which carotenoids may function include antioxidant action, singly or in combination with other antioxidants, metabolism to retinoids and/or carotenoids, and modulation of enzyme activity. By any one or a combination of these mechanisms, carotenoids may function in the regulation of biological pathways. The relationship of carotene function to its metabolism by enzymes of oxygen metabolism is discussed below.

Carotenoids are widely distributed in nature. Over 600 have now been identified and characterized. A few of the more biologically important carotenoids are shown in Fig 1. Due to their highly conjugated bond system, carotenoids are among the most efficient quenchers of singlet oxygen and have been extensively studied in bacteria and plants (16,17). By quenching UV reactions of singlet oxygen induced by UV light, carotenoids also protect against erythropoietic protoporphyria and other diseases of light sensitivity (18). However, except for these reactions, although singlet oxygen is produced in high yield by the reaction of ozone with biological molecules in vitro (19), a significant role for singlet oxygen in vivo has not yet been demonstrated.

Possibly of great importance in biological reactions are reactions of carotenoids with peroxyl radicals (20,21). A number of epoxide products of these reactions have now been characterized, including the 5,6 epoxy β,β carotene and the 15,15' epoxy β,β carotene (22) (Fig 2). These products appear to be formed by radical addition to the carotenoid molecule in contrast with the electron or hydrogen transfer reactions of other cellular antioxidants such as vitamin E. A possible mechanism has been presented (Fig 3). In addition, a variety of apocarotenals have been identified as the product of radical-initiated autoxidation (21).

A number of carotenoid metabolites have been identified in fish, including astaxanthin, canthaxanthin and echinenone (23). In addition, in some species, the reactions from β-carotene to canthaxanthin are fully reversible. Possible pathways for these interconversions are shown in Fig 4. Mechanisms for these reactions have not been proposed.

In 1954, Glover and Redfern proposed a mechanism for carotene metabolism based on the well-known pathways of oxidative metabolism of fatty acids (Fig 5) and the observed products in plants and murine species (24). It now appears that carotenoids are metabolized by dioxygenases which are ubiquitous in animals, plants and bacteria. However, enzymatic reactions of carotenoids have been studied in detail in only two enzymes; carotene dioxygenase and soybean lipoxygenase.

Carotene Dioxygenase (EC 1.13.11.21)

Metabolism to Retinal

Goodman and Olson first reported the metabolism of β-carotene to retinal in intestinal mucosa of a variety of species (25,26). As retinal was the only product detected, it was proposed that β-carotene was specifically cleaved at the central bond and the enzyme was therefore named 15,15' carotene dioxygenase (Fig 5). The partially purified enzyme from rat intestinal mucosa requires molecular oxygen and is stimulated by thiols and chelators indicating a sulfhydryl binding site and a role for metals (25-27). In early experiments, ratios of retinal formed to β-carotene consumed of 1 to 2 were reported (28-30). Studies of the substrate specificity of carotene dioxygenase have produced conflicting results. β-Apo-10' carotenal has been reported to be either 10x more effective a substrate for the dioxygenase than β-carotene (28) or only about 1/2 as effective (29). It has generally been agreed that canthaxanthin is inert to the dioxygense; it is typically used to assay non-retinoid effects of carotenoids. However, recent data obtained in chick intestine with a sensitive HPLC assay indicate that canthanxanthin may in fact be a substrate for the enzyme (31). Alternatively, it is possible that canthaxanthin

is metabolized to β-carotene in birds similar to findings in murine species for the interconversion of canthaxanthin and β-carotene (23). Carotene cleavage activity is widely distributed throughout the vertebrates, with the highest activities in ferret, guinea pig and rabbit. The major site of action of carotene metabolism is intestinal mucosa (28,29) although activity has been reported in rat liver, lung and kidney (32-34). Carotene metabolism is apparently sensitive to dietary manipulation. Increasing dietary protein increases conversion of β-carotene to retinol in rats and humans (35,36) apparently by increasing enzyme synthesis. In addition, retinol status may influence carotene metabolism. Increasing dietary retinol (~ 10X) produces a corresponding 3-fold inhibition in rat intestinal dioxygenase activities (37), and addition of β-carotene (100 mg/kg) to the diet of rabbits drastically increased (150 fold) the amount of vitamin A in liver (38). As retinol does not accumulate in serum in response to carotene administration in normal individuals, when retinol is sufficient, carotene is apparently transported intact to the liver, metabolized, and stored as retinol. Conversely, in vitamin A low-to-deficient children, administration of 1.2 mg/day of β-carotene resulted in 1.5 to 2 fold increases in serum retinol levels (39). Additionally, as prolonged administration of excess carotene does not result in retinol toxicity (18), apparently there is hepatic control of metabolism of carotene to retinol.

Recently, production of apocarotenoids and retinoic acid (34) as well as retinal from carotenoids in rat and ferret intestine has been reported (33). Most workers have assumed that only one enzyme exists for carotene cleavage, the putative 15,15' dioxygenase, although workers are not in agreement as to whether the enzyme is cleaved at single or multiple sites. However, the enzyme has not been purified, thus the possibility that more than one enzyme exists cannot be ruled out at present. The observation that β-carotene is less effective biologically than retinol has been taken to imply an asymmetric mechanism; however, differences in effectiveness of absorption cannot be ruled out. In all discussion of this enzyme activity, the astonishingly small conversion of substrate to product must be considered; i.e., 10E-4 % for retinoic acid (34) to 3%. for apocarotenals (including retinal, 33).

Lipoxygenases

Lipoxygenases catalyze the formation of allylic hydroperoxides of polyunsaturated fatty acids and require a catalytic amount of peroxide for initiation of the reaction. They are ubiquitous in plants and animals. The soybean enzyme has been extensively studied and has been successfully employed as a model enzyme for in vivo lipoxygenases (40). In addition, cooxidation reactions of carotene and linoleic acid by soybean lipoxygenases have been known for over 50 years (40-43), although neither the products nor the mechanism has been systemically studied previous to this report. Thus we investigated the reaction of carotenoids with lipoxygenases using soybean lipoxygenase as a model.

Results and Discussion

As shown in Fig 6, β-carotene inhibits the rate of peroxide formation (A234) and this inhibition is proportional to the loss of peroxide product (Fig 7), presumably 13 hydroperoxy, 9, 11-octadecadienoic acid (64). At 35 μM β-carotene and linoleic acid the absorbance of β-carotene at 452 nm is significantly diminished (bleached) by 5 minutes, as has previously been noted by others (40-43, Fig 8) Although some diminution in the 452 signal is observed in the presence of linoleic acid, the diminution is greatly enhanced by the enzyme.

We detect two major autoxidation products of β-carotene in aqueous buffer; an unidentified polar fraction, possibly aldehydes or apocarotenals (20) and a more hydrophobic fraction which migrates in the region containing the epoxide products identified by reaction of carotene with peroxyl radical (20,21). However, at no concentration of β-carotene over the range 20 to 300 μM have we been able to detect enzyme-dependent carotenoid product formation over the range 300 to 600 Nm (Fig 5). These same products have recently been reported by others (20,21). The enzymatic oxidation products could be polar products which are not retained on our HPLC column, compounds which have absorption maxima below 300 Nm or adducts that do not enter the

column. It has been proposed previously that enzymatically produced peroxyl radicals oxidize carotene directly, resulting in carotene bleaching. others have proposed (40) that cooxidation occurs concurrently with peroxide formation but by a separate, as yet unidentified, mechanism. Indeed, a carotenoid bleaching reaction in photosensitized liposomes has been observed which was not explained by reaction with peroxyl radical. At present, insufficient data exist to differentiate between the two mechanisms.

Our data suggest that carotenoids may function by any one or combination of three mechanisms: (a) by competing for fatty acid substrate, (b) by inhibition of enzyme activity by oxidation products, or (c) by scavenging catalytic peroxide needed to activate the enzyme (Fig 6). Experiments are in progress in our laboratory to differentiate among these possibilities.

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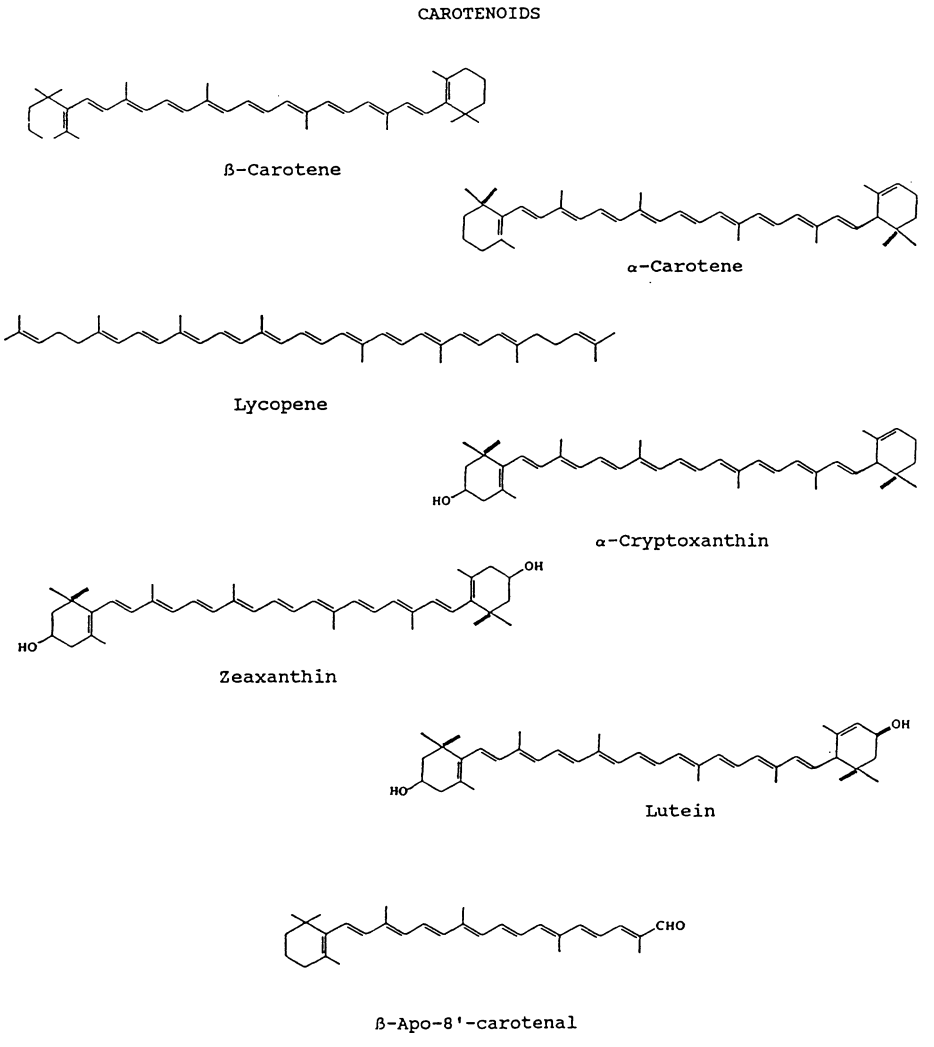

Fig. 1. Biologically important carotenoids

Products of Chemical Reactions of Organic Peroxides and β-Carotene

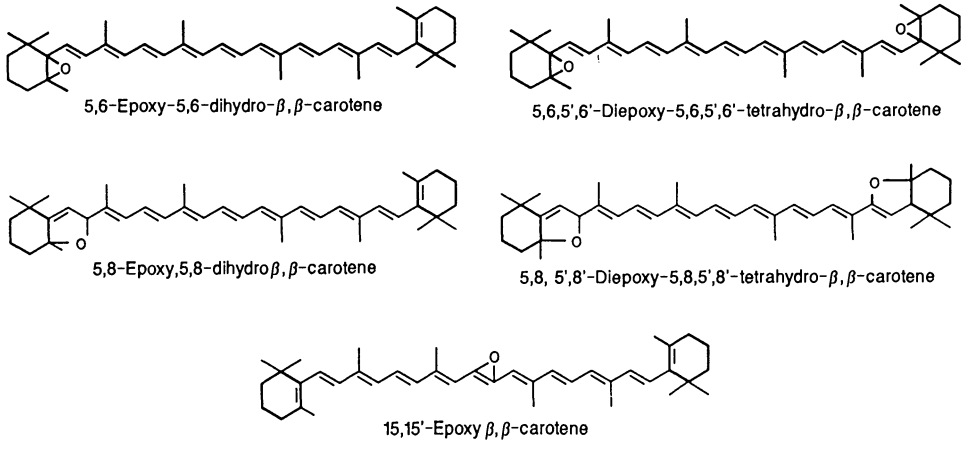

Fig. 2. Products of chemical reactions of organic peroxides and β-carotene [Kennedy, T.A. and Lublin, D.C., 1991, Chem. Res. Toxicol., 4, 290-295, Teroa, J, 1989, Lipids, 24. 659-661.]

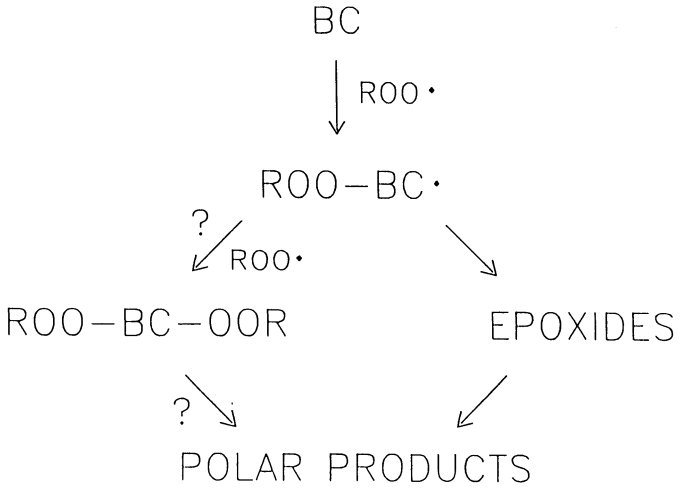

Fig. 3. Proposed mechanism for reactions of carotenoids with peroxyl radicals. [Kennedy T.A. and Liebler D.C.,(1991) Chem. Res. Toxicol., 4, 290-95]

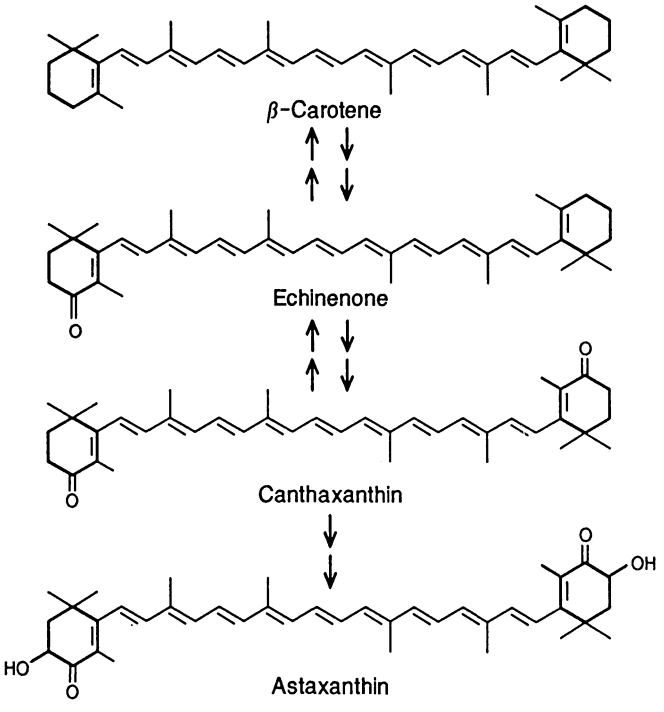

Fig. 4. Proposed Carotenoid Metabolism in fish. [Goodwin, T.W. Ann. Ren. Nutr., 1986. 6, 276-97.]

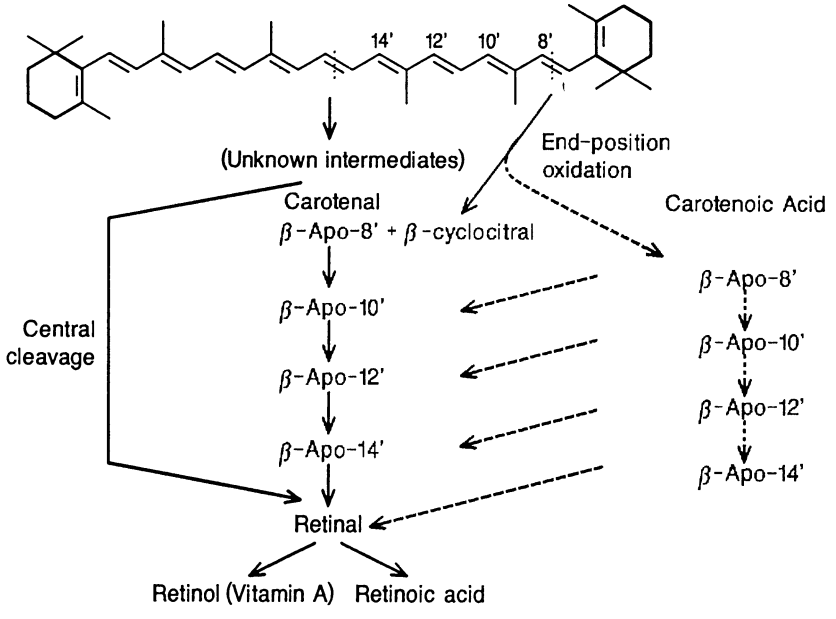

Fig. 5. Metabolism of β-carotene: Glover-Redfearn Hypothesis. [Thommen, H., in Carotenoids, Isler, O., ed., Birkhauser Verlag, Basel, pp 639, 1971]

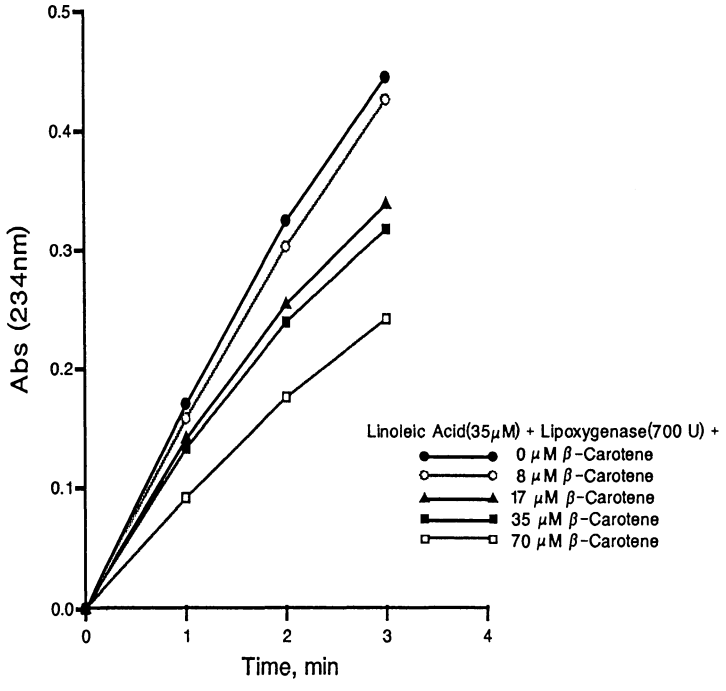

Fig. 6. Effect of β-carotene on peroxide formation.

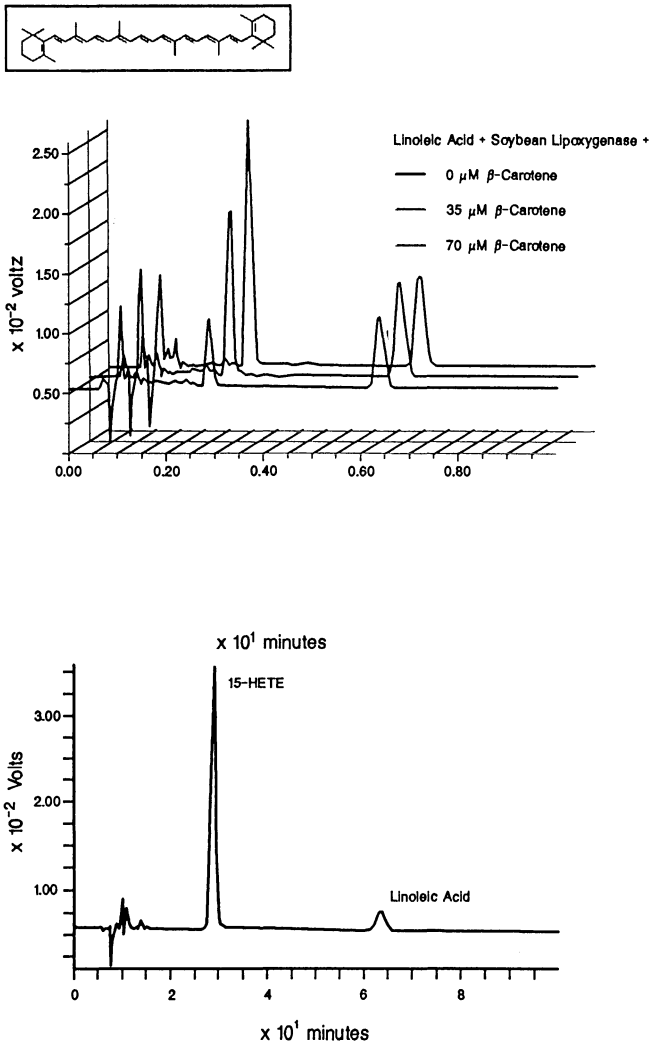
Linoleic Acid + Soybean Lipoxygenase +
0 μM β-Carotene
35 μM β-Carotene
70 μM β-Carotene
x 10⁻² voltz
2.50
2.00
1.50
1.00
0.50
0.00
0.20
0.40
0.60
0.80
x 10¹ minutes
15-HETE
Linoleic Acid
x 10⁻² Volts
3.00
2.00
1.00
0
2
4
6
8
x 10¹ minutes

Fig 7.

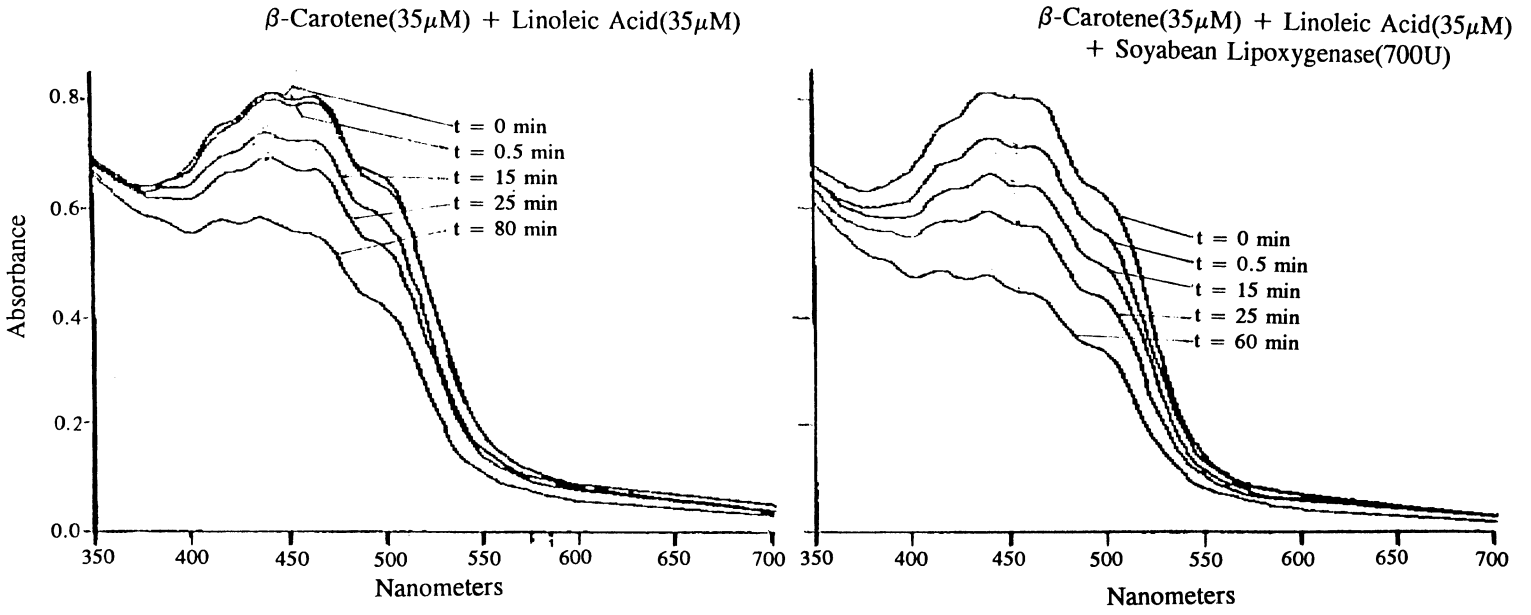
β-Carotene(35μM) + Linoleic Acid(35μM)
Absorbance
0.8
0.6
0.4
0.2
0.0
t = 0 min
t = 0.5 min
t = 15 min
t = 25 min
t = 80 min
350
400
450
500
550
600
650
700
Nanometers
β-Carotene(35μM) + Linoleic Acid(35μM)
+ Soyabean Lipoxygenase(700U)
t = 0 min
t = 0.5 min
t = 15 min
t = 25 min
t = 60 min
350
400
450
500
550
600
650
700
Nanometers

Figure 8

Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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ROLE OF β-CAROTENE IN DISEASE PREVENTION WITH SPECIAL REFERENCE TO CANCER

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Summary

β-carotene occurs widely in nature, particularly in green and yellow vegetables and fruits. In addition to being an important and safe source of vitamin A, it is a potent quencher of singlet oxygen and an antioxidant which can scavenge free radicals. There is overwhelming epidemiological evidence that consumption of foods rich in β-carotene can reduce the risk of certain cancers. Evidence for lung cancer is particularly strong. Data on cancers of the stomach, esophagus and cervix are limited but consistent. In three studies, use of a β-carotene supplement singly or in combination with vitamin A or antioxidant vitamins C and E caused regression of oral leukoplakia in a significant proportion of the study population. β-carotene is an immunoenhancer. Preliminary findings from a double-blind, placebo-controlled clinical intervention trial and an epidemiological study support the role of β-carotene in reducing the risk of cardiovascular disease. In one study, high intake of foods rich in carotenoids and a high level of carotenoids in blood were found to be associated with reduced risk of senile cataract. Certain findings suggest that the protective role of β-carotene against cancer and perhaps other degenerative diseases is independent of its provitamin A role. β-carotene is safe even when consumed in large quantities. In the U.S. population, dietary intake of β-carotene is very low as compared to that likely to be achieved if dietary guidelines for healthy living are followed. Dietary modifications or use of food fortified with β-carotene, or use of β-carotene supplements may be necessary to meet the need for optimal β-carotene intake.

Introduction

The yellow, orange and red colors of fruits and vegetables are due to carotenoid pigments. These pigments also occur in all photosynthetic organisms, in some non-photosynthetic bacteria and in certain fungi. Many dark green leafy vegetables contain high levels of carotenoids, but their color is masked by chlorophyll (Bramley and Mackenzie, 1988). Humans and animals are incapable of synthesizing carotenoids (Goodwin, 1984). The beautiful colors of lobsters, flamingoes and salmon are due to carotenoids in the animals' diets. More than 600 carotenoids have been identified in nature (Straub, 1987). About 60 of these, including certain apocarotenoids, can potentially yield vitamin A activity (Simpson and Tsou, 1986). However, only 5 or 6 of the provitamin A carotenoids occur in significant amounts in commonly-consumed foods. β-carotene is the most abundant and most efficient provitamin A in foods.

β-carotene has been traditionally recognized for its role as a precursor of vitamin A. However, the last decade has witnessed rapid accumulation of evidence suggesting that β-carotene is more than a safe source of vitamin A (Gaby and Singh, 1991). A large number of epidemiological, animal, and in vitro, as well as a limited number of clinical intervention (precancerous lesions) studies have yielded findings supporting the protective role of β-carotene against certain types of cancers. Evidence is also beginning to emerge suggesting roles of β-carotene in protecting against cardiovascular disease (Gaziano et al., 1990; Street et al., 1991) and of carotenoids in reducing the risk of cataract (Jacques and Chylack, 1991). Some findings suggest that the role of β-carotene in reducing the risks of degenerative diseases such as cancer and cardiovascular disease may be independent of its provitamin A role.

Absorption and Transport of β-Carotene

β-carotene is very poorly (10-50%) absorbed (Erdman, 1988). In the mucosal cells of the small intestine, it is partly absorbed intact and partly converted to retinol

(vitamin A) by a highly regulated process. An individual's vitamin A status and intake level are two important factors which determine the proportion of the absorbed β-carotene that is converted to vitamin A. Thus, consumption of even extremely large doses (up to 180 mg daily) for long periods does not cause vitamin A toxicity (Mathews-Roth, 1981, 1989). Like preformed retinol, the retinol derived from β-carotene is esterified (mostly to retinyl palmitate) and incorporated mainly into chylomicrons and transported to the liver via the lymphatic route. The intact β-carotene is also absorbed via the lymphatic route (Goodman et al., 1966; Blomstrand and Werner, 1967). A small proportion of β-carotene may also be converted to vitamin A in the liver. However, unlike vitamin A, most of the intact β-carotene is released from the liver into the blood circulation, where it is transported primarily in the low density lipoprotein (LDL) cholesterol fraction (Krinsky et al., 1958; Bjornson et al., 1976). The absorption of β-carotene is modulated by the presence of several other dietary components such as fat (increased absorption), and fiber (decreased absorption) (Erdman, 1988).

Synthetic β-carotene is absorbed more efficiently than the β-carotene in vegetables (broccoli, carrots) (Brown et al., 1989). The efficiency of absorption of β-carotene varies widely from subject to subject (Dimitrov et al., 1988; Brown et al., 1989; Mathews-Roth, 1990; Nierenberg et al., 1991), and seems to be influenced by gender, body mass index (BMI), smoking, alcohol consumption, and serum levels of HDL cholesterol and triglycerides (Costantino et al., 1988; Nierenberg et al., 1991). Females, nonsmokers, lean subjects and subjects with high blood levels of β-carotene seem to absorb β-carotene more efficiently.

Biological Roles of β-Carotene

Broadly speaking, biological roles of β-carotene in man can be divided into two categories: (I) provitamin A role and (II) non-provitamin A roles.

I. β-Carotene as Provitamin A

β-carotene is the most abundant and most efficient provitamin A in foods. It is a 40-carbon hydrocarbon with a beta-ionone ring at each end of a central polyene chain. To be a provitamin A, a carotenoid should have at least one unmodified beta-ionone ring at one end of an 11-carbon or longer polyene chain (Simpson, 1983). The provitamin A carotenoids other than β-carotene are considered to possess about 50% of the vitamin A activity of β-carotene. Conversion of all-trans β-carotene to cis-isomers also lowers the vitamin A potency of the carotenoid (Simpson and Tsou, 1986).

Although β-carotene in raw fruits and vegetables is known to occur predominantly in the all-trans form, a varying proportion of the carotenoid gets converted to cis-isomers during food processing and exposure to high temperatures and solvents. Similarly, synthetic β-carotene in crystalline form is all-trans, but a certain proportion can change to cis-isomers due to exposure to heat during preparation of certain product forms.

In biological systems, conversion of β-carotene to vitamin A is very inefficient. Traditionally, vitamin A activities of both preformed vitamin A (retinol) and β-carotene (carotenoids) have been expressed in International Units (IU). One IU of vitamin A activity has been defined as equal to 0.33 μg of all-trans retinol or 0.60 μg of all-trans β-carotene or 1.2 μg of provitamin A carotenoids other than β-carotene (Simpson and Tsou, 1986). However, this conversion factor is based on animal data and does not take into account the very poor bioavailability of β-carotene in humans. The expression of vitamin A activity of carotenoids in foods is misleading and is highly overestimated. Thus, in 1967, an FAO/WHO Expert Committee proposed that vitamin A activity of foods be expressed as retinol equivalents (RE) (FAO/WHO, 1967). This takes into consideration both the low bioavailability and inefficiency of conversion of β-carotene to vitamin A. One RE is defined as = 1 μg retinol or 6 μg of β-carotene or 12 μg of provitamin A carotenoids other than β-carotene. Using food consumption data, USDA has estimated that about one-third of the total vitamin A activity in the U.S. diet is derived

from carotenoids (National Research Council, 1989). In the developing countries where consumption of meat, eggs and dairy products is very low, a significantly higher percentage of vitamin A activity is derived from carotenoids.

II. Non-Provitamin a Functions of β-carotene

A. β-carotene as a Singlet Oxygen Quencher and an Antioxidant

β-carotene is one of the most potent singlet oxygen ($^1O_2$) quenchers in biological systems (Krinsky, 1989). Occurrence of 9 or more conjugated double bonds seems to be essential for the quenching characteristic of a carotenoid (Foote et al., 1970). On reaction with $^1O_2$, β-carotene changes to a triplet state. The excitation energy from the triplet carotenoid is subsequently dissipated in the form of heat energy, resulting in the regeneration of the original β-carotene molecule. Thus, theoretically β-carotene can neutralize $^1O_2$ in a catalytic manner (see Krinsky, 1989). The catalytic nature of this reaction would suggest that an initial supply of the carotenoid in the body could provide life-long protection against singlet oxygen damage. However, there is some indication that during this process certain proportions of the carotenoid molecules are destroyed (Hasegawa et al., 1969).

In addition to acting as a $^1O_2$ quencher, β-carotene can also act as an antioxidant and thus protect cells against damage from oxidative stress (Burton and Ingold, 1984; Krinsky, 1989). The oxidative damage in cells is caused mostly by free radicals, the chemical species with one or more unpaired electrons. Free radicals are produced endogenously in the body as intermediates in a number of essential metabolic reactions (Freeman and Crapo, 1982; Slater et al., 1987; Cross et al., 1987). For example, the monovalent reduction of oxygen during oxidative phosphorylation in the mitochondria generates superoxide (O_2^-) and hydroxyl ($^{\bullet}OH$) radicals, as well as hydrogen peroxide (H_2O_2) (Cross et al., 1987) Similarly, phagocytic leukocyte activity, particularly during inflammation, generates significant amounts of free radicals (Weiss and LoBuglio, 1982).

Ingestion of certain agents/drugs (anthracyclic antineoplastic agents, pesticides) and exposure to radiation can also generate free radicals. Autoxidation of small molecules capable of undergoing oxidation-reduction reactions, such as catecholamines, hydroquinones, flavins, etc., is also known to generate free radicals. In addition, exposure to cigarette smoke and certain environmental agents such as photochemical air pollutants, ozone, NO_2 and certain aromatic hydrocarbons can enhance the free radical burden in the body, either because some of these air pollutants are already free radicals (e.g., NO2) or can initiate reactions in the body (ozone --> lipid peroxidation, detoxification of certain xenobiolics) that produce free radicals (Freeman and Crapo, 1982).

Free radicals are highly reactive and if not neutralized, they can react with and seriously damage cell membranes and important cellular constituents including DNA, leading eventually to extensive cell and tissue destruction.

The body possesses defence mechanisms to protect itself against free radical damage. Basically, these mechanisms consist of (i) low molecular weight free radical scavengers (alpha-tocopherol, ascorbate, β-carotene/carotenoids and glutathione peroxidase); and (ii) enzyme systems (superoxide dismutase, catalase and glutathione peroxidase). Normally, these defense mechanisms can keep free radicals in check and thus can protect the body from oxidative damage. However, under certain conditions, such as chronic exposure to cigarette and certain environmental pollutant, use of certain drugs including alcohol and increased activity of phagocytic leukocytes, etc., free radicals can overwhelm the cellular defense mechanism. Under such conditions, excessive oxidative stress can potentially cause cell and tissue damage, including damage to DNA, resulting in certain degenerative disease. Indeed, free radical damage has been implicated in aging process and is considered to contribute to the etiology of many degenerative disorder such as cancer, cataract, cardiovascular disease and emphysema.

B. β-Carotene and Cancer/Precancerous Lesions

1. Precancerous Lesions

The formation of precancerous lesions is the first clinically identifiable change in the tissue during the development of cancer. Treatments resulting in regression or an inhibition of progression of precancerous lesions represent an important strategy in cancer chemoprevention. Currently, epidemiological and biochemical studies of precancerous lesions are limited. However cervical dysplasia and oral leukoplakia have been the focus of some recent studies.

Some reports suggest that β-carotene/carotene may reduce the risk or cause regression of certain precancerous lesions (e.g. cervical dysplasia and oral leukoplakia) (see Singh and Gaby, 1991).

Cervical dysplasia, a precancerous lesion of the uterine cervix is associated with increased risk of cervical cancer. It is generally agreed that early detection of this precancerous lesion may significantly reduce the incidence of and mortality from cervical cancer (Celentano et al., 1989).

Two epidemiological studies reported a high incidence of cervical dysplasia/carcinoma in situ in subjects with low consumption of foods rich in carotenoids. Similarly, three reports noted lower blood levels of β-carotene in women with cervical dysplasia, as compared to normal controls (see Singh and Gaby, 1991). In one study, risk of cervical dysplasia or carcinoma in situ was 2 to 3 times greater in women consuming dietary carotene below the median intake, as compared to those in women with the highest dietary intake (Wylie-Rosett et al., 1984). Similarly, in another study, women with the highest quartile of plasma β-carotene had 80% less risk of cervical carcinoma in situ, as compared to women in the lowest quartile of plasma β-carotene (Brock et al., 1988).

Another precancerous lesion that has, in recent years, been the focus of attention is oral leukoplakia. A number of small-scale clinical intervention studies have reported that β-carotene supplementation singly, or in combination with vitamin A or with vitamins C and E, caused a partial or complete regression of oral leukoplakia in a significant percentage of the population under study (Garewal, 1990, 1991; Stich et al.,

1988; Kaugars et al., 1990). In a study conducted in India, use of a 180 mg β-carotene supplement weekly for 6 months caused a marked reduction in micronucleated cells (an indicator of early events in precancerous lesions) and decreased the appearance of new precancerous lesions (Stich et al., 1988). In another study in the U.S., use of a β-carotene supplement (30 mg daily) for 6 months produced a response rate of 71% (partial or complete regression) in subjects with oral leukoplakia (Garewal, 1990). In yet another study, 6 patients with oral epithelial dysplasia and 2 with hyperkeratosis were supplemented with a combination of antioxidants (30 mg of β-carotene, 1 g of vitamin C and 800 IU of vitamin E in 4 equal doses per day). Of the 6 of these patients who had received the supplement for at least 1 month, 3 showed complete regression, one 75% regression and two 50% regression of the oral lesions (Kaugars et al., 1990).

These findings are extremely promising and encouraging. While several long-term clinical intervention trials using β-carotene supplementation and measuring cancer incidence and/or cancer mortality as the end points are still ongoing, these are the first human studies demonstrating efficacy of pure β-carotene as a potential cancer preventive agent. Use of β-carotene supplementation in the treatment of oral leukoplakia offers tremendous opportunity since the modality of oral leukoplakia treatment currently available is surgical incision, which causes severe morbidity.

2. Cancer

It is estimated that about 35% (range 10-70%) of all cancers can be attributed to diet and 30% to smoking (Doll and Peto, 1981). According to other investigators, 40% of all cancers in men and 60% of all cancers in women can be attributed to diet (Wynder and Gori, 1977). Thus, a significant proportion of cancer incidence is preventable. While consumption of too much of certain nutrients, such as fat, may contribute to the risk of some types of cancers, intakes of foods rich in certain other substances such as carotenoids, some vitamins and fiber have been found to be associated with reduced risk of certain cancers.

In a large number of prospective and retrospective epidemiological studies intakes of foods rich in carotenes/β-carotene, or high levels of β-carotene in the blood, have been found to be strongly associated with a reduced risk of certain types of cancers (Gaby and Singh, 1991; Ziegler, 1991). The association with lung cancer is particularly strong and consistent. Studies on cancer of the stomach, esophagus, cervix, and throat, although limited in number are relatively consistent in showing a protective role of β-carotene against these cancers. The protective effect of carotenoid-rich foods on other cancers such as cancers of the colon, breast, ovary and prostate is less clear.

Of the more than two dozen studies that have examined the relationship between carotenoid-rich food intake and lung cancer incidence or mortality, six were prospective studies (Shekelle et al., 1981; Kvale et al., 1983; Hirayama, 1985; Wang and Hammond, 1985; Paganini-Hill et al., 1987; Knekt et al., 1991). Five of these studies found decreased risk of lung cancer with increased consumption of foods rich in β-carotene/carotenoids (Shekelle et al., 1981; Kvale et al., 1983; Hirayama, 1985; Wang and Hammond, 1985; Knekt et al., 1991). In 1981, Shekelle and his colleagues published results of a 19-year longitudinal study conducted on 1,954 employees of the Western Electric Co., Chicago. These investigators developed a carotenoid index from dietary history and for the first time evaluated the effect of the intake of preformed vitamin A and that of carotenoids independently. Three noteworthy findings emerged: (i) Smokers had markedly greater incidence of lung cancer than nonsmokers--the longer the duration of smoking, higher was the incidence of lung cancer; (ii) Intake of preformed vitamin A was not associated with a reduced risk of lung cancer; and (iii) The highest incidence of lung cancer was in the quartile of lowest carotene intake. The risk of lung cancer in the lowest quartile was more than 6 fold greater than in the highest quartile. An estimated consumption of β-carotene in the lowest quartile was roughly up to 2 mg and that in highest quartile 4-19 mg daily. One prospective study failed to record a significant association between foods rich in carotene and lung cancer (Paganini-Hill et al., 1987). However, as the investigators have pointed out, the dietary intake of vitamin A (carotenoids + retinol) in this population was quite high; the median intake being 17,206

IU. Thus, subjects even in the lowest tertile had consumed relatively high levels of vitamin A. Furthermore, a careful validation of the self-reported information on the frequency of consumption of selected food items led the investigators to suggest that considerable inaccuracy in the data pertaining to β-carotene intake might have occurred.

Similarly, five prospective epidemiological studies have examined the association between blood levels of β-carotene and lung cancer risk. All of these five studies found reduced risk of lung cancer among subjects with high levels of β-carotene in the blood (Stahelin et al., 1984; Nomura et al., 1985; Menkes et al., 1986; Wald et al., 1988; Connett et al., 1989). One prospective study that measured total carotene level in the blood failed to find any association between the blood carotenoid level and lung cancer incidence (Willett et al., 1984).

In most of the prospective studies, the dietary intake or blood analysis data were collected long (up to 20 years) before cancer incidence/mortality evaluation. Thus, it is unlikely that carotene/β-carotene status in these studies was influenced by preclinical disease. Two studies with long-term follow-up examined the effect of time lapsed between the collection of dietary intake data and incidence of lung cancer, and concluded that dietary intake did not appear to be influenced by a preclinical disease (Shekelle et al., 1981; Knekt et al., 1991).

In addition to the above-discussed prospective studies, 18 retrospective studies have examined the association between consumption of carotenoid-rich foods or blood levels of β-carotene and cancer incidence and/or mortality (Ho et al., 1988; Dartigues, et al., 1990; Gaby and Singh, 1991; Harris et al., 1991). Of these, 17 studies found reduced risk of lung cancer with high intakes of fruits and vegetables rich in carotenes/β-carotene. Similarly, in a recent retrospective study, serum levels of β-carotene in the family members of the lung cancer patients were lower than those in the matched controls and their family members. The family members of the patients were used as surrogates in order to minimize the possibility that the lowered serum levels of β-carotene might be due to changes in diet and metabolism of the patients caused by the disease (Smith and Waller, 1991).

a) Protective Role of β-carotene Against Cancer Independent of Provitamin A Role:

In three prospective (Shekelle et al., 1981; Paganini-Hill et al., 1987; Knekt et al., 1991) and ten retrospective (Hinds et al., 1984; Ziegler et al., 1984; Ziegler et al., 1986; Samet et al., 1985; Wu et al., 1985; Byers et al., 1987; Bond et al. , 1987; Pastorino et al. , 1987; Ho et al., 1988; Marchand et al., 1989) intake of dietary carotene, but not that of preformed vitamin A (retinol), was associated with reduced risk of lung cancer. In one study, both dietary carotene and preformed vitamin A were associated with reduced risk of lung cancer, but the vitamin A effect was limited to adenocarcinoma (Fontham et al., 1988). Similarly, in the 5 prospective studies where both blood retinol and β-carotene were evaluated, high blood levels of β-carotene were associated with reduced risk of lung cancer. (Stahelin et al., 1984; Nomura et al., 1985; Menkes et al., 1986; Wald et al., 1988; Connett et al., 1989). But, in none of these studies was blood retinol level ever associated with reduced risk of lung cancer. Collectively, these findings suggest that the protective role of β-carotene against lung cancer may be independent of its role as provitamin A.

b) Effect of β-carotene/Carotene on Lung Cancer: Men vs. Women

In 9 prospective (4 dietary intake and 5 blood levels) and 7 retrospective (dietary intake) studies conducted with men, consistent associations between high carotene intakes or high blood levels of β-carotene and reduced risk of lung cancer were found. Four retrospective studies showed a similar association for women, but 3 did not (see Ziegler, 1991).

c) Effect of β-carotene/Carotene on Relative Risk of Lung Cancer

In studies using a carotene index, smoking-adjusted relative risks of lung cancer in the lowest, as compared to the highest quartile or tertile, were found to be 1.5-2.7 (Ziegler, 1991).

d) Potential Mechanisms of Cancer Preventive Action of β-carotene

i) Antioxidant/Singlet Oxygen Quenching

There is a growing body of evidence that free radicals may contribute to cancer etiology by acting as carcinogens and by modulating various stages of carcinogenesis (i.e., initiation, promotion and progression) (Cerutti and Trump, 1991; Trush and Kensler, 1991; Troll and Wiesner, 1985). The exact mechanism by which free radicals influence these processes is not yet fully understood. However, there is some suggestive evidence that free radicals may modify the phenotypic expression of cells by influencing signal transduction cascades. Free radicals can cause DNA damage (Birnboim, 1982), chromosomal aberrations (Emerit and Cerutti, 1981; Weitberg et al., 1983) and even mutations (Cerutti and Trump, 1991; Trush and Kensler, 1991). There is some indication that mutagenic modifications of DNA may activate proto-oncogenes and inactivate tumor suppressor genes leading to "response modifications" of target cells. Such "response modifications" of target cells is considered to play an important role in tumor promotion and progression (Cerutti and Trump, 1991). Thus, β-carotene could reduce the risk of cancer by scavenging free radicals and/or quenching singlet oxygen.

ii) Stimulation of Immune Response

Several immune system components such as tumor necrosis factor and natural killer cells play important roles in immunosurveillance and destruction of tumor cells. A number of animal studies and some human studies have shown that β-carotene can enhance natural killer cell activity (Oliver et al., 1989; Prabhala et al., 1989; Watson et al., 1991) and stimulate formation of tumor necrosis factor. B-carotene has been shown also to enhance tumor cell killing ability of macrophages and enhance the function of cytotoxic T-cells in mice (see Bendich, 1988).

iii) Antimutagenic Effect

There are several lines of evidence that beta carotene is antimutagenic (see Krinsky, 1990). For example, in one study, exposure of S. typhimurium (TA100) to 8 methoxypsoralen and UV light increased the number of histidine revertants, but the increase was blocked by the addition of β-carotene to the culture medium. Similarly, in another study, carotenoids prevented the appearance of mutants of certain bacteria when they were exposed to the mutagen, cyclophosphamide. In a study using strains of C 127 cells, transformation of several strains of this cell line by bovine papillomavirus caused a high incidence of mitotic irregularities and elevated frequency of cells with micronuclei. A 3-day exposure of these cells to β-carotene greatly reduced the chromosomal irregularities (Stich et al., 1990). B-carotene feeding to Chinese hamsters was found to cause an anticlastogenic effect on chromosomal aberrations in bone marrow cells induced by direct acting mutagens (Renner, 1985). In another study, β-carotene inhibited the clastogenic action of methyl methanesulphonate and 4-nitroquinoline in Chinese hamster ovary in culture (see Stich et al., 1990). In two human studies, use of a β-carotene supplement markedly reduced the frequency of cells with micronuclei in the oral mucosa of betel quid chewers and snuff dippers (see Stich et al., 1990).

Addition of β-carotene to mouse mammary glands in organ culture during the exposure to 7, 12-dimethylbenz(a) anthracene (DMBA), N-nitrosodiethylamine (DENA) and N-methylnitrosourea (MNU) caused a marked reduction in the sister chromatid exchanges induced by these carcinogens (Manoharan and Banerjee, 1985). Feeding of β-carotene to mice inhibited the benzo(a)pyrene and mitomycin C-induced chromosomal breaks in the bone marrow (Raj and Katz, 1985). These findings suggest that β-carotene acts as an antimutagenic agent.

3. Cardiovascular Disease

Two recent population based, case-control studies (Street et al., 1991; Riemersma et al., 1991) and preliminary findings of a clinical intervention trial (Gaziano et al., 1990)

showed that β-carotene may reduce the risk of cardiovascular disease. In one of the epidemiological studies, blood samples from 125 cases with myocardial infarction (MI), 125 community controls and 125 hospital controls, which had been collected about 15 years earlier, were analyzed for β-carotene (Street et al., 1991). When β-carotene levels were divided into quintiles, the risk of MI decreased with increasing levels of β-carotene. An odds ratio of 0. 54 was obtained when the highest quintile of β-carotene was compared to the lowest quintile for case: community control. Similarly, in another study, 110 cases of angina and 394 controls were evaluated for the plasma levels of vitamins A, C and E and carotene (Riemersma et al., 1991). High plasma levels of antioxidant vitamins C and E and carotene were associated with low risk of angina. However, after adjusting for smoking as a confounding ffactor, the protective effect of carotene became insignificant.

Preliminary findings from a small subgroup of a large ongoing randomized, placebo-controlled, double blind clinical intervention trial (the Physicians' Health Study) showed that use of β-carotene supplements may markedly reduce the risk of cardiovascular events in a population with chronic angina and/or in those who have had coronary bypass surgery or angioplasty (Gaziano et al., 1990). In this study, 333 subjects out of a total of 22,071 male physicians had, at the time of recruitment, a history of stable angina pectoris and/or coronary revascularization (coronary bypass surgery or angioplasty). One hundred and sixty of these were supplemented with β-carotene (50 mg on alternate days) and 173 subjects received placebo for about 5 years. Subjects consuming β-carotene, as compared to those on placebo, had about a 50% reduction in the major cardiovascular events (stroke, myocardial infarction, revascularization, cardiovascular mortality).

The etiology of cardiovascular disease, like other degenerative diseases associated with old age, is multifactorial. Among other factors, free radical-mediated lipid peroxidation is considered to play an important role in the etiology of atherosclerosis (Cross et al., 1987). Oxidative modification of low-density lipoprotein (LDL) in particular is considered to contribute to the etiology of atherosclerosis (Steinberg et al.,

1989). On the other hand, high serum levels of high-density lipoprotein (HDL) cholesterol have been associated with reduced risk of cardiovascular disease.

Some animal, ex vivo and human studies have suggested that β-carotene may reduce lipid peroxidation (Kunert and Tappel, 1983; Dixit et al., 1983; Mobarhan et al., 1990).

In a preliminary study, β-carotene level in the LDL seemed to protect the lipoprotein against oxidative stress (Quintao et al., 1989). In a double-blind, placebo-controlled study, men with low antioxidant status were supplemented daily with an antioxidant combination containing 2 mg β-carotene, 300 mg alpha-tocopherol, 600 mg vitamin C and 75 μg selenium in yeast. After 5 months of supplementation with the antioxidants, a reduction in serum lipid peroxides, ADP-induced platelet aggregation and serum thromboxane B_2 was observed (Salonen et al., 1991).

Recently, supplementation with a high dose of β-carotene (300 mg daily) for 1 month was found to increase the level of HDL cholesterol in the blood (Ringer et al., 1991). Similarly, preliminary findings from a placebo-controlled cancer chemoprevention study showed a significant increase in the blood levels of HDL cholesterol following treatment with β-carotene (20 mg daily) for 2 years (Gaffney et al., 1990). The β-carotene treatment-mediated increase in the HDL cholesterol was particularly impressive in the subjects who had relatively low levels of HDL cholesterol before the treatment.

Thus, among other factors, an inhibition of lipid peroxidation, including inhibition of LDL oxidation and increased blood HDL cholesterol, may provide two important mechanisms for the β-carotene-induced reduction of cardiovascular disease risk.

4. Cataract

The eye lens is a transparent organ consisting of about 63% water and 35% protein (Taylor, 1989). Unlike other organs, protein in the lens does not undergo rapid turnover; in fact, it may remain in situ for decades. An aggregation, polymerization and precipitation of the protein in the lens due to exposure to light, oxygen and other factors

causes opacity of the lens (cataract). Cumulative oxidative damage to the lens protein is considered an important factor in the etiology of cataracts (Taylor, 1989; Marak et al., 1990). There is a large body of data from animal and in vitro studies to suggest that antioxidant vitamins C and E may protect the lens from oxidative damage and thus reduce the risk of cataracts (Varma, 1991; Robertson et al., 1991). In a study conducted at the USDA Human Nutrition Research Center on Aging, at Tufts University relative risk of senile cataract was 86% lower in the highest quintile as compared to the lowest quintile of carotenoid consumption (Jacques and Chylack, 1991). The same investigator also reported an inverse association between the blood level of carotenoids and the incidence of senile cataract (Jacques and Chylack, 1991.)

III Dietary Intake of Carotenoids in U.S.

The role of diet in prevention of disease and promotion of health has become a major focus of public health policy and research. Various dietary guidelines advising liberal intakes of fruits and vegetables rich in carotenoids/β-carotene have been issued in the United States (Surgeon General, 1988; Butrum et al., 1988; U.S. Department of Agriculture, 1990).

However, various national food consumption and nutrition surveys continue to show that dietary intake of such fruits and vegetables is very poor in the U.S. A 24-hr dietary recall data from 11,658 adults in the second National Health and Nutrition Examination Survey (NHANES II) collected during 1976-80 showed that on the day of the interview about 80% of this population did not eat any fruit or vegetables rich in β-carotene (Patterson and Block, 1988). Similarly, 1984 food consumption data based on food disappearance information on vegetables and fruits in the United States showed that consumption of β-carotene-rich fruits and vegetables was extremely poor (Lachance, 1988). An evaluation of data from Continuing Survey of Food Intake by Individuals showed that U.S. women (19-50 years old) consumed on the average 1.5 mg of β-carotene per day (Lachance, 1988). Lachance estimated that if the dietary guidelines

issued by U.S. Department of Agriculture and National Cancer Institute were followed, the β-carotene intake would be nearer to 6.0 mg per day. In the 19-year longitudinal study by Shekelle et al. (1981), an estimated consumption of β-carotene in the lowest quartile was below 2 mg and that in the highest quartile was about 6.0 mg on the average. Thus, the average consumption of carotenoids in the U.S. population seems to be similar to that in lowest quartile in the study of Shekelle et al., (1981), which had a 6 times higher risk of lung cancer than people in the highest quartile of carotene consumption. it is clear that the U.S. population has to either substantially increase the consumption of fruits and vegetables rich in carotenoids, or judiciously use supplements and/or β-carotene fortified foods to reach a level of intake associated with reduced cancer risk.

IV. Public Health Implications

The economic and social impact of reducing the risks of cardiovascular disease and cancer can be appreciated by the fact that these two diseases are the number one and number two killer diseases in the industrialized countries. Cardiovascular disease and cancer are responsible for more than 40% and over 20% respectively of all the deaths in the U.S. The estimated cost (health care plus lost productivity) to the U.S. in 1987 was more than $60 billion from cardiovascular disease and about $72 billion from cancers (Surgeon General, 1988). Similarly, in the U.S. alone, every year about 400,000 persons develop cataracts and about 4,700 become blind due to cataracts (Taylor, 1989). If early lens changes are added to the list of cataracts, the cataract incidence will double (see Taylor, 1989). It is estimated that a delay of cataract surgery by 10 years can save $608 million yearly in the U.S. alone.

In the year 1980, the life expectancy in the U.S. was 73.7 years, of which 62 years were healthy life and the remaining 11.7 years were dysfunctional life, mostly due to degenerative disease (U.S. Department of Health and Human Services, 1990). About 40% of the population in the U.S. over 65 years of age have limitations of activity, primarily due to chronic illness or impairments. It is estimated that by the year 2030,

those 65 years and older will constitute more than 20% of the U.S. population (U.S. Department of Health and Human Services, 1990). The increase in the older population will bring higher incidence of degenerative diseases associated with old age. Thus, preventive measures, including good nutrition are critically important in order to avoid or reduce the devastating impact of this change in population demography.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

ANTI-TUMOR AND ANTI-TUMOR PROMOTING ACTIVITY OF α- AND β-CAROTENE

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Summary

Carotenoids are known to inactivate certain reactive oxygen species, such as singlet oxygen. Since antioxidants have been suggested to be one of the promising agents for cancer control, it is worthwhile to evaluate the anti-cancer potency of these carotenoids.

In the course of the study, we found that palm oil derived natural carotene which contains 60% β-carotene, 30% α-carotene and 10% others, showed potent anti-tumor activity. Therefore, we compared the activity of α- and β-carotene, and found that α-carotene showed more potent anti-proliferative effect than β-carotene. α-Carotene was also proved to have higher potency than β-carotene to suppress tumor promotion in two-stage skin and lung carcinogenesis experiments. Thus, α-carotene seems to be a promising agent for cancer control.

Further study to find other types of useful carotenoids in natural materials, especially in our daily foods, was also carried out.

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Introduction

Carotenoids, which contain many conjugated double bonds, can inactivate certain reactive oxygen species, such as singlet oxygen. Since antioxidants and radical scavengers have been suggested to be one of the promising agents for cancer control, we evaluated the anti-cancer potency of natural carotenoids.

β-Carotene, one of the most widespread natural carotene, has been reported to have cancer preventive activity (1). Thus, first of all, we examined biological activities of β-carotene (2). However, in natural materials including our daily foods, β-carotene is often associated with various other types of carotenoids. In fact, besides β-carotene, α-carotene, lycopene, lutein and cryptoxanthin are detectable in human blood and tissues (3-5). Therefore, we are especially interested in these carotenoids.

In the course of the study, we found that natural carotene extracted from palm oil suppressed very effectively the proliferation of human malignant tumor cells. The proportion of components in palm carotene are known to be almost the same as that in carrot carotene; i.e., palm carotene consists of 60% β-carotene, 30% α-carotene and 10% others. Thus, we compared the effect of α-carotene with that of β-carotene on the proliferation of cancer cells. Furthermore, we also compared the anti-tumor promoting activity of α-carotene with that of β-carotene. In both cases, α-carotene showed higher potency than β-carotene.

In addition to β- and α-carotene, lycopene, lutein and cryptoxanthin distributed in human blood and tissues may also be important. Furthermore, in our daily foods, other types of carotenoids are also found; e.g., fucoxanthin, halocynthiaxanthin and so on. Although the amount of these natural carotenoids in human has not been determined yet, it seems worthwhile to evaluate anticancer potency of these carotenoids. In fact, in the present study, these carotenoids showed higher potency than β-carotene in anti-cancer activity.

Materials and Methods

Chemicals

α-Carotene, purified from palm carotene concentrates, was provided by Lion Oleo-chemical Co. LTD. β-Carotene was purchased from Sigma. α-Carotene or β-carotene was prepared as emulsion with 0.5% sucrose ester P-1570 (Mitsubishi-Kasei Food Co.), 1.0% Sansoft 8000 (Taiyo Co.), 0.2% L-ascorbyl stearate and 4.0% peanut oil.

Cryptoxanthin was purchased from Extrasynthese. Fucoxanthin was isolated from the brown algae, Hijikia fusiforme, and its purity was 92%, as determined by HPLC. Halocynthiaxanthin was isolated from the sea squirt, Halocynthia roretzi, and its purity was 100%. Cryptoxanthin, fucoxanthin and halocynthiaxanthin were dissolved in dimethyl sulfoxide. Structures of these natural carotenoids are shown in Fig. 1.

7,12-Dimethylbenz[alanthracene (DMBA) was purchased from Wako Pure Chemicals Industries. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Pharmacia PL Biochemicals. 4-Nitroquinoline 1-oxide (4NQO) was purchased from Nacalai Tesque.

[α-^{32}P]2'-Deoxycytidine 5'-triphosphate was obtained from Amersham Japan.

Culture of Human Malignant Tumor Cells

GOTO cells (neuroblastoma), PANC-1 cells (pancreas cancer), HGC-27 cells (gastric cancer) and COLO320DM cells (colon cancer) were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% foetal bovine serum. HeLa cells were maintained in Eagle's minimum essential medium supplemented with 10% calf serum. Cultures were incubated at 37°C under the humidified atmosphere of 5% $C0_2$ in the air.

Measurement of N-myc Messenger RNA

Total cellular RNA of GOTO cells was isolated by the guanidinium thiocyanate method, electrophoresed on a formaldehyde gel, transferred to nitrocellulose, and then

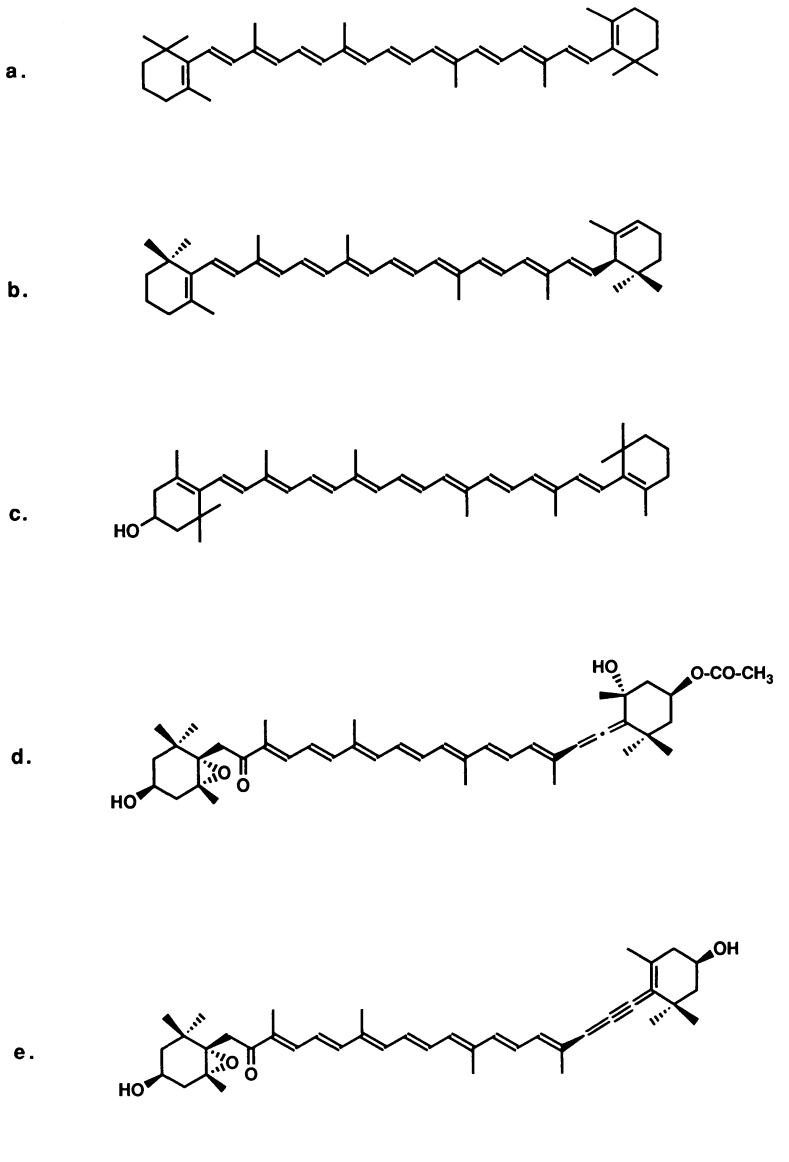

Fig. 1 .Structure of carotenoids. a, β-Carotene; b, β-carotene; c, cryptoxanthin; d, fucoxanthin; e, halocynthiaxanthin.

hybridized with a nick-translated ^{32}P-labelled probe of the N-myc gene. After washing, autoradiogram was prepared.

Analysis of Cell Cycle

Sample cells (5 x 10^5 cells) were collected, and resuspended in 4 ml of ice-cold 0.1% Triton X-100 in 0.3 M NaCl-0.01 M glycine, and 0.2 ml of 1 % ribonuclease solution was added. DNA of the cells was stained with 0.1 ml of ethidium bromide solution (0.2 mg/ml). DNA content was analyzed by flow cytometer.

Two-Stage Skin Carcinogenesis Experiment

The back of female ICR mice (purchased from Shizuoka Laboratory Animal Center) at 7 weeks of age was shaved with an electric shaver. After 2 days, initiation was accomplished by a single application of 100 μg of DMBA on the shaved area. TPA, at the dose of 1 μg per painting, was applied twice a week starting 1 week after the initiation. The experiment was continued for 20 weeks. α-Carotene or β-carotene (400 nmol), mixed in 200 μl of acetone, was applied with each promoter application. Control mice were treated with vehicle in acetone. The number of tumors was determined once a week.

Two-Stage Lung Carcinogenesis Experiment

Experiment for two-stage mouse lung carcinogenesis was performed as reported by Inayama (6). The animals used were 6-week-old male ddY mice (purchased from Shizuoka Laboratory Animal Center). As the initiator, 4NQO (0.3 mg per mouse), dissolved in a mixture of olive oil and cholesterol (20 : 1), was given by a single subcutaneous injection on the first experimental day. As the tumor promoter, 10% glycerol was given as drinking water ad libitum from the beginning of experimental week 5 for 25 weeks continuously. α- or β-Carotene (at the concentration of 0.05%) or vehicle as control, was mixed in drinking water. Mice were killed at week 30, and the lungs were fixed via intratracheal instillation of 10% formaldehyde. After separation of each pulmonary lobe, the number of tumors was counted under a microscope.

Results

Effect of palm carotene on the proliferation of human malignant tumor cells

As shown in Table 1 , palm carotene (20 μM) inhibited the growth of neuroblastoma GOTO cells, pancreas cancer PANC-1 cells, stomach cancer HGC-27 cells and cervic cancer HeLa cells. Among these human malignant tumor cells, GOTO cells were most sensitive to the treatment with palm carotene. The suppression of the proliferation of GOTO cells by palm carotene was found to be dose- and time-dependent (Fig. 2). The inhibitory effect of palm carotene was detectable at the concentration of as low as 5 μM, and ID_{50} was calculated to be around 15 μM, at day 6 after the treatment.

Effect of α- and β-Carotene on the Proliferation of GOTO Cells

Since main components in palm carotene are α- and β-carotene, we compared the effect of these carotenes on the proliferation of GOTO cells. As shown in Fig. 3, α-carotene inhibited the growth of GOTO cells in a dose- and time-dependent manner. β-Carotene also inhibited the growth of GOTO cells, but it was less potent than α-carotene; α-carotene was 10 times more inhibitory than β-carotene.

Furthermore, we observed that morphologically differentiated cells were induced by α-carotene; the treatment with α-carotene at the concentration of 2 μM for 5 days induced differentiated phenotype by 74%, while in the control culture, little cell population showed morphological differentiation.

Effect of α-Carotene on the Level of N-myc Messenger RNA in GOTO Cells

It is of interest to elucidate the mechanism of potent anti-tumor action by α-carotene. As one of the approaches to this problem, we determined the effect of α-carotene on the expression of cell growth-related oncogene, N-myc gene, which is known to be amplified in GOTO cells. As shown in Fig. 4, the expression of N-myc gene was suppressed by the treatment with α-carotene; 5 μM α-carotene caused an 82% decrease in the level of N-myc messenger RNA at 18h after the treatment. However, within 48h, the level of N-myc messenger RNA returned to the level of untreated cells (data not shown).

TABLE 1 .

Effect of palm carotene on the proliferation of human malignant tumor cells

Cell (20 μM)	Condition Palm carotene	Cell number x105 cells per dish	(% of control)
GOTO	-	6.78	
	+	3.01	(44.7)
PANC-1	-	5.80	
	+	3.21	(55.2)
HGC-27	-	23.82	
	+	14.51	(60.9)
HeLa	-	9.30	
	+	6.40	(68.8)

After 2 days of the inoculation of cells, palm carotene or its vehicle was added into cultured medium. Viable cells were counted after 5 days. Data are mean values of duplicate experiments.

Effect of α-Carotene on Cell Cycle

Analysis of the effect of α-carotene on cell cycle was also carried out. Analysis of DNA contents of both the cells treated with α-carotene and the control cells by flow cytometry, revealed that α-carotene caused cell cycle arrest in G_0/G_1 phase. Thus, the time course of the changes in percentage of cells in the G_0/G_1 phase was calculated. As shown in Fig. 5, α-carotene caused G_0/G_1 arrest within short time after the treatment, but the effect was transient.

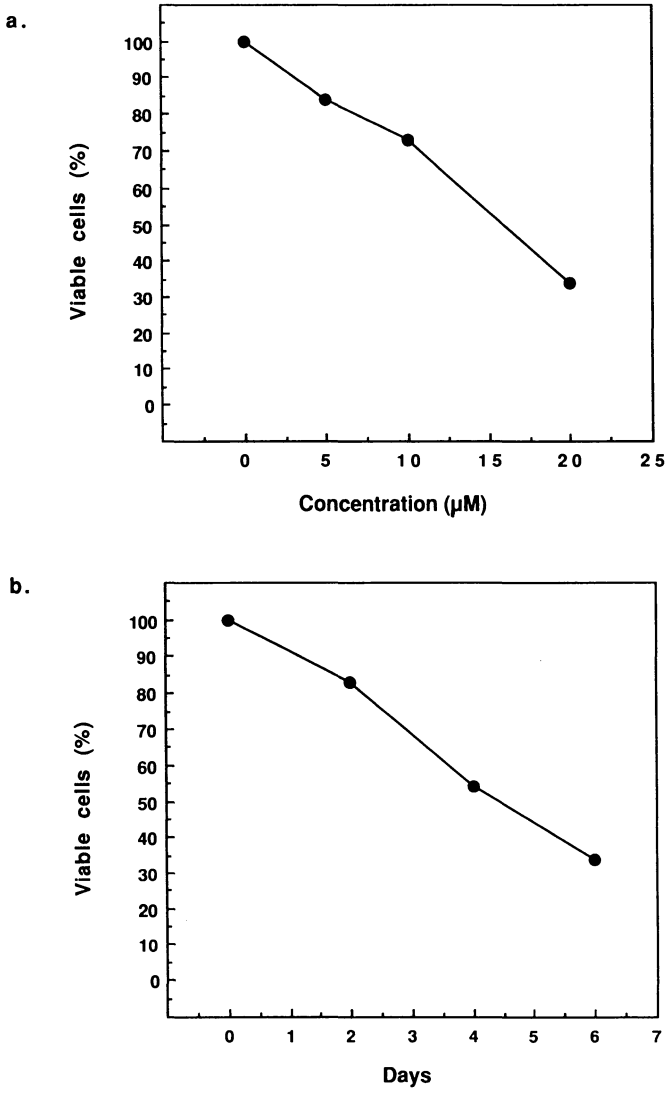

Fig. 2. Effect of palm carotene on the growth of GOTO cells. After 2 days of the inoculation of GOTO cells, palm carotene or its vehicle as control was added into culture medium. Viable cells were counted at 5 days (a), or at every 2 days (b). Data are mean values of duplicate experiments, and expressed as % of control; the number of viable cells which was observed in the control culture without palm carotene treatment was defined as 100%.

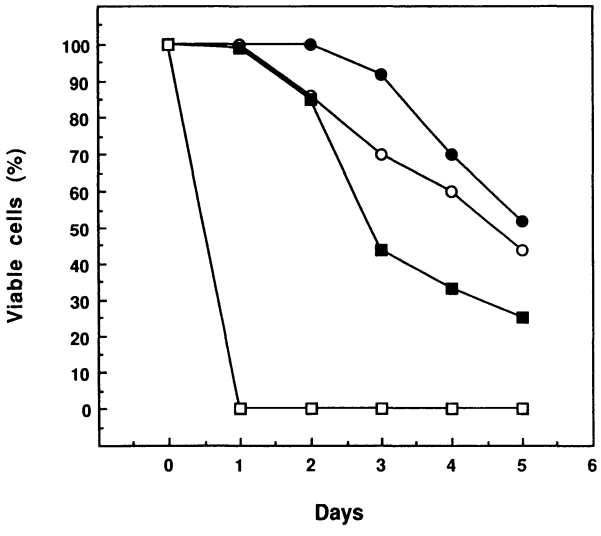

Fig. 3. Effect of α- and β-carotene on the proliferation of GOTO cells. After 2 days of the inoculation of GOTO cells, α- or β-carotene,, or vehicle as control, was added into culture medium. Viable cells were counted every day for 5 days. Data are mean values of triplicate experiments, and expressed as % of control; the number of viable cells which was observed at each day in the control culture without carotene treatment was defined as 100%.
○, 2 μM α-carotene; ■, 5 μM α-carotene; □, 10 μM α-carotene; ●, 20 μM β-carotene.

Effect of α- and β-carotene on the tumor promotion in the two-stage carcinogenesis of skin and lung

Fig. 6 shows the time course of skin tumor formation of the groups treated with DMBA plus TPA, with or without α-carotene or β-carotene. The first tumor appeared at week 8 in the control group. In the groups treated with α- or β-carotene, the first tumor appeared at week 13 and 9, respectively. The development rate of tumors was also higher in the control group than carotenoid-treated groups. The treatment with α-carotene

resulted in a statistically significant decrease in the number of tumors at week 20 compared with the control group ($p < 0.01$, t test). β-Carotene showed also inhibitory effect, but the difference from the control group was not statistically significant. These results suggest that α-carotene has a stronger anti-tumor promoting effect than β-carotene.

The higher potency of α-carotene than β-carotene to suppress tumor promotion was confirmed by other experimental systems of two-stage carcinogenesis in vivo. As shown in Table 2, administration of α-carotene during the promoting stage of lung carcinogenesis in 4NQO-initiated mice resulted in the decrease of the mean number of tumors per mouse to about 30% of the control group ($p < 0.001$, t-test). On the other hand, the β-carotene treated group did not show any such significant difference from the control group. α-Carotene, but not β-carotene, was also found to show the tendency to decrease the percentage of tumor-bearing mice, though the difference was not statistically significant.

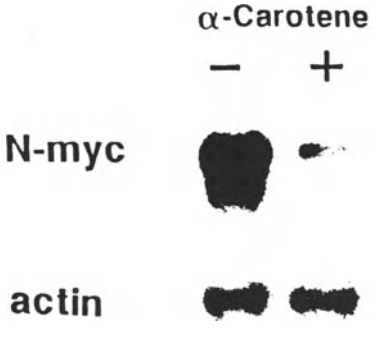

Fig. 4. Effect of α-carotene on the level of N-myc messenger RNA in GOTO cells. Northern blot analysis of cytoplasmic RNA from control cells (-) or from cells pretreated for 18h with 5 μM α-carotene (+), probed with ^{32}P-labelled N-myc or actin DNA (internal control), is shown.

TABLE 2.

Effect of α- and β-carotene on the promotion of lung tumor formation by glycerol in 4NQO-initiated mice

Group	Percent of tumor-bearing mice	Mean number of tumors a) per mouse
Control	94	4.06 ± 0.18
+ α-Carotene	73	1.33 ± 0.08[b]
+ β-Carotene	93	4.93 ± 0.28

4NQO (0.3 mg per mouse), dissolved in a mixture of olive oil and cholesterol (20 : 1), was given by a single subcutaneous injection on the first experimental day. Glycerol was dissolved in water and the 10% solution was given as drinking water ad libitum from the experimental week 5 to 30 continuously. α- and β-Carotene (at the concentration of 0.05%) or vehicle as control was mixed in drinking water. Each experimental group consisted of 16 mice.
a) Values are mean ± SE
b) $p < 0.001$ (t-test)

Anti-Cancer Activity of Various Kinds of Carotenoids

The results, which show that α-carotene has higher anti-cancer activity than β-carotene, prompted us to examine the biological activities of other types of carotenoids, especially of those distributed in human blood and/or in our daily foods. As shown in Table 3, not only α-carotene, but also cryptoxanthin, fucoxanthin and halocynthiaxanthin, showed higher activity than β-carotene to suppress the proliferation of GOTO cells. Among them, halocynthiaxanthin was shown to have comparable potency to that of α-carotene.

Discussion

The present findings clearly show that various types of carotenoids have anti-cancer activity. It is noteworthy that these carotenoids possess not only therapeutic activity against malignant tumor cells, but also protective activity against carcinogenesis. The present result is the first report showing the anti-tumor-promoting activity of α-carotene. Recently, we also found that fucoxanthin, halocynthiaxanthin and lutein showed anti-tumor-promoting effect in vitro (unpublished data). In the case of fucoxanthin, preventive effect against tumorigenesis in vivo has also been proved (7).

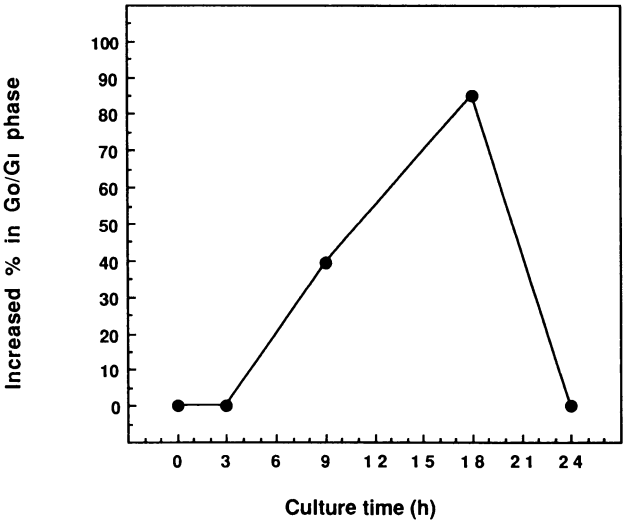

Fig. 5. Changes in percentage of GOTO cells in G_0/G_1 phase during 3 - 24 h of exposure to α-carotene.
Percentage of GOTO cells in G_0/G_1 Phase in culture treated with 5 μM α-carotene is compared with that in control culture. Data are expressed as the increased % of G_0/G_1 population in α-carotene-treated cells.

TABLE 3.

Effect of various types of carotenoids on the growth of GOTO cells

Carotenoid	(Concentration)	Inhibition %	
		2 days	5 days
β-Carotene	(2 μM)	0	2.8
	(20 μM)	1.2	47.8
α-Carotene	(2 μM)	15.2	52.4
	(20 μM)	100	100
Fucoxanthin	(2 μM)	3.9	8.9
	(20 μM)	49.8	80.2
Halocynthiaxanthin	(2 μM)	12.1	32.2
	(20 μM)	100	100
Cryptoxanthin	(2 μM)	3.1	6.2
	(20 μM)	39.9	70.2

After 2 days of the inoculation of GOTO cells, carotene or its vehicle was added into culture medium. Viable cells were counted after 2 and 5 days. Data are mean values of duplicate experiments, and expressed as % of inhibition.

The mechanism of action of these carotenoids remains to be elucidated. One possible explanation is the radical scavenging action of these carotenoids. On the other hand, it is unlikely that the activities of these carotenoids reflect their vitamin A activity, since β-carotene, which has the highest pro-vitamin A activity among the carotenoids tested in this study, showed less potency than other carotenoids in anti-cancer activity.

Inhibitory effects of α-carotene on cell growth-related oncogene expression, and cell cycle progression were also demonstrated, but the significance of these findings by way of the explanation for the anti-cancer action of the carotenoid is still not so clear, and further investigations should be carried out.

In any case, these carotenoids appear to be promising agents for the purpose of cancer control, and more extended studies should be continued.

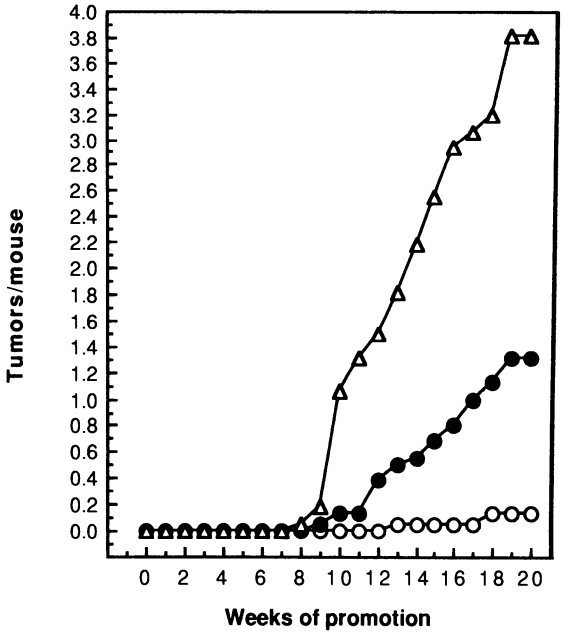

Fig. 6. Effect of α- and β-carotene on the promotion of skin tumor formation by TPA in DMBA-initiated mice. From 1 week after the initiation by 100 μg of DMBA, 1.0 μg of TPA was applied twice a week for 20 weeks. α- or β-Carotene (400 nmol) was applied with each TPA application. Each experimental group consisted of 16 mice.
Δ, Group treated with DMBA plus TPA and vehicle as control; $\circ$ and $\bullet$, groups treated with DMBA plus TPA and α- or β-carotene, respectively.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

PRODUCTION OF PALM OIL CAROTENOID CONCENTRATE AND ITS POTENTIAL APPLICATION IN NUTRITION

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Summary

Crude palm oil is known to be the richest natural plant source of carotenoids in terms of retinol (provitamin A) equivalents. Currently, however, these carotenoids are destroyed during physical refining of the oil. In view of the physiological importance of these carotenoids, various methods of isolation have been attempted and these include saponification, urea inclusion, selective solvent extraction, adsorption, molecular distillation and transesterification followed by distillation of esters. This paper will describe the processes developed in PORIM which yield carotenoid concentrates of > 80,000 ppm. These are based on (a) adsorption and (b) molecular distillation methods. Another method for the production of red palm oil will also be described. Chemical analysis of the carotenoid concentrates and red palm oil reveals eleven components viz α-carotene, β-carotene, lycopene, phytoene, phytofluene, α-zeacarotene, β-zeacarotene, ς-carotene, γ-carotene, δ-carotene and neurosporene. The presentation of the carotenoid concentrate in different forms (capsules, powder and emulsion) has been developed and the stability of carotenoids in these forms has been evaluated. Toxicological tests showed that the carotenoid concentrate is safe. A review of the biological activities of these carotenoids e.g. their ability to quench singlet oxygen, anti-tumour activity and anti-atherosclerotic activity, is presented.

Introduction

Carotenoids, a class of C40 polyunsaturated hydrocarbons, impart an orangy-red colour to palm oil. Crude palm oil contains the highest concentration of agro-derived carotenoids with a total concentration in the range of 500 - 700 ppm (Goh et al., 1985). It is in fact the world's richest natural plant source of carotenoids in terms of retinol (provitamin A) equivalents, having 15 times more retinol equivalents than carrots and 300 times more than tomatoes (Tan et al., 1987). Carotenes, in particular β-carotene, are known for their provitamin A activities as they can be transformed into vitamin A in vivo. The vitamin A equivalents of α-, β-and γ-carotenes and β-zeacarotene which are present in crude palm oil are 0.9, 1.67, 0.75 and 0.42 respectively (Isler, 1971; Morton, 1970). β-Carotene, besides being a precursor of vitamin A, has been shown to be an efficient quencher of singlet oxygen and as such is an effective antioxidant (Krinsky et al., 1982; Santamaria et al., 1988; Wefers et al., 1988; Machlin et al., 1987). α-Carotene and lycopene have also been reported to be effective singlet oxygen quenchers (Mascio et al., 1989). In fact, epidemiological studies in the 1980s strongly associate β-carotene with the prevention of certain types of cancers such as oral, pharyngeal, lung and stomach cancers (Sundram et al., 1989; Norman et al., 1988; Suda et al., 1986; 1986; Mathews-Roth et al., 1987; Peto et al., 1989; Mettlin, 1984). In this connection, the National Institute of Health has identified β-carotene as one of the first top ten cancer preventive agents. What is more interesting is the recent report on α-carotene which has been shown to be tenfold more potent as an anti-cancer agent than β-carotene (Murakosh et al., 1989). Of late, research has also indicated that β-carotene has a positive effect in the reduction of atherosclerosis (Gaziano et al., 1990).

Since carotenoids have grown in importance and value, their recovery from crude palm oil and palm oil by-products is of interest. The commercially planted oil palm in Malaysia is the Tenera palm, a cross between Dura and Pisifera, all belonging to Elaeis guineensis family, originating from West Africa. A higher concentration of carotenoids can be obtained from oils of Melanococca and their crosses with Elaeis guineensis palm

(Yap et al., 1992; Tam et al., 1976). Carotenes can also be obtained from a palm oil by-product such as palm pressed fibre (Choo et al. , 1991) and oil from a second pressing in the mills (Choo et al., 1989).

In the current technology of physical refining, the carotenoids in crude palm oil undergo thermal decomposition during deodorisation/deacidification processes (240°-270°C). As a result, the processed products, normally known as refined, bleached and deodorised (rbd) palm oil contain no carotenoids at all. This represents a tremendous loss of pro-vitamin A. It has been estimated that the loss of carotenoids through thermal destruction in 1991 is about 3,660 tonnes. This is certainly a paradoxical situation in view of the fact that carotenoids have been found to have important nutritional and pharmacological properties as mentioned earlier.

In view of the importance of carotenoids for public health applications, three methods have been developed to extract and concentrate them from palm oil and palm oil products.

Material and Methods

Preparation of Alkyl Esters Through Transesterification

Malaysian crude palm oil from the commercially planted Tenera oil palm species was transesterified with methanol or ethanol (AR grade) at a molar ratio of oil to alcohol 2:1, catalyzed by 0.5% (w/w) sodium hydroxide (AR grade) after the free fatty acid had been neutralized. The reaction mixture was stirred, heated to reflux and monitored by thin layer chromatography (TLC) (silica gel, solvent chloroform/hexane 1:1 (v/v)) until all the triglycerides were converted to alkyl esters. The ester layer was then separated from the glycerol layer, and was washed with distilled water until the washings became neutral. The final ester product was dried with Na_2SO_4 and the solvent removed under reduced pressure.

Concentration via Removal of Alkyl Esters

The carotenoids in the volatile alkyl esters obtained from transesterification were recovered through two different methods: (i) The alkyl esters were distilled under high

vacuum using a Sibata falling film molecular apparatus at a pressure of $< 10 \times 10^{-3}$ torr with a temperature ranging from 100 - 170°C; the carotenoid concentrate was collected as a residue. (ii) Alkyl esters obtained were dissolved in alcohol (methanol or ethanol, depending on whether methyl or ethyl esters were used) (1:2 v/v), and the mixture introduced onto the glass column packed with C_{18} reverse phase Silica gel. The colorless esters were eluted first, and excess alcohol was introduced until the carotenoids' band (red color) was about to be eluted out; hexane and alcohol (methanol or ethanol 98:2 v/v) or chloroform were then used to elute out this high carotenoid fraction adsorbed in the reverse phase.

Carotene Composition of Carotenoid Concentrate and Red Palm Oil

Qualitative and quantitative carotene profiles of carotenoid concentrates and red palm oil were carried out using a Varian 5000 HPLC equipped with a variable wavelength UV-100 detector. Isocratic separation was performed on a 5 μm Zorbax ODS column (4.6 mm ID X 25 cm) with a solvent system of acetonitrile (89%) and dichloromethane (11%) at a flow rate of 1 ml/min. α- and β-carotenes were used as external standards for quantitative studies.

Stability of Carotenoid

Carotenoid stability of the prepared oil capsule and powder forms has been determined for a period of one year at ambient and freezer (-15°C) temperatures.

Preparation of Deacidified and Deodorised Red Palm Oil

The oil sample was pretreated with 20% phosphoric acid (0.5% wt. of oil) at 90°C for 10 minutes, followed by bleaching earth (0.5% wt. of oil) at 110°C for 30 minutes. The oil was then filtered to remove the bleaching earth. The pretreated oil was then subjected to deacidification and deodorisation through molecular distillation, the oil was deacidified and deodorised and recycled from 130°C to 170°C at a flow rate of about 8 - 12 kg/hr. The various quality parameters of the pretreated deacidified and deodorised red palm oil were determined following either the AOCS or IUPAC methods.

Results and Discussion

Carotenoids have been recognised to be important nutritionally and thus numerous methods of extraction have been developed to recover them from crude palm oil; These include saponification methods (Tabor et al., 1948; Blaizot et al., 1953), urea process (Knafo, 1952), adsorption (Ong et al., 1980, Unilever Ltd., 1953; Mamuro, et al., 1986; Tanaka et al., 1986) selective solvent extraction (Tanaka et al., 1986; Passino, 1952), molecular distillation (Ooi et al., 1986) and transesterification followed by distillation of esters (Lion Fat and Oil Company, 1976; Eckey, 1949; Hara et al., 1988; Hama et al., 1986).

In this paper, three methods of extraction and concentration of carotenoids from palm oil and palm oil products are reported. The first method which involves transesterification of crude palm oil to alkyl esters (mainly methyl and ethyl esters) followed by molecular distillation of the volatile esters has led to the production of carotenoid concentrate of > 80,000 ppm. As the process involves very mild distillation conditions with pressure < 10×10^{-3} torr, temperature < 170°C and short residence time for ester in the heater, the valuable minor components such as carotenoids and vitamin E originally present in crude palm oil are still found unchanged (Ooi et al., 1988). This process has also been demonstrated on a pilot plant scale giving 75% recovery of carotene based on 17 kg of ester per batch. Analytical data of the carotenoid concentrate by HPLC is shown in Table 1 and a typical HPLC chromatogram of the palm oil carotenoid concentrate is shown in Fig. 1. 11 types of carotenes have been detected of which α- and β-carotenes constitute about 90% of the total carotenes present. It can be seen from Table 1 that the carotene profile of the carotenoid concentrate is similar to that of the starting material, indicating that the process has not destroyed the carotenes. The carotenoid concentrate prepared by this process has also been subjected to a toxicological study (Tan et al., 1991). This study which involved 4 groups of Spraque-Dawley rats (n=12 per group) were fed on a semi-purified diet supplement with 0.2% palm oil based carotenoid concentrate (20,000 ppm), methyl ester, ethyl ester and a control diet for 16 weeks. Histopathological examinations of the major organs such as heart, lungs , adrenals, kidneys, liver and spleen were found to be normal in all dietary groups. No extensive or

significant amount of fat was deposited in the heart and the coronary vessels and aorta was found to be normal in all dietary groups. It was concluded that the carotenoid concentrate and other dietary test groups do not have any toxicological effects on the major organs of the male rats.

The carotenoid concentrate prepared by this process has also been presented in three different forms for pharmaceutical application. These include capsules (both soft and hard) , powder and emulsion. The powder formulation has been successfully formulated and it could be made into carotene tablets, or encapsulated in hard capsules. Preliminary results on the storage stability tests show that the carotenoids in powder form during storage at room temperature were not as stable as carotenoid concentrate in the capsules. This could be due to greater exposure to light and air in the powder form, leading to increased oxidation or degradation of carotenoids. However, only a slight decline (< 4%) in carotenoid content was observed for the powder if it was kept in the freezer (-15°C) for a period of one year (see Figures 1 and 2).

The second method which involves preparation of carotene enriched palm oil has been effected and the process involves degumming of the oil with phosphoric acid followed by treatment with bleaching earth. The treated oil is then subjected to deodorisation and deacidification at mild reaction temperature to remove odoriferous materials as well as free fatty acids (Choo et al., 1988). More than 80% of the carotenes originally present in crude palm oil is retained. Analysis by HPLC shows that the profile of the carotenes is similar to that of the starting material, again indicating that carotenes are not destroyed during the process (see Table 1). The quality of this red palm oil (as shown in Table 2) has also been found to be good. It has been determined by sensory panel that it is suitable in food application (Nor Aini Idris, 1991). This process has also been upgraded to pilot scale operation and under these conditions, 75% of carotene in red palm oil is retained. It is observed that more than 80% of vitamin E originally present in crude palm oil is also retained.

It must be noted that in the second process, the triglycerides remain intact unlike the first process where all the triglycerides have been converted to alkyl esters. However, the first process could yield a higher concentration of carotenoids after the alkyl esters are removed.

Table 1. Carotene Composition (%) of Carotenoid Concentrate, Red Palm Oil and Crude Palm Oil.

Carotene	Carotenoid concentrate	Red palm Oil	Crude palm
phytoene	1.5	2.0	1.3
phytofluene	0.3	1.2	0.1
cis-β-carotene	0.9	0.8	0.7
β-carotene	49.9	47.4	56.0
α-carotene	33.3	37.0	35.1
cis-α-carotene	5.5	6.9	2.5
ς-carotene	1.7	1.3	0.7
γ-carotene	1.3	0.5	0.3
δ-carotene	0.6	0.6	0.8
neurosporene	0.1	trace	0.3
β-zeacarotene	1.3	0.5	0.7
α-zeacarotene	0.4	0.3	0.2
Lycopene	3.4	1.5	1.3
Total (ppm)	80,560	545	673

Table 2 Quality Parameters of Red Palm Oil

Carotenes	> 80% intact
Tocopherol and Tocotrienols	>80% intact
Free fatty acids	< 0.1%
Peroxide value	< 0.2
Phosphorus content	< 2 ppm
Moisture and impurities	< 0.1%

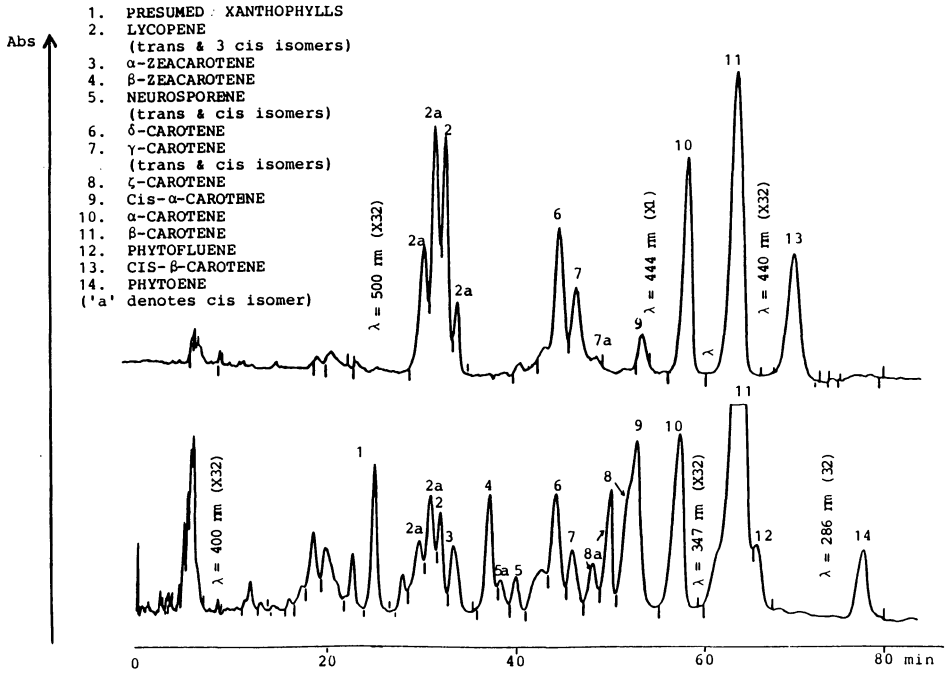


FIGURE 1. HPLC OF CAROTENOIDS OF PALM OIL

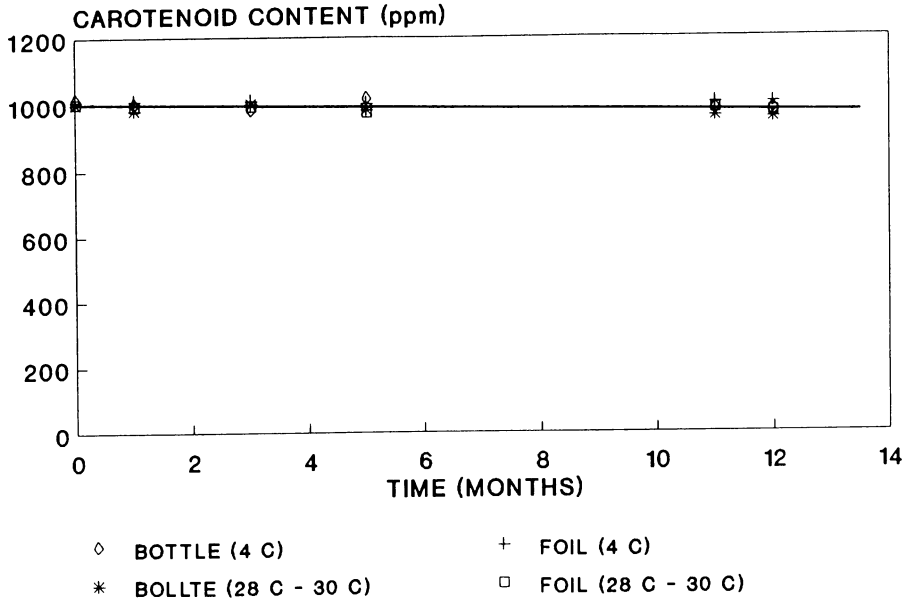
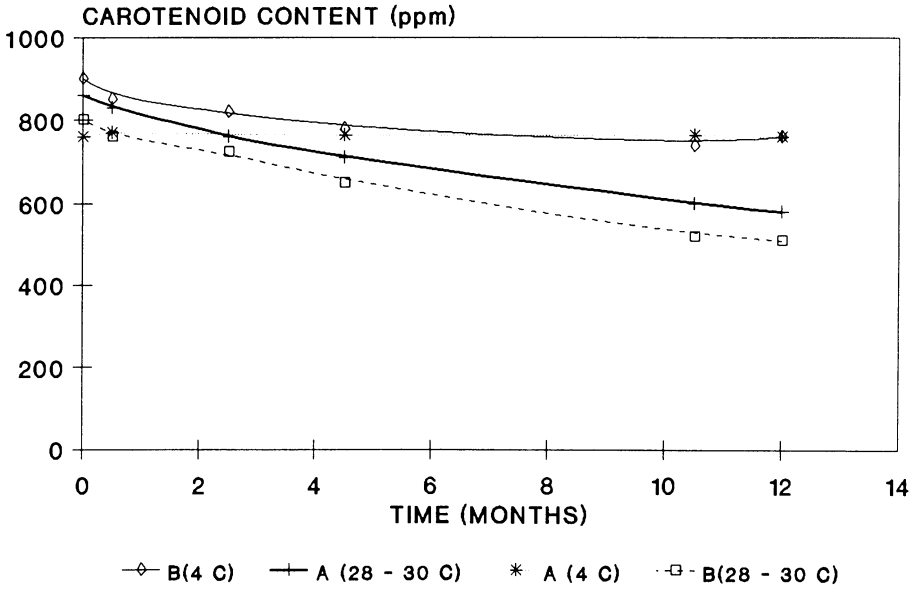


FIGURE 2. STORAGE STABILITY OF CAROTENOIDS IN CAPSULE FORM



A=Samples kept in sample bottle
B=Samples kept in clear bottle

FIGURE 3. STORAGE STABILITY OF CAROTENOID IN POWDER FORM

The third method of carotenoid concentrate has been investigated using C_{18} reverse phase column chromatography (Choo et al., 1991). A recovery of >90% (w/w) can be obtained through this method and the column can be reused for >50 times without any loss of activity. This process, however, requires further work.

Conclusion

Palm oil carotenoids can be successfully obtained for food and pharmaceutical applications. The safety of application is assured by the results of the toxicological study.

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CAROTENOIDS, NOVEL POLYENE POLYKETONES AND NEW CAPSORUBIN ISOMERS AS EFFICIENT QUENCHERS OF SINGLET MOLECULAR OXYGEN

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Summary

The singlet molecular oxygen ($^1O_2$) quenching activity of carotenoids and newly synthesized polyene polyketones and capsorubin isomers was examined using the thermodissociable endoperoxide of 3,3'-(1,4-naphthylene) dipropionate ($NDPO_2$) as $^1O_2$ source and a germanium diode to monitor $^1O_2$ photoemission. C_{28}-polyenetetrone (1) exhibits the highest physical quenching rate constant with $^1O_2$ (k_q = 16 x 10^9 $M^{-441}s^{-1}$). For comparison, the rate constant for the most efficient biological carotenoid, lycopene (3) is k_q = 9 x 10^9 $M^{-1}s^{-1}$ and that of β-carotene (5) k_q = 9 x 10^9 $M^{-1}s^{-1}$. The presence of two oxalyl chromophores at the ends of the polyene chain seems to enhance the $^1O_2$ quenching ability in the C_{28} -polyene-tetrone (1). C_{28}-polyene-tetrone-diacetal (2) (k_q = 9 x 10^9 M^{-1} s^{-1}) and C_{40}-epiisocapsorubin (4) (k_q = 8 x 10^9 M^{-1} s^{-1}) also have high $^1O_2$ quenching abilities.

Due to the high singlet oxygen quenching abilities, C_{28}-polyene-tetrone (1), C_{28}-polyene-tetrone-diacetal (2) and C_{40}-epiisocapsorubin (4) may have potential use in preventing $^1O_2$-induced damage in biological and nonbiological systems.

Introduction

Biological systems produce singlet molecular oxygen ($^1O_2$) by photooxidation of endogenous and exogenous compounds and by enzyme-catalyzed reactions (Sies, 1986; Kanofsky, 1989). $^1O_2$ is responsible for photodynamic destruction of biologically and commercially important molecules, including DNA damage (for review see Piette, 1990), measured as single-strand breaks in plasmid DNA, resulting in the loss of biological activity and mutagenesis (Wefers et al., 1987; Schneider et al., 1990; Devasagayam et al., 1991a). Hence there is an interest in efficient quenchers of $^1O_2$. Carotenoids and other polyenes are among the most efficient $^1O_2$ quenchers (Foote, 1979; Monroe, 1985). Because of the efficiency with which they protect biological systems from photodynamic and other forms of oxidative damage (Krinsky, 1989), carotenoids are of considerable interest in health and nutrition (Ames, 1983; Di Mascio et al., 1991a). The discovery that carotenoids quench $^1O_2$ was an important advance in our understanding of the effects of carotenoids in preventing damage in biological systems (Foote and Denny, 1968; Foote et al., 1970).

A wide variety of foods owe their color mainly to carotenoids. Capsorubin is a carotenoid with optically active cyclopentanol end groups, isolated from red pepper (Capsicum annuum). Red pepper is an important spice used in the curries in Indian and Mexican cooking. The deep red extract of red pepper containing capsorubin is added to a variety of food products (Isler, 1971). The advent of synthetic carotenoids has increased interest in coloring of food (Ruttimann, 1982).

It is generally accepted that an increasing number of conjugated double bonds is associated with a better quenching ability against $^1O_2$ (see reviews Krinsky, 1979; Mathis and Schenck, 1982 and refs. therein). Recently, Di Mascio et al. (1989), Truscott (1990) and Conn and Truscott (personal communication) have studied the $^1O_2$ quenching abilities of carotenoids with similar numbers of conjugated double bonds, however revealing differences in their $^1O_2$ quenching abilities, e.g. for β-carotene and lycopene, which may

be taken as indication for a role of the spectral properties. To examine whether the different behavior could be due to structural differences of the end groups, we studied a number of new carotenoids. This work has been presented in full by Devasagayam et al. (1991 b)

Materials and Methods

Materials

β-Carotene (5) and lycopene (3) were gifts from Dr. Bausch, Hoffmann-La Roche (Basel, Switzerland). Novel polyene polyketones (1, 2, 6, 9, 10) and synthetic capsorubin analogues (4, 7, 8, 1) were synthesized and their structures ascertained by NMR-, MS-, UV- and IR-spectroscopic analyses (Martin, Ippendorf, Werner, manuscript in preparation). The compounds used in this study whose structures are given in Fig. 1 are as follows:

6,10,15,19-tetramethyl-tetracosa-4,6,8,10,12,14,16,18,20-nonaene-2,3,22,23-tetraone (1) (C_{28}-polyene-tetrone); 6,10,15,19-tetramethyl-2,2,23,23-tetramethoxy-tetracosa-4,6,8,10,12,14,16,18,20-nonaene-3,22-dione (2) (C_{28}-polyene-tetrone-diacetal); lycopene (3); C_{40}-epiisocapsorubin (4); β-carotene (5); 8,13-dimethyl-2,2,19,19-tetramethoxy-eicosa-4,6,8,10,12,14,16-heptaene-3,18-dione (6) (C_{22}-polyene-tetrone-diacetal); C_{34}-epiisocapsorubin 7); C_{34}-capsorubin (8); 6,11-dimethyl-hexadeca-4,6,8,10,12-pentaene-2,3,14,15-tetraone(9) (C_{18}-polyenetetrone); 6,1 -dimethyl-2,2,15,15-tetramethoxy-hexadeca-4,6,8,10,12-pentaene-3,14-dione (10) (C_{18}-polyene-tetrone-diacetal); C_{30}-epiisocapsorubin (11).

The endoperoxide of the disodium salt of 3,3'-(1,4-naphthylene) dipropionate $NDPO_2$ was prepared as described (Di Mascio and Sies, 1989).

Generation and Quenching of Singlet Molecular Oxygen

Singlet oxygen was chemically generated by using the thermodissociation of the endoperoxide of 3,3'(1,4-naphthylene) dipropionate ($NDPO_2$) yielding 3,3'-(1,4-naphthylene) dipropionate (NDP), $^1O_2$ and $^3O_2$ (Di Mascio & Sies, 1989). At

37°C, 3mL of deaerated ethanol/chloroform (1 : 1) was placed in a thermostated, closed glass cuvette. The reaction was started by adding 30 to 50 μL of $NDPO_2$ dissolved in D_2O to give a final concentration of 5 mM $NDPO_2$.

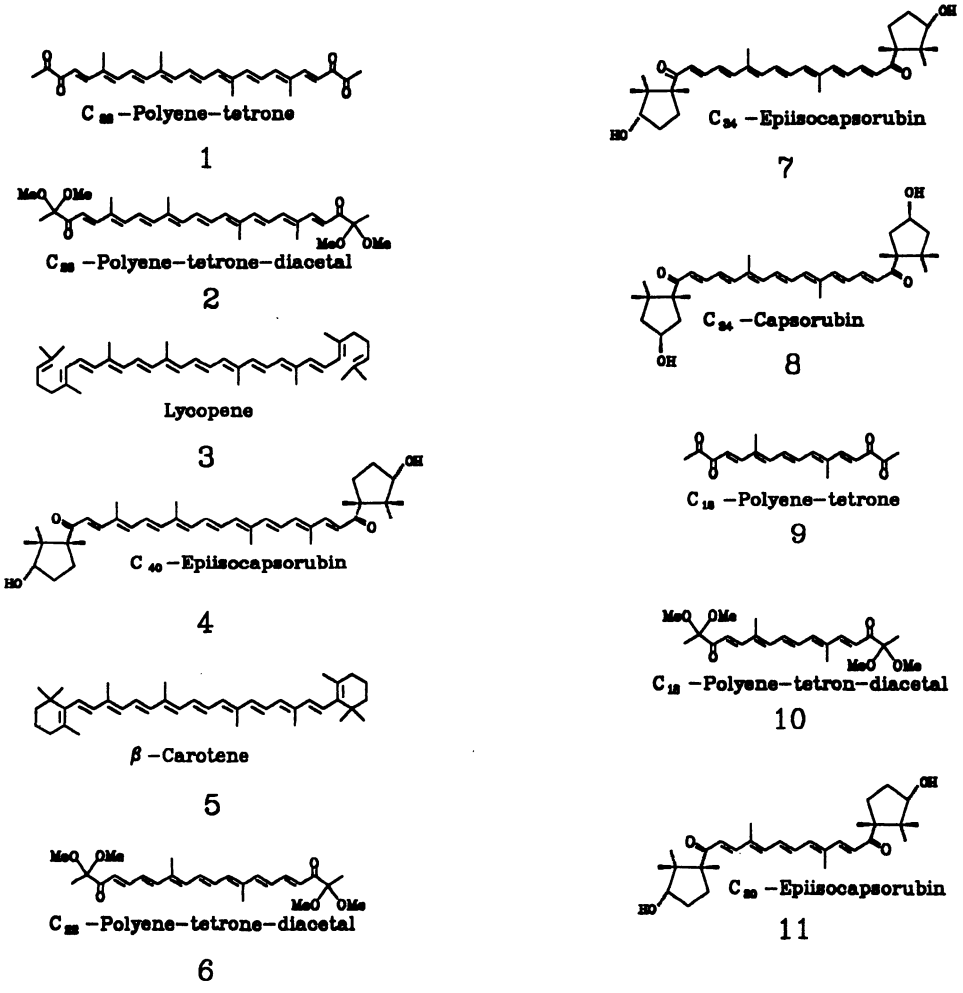

Fig. 1: Chemical structures of the compounds used.

$$O_2(^1\Delta_g) \text{----------} > O_2(^3\Sigma_{-g}) \quad + \quad h\nu \ (1270 \text{ nm})$$

Infrared photoemission of $^1O_2$ (reaction 1) was monitored at 1270 nm using a liquid nitrogen-cooled germanium diode photodetector (Model EO-817L, North Coast Scientific Co., Santa Rosa, CA, U.S.A.), as described in detail (Di Mascio & Sies, 1989; Di Mascio et al., 1991b). The stock solutions of the carotenoids were freshly prepared in deaerated chloroform and kept on ice until use to minimize oxidation.

At the maximum of the monomol emission signal, which was achieved within 5-6 min, 10 to 50 μL of the quencher (carotenoids) solution were added (see Fig. 2) and the overall quenching constant ($k_q + k_r$) calculated from Stern-Volmer plots, using equation (2):

$$So/S = 1 + (k_q + k_r) \ \tau[Q] \qquad (2)$$

where So and S are the photoemission intensities in the absence and presence of the quencher, respectively; k_q physical quenching rate constant; k_r, chemical reaction rate constant; r, lifetime of $^1O_2$ in the solvent; and [Q], quencher concentration. Fig. 3 shows Stern-Volmer plots for three polyenes with high quenching abilities and for β-carotene. The $^1O_2$ lifetime was estimated using time-resolved spectroscopy according to Valduga et al., (1988). The r-value in the solvent system (chloroform : ethanol water: deuterium oxide, 75: 75: 1 : 1) is 33.2 $\pm$ 0.9 μs. As $k_q >> k_r$, k_r was negligible.

Results

As shown in Table I and Fig. 3 C_{28}-polyene-tetrone (1) was the most effective compound with a k_q value of 16 x 10^9 M^{-1} S^{-1}, 1.8-fold higher than that for lycopene (3) (k_q = 9 x 10^{-9} M^{-1} s^{-1}), the biological carotenoid with the highest quenching ability (Di Mascio et al., 1989; due to the lifetime of 33.2 μs, this value is lower than that reported previously, calculated with 10 μs). The presence of the oxalyl end groups in the polyene chain of (1) apparently enhance the quenching ability. Polyenes with similar carbonyl end groups (2) and (4) have nearly identical $^1O_2$ quenching abilities, with a k_q value of 8 x 10^9 M^{-1} s^{-1} and 9 x 10^9 M^{-1} s^{-1}.

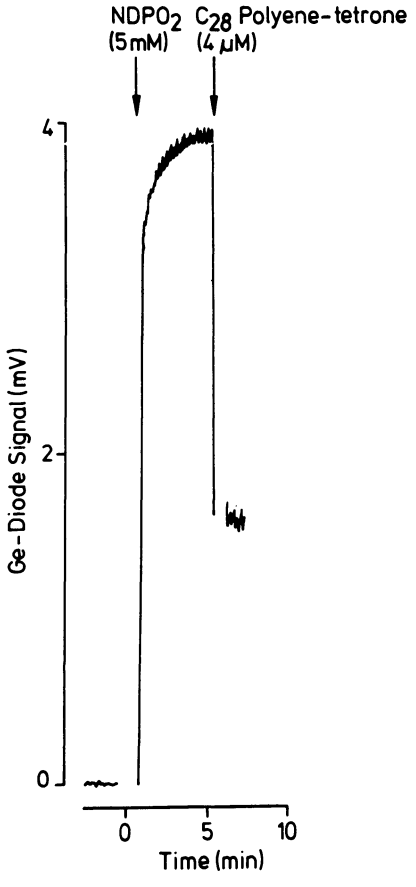

Fig. 2: Loss of the $NDPO_2$-generated singlet oxygen monomol emission signal at 1270 nm by various polyenes in dependence on quencher concentrations.

The polyenes with lower number of conjugated double bonds exhibit much less quenching. C_2-polyene-tetrone-diacetal (6), C_{34}-epiisocapsorubin (7) and C_{34}-capsorubin (8) have k_q values around 2×10^9 M^{-1} s^{-1}. The percent loss of germanium diode signal at 1 mM for other compounds are as follows: C_{18}-polyene tetrone (9), C_{30}-epiisocapsorubin (11), C_{18}-polyene-tetrone-diacetal (10), 15-25.

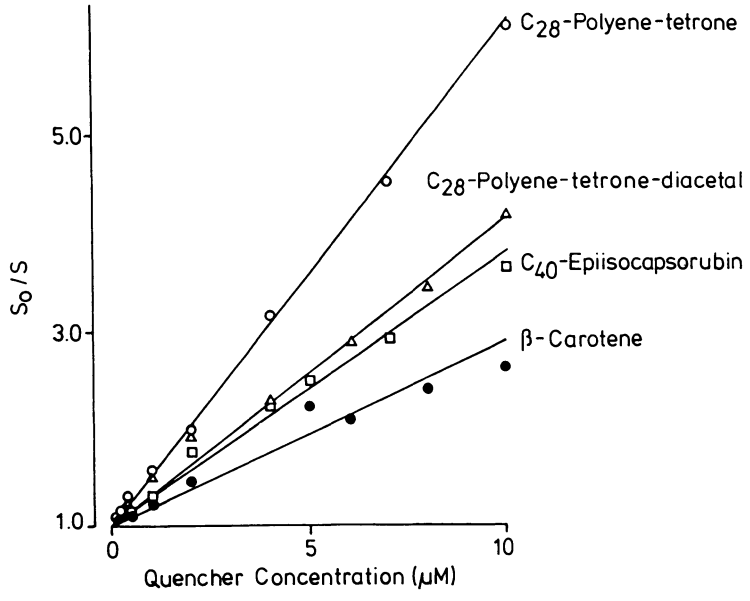

Fig. 3: Stern-Volmer plots for the quenching of singlet oxygen by C_{28}-polyene tetrone, C_{28}-polyene tetrone diacetal and C_{40}-epiisocapsorubin as compared to β-carotene in chloroform : ethanol (1 : 1).

The absorbance maximum corresponding to the lowest excited vibrational state is also given in Table I for each compound.

Discussion

In agreement with the former studies mentioned in the introduction, we found that a relationship exists between $^1O_2$ quenching ability and structure of the carotenoid : compound (1), the carotenoid with the largest red shift, is the most efficient $^1O_2$ quencher.

Table I

Singlet oxygen quenching abilities and $\pi\pi^*$ absorbance maxima of carotenoids and new synthetic carotenoids as compared to lycopene and β-carotene.

Compound		$k_q * 10^9$ [$M^{-1} S^{-1}$]	$\pi\pi^*$ absorbance maximum [nm]
C_{28}-polyene-tetrone	(1)	16 ± 1	533
C_{28}-polyene-tetrone-diacetal	(2)	9 ± 1	525
Lycopene	(3)	9a	515
C400-Epiisocapsorubin	(4)	8 ± 1	523
β-Carotene	(5)	5a	490
C_2-Polyene-tetrone-diacetal	(6)	≈2	482
C_{34}-Epiisocapsorubin	(7)	≈2	475
C_{34}-Capsorubin	(8)	≈2	475
C_{18}-Polyene-tetrone	(9)	0.01	450
C_{18}-Polyene-tetrone-diacetal	(10)	<0.01	432
C_{30}-Epiisocapsorubin	(11)	<0.01	428

The absorbance maxima are the lowest vibrational state of the $\pi\pi^*$, absorbance maximum. Data are in $CHCl_3$. The k, values are means ± S.E.M. of three or four independent titrations, or of two titrations (at least 10 data points per titration). The k_q values are derived from Stern-Volmer plots.

[a]Modified from Di Mascio et al. 1989 (calculated for measured $^1O_2$ lifetime τ=33.2 μS in the solvent system of this work).

The origin of the bathochromic absorption of such unsaturated α-diketones has been discussed recently (Martin et al., 1991a,b). The carotenoids (2), (3) and (4) or (6), (7) and (8) with structurally different end groups but very similar λ_{max} values have nearly identical quenching abilities. Substantially smaller quenching rate constants were obtained for the carotenoids (9) - (11). Compounds with 4. values lower than 475 nm are unable to quench $^1O_2$ efficiently, as shown in Table 1.

Moreover, the relationship between k_q and λ_{max} can be assigned to other carotenoids (Di Mascio et al., 1989; Conn and Truscott, personal communication). Even though the λ_{max} value (S_0-S_2 energy gap) of carotenoids correlates well with their $^1O_2$ quenching ability, it is most likely triplet state T_1(S_0-T_1, energy gap) of the carotenoid that is involved in the energy transfer process. Gijzeman and Sykes (1973) arid Truscott et al. (1973) showed that an approximate linear relationship exists between the energies of the triplet-triplet absorption maxima and their ground state maxima. Similar observations were made by Mathis and Kleo (1973) and Bensasson et al. (1976), reporting a linear relationship between the inverse of the estimated triplet energy level ($1/E_T$) and the -length of the conjugated polyene chain.

Acknowledgments

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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MODIFICATION OF ALLOXAN DIABETES IN RATS BY VITAMIN A STATUS

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Summary

The source of alloxan and its role in the aetiology of diabetes mellitus in man is unclear. Alloxan can be found in body fluids in man during diseases and may be formed from a related industrial material. Its structure closely resembles pyrimidine and may be derived from uric acid. Alloxan-induced diabetes is mediated via free radical damage of pancreatic β-cells. Therefore, antioxidants or free radical scavengers may alter the diabetogenic action of the chemical. The aim and the design of the study is to test the susceptibility of the animal with various vitamin A status to diabetes after a challenge of 100 mg/Kg body weight of alloxan. Physiological and histological parameters, fasting plasma glucose and islet size and number respectively, are monitored as indexes of the severity of the induced diabetes over a period of three weeks. Since retinol has been found to stimulate β-cancer cell but not normal β-cell to secrete insulin in vitro, the effect of vitamin A supplementation on the established diabetes in vivo is also studied for three more weeks. It is concluded that vitamin A deficiency increases the susceptibility of rats to alloxan. Supplementation of retinyl palmitate at twice of normal level of intake confers no additional protective effect against alloxan damage over the control. In the vitamin A depleted rat, replenishment of retinoid does not ameliorate the elevated fasting glucose level, but does restore the total islet size (size x number of islet) comparable to the supplemented group.

Introduction

Alloxan has been widely used to induce diabetes mellitus in experimental animals (Fischer and Rickert, 1975; Malaisse, 1988) since its β-cytotoxic (Dunn, Sheehan and McLetchie, 1943) and diabetogenic (Dunn and McLetchie, 1943) effect was discovered nearly half a century ago. The chemical may be formed directly from either uric acid or alloxantin, an industrial material related to alloxan (Windholz, Budavari, Blumetti and Otterbein, 1983). Also, alloxan and alloxantin have been found in body fluids under pathological conditions. Furthermore, the chemical structure of alloxan (2,4,5,6-tetraoxohexahydropyrimidine) closely resembles that of pyrimidine. The role of alloxan and its related substances in the pathogenesis of diabetes in man remains unanswered and whether the source of alloxan is exogeneous or endogeneous stays unclear (Lukens, 1948; Lazarow, 1949). Epidemiological data of environmental alloxan are still lacking, though of other chemicals are well documented (Miller, Stokes and Silpipat, 1978; Helgason and Jonasson, 1981; Bouchard, Sai, Reach, Caubarrere, Ganeval and Assan, 1982).

Alloxan-induced diabetes is mediated via free radical damage of the pancreatic , β-cell (Rerup, 1970; Okamoto, 1990) and antioxidants or free radical scavengers (Lukens, 1948; Lazarow, 1949; Sumoski, Baquerizo and Rabinovitch, 1989) have been implicated to prevent the alloxan diabetes. Nutritional antioxidants such as vitamin A, E and C have been tested for this effect. Vitamin A given immediately after alloxan injection (Chertow, Webb, Leidy Jr. and Cordle, 1989) or a long term supplementation of vitamin E (Slonim, Surber, Page, Sharp and Burr, 1983) have been shown to ameliorate the animals from the toxicity of alloxan. On the other hand, water soluble antioxidant, vitamin C, has no protective effect on the toxicity of alloxan when they are mixed prior to injection (Lukens, 1948).

The protective effect of vitamin A against alloxan-induced diabetes is dependent on the route of administration, which is much more evident if both are injected intraperitoneally than intravenously. The aim of the study is to investigate if the nutritional status of vitamin A, either supplemented or depleted, will alter the susceptibility of the animals to the same diabetogenic dosage of alloxan. This study will

pose an interesting relation between nutrition and free radical damage to the pancreas, which may arise due to autoimmune disorders (Sumoski, Baquerizo and Rabinovitch, 1989) and environmental triggering factors such as toxins or pollutants.

Since retinoids have been reported to stimulate insulin secretion in β-cancer cell line in vitro (Chertow, Moore, Blaner, Wilford and Cordle, 1989) but not in normal islets (Chertow, Webb, Leidy Jr. and Cordle, 1989), the effect of vitamin A supplementation on the alloxan-diabetic rats in vivo is also studied. Fasting plasma glucose and size of the islets of Langerhans will respectively serve as the physiological and histological indexes of the recovery of alloxan diabetes, if any, after the supplementation.

Methods and Materials

Experimental Protocol

Sprague-Dawley rats were accustomed to either of the following treatments for two weeks since weaning, being fed on normal rat chow (Purina 5010 diet, St. Louis, MO), vitamin A deficient diet (ICN Biomedicals, Cleveland, OH) or the same diet but supplemented with a weekly intramuscular injection of retinyl palmitate (Sigma, Chemicals, Co., St. Louis, MO) in olive oil at a dosage of 20mg/Kg body weight. This supplied twice of the normal weekly intake of retinol. Size of the normal control, vitamin A deficient and vitamin A supplemented groups were 10, 12 and 12 respectively.

The rats were housed in an environment with controlled temperature and humidity, and with an alternative 12 hour light/dark cycle. Tap water and rat chow were consumed ad lib.

Induction of Diabetes with Alloxan

When the rats were about 2 month old and weighed 120-150g, they were induced diabetic by injecting intraperitoneally 100mg/Kg body weight of alloxan (Sigma Chemicals

Co., St. Louis, MO), which had been freshly dissolved in deionised distilled water. Injection of alloxan was 3 days apart from the last vitamin A injection to reduce any chemical interaction. Fasting plasma glucose level was monitored weekly with the Sera-Pak (Ames division, Miles Italians, Milano, Italy) since the induction of diabetes mellitus in week 0 up to week 6. From week 3 to week 6, the vitamin A deficient rats were given the same dose of retinol as the supplemented rats were receiving. The control continued to receive weekly the vehicle of O.1ml of olive oil whereas the supplemented rats continued with the retinol supplementation until week 6.

Histologic Study

Pancreases of two rats were excised from each of the three groups in week 0, 3 and 6, before and after vitamin A supplementation in deficient and supplemented groups as compared to the control. Islets were stained with aldehyde fuchsin and nuclei with celestone blue and haemalum (Bancrofit and Cook, 1984). Thirty slides from each pancreas were randomly selected. Photomicrographs of all islets observed were taken at 400x manification and islet size was traced on paper and weighed for comparison.

Fasting Plasma Glucose Determination

Rats were fasted overnight from 17:00 of previous day to 13:00 when fasting plasma glucose was determined. The rats were refrained from agitation in a tight-fit restrainer cage for about 20 minutes before blood was collected from tail veins. Plasma was prepared by the same method as determining haematocrit. About 50μl of blood was collected in heparinized capillary tubes (Bilbate Ltd., Daventry, England) and then sealed with plasticine. Red blood cells were centrifuged down in a micro-haematocrit centrifuge (Hawksley, England) for 2 minutes and the part of the capillary tube with plasma was cut off with a glass cutter. Plasma was blown out into an Eppendorf tube. 20μl of plasma was mixed with 2.5ml of the reagent solution containing glucose oxidase, peroxidase and a chromogen which absorbs 505nm light for the end-point determination of plasma glucose.

Statistical Analysis

Statistical analyses consisted of two-sample t-tests for the two-tailed comparisons of vitamin supplemented or depleted group with control (Zar, 1984).

Results

Administration of alloxan led to an increase of the fasting plasma glucose level which peaked in week 3 in all groups of rats. Thereafter, recovery was observed and fasting glucose level gradually declined, irrespective of the vitamin A status.

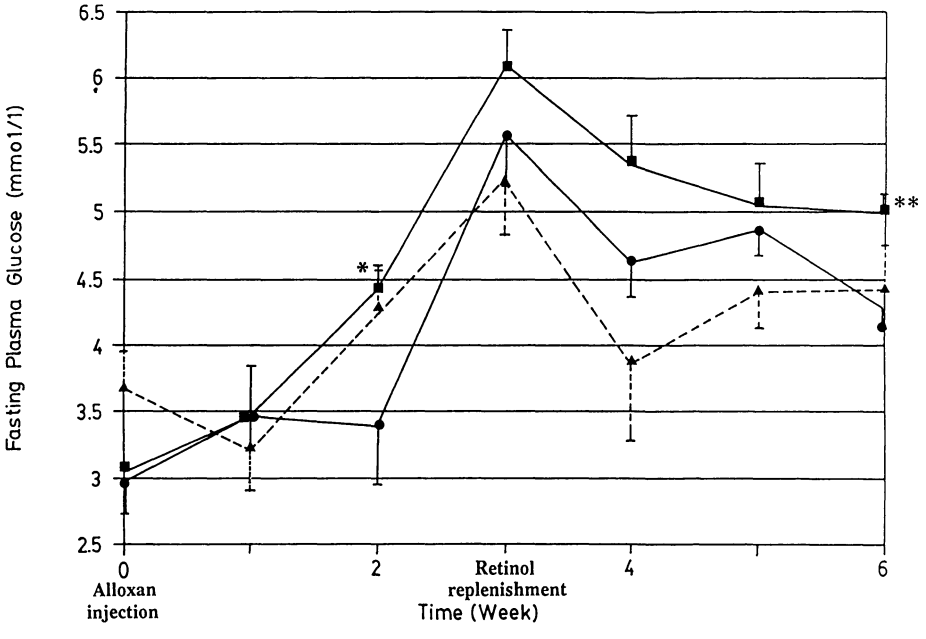
The severity of alloxan diabetes in rats with various status of vitamin A was reflected by the level of fasting plasma glucose (Chertow, Webb, Leidy Jr. and Cordle, 1989) and inversely by the islet size (Dunn and McLetchie, 1943). The time course of the change in fasting glucose was shown in figure 1. The fasting glucose level of the vitamin A depleted group was statistically significant from the control in week 2 and week 6, $p < 0.05$ and $p < 0.1$ respectively with the control rats.

The increase in the fasting glucose level was reflected by the reduction of the islet size. By week 3, islet size was reduced about 10 times and 30 times of the prediabetic stage of the control and of the supplemented rats, respectively. The reduction of islet size was more prominent, being 250 times in the depleted group.

When vitamin A supplementation was started in the deficient rats, the deficient group still maintained the highest fasting plasma glucose which was statistically greater than the other two groups by week 6. Total islet size (Number x size of islet) of the supplemented rats in both supplemented and deficient groups was the same.

Discussion

Injection of alloxan caused a steady increase of fasting plasma glucose over the first three weeks. Thereafter, spontaneous recovery was observed in the control rat (Patent and Alfert, 1967) and the fasting glucose level gradually dropped. Over 90% of the total islet size (and function) was destroyed by alloxan (Table 1) and led to diabetes.



(*p<0.05; **p<0.1)

Figure 1. Time course of the fasting plasma (Mean ± SEM) in Vitamin A Deficient rat (■) and supplemented rat (▲) versus control (●)

TABLE 1

Change of total islet size [number(n) x size of islet (in arbitrary unit)] after the induction of diabetes in week 0 and retinoid replenishment to the depleted rat in week 3

Week	0	3	6
Control	10 (n = 80)	1.11 (n =11)	1.11 (n = 11)
Depleted	10 (n = 82)	0.04 (n = 2)	0.45 (n = 6)
Supplemented	10 (n = 78)	0.35 (n = 6)	0.45 (n = 10)

The reduction in the total islet size is comparable to the standard procedure of 90% pancreatectomy in experimental diabetes induced by surgical means (Martin and Lacy, 1963). The size and the number of the islet in the control remained the same at week 3 and 6.

In the vitamin A depleted rats, same dosage of alloxan caused a much more elevated fasting glucose level, implying that the damage to the β-cells was more profound. The fasting plasma glucose was the highest of the three groups. The physiological change of fasting glucose level is also reflected by the reduction of the islet size and number. This suggests that vitamin A deficiency increases the susceptibility of rats to alloxan. After the depleted group was later replenished with retinyl palmitate, the fasting glucose level slightly improved but it was still the most elevated of the three groups. Therefore, vitamin A supplementation does not ameliorate the established diabetes, which supports the findings that retinoids does not stimulate insulin secretion from intact islets in vitro (Chertow, Webb, Lxidy Jr. and Cordle, 1989).

Although supplementation of vitamin A to the deficient rats led to a restoration of the total islet size comparable to that of the supplemented group, but fasting glucose of the former group remained significantly higher. A well-known function of vitamin A is its role in maintaining the normal differentiation of epithelial tissues (De Vet, 1989) and

since the pancreas is originated from the epithelium of duodenum (Langman, 1979), the supplementation may regenerate the islet of Langerhans (Patent and Alfert, 1967) but the secretion of insulin remains impaired.

In the retinol supplemented group, the vitamin A supplementation throughout the study period conferred no addition protective effect against alloxan toxicity over the control. The fasting glucose level was not statistically different from the control, even though the reduction of the total islet size was slightly more.

As a conclusion, vitamin A deficiency increases the susceptibility of the animals to alloxan diabetes. On the other hand, vitamin A supplementation at twice of the daily level of intake does not confer any additional protection over the control against the alloxan toxicity. The protective effect of retinyl palmitate given immediately after alloxan injection is not observed when the vitamin A is slowly released from the intramuscular depot. Probably the discrepancy is due to the slow rate of the depot releasing the retinoid and thus the toxicity of the alloxan challenge is not attenuated by sufficient amount of vitamin A in the blood. Vitamin A must be given within a short span of time from the alloxan-insult in order to exert its protective effect over the alloxan diabetes. Supplementation of retinoid has no effect in ameliorating the elevated fasting glucose level in vivo. This observation is in agreement with the findings that retinoids do not stimulate insulin secretion in normal intact islets in vitro.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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FLAVONOIDS IN FOODS: THEIR SIGNIFICANCE FOR NUTRITION AND HEALTH

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Summary

Flavonoids are regular components of the human diet found in vegetables and fruits that have long been considered inert and nonessential for human health. In the last decade, however, they have become the subject of intensive investigations because of their possible beneficial as well as deleterious effects. These studies have revealed that many flavonoids possess a variety of biological activities. Although some flavonoids are mutagenic in short term in vitro assays, they may also have certain "protective" effects on some human diseases, including cancer. Since flavonoids are natural antioxidants, they play a role in preserving many biologically beneficial compounds in foods, as well as scavenging electrophilic species and oxygen free radicals formed during metabolic processes in humans. Studies have been conducted to investigate the therapeutic potential of flavonoids for the prevention or treatment of certain human diseases. In contrast, however, there is also evidence of the potentially hazardous nature of flavonoids (e.g. enhancement of nitrosation) . The main obstacle to explaining their effects (either beneficial or hazardous) is the lack of information on their mechanism of action. We explored the effect of 3 plant polyphenols: quercetin, ellagic acid and chlorogenic acid when consumed with two carcinogens: benzo(a)pyrene [B(a)P] and 2-amino-3-methylimidazo(4,5-f)quinoline [IQ], using short term in vivo studies with mice and rats. We used mice in a host-mediated mutation assay and to study the uptake of (^{14}C)-B(a)P, and bile-cannulated rats for uptake of (^{14}C)-B(a)P and (^{14}C)-IQ. Our results indicate that flavonoids may exhibit a "protective" effect against carcinogens not only at cellular or enzymatic levels, but also by reducing their bioavailability.

Introduction

The extent to which diet and dietary habits can modify the development of chronic diseases in humans, such as cancer and cardiovascular diseases, has been the subject of widespread debate and extensive investigations. In the case of cancer, there is a great deal of epidemiological evidence suggesting that dietary factors are responsible for the occurrence of many major human cancers (Alfin-Slater and Kritchevsky, 1991). On the other hand, there are also numerous reports in the literature (for a review see: Birt and Bresnick, 1991), which indicate that the incidence of neoplasia induced by known carcinogens in experimental animals (rats, mice) can be significantly reduced if the animals' diet is supplemented (at an average of 20%) with cauliflower, cabbage, brussels sprouts or alfalfa. Furthermore, epidemiological studies also indicate that daily consumption of vegetables appears to be associated with a reduction of many types of cancer (Negri et al.,1991), and also with a reduced incidence of neoplasia in a number of regions of the world (Hirayama, 1986). Since vegetables, which are regular components of the Western diet, contain a variety of both nutritive and non-nutritive ingredients, it is difficult to identify the active component(s) (e.g. vitamins, dietary fibre, minerals, polyphenols, chlorophyll, antioxidants, carotenoids) that may be responsible for the inhibitory effect on neoplasia (Birt and Bresnick, 1991). It is most likely that this protective effect is not due to a single agent, but to a combination of many ingredients. This is in agreement with experimental observations in laboratory animals, in which "vegetables, or mixtures of agents, have generally been found to have greater cancer inhibitory ability than single substances that have been isolated therefrom" (Birt and Bresnick, 1991). Nevertheless, it would be worthwhile to identify at least the type(s) of dietary constituent, that may act as anticarcinogens or contribute toward the inhibition of human cancers (Wattenberg,1983). Food polyphenols, and especially flavonoids, which have long been thought to be nonessential for human health, may be just such a group of food ingredients (Stich and Rosin, 1984; Birt and Bresnick, 1991) . Recently it has been suggested that flavonoids might be "useful as new chemopreventive agents in human carcinogenicity" (Gabor, 1988). The evidence for this, however, is fragmentary and, so

far, controversial. This review will summarize the highlights of investigations regarding the biological properties of flavonoids, concentrating mainly on the antimutagenic and anticarcinogenic aspects.

The first part of the report will review the recent information regarding the presence and general biological activities of flavonoids in foods, emphasizing mechanisms that have been suggested for their apparent beneficial influence on human health. Subsequently, we will present an overview of the work being carried out in our laboratories on the influence of polyphenols on the bioavailability of xenobiotics.

Flavonoids in Human Diet

Intake

Flavonoids, as a large group of plant polyphenols, are widely distributed in variable amounts in foods consumed by humans. They have little nutritional value, and generally are considered to be inert and nonessential for human health, although they provide certain benefits to plants. Eating a regular Western diet it is practically impossible to avoid consuming flavonoids. Edible plants such as fruits or vegetables contain significant amounts of flavonoids, and their concentrations are consistently and significantly greater in the leaves, skin and peel than in their deeper tissues (Herrmann, 1976). For instance, in the outer skins of certain onions, e.g. Allium cepa, the flavonol content may reach up to 6.5% of the fresh weight (Herrmann, 1976). In soft parts of fruits and their juices, the flavonol concentration (usually as the o-glycosides) may be 100 mg/kg fresh weight (Pierpoint, 1986). Apples, prunes, citrus fruits, cabbage, lettuce and potatoes are only a few examples of fruits and vegetables in which flavonoids are found in substantial amounts (IARC, 1983). A cup of brewed black indian tea may contain over 40 mg of flavonoids.

Estimates for the average daily intake of flavonoids in the western diet vary from 50 mg (Brown, 1980) to about 1 g/person (Pierpoint, 1986). Quercetin and kaempferol are the two most abundant flavonols in our diet. Pierpoint (1990) suggested that the daily

intake of the polymerized flavonoids could be considerably higher. He estimates the daily intake of these polyphenols to be almost 900 mg, from tea alone, for an adult in the UK.

Biological Effects

Since the flavonoids were generally considered to be nontoxic, "inert" or "semi-essential" components of the diet (Kuhnau,1976), up till recently little attention had been devoted to their potential nutritional, biochemical, physiological or pharmacological role in human health.

There is well documented experimental evidence (Birt and Bresnick, 1991 and refs. therein; Stich, 1991 and refs, therein), mainly from the in vitro assays, that many flavonoids may activate or inhibit enzymatic processes of the liver and other organs. For illustration, only a few of the recently reported effects will be mentioned here (for a review see Newmark, 1987).

Certain flavonoids have been found to inhibit protein phosphorylation by protein kinases in human neutrophils and platelets. Protein phosphorylation is involved in the activation of these cells during inflammation (Wallace, 1990). Similarly, quercetin inhibits protein kinase C, an enzyme involved in the transduction of growth factor signals to the nucleus, an effect which has been suggested as a "novel therapeutic target for anticancer agents" (Hoffman et al. , 1989) . Flavonoids have also been shown, by both in vitro and in vivo experiments, to inhibit platelet aggregation (Gryglewski et al., 1987), and to modify the activity of enzymes involved in arachidonic acid metabolism (Alcaraz and Ferrandiz, 1987), thereby acting as antiinflammatory agents (Alcaraz and Jimenez, 1988). Furthermore, they have been found to inhibit histamine release from basophilic leukocytes and other mediators of allergic hypersensitivity reactions (Middleton et al., 1987). Flavonoids inhibit the activities of hyaluronidases, enzymes that are involved in a number of processes, including allergic reaction, inflammation, migration of cancer cells and malignant cell proliferation (Kuppusamy et al., 1990).

The ability of rutin and quercetin to react with superoxide anion and lipid peroxy radicals and also to form iron complexes that are unable to catalyze the formation of

active oxygen radicals makes them useful for possible therapy in combatting cellular damage caused by radicals ("free radical pathologies") (Afanas'ev et al., 1989).

Despite the consensus among most investigators today that quercetin (or other flavonoids) is not a carcinogen (see latter), a controversy surrounds the other biological effects, which could adversely affect health; for instance, the effects of flavonoids on the formation of nitrosamines and oxygen radical species. The majority of N-nitrosamines and N-nitrosamides found in foods,mainly in smoked and pickled products, have been shown to be carcinogenic, producing tumors at various sites in experimental animals (Druckery et al., 1967). Nitrosamines can also be formed in the stomach under acidic conditions from amines and nitrite which may be available through ingestion of certain foods, drugs and drinking water. It has been observed that vegetables can reduce the formation of N-nitroso compounds. This is presumably due to their content of vitamin C, since vitamin C is a scavenger of nitrite. Other naturally occurring phenolic compounds in vegetables also inhibit the formation of N-nitrosamines (Stich and Rosin, 1984). However, some polyphenols and particularly some flavonoids (quercetin, kaempferol, naringenin) can actually accelerate the process of N-nitrosamine formation (Walker et al., 1982). The precise mechanism of action for the enhancement or inhibition of N-nitrosation by natural polyphenolic compounds is still unknown (Gichner and Veleminsky, 1988).

Both quercetin and myricetin produce reactive oxygen species (superoxide, hydrogen peroxide, and hydroxyl radical) through autoxidation and redox cycling (Hodnick et at al., 1986; Canada et al., 1990; Sahu and Washington, 1991). It appears, however, that this reaction is highly pH dependent, and no autoxidation of quercetin can be detected at physiological pH (Canada et al., 1990), therefore this may not be a significant problem. However, it was also suggested that this autoxidation of flavonoids, in a long exposure, may potentially produce intestinal injury (Canada et al., 1989).

Antibacterial and antifungal activities of flavonoids against plant pathogenic organisms are also effective against human pathogens. Fungi, bacteria and viruses associated with human diseases are frequently susceptible to polyphenols (Mitscher and Gollapudi, 1990).

Recently, it has been found that tea polyphenols were selective growth inhibitors of clostridia. This in turn could indicate an influence of tea polyphenols on the intestinal microflora, and their use for the possible prevention of certain human diseases associated with clostridia (Ahn et al., 1991).

Flavonoids are known to have a number of biological effects which have pharmacological significance. For a long time it has been thought that flavonoids can maintain or restore the normal integrity of the blood vessel wall. This is the main reason for clinical applications of flavonoids for treatment of decreasing capillary fragility (cataract prevention, recovery from frostbite, myocardial infarction, bruising in contact sports, etc.) (Havsteen, 1983). Quercetin and rutin can reduce the level of serum triglycerides and are antithrombotic (Kato N. et al., 1983), while other flavonoids are effective in the treatment of arteriosclerosis, hyperlipidemia and atherosclerosis (Middleton, 1986; Khushbaktova et al., 1991). Additionally, a number of beneficial pharmacological effects (antiallergic, antiinflammatory) were also found for flavonoids from citrus fruits (Kumamoto et al., 1986; Middleton et al. 1987).

As a result of the above findings, there is considerable interest and effort in research on the therapeutic potential of flavonoids as drugs for the prevention or treatment of certain human diseases (Singleton, 1981; Farkas et al., 1986). In addition, some flavonoids are used as "model" compounds for developing drugs with more efficient pharmacological effects (Middleton, 1990). For instance, flavone acetic acid, a synthetic flavonoid, is currently under clinical evaluation for its antitumor and immune-modulation activities (Ching and Baguley, 1990), while 3-methoxyflavones are tested as antiviral (e.g. against human rhinovirus) compounds (De Meyer et al., 1990).

As mentioned above, some enzymatic effects of flavonoids on cell physiology, are being explored as novel therapeutic targets as anticancer agents. It has been observed in in vitro tests, that the combination of the protein kinase inhibition achieved by quercetin with the activity of busulphan (an antileukaemia chemotherapeutic agent), acts synergistically in inhibiting the proliferation of human leukaemia cells (Hoffman et al., 1989).

The controversy is still unresolved however, whether food flavonoids should be considered to be beneficial or hazardous agents in the human diet (Stich, 1991). The consensus of opinion currently appears to support the view that food flavonoids are more beneficial than hazardous.

Genotoxic and Tumorigenic Effects

Interest in research with food flavonoids heated up following two reports published in 1977 that the most abundant food flavonoid, quercetin, had been found to be mutagenic in in vitro tests (Bjeldanes and Chang, 1977; Sugimura et al., 1977). Concern about the potential health hazard from flavonoids in foods increased still further after the publication of results from the first feeding study with rats, when 0.1% of quercetin produced an increased incidence of intestinal and bladder cancers (Pamucku et al., 1980). Other investigations, mainly done in vitro using various microorganisms and mammalian cells in culture (MacGregor, 1984), revealed that some flavonoids possess mutagenic and genotoxic effects. However, the carcinogenic effect of quercetin in rodents, shown previously, could not be confirmed, even when a diet containing 10% of quercetin was used (for review see Stavric, 1984) . There is little information on the carcinogenic potential of other flavonoids. Furthermore, it was found in animal tests, that certain flavonoids exhibited some antitumorigenic activity and a number of other potentially beneficial effects for human health (Stich and Rosin, 1984; Birt and Bresnick, 1991).

In mammalian cell cultures, several polyphenols have proven to be potent inducers of chromosome/chromatid aberrations (Stich, 1991). However this strong clastogenic effect in mammalian cells is not matched by a strong mutagenic effect in Salmonella typhimurium (MacGregor and Jurd, 1978) or Saccharomyces cerevisiae (Rosin, 1984) for most of the flavonoids studied. Various modulating factors, some known (e.g. the presence of transition metals Mn^{2+}, Cu^{2+} and Fe^{2+}; or high pH level) , others still unknown, have been shown to influence the extent of genotoxicity of various flavonoids and need to be considered when extrapolating results to human situations.

Recently it has been reported that quercetin induced malignant cell transformation in mammalian cell culture. This finding suggests that daily intake of quercetin (throughout life) may produce genetic effects in somatic cells, "resulting in increased risk of cancer with aging" (Sakai et al., 1990).

On the other hand, the remarkable ability of several flavonoids to suppress the molecular action of a variety of chemical mutagens in in vitro experiments is further indication that food flavonoids, under experimental conditions, may not be completely inert, agents. Therefore, it is expected that these in vitro observation may reflect on the ability of flavonoids to reduce or inhibit the adverse biological effects of the carcinogens (Bhattacharya, 1990).

Interesting observations have been reported recently for the biological effects of anthraflavic acid, another plant-derived polyphenol. Anthraflavic acid inhibited the mutagenicity of IQ, when incorporated into the Ames Salmonella assay. However, when administered in vivo to rats, the opposite effect occurred. Moreover, hepatic preparations from anthraflavic acid-treated rats were more efficient than those from control preparations in converting IQ to mutagenic species in vitro. This would suggest that although anthraflavic acid is antimutagenic for IQ in the Ames test, it may potentiate its carcinogenicity in in vivo experiments (Ioannides et al., 1991). This is an example that shows that in vitro experiments do not always correlate well with in vivo results.

Citrus flavonoids were found to possess antimutagenic potential against a number of mutagens using the Salmonella test (Bala and Grover, 1989), and also to have antiproliferative effects on a human squamous cell carcinoma in in vitro studies (Kandaswami et al., 1991).

Quercetin has been reported to inhibit proliferation of human ovarian cancer cells in vitro, which contain type II oestrogen binding sites. Since the primary ovarian tumors contain the same type of binding sites, there is a possibility that quercetin could also be active in inhibiting ovarian tumors in vivo (Scambia et al., 1990).

Nevertheless, the question arises: why are quercetin and other flavonoids genotoxic in in vitro tests, but they are not animal carcinogens? Several investigators have suggested

that perhaps "these agents are initiators but also possess antipromotional activity, thus negating any potential role as a carcinogen" (Birt and Bresnick, 1991). To understand the complexity of this question, the next two sections, will provide an overview of animal tests and suggested mechanisms by which flavonoids may act in reducing carcinogenicity.

Protective Effects of Flavonoids in Suppressing Tumorigenicity

This section will summarize results with some polyphenols, which have been found to exhibit certain beneficial or "protective" effects in suppressing tumorigenicity.

There are a large number of regular food ingredients, that have been found to exhibit either antimutagenic or anticarcinogenic properties (Fontham, 1990; Birt and Bresnick, 1991; Stich, 1991). Since all vegetables and fruits contain flavonoids, and no detrimental effects of flavonoids have been observed in humans and in in vivo studies (Wattenberg, 1983; Scambia et al., 1990; Daniel and Stoner, 1991; Kandaswami et al., 1991), there is an increased interest and expectation in possible use of flavonoids as chemopreventive agents in human carcinogenesis (Gabor, 1988; Birt and Bresnick, 1991).

Using experimental animals, flavonoids have been shown to modulate the activity of known carcinogens. Several feeding studies with mice using several polyphenols and known chemical carcinogens, produced encouraging, although in some instances, disappointing results (Nishino, et al., 1984; Smart et al., 1986; Wattenberg et al., 1980; Hirono et al., 1980). For example, quercetin showed an antitumor effect in mouse skin after topical application (Fujiki et al., 1986; Kato R., 1983) but not after oral administration (Horiuchi et al., 1986). Other experiments similarly reported little or no "protective" effect of quercetin when given orally, while the "protective" capacity was observed if quercetin was applied topically. The lack of activity may be due to the fact that quercetin may not have been absorbed in sufficient amounts to exhibit the protective effect. However, other polyphenols, such as ellagic acid, exhibited protective effect when given orally (Nakadate et al., 1984; Mukhtar et al., 1986).

Polyphenols from green tea significantly protected against tumorigenicity of PAHs and 3-methylcholanthrene (3-MC) administered by various routes in different animal experiments using different protocols (topical administration or oral feeding in drinking water) (Wang et al., 1989).

Acidic components incorporating phenolic groups have been repeatedly implicated as active antioxidants (Larson, 1988). Many foods derived from plants contain such acidic polyphenols, like caffeic acid, chlorogenic acid, ellagic acid and others. For instance, coffee contains relatively large amounts of chlorogenic acid. In some green coffee, up to 10 % of the weight is chlorogenic acid, and a substantial amount of it survives the roasting process (IARC, 1991). Chlorogenic acid was found to be the most abundant phenolic acid in plants, and also it has the most active antioxidant property (Larson, 1988). Dietary chlorogenic acid was found to inhibit liver and large intestine tumors in hamsters induced by the chemical carcinogen methylazoxymethanol [MAM] (Mori et al., 1986). Since the carcinogen in these experiments was introduced intravenously, the inhibition may have been due to modification of the activity of detoxification enzymes.

Ellagic acid, a polyphenol present in many fruits and vegetables, has been shown to be a potent antagonist of the carcinogenic effects of several polyaromatic hydrocarbons (PAHS) (Wood et al., 1982; Mukhtar et al., 1984). Ellagic acid was reported to exert a "protective" effect also against other food carcinogens. For instance, 4% of ellagic acid fed to rats receiving subcutaneous injections (for 15 weeks) of N-nitroso-benzylmethylamine (a compound which induces esophageal tumors in 100% of rats) inhibited significantly (60%) the formation of tumors (Daniel and Stoner, 1991). In contrast, in some cases, ellagic acid (and some other polyphenolic acids, like chlorogenic and ferulic) failed to produce in in vitro assay any inhibitory effect on the mutagenicity of some other food mutagens/carcinogens, like AIAs (amino-imidazoazaarenes) (Alldrick et al., 1986) . It is still unclear why polyphenolic acids in some cases inhibit the mutagenicity of food xenobiotics, while in other instances they do not. It has been postulated that the inhibitory effect of ellagic acid may be due to its interaction with hydroxylated metabolites of the xenobiotics. This reduction in the chemical reactivity

would diminish the capacity of the xenobiotic [e.g. B(a)P] to undergo covalent binding to DNA (Wood et al., 1982). It has been demonstrated, that in addition to the mutagen itself (its chemical structure), the source of the hepatic S9 mixes used in in vitro assays (e.g. obtained either from induced or non-induced mouse or hamster) could play a role in the mutagenic response or inhibitory capacity of the individual flavonoid (Alldrick et al., 1986).

Quercetin, when fed to mice, inhibited B(a)P-induced nuclear-damage in colonic epithelial cells (Wargovich et al., 1985). Similarly, dietary quercetin significantly reduced the number of palpable rat mammary tumors/rat and also the number of rats with tumors, induced by intragastric instillation of 7,12,dimethylbenz(a)anthracene (DMBA) and by i.v. injection of N-nitrosomethylurea, both confirmed chemical carcinogens (Verma et al.,1988). The same xenobiotic, DMBA, induces chromosome aberrations in rat bone marrow. However, fresh or boiled juices from vegetables (e.g. onion, cabbage, egg plant) significantly suppressed the incidence of aberrations (Ito et al., 1986).

The Mechanisms by Which the Food Flavonoids Can Exert Their 'Protective' Effect in Reducing Carcinogenicity

In spite of the progress made by many investigators in the last several years to elucidate the mechanism by which food flavonoids exert their protective effects in reducing carcinogenicity, the precise mechanism of action is still unclear (Stich, 1991). Studies to identify agents (principles) in food with anticarcinogenic potential, have shown that most of these compounds (e.g. vitamins C, E, A, beta-carotene, selenium) possess antioxidative potential (Amstad et al. , 1990) . Since many flavonoids are also antioxidants, it is likely that the antioxidative property of flavonoids is, at least in part, responsible for their reported anticarcinogenic potential. Nevertheless, overwhelming evidence from research conducted in many laboratories in the last several years, indicates that the mode of action of flavonoids extends much wider than their antioxidative properties. This section will review the mechanisms, suggested by different studies, by which food flavonoids may exhibit their "protective" action. They are as follows:

- by their action as antioxidants. Flavonoids could protect certain biologically beneficial, but easily oxidizable compounds in foods (i.e. vitamins) by reducing

their oxidative degradation (Fraga et al., 1987). Similarly, they exhibit a quality-preserving effect on raw or cooked meat (Herrmann, 1976), and extend the shelf-life of lard, edible oils and fruits (Dick et al., 1985).

- by their action as scavengers of free radicals formed either during preparation of food, or as a result of certain metabolic processes in the body (Husain et al., 1987; Robak and Gryglewski, 1988). The deleterious effect of free radicals in cells is assumed to be responsible for many chronic diseases, including aging and cancer. However, there is a controversy concerning the correlation between antioxidative and scavenging activities of flavonoids. Some authors found that the antioxidative properties of flavonoids are mainly due to their free radical scavenging capacity (Cillard et al., 1990), while others could not find that correlation (Yuting et al., 1990).
- by their action as chelating agents. Flavonoids can form complexes with transition metals, e.g. with copper, thus preventing destruction of ascorbic acid, or with iron or copper thus preventing the initiation of free radical reaction (Pincemail et al., 1990).
- by inhibiting lipid peroxidation, through combining the (above described) chelating and antioxidative properties of flavonoids. Rutin and quercetin were found to be effective inhibitors of iron ion-dependent lipid peroxidation (Afanas'ev et al., 1989).
- by blocking or trapping ultimate carcinogen electrophiles by forming innocuous products in a nucleophilic chemical reaction. To carry out this function, flavonoids need to be absorbed and present in the target cell, either unmodified or metabolized. However, even if not absorbed, flavonoids could still be useful in blocking chemical carcinogens in the lumen of the gastrointestinal tract (Newmark, 1987).
- by interaction and subsequent binding to the mutagenic/carcinogenic metabolite(s) to render them ineffective to bind covalently with DNA (Wood et al., 1982; Mukhtar et al., 1984; Das et al., 1985).

- by forming adducts with DNA, thus 'masking' binding sites and rendering them unavailable for reaction with mutagens or carcinogens. This mechanism was observed in a series of in vitro tests with ellagic acid and explants of different organs of the rat (Teel, 1986).
- by inhibiting the invasiveness of tumor cells. Using in vitro assay it was found that the flavonoid, (+)catechin, possessed an antiinvasive activity in tumor cells (Bracke et al., 1988). Oral administration of catechin reduced the B(a)P induced forestomach tumors in mice (Nagabhushan, 1990).
- by inhibiting the promotion phase of carcinogenesis. Several investigators have observed that some flavonoids (e.g. quercetin, luteolin, apigenin, aqueous extracts of green tea) when administered topically, act as antipromoters against known promoters of carcinogenesis (Nishino et al., 1984; Wang et al., 1989; Wei et al., 1990). However, only limited antipromoting activity of quercetin was observed in tests with mice receiving a diet with 1-4% quercetin (Fujiki et al., 1986). This low activity could be explained by intensive degradation of quercetin by intestinal flora, which is partly the reason for its limited absorption. There is a good correlation between the ability of some of the flavonoids to inhibit certain promoter-stimulated biochemical processes and their activity as antipromoters (Birt and Bresnick, 1991).
- by decreasing the production of prostaglandin E_2, which in turn may reduce tumorigenicity. After in vitro experiments exposing mouse fibrosarcoma cells to quercetin, diminished tumorigenicity was observed (Okada et al. 1990).
- by inhibiting endogenous nitrosation in the stomach of man. Plant extracts rich in (+)catechin suppressed the endogenous formation of nitrosoproline (Stich and Rosin, 1984).
- by influencing the immune system. Some flavonoids can interfere with tumor development (Wiltrout and Hornung, 1988). It has been shown that some flavonoids can enhance natural killer cell activity and induce interferon production and act synergistically with interleukin-2 (Wiltrout and Hornung, 1988).

- by inhibiting enzymes or blocking biosynthesis of enzymes involved in reaction sequences required for transforming procarcinogenic compounds into ultimate carcinogens, e.g. by inhibiting the arachidonate cascade mechanism or lipoxygenase (Wheeler and Berry, 1986).
- by modulating the balance between activation and inactivation processes of specific enzymes in the liver. It has been found that tangeretin (polymethoxylated flavonoid) inhibits aflatoxin B_1 induced hepatocarcinogenicity in the rat, while quercetin is ineffective (Suschete et al., 1991).

The experimental evidence, as presented above, suggests that there are many plausible mechanisms of action by which flavonoids and other polyphenols can reduce carcinogenicity. It is expected that many of the above mentioned observations, mainly from in vitro experiments, may reflect the ability of flavonoids to counteract the adverse biological effects of the carcinogens (Bhattacharya, 1990) . However, not all modes of action are applicable to all flavonoids and it is conceivable that in some cases the same flavonoid may act in more than one way, or may not act at all.

Investigation of the Influence of Polyphenols/Flavonoids on the Bioavailability of Benzo(a)Pyrene [b(a)p] and 2-Amino-3-Metrylimidazo(4,5-f)Quinoline [IQ]

Benzo(a)pyrene [B(a)P] and IQ are potent mutagens and carcinogens found in foods, both formed during the exposure of meat to heat, during cooking. While B(a)P is the most potent mutagen among the polycyclic aromatic hydrocarbons (PAHs), IQ belongs to the group of heterocyclic aromatic amines (AIAs) (Howard and Fazio, 1980; Pariza et al., 1990).

As mentioned above, various substances occurring in foodstuffs significantly inhibit the mutagenicity of xenobiotics in vitro and may reduce carcinogenicity of these compounds in some experimental animals. The precise mechanism for these inhibitions is not elucidated. Among foods that have been shown to reduce the mutagenicity of AIAs and PAHs are juices of vegetables such as cabbage, radish and turnip (Kada et al., 1978; Shinohara et al.,1988), and these are known to contain relatively large amounts of flavonoids (Kada et al., 1978).

We have been interested in finding out if polyphenols can play a role in bioavailability of these two xenobiotics, B(a)P and IQ. The three plant polyphenols tested were: quercetin, ellagic acid and chlorogenic acid. For these studies we used short term in vivo studies with mice and rats. Here will be presented the highlights of these studies, which are still in progress and will be reported elsewhere (Stavric et al., 1991).

Both xenobiotics were tested either as 'cold' or as ^{14}C-labelled chemicals. Mice were used in two different types of test: (a) for the intrasanguineous host-mediated assay (Ami et al., 1977), in which recovered bacteria from liver homogenates were used for the mutation assays in the Ames test (Maron and Ames, 1983); and (b) for uptake of (^{14}C)-B(a)P from the intestine. Rats were used after bile-cannulation to investigate the recoveries of the radioactivity [(^{14}C)- counts] excreted in the bile for 14 hours after dosing with (^{14}C)- labelled B(a)P or IQ. In all experiments, animals were dosed orally, either with the tested xenobiotic alone (controls) , or in combination with the tested polyphenol. Polyphenols were administered at 2% level blended with the regular diet.

The Intrasanguineous Host-Mediated Assay

The objective of this experiment was to investigate the effects of polyphenols on the in vivo mutagenic activity of B(a)P and IQ. The working hypothesis was that if the polyphenols interact with B(a)P or IQ in the gastrointestinal tract, then a probable reduction in their uptake will occur, which should be reflected in reduced mutagenicity of Salmonella recovered from the liver. Quercetin, ellagic acid and chlorogenic acid, all reduced the mutation rate of B(a)P by 52, 66 and 72 percent, respectively. No differences were observed in the mutagenic activities of the livers of mice treated with IQ alone, or in combination with either quercetin or ellagic acid.

The Uptake of (^{14}C)-B(a)P in the Mouse

The aim of this experiment was to investigate the possible interaction of flavonoids and B(a)P in the intestinal tract, which could reduce the uptake of B(a)P. All three tested polyphenols, namely, quercetin, chlorogenic acid and ellagic acid reduced the uptake of B(a)P by 17, 29 and 26 percent respectively, in comparison with the uptake of B(a)P if given alone.

Studies with Bile-Cannulated Rats

The previous experiments with mice indicated that polyphenols, quercetin, ellagic acid and chlorogenic acid, influenced the bioavailability of B(a)P, but not of IQ from the gastrointestinal tract. Bile-cannulated rats were used to examine if the same effect could be observed in the rats. The results obtained indicated that all three polyphenols reduced the bioavailability of B(a)P by 21, 16 and 59 percent, respectively, in comparison with the uptake of B(a)P if given alone. In tests with IQ, only ellagic acid has been tested so far and it was found that it did not influence the uptake of IQ.

Assessment

Epidemiologic evidence and results from studies with animals suggest that diet is a major factor in the development of certain human cancers (Alfin-Slater and Kritchevsky, 1991). Although no one compound has been identified as being responsible for this, many food ingredients have been observed to possess a variety of biological activities, including both carcinogenic and anticarcinogenic properties. Since the human diet is a complex mixture, which may contain, besides nutritional, also nonnutritional components, some with detrimental and others with beneficial influence, the balance between them is what actually determines the overall effect on human health (Amstad et al., 1990). These agents could either enhance the effect of carcinogens or their formation from precursors, or acting in a "beneficial" way, lessen or eliminate the effects of carcinogens (Fiala et al., 1985).

As discussed above, flavonoids have a variety of effects on enzymatic systems and biological processes. Although many of these effects, per se, are not very dramatic, most of them can be classified as 'beneficial' for human health, this in spite of the fact that they are nutritionally nonessential. This net beneficial effect of the flavonoids appears to result from the contribution of many individual flavonoids (and many other ingredients in fruits and vegetables) acting in concert at many different sites and involving a number of mechanisms of action. The performance of the flavonoids for human health, could be compared to the work of an orchestra, in which the sound from each individual instrument contribute, just a little, to the resulting musical performance.

The main reason for the difficulty in explaining the biological effects of flavonoids is the lack of precise information on their mechanism of action (Stich, 1991). As discussed, it appears that there is more than one mechanism by which flavonoids can exert their activity. Recently, De Flora and Ramel (1988) provided an excellent discussion of the overall mechanism of antimutagenesis and anticarcinogenesis. In their scheme, they classified the inhibitors by the possible mechanisms of action into three broad categories: (a) those that act extracellularly, either by hindering uptake of carcinogens, or by favoring removal or deactivation of mutagens/carcinogens; (b) those that act intracellularly, modulating metabolic processes in several different ways, and by blocking DNA-carcinogen interaction; and (c) those that act on already initiated neoplastic cells by modulating tumor promotion or by preventing malignant cell invasion. It appears that the mechanisms by which flavonoids, as a group, perform their anticarcinogenic effects could be ascribed to all three categories which gives further support for the likelihood of a diversified mode of action on biological systems. It appears that the agents that can act extracellularly are of more practical significance (Hayatsu, 1991), i.e. they counteract the activity of carcinogens before they can enter target cells. our studies support this view.

In many investigations to elucidate the mechanism for the in vivo activities of flavonoids, quercetin was the most frequently used flavonoid. However, information obtained with quercetin, may not be applicable to other flavonoids. Although it is the most abundant flavonoid in our diet, it appears that quercetin may also be the least absorbable from the intestine (Gugler et al., 1975; Ueno et al., 1983; Crebelli et al.,1987). Quercetin is intensively metabolized by the intestinal flora, and only its metabolites, which are regular phenolic acids or their lactones (Kuhnau, 1976) are absorbed from the gut. For a number of effects observed in in vitro studies, the concentration of quercetin used was relatively high. A comparable concentration of quercetin, as such, in the liver or other body tissues has never been observed. Actually, the amount and concentration found was very low (Crebelli et al., 1987). However, dietary quercetin still displays a beneficial role in reducing carcinogenesis in experimental animals. Therefore, at least for quercetin, and probably for some other flavonoids that

are poorly absorbed from the GI tract, the mechanism of action is mainly related to its ability to interact physico-chemically with the carcinogens in the intestine, thus rendering them less bioavailable.

Conclusion

Polyphenols and flavonoids are common dietary components with certain biological activities and pharmacological potencies. In many in vitro experiments, they exert variable genotoxic effects. However, their overall effects could be considered non-toxic, nongenotoxic in vivo and non-carcinogenic and thus may even possess beneficial effects for human health. The exact mechanism for their beneficial effects is not yet elucidated, although it is thought that they may act simultaneously in several different ways.

Data from our experiments with animals suggest that polyphenols could hinder the uptake of some xenobiotics from the gastrointestinal tract. Our results with quercetin, ellagic acid and chlorogenic acid indicate that their beneficial effect [on B(a)P] could best be explained, at least in part, by their reduction of the bioavailability of carcinogens.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

STUDIES ON FLAVONOIDS AND RELATED COMPOUNDS AS ANTIOXIDANTS IN FOOD

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Summary

Flavonoids are naturally occuring benzo-γ-pyrone derivatives, ubiquitous in vascular plants. These plant polyphenols have been reported to act as antioxidants in various biological systems. They do so by acting as free radical scavengers and/or as metal ion chelators. The other commonly known antioxidants, α-tocopherol, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) each possesses a phenolic structure which is a feature also shared by the flavonoids. We have found that the plant polyphenols were more effective in inhibiting lipid oxidation than the commonly known antioxidants on raw or cooked fish. The order of potency is; tannic acid = ellagic acid > myricetin > quercetin > morin > kaempferol > rutin.

The enhanced lipid oxidation induced by divalent metal salts ($CuSO_4$, $FeSO_4$, $ZnSO_4$, $NiSO_4$ and $MgSO_4$) on the cooked fish was inhibited to a varying degree by the plant polyphenols (100 ppm). The antioxidative potency of these compounds was independent of the type of metal ion-induced lipid oxidation. Arising from our investigations we are able to conclude that polyhydroxylations on rings A and B of the flavonoid structure as well as the presence of a 2, 3-double bond, a free 3-hydroxyl substitution and a 4-keto moiety will confer potent antiperoxidative properties upon the flavonoid molecule.

Introduction

Flavonoids are ubiquitously distributed in vascular plants. They are benzo-γ-pyrone derivatives and are usually responsible for the myriad bright colours found in plant tissues. These colours are easily changed to different shades and tones when the surrounding pH of the medium is altered (Brouillard & Cheminat, 1988).

Animals cannot synthesize flavonoids, which enter into the animal systems through their diets. It is estimated that man consumes about lg/day of flavonoids from the intake of foods containing plant tissues such as vegetables, fruits, cereals, tea cocoa, coffee, wine, beer, cola and peanuts (Kuhnau, 1976; Wollenweber & Dietz, 1981).

Lipid membranes are usually the main targets for free radical attack and often is the cause of non-enzymatic lipid peroxidation. Flavonoids have been reported to act as antioxidants in both non-aqueous lipid and aqueous lipid systems (Kimuya et.al., 1981; Sorata et.al.,1984; Jha et.al., 1985; Ratty & Das, 1988). Their antioxidative properties are mainly due to their abilities to act as free radical scavengers and/or as metal chelators (Thompson et.al., 1976; Ratty et.al.,1988; Robak & Gryglewski, 1988). In addition, flavonoids have been shown to exhibit a quality-preserving and antioxidative effect on raw and cooked meat (Herrmann, 1976), on edible oils (Das & Pereira, 1990) and on milk fat and lard (Nelson, 1982). Flavonoids in particular can stabilize ascorbic acid (vitamin C), especially in the presence of heavy metal ions, such as copper, which can destroy ascorbic acid rapidly (Herrmann, 1976).

The flavonoids possess a phenolic structure, which is a feature shared by the commonly known natural (α-tocopherol) and synthetic (butylated hydroxyanisole, BHA and butylated hydroxytoluene, BHT) antioxidants. The purpose of this report is to demonstrate the potential use of these plant polyphenols for the control of oxidative rancidity that occurs frequently in raw and cooked ground fish. A structure-activity relationship of the potent antioxidative flavonoids was also made. Their antioxidative potencies were also compared with the commonly used food antioxidants such as α-tocopherol, ascorbic acid and BHT.

Materials and Methods

The flavonoids, rutin, quercetin, morin, myricetin and kaempferol, and the polyphenols, tannic acid and ellagic acid were obtained from Extrasynthese-Genay, France. The antioxidants, L-ascorbic acid, α-tocopherol and butylated hydroxytoluene were purchased from Sigma chemical Co., St. Louis, Missouri. All other chemicals used were of analytical grade.

The fish (Ikan tinggerri; Scomberomorus commersoni) obtained from the local wet market, was deskinned, deboned and the flesh homogenised. The ground fish was then divided into 10g aliquots prior to the addition of the test compounds.

Relative amounts of the test compounds (Fig.1) were dissolved separately in 50% ethanol and aliquots (0.5 ml) were added to the ground fish samples (10g) and mixed well to give a final concentration of either 30 or 200 ppm. All the samples were then stored (individually wrapped in aluminium foil) at either -20°C or 4°C until analysed.

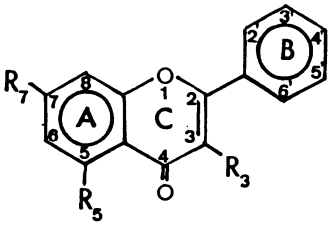
The extent of lipid oxidation was assayed by the thiobarbituric acid (TBA) test. This test is commonly used as a measure for the extent of lipid oxidation in muscle foods (Gray, 1978; Rhee, 1978) . The procedure used here was that described by Siu and Draper (1978).

The degree of lipid oxidation was expressed as:-

(i) TBAN (TBA No. = mg malonyldialdehyde (MDA)/kg fish) calculated using the molar extinction coefficient of MDA, $\epsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Sinnhuber & Yu, 1958) or as

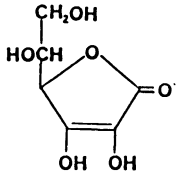
(ii) % TBARS (thiobarbituric acid reactive substances) determined using

$$\frac{A_{532\ test}}{A_{532\ control}} \quad X \quad 100\%$$

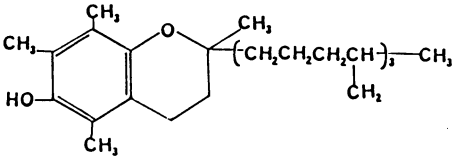


Flavonoid

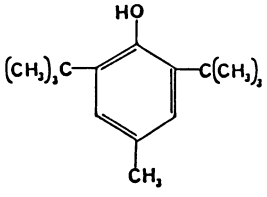
Compounds	Structure						
	Ring A		Ring C	Ring B			
	R_5	R_7		2'	3'	4'	5'
Rutin	OH	OH	O-rutinose	H	OH	OH	H
Quercetin	OH	OH	OH	H	OH	OH	H
Morin	OH	OH	OH	OH	H	OH	H
Myricetin	OH	OH	OH	H	OH	OH	OH
Kaempferol	OH	OH	OH	H	H	OH	H



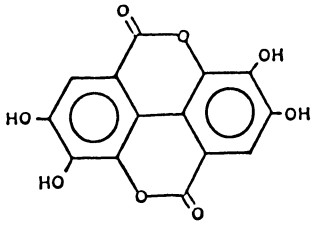
Vitamin C
(L-ascorbic acid)



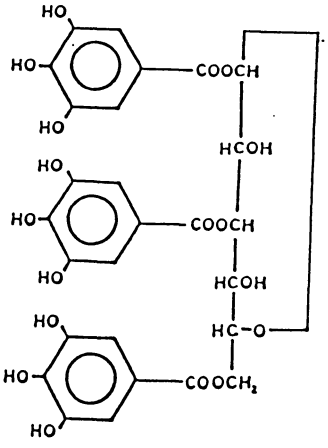
Vitamin E
(α-tocopherol)



BHT
(butylated
hydroxytoluene)



Ellagic
acid



Tannic
acid

Fig.1: Structures of flavonoids, polyphenols and other commonly used antioxidants.

Treatment of Fish

(i) Untreated fish: Weekly over 3 weeks, duplicate packets of untreated raw and cooked fish samples, stored at either -20°C (frozen) or 4°C (refrigerated) were assayed for TBARS. (ii) Raw fish pretreated with antioxidants: After 14 days of storage at 4°C, duplicate packets of antioxidant treated raw fish samples were assayed for TBARS. (iii) Cooked fish pretreated with antioxidants: After 24 hours of storage at -20°C the antioxidant pretreated raw fish samples were transferred to glass petri dishes and cooked by either steam (15 mins) or microwave (30s), or left raw. The TBARS was then determined. (iv) Stored cooked fish pre-treated with antioxidants The antioxidant pretreated raw fish samples (stored at -20°C for 24 hours) were steam cooked and then further stored at 4°C for 1 week prior to analyses of TBARS. (v) Metal ion-induced lipid oxidation: Aliquots (0.1 ml) of the divalent metal salts ($CuSO_4$, $FeSO_4$, $ZnSO_4$, $NiSO_4$ and $MgSO_4$) were added to ground fish (10g) together with aliquots (0.1ml) of either quercetin, myricetin, tannic acid or ellagic acid, and then steam-cooked. The final concentration of each divalent metal salt was 10^4 pmol/10g fish and the final concentration of each polyphenol was 100 ppm. The TBARS was then determined.

Results and Discussion

(i) Untreated Fish

The inherent lipid oxidation was demonstrated in untreated, raw and steam-cooked ground fish, stored at either -20°C or 4°C over 3 weeks (Fig.2) . Untreated raw or cooked fish stored at -20°C showed minimal lipid oxidation over the 3 weeks of storage. Birch and Lindley (1986) have reported that biological materials stored at low temperatures would undergo low oxidative deterioration. Untreated, raw or cooked fish stored at 4°C, however, exhibited a greater extent of lipid oxidation compared to those stored at -20°C, over the same period of storage. The extent of lipid oxidation of stored

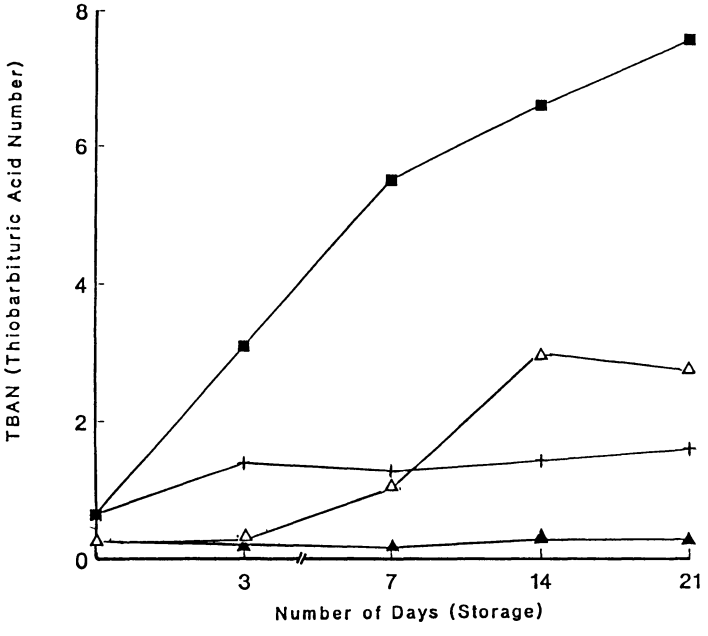


Fig. 2 Effect of storage (4°C and -20°C) of raw and cooked fish on lipid oxidation.
● = raw fish stored at -20°C, ○ = raw fish stored at 4°,
■ = cooked fish stored at -20°C, † = cooked fish stored at 4°

raw fish increased from day 0 to day 14 and then decreased slightly on day 21. Temperature has been shown to promote the extent of lipid oxidation, probably by increasing the rate of alkyl peroxide propagation and decomposition (Troller & Christian, 1978).

(ii) Antioxidant Pretreated Raw Fish

The effect of antioxidant-treated raw fish stored at 4°C for 14 days is shown in Table I. All the antioxidants, with the exception of rutin (30 and 200 ppm and α-tocopherol (30 ppm) exhibited < 50 % TBARS compared with the control samples. In addition, all the antioxidants, with the exception of myricetin, L-ascorbic acid and BHT were more potent at the higher concentration (200 ppm).

TABLE I.

Effect of storage of antioxidant-treated raw fish at 40C for 14 days.

Compounds	% TBARS	
	30 ppm	200 ppm
Rutin	56	50
Quercetin	11	6
Morin	22	11
myricetin	6	6
Kaempferol	17	11
Tannic acid	17	11
Ellagic acid	33	28
L-ascorbic acid	11	11
α-tocopherol	50	33
BHT	11	11

Each value is the mean of 4 determinations (2 different experiments). S.D.values ranged between 10-20 %.

(iii) Antioxidant Pretreated Cooked Fish

The effects of 2 different methods of cooking on lipid oxidation in ground fish, with and without additives were studied. Both the steam and microwave-cooked fish samples, without antioxidant treatment (i.e. controls), gave higher TBA Nos. than the uncooked samples (Fig.3). This agrees with results obtained by Sato and Hegarty (1971), who postulated that any process (such as cooking) which L-Ascorbic acid acted as a prooxidant (> 100 % TBARS compared with the control samples) in both steam-and microwave-cooked fish (Fig.3). This prooxidant effect of L-ascorbic acid in meat has also been reported by other workers (Sato & Hegarty, 1971; Benedict et.al., 1975).

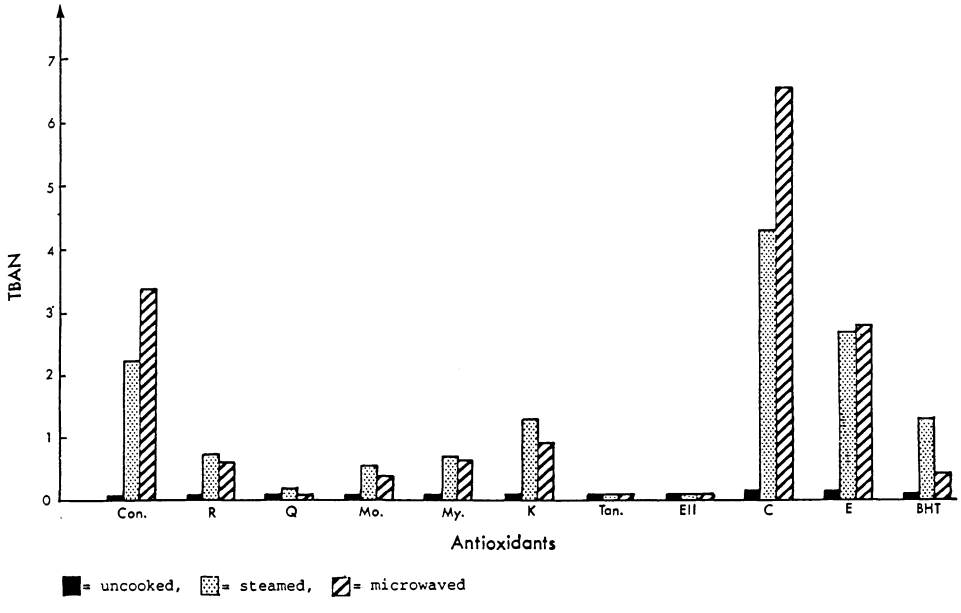


Fig. 3 Effect of antioxidants (200 ppm) on lipid oxidation of uncooked, steam-cooked and microwave-cooked fish.
Con = control (50 % ethanol), R = rutin, Q = quercetin, Mo = morin My = myricetin, Tan = tannic acid, Ell = ellagic acid, vit C = Vitamin C (L-ascorbic acid), vit e = vitamin E (α-tocopherol), BHT = butylated hydroxytoluene

The other test compounds used in this study acted as antioxidants in cooked fish (regardless of the mode of cooking). The plant polyphenols were generally more effective than the commonly used antioxidants. Quercetin, morin, myricetin, tannic acid and ellagic acid interestingly were effective in inhibiting the increased extent of lipid oxidation in both steam and microwave-cooked fish (Fig.3).

In view of earlier reports that polyhydroxy flavones possess molecular structural features that enable them to act as free radical acceptors as well as metal chelators (Crawford et al., 1960; Ratty et al., 1988), it is therefore pertinent to suggest that the flavonoids and polyphenols inhibited lipid oxidation in cooked fish by scavenging the free radicals as well as by chelating the metal ions that produced them.

(iv) Antioxidant Pretreated Stored Cooked Fish

After one week storage at 4°C, there was a large increase in lipid oxidation in the cooked samples in the absence of added antioxidants (i.e. control; Fig.4) . According to Labuza (1971), a TBA No. of 1.0 represented the onset of oxidative rancidity. Tannic acid and ellagic acid, at both 30 and 200 ppm addition levels were effective in retarding the onset of oxidative rancidity in cooked fish even after 1 week of storage at 4°C. Myricetin and quercetin were only effective at 200 ppm (TBA No. < 1.0) , while morin, kaempferol, rutin, L-ascorbic acid, α-tocopherol and BHT were ineffective in retarding the onset of oxidative rancidity at both the concentrations used (Fig.4). L-Ascorbic acid continued to act as a prooxidant in the one week stored steam cooked fish.

(v) Metal Ion-induced Lipid Oxidation

Different divalent metal ions (each at 10^4 pmol/10g fish) -induced lipid oxidation systems, in the presence of quercetin, myricetin, tannic acid, ellagic acid and EDTA (each at 100 ppm) was studied (Table II) The order of antiperoxidative potency of the polyphenols was ellagic acid (16-25 % TBARS of the control), followed by tannic acid (23-40 %), myricetin (30-47 %) and quercetin (56-67 %). Their antiperoxidative potency was exerted irrespective of the type of metal ion-induced lipid peroxidation. EDTA, a known metal chelator, exhibited an antioxidative potency of only (30-48 %) .

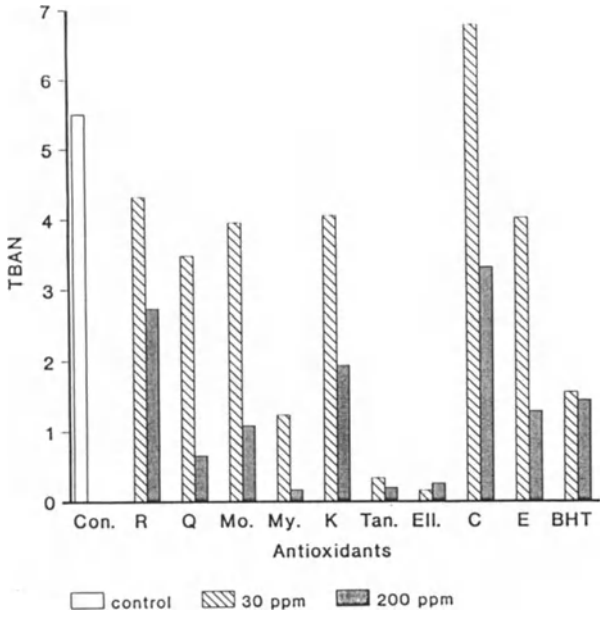


Fig. 4 Effect of antioxidants (30 ppm and 200 ppm) on lipid oxidation of steamed-cooked fish stored for one week at 4°C.

Con = control (50 % ethanol), R = rutin, Q = quercetin, Mo = morin, My = myricetin, Tan = tannic acid, Ell = ellagic acid, vit C = vitamin C (L-ascorbic acid), vit E = vitamin E (α-tocopherol), BHT = butylated hydroxytoulene.

Heimann and Reiff (1953) reported that (a) the α-β unsaturated ketone structure of the pyrone ring, (b) the free hydroxyl group on the C ring and (c) the o-hydroxyl groups on the phenyl side ring, increase the antioxidant effect of flavones in commercial ethyl linoleate. From our data, we also observed that rutin which lacks a free hydroxyl group on the C ring; kaempferol which has only a single hydroxyl group on the phenyl side ring (B ring); and morin which has a p-hydroxyl group on the B ring, are less potent antioxidants than quercetin and myricetin.

TABLE II.

Effect of test compounds on metal ion-induced lipid oxidation.

Compounds 100 ppm	% TBARS Metal ions(104 pmol/10g)				
	Cu^{2+}	Fe^{2+}	Zn^{2+}	Ni^{2+}	Mn^{2+}
Quercetin	65	67	59	59	56
Myricetin	47	37	30	45	40
Tannic acid	29	28	23	39	40
Ellagic acid	24	18	25	22	16
EDTA	48	30	47	43	35

Each value is the mean of 4 determinations (2 different experiments). S.D. values ranged between 10-20 %.

It has been reported that polyhydroxy flavones possess bimodal activity, thus enabling them to function both as free radical scavengers and metal chelators (Lea, 1958; Ratty & Das, 1988). In a biological system, however, it is difficult to evaluate the exact contribution of each mode of action to their total antioxidant capacity (Crawford et.al., 1960).

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

AUTOCOID-IMMUNOPHARMACOLOGY OF FLAVONOIDS

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Summary

Biologically active flavonoids are conspicuous constituents of many a medicinal and edible plants of natural abundance. Traditional use of such plants is common for diseases understood to have an immune dysfunction. Various reports indicate the influence of flavonoids on pluralistic molecular events in the immune process. Anti-mediator effect on target tissues as well as suppression of immunologic release process (i.e. degranulation of mast cells) and modulation of developmental events of immune response have been observed with flavonoid treatments. A simplistic and unified explanation for the effects through some common biochemical of flavonoids, reported widely, was subjected to simultaneous evaluation vis-a-vis the immunomodulatory profile, to test the hypothetic mechanistic linkage, and the results suggest such a possibility.

Introduction

The flavonoids are widely distributed plant constituents known to execute defense functions (Horborne et al., 1975), and display a host of activities, viz. anti-inflammatory, anti-allergic, inhibiting capillary permeability and experimental gastric ulcers, anti-microbial and anti-tumour actions as well as mutagenic potential in the animal species (Kuhnau, 1976; Gabor, 1979; Middleton et al., 1981; Pamukeu et al., 1980). Their

in-vitro antagonistic potential against several mediators of allergy and inflammation (Ramaswamy et al., 1979; Chau and Haley, 1969; Capasso et al., 1988) and inhibition of antigen-induced anaphylaxis on guinea pig ileum (Fanning et al., 1983), and against in-vivo anaphylaxis in guinea pigs (Raiman et al., 1947; Moss et al., 1950) indicate a probable immunomodulatory effect, which might explain the use of flavonoid-rich plants by the physicians of traditional system in diseases like bronchial asthma and dermal allergies. However, there is a relative scarcity of systematic studies in this area. The results of our studies with flavonoids on some humoral immunity parameters are being presented in this communication.

Materials and Methods

The studies were conducted in non-sensitized and seensitized animals. Colony-bred adult guinea pigs and albino rats (Charles Foster) were used. The animals were acclimatised to the laboratory conditions for one week before being used and were maintained on pellet diet (Lipton India) with additional provision of green vegetables in case of guinea pigs.

1. Anti-mediator Studies

Experiments were conducted on isolated ileum of guinea pigs mounted in 10 ml organ bath using Tyrode's solution (NaCl 8.0, KCl).2, $CaCl_2$ 0.2, $MgCl_2$ 0.01, NaH_2PO_4 0.05, $NaHCO_3$ 1.0, and dextrose 1.0 g/1) maintained at 36 degrees Celcius and aerated with a miniature pump. Thirty sec drug contact period with the spasmogen and a 3 min response cycle was maintained.

2. Studies On Humoral Immunity

Studies on immediate hypersensitivity were conducted in sensitized guinea pigs and albino rats. Following parameters were used :-

Dale Schultz reaction: Guinea pigs, sensitized with chicken egg albumin (1 mg/animal)$^{+}$ Freun's complete adjuvant (0.5 mg/animal) s.c., were sacrificed on day 21, and their ileum was mounted as described for antimediator studies above. The antigen challenge was given with chicken egg albumin (24 ug/ml) and the maximal response thus obtained was expressed as percent of maximal histamine induced response in the same tissue.

The studies on Dale Schultz reaction were conducted both in actively sensitized (Active Dale Schultz) or passively sensitized (Passive Dale Schultz) animals. The passive sensitization was carried out by administering i.p. 1 ml of serum from the actively sensitized guinea pigs, and then sacrificing them 72 h later for Dale Schultz reaction study.

Mast cell degranulation: Rat's mesenteric mst cells, as described by Norton (1954), from either nonsensitized animals or from the rats sensitized with chicken egg albumin (0.5 mg/rat s.c.)+ aluminium hydroxide gel (12 mg/rat s.c. on day 1,3 and 5) were used. The rats were sacrificed on day 10 and the mesentery taken out. Rats were also sensitized with horse seum and B.pertussis as per the method of Gupta and Tripathi (1973) in which case rats were sacrificed on day 14. Some experiments were conducted on the rats passively sensitized with i.pl injection of 1.0 ml of serum from the actively sensitized rats. The mesenteric mast cells were challenged in vitro with appropriate antigen or compound 48/80.

Survival period: Time lag in min between the intravenous antigen challenge on day 14 and the death, was recorded in albino rats sensitized with horse serum and B.pertussis vaccine as described above.

Hematocrit volume: It was calculated by subtracting volume of plasma from total volume of blood in same animals in which survival period was calculated.

3. Free Radical Biology Studies

Malonaldialdehyde (MDA) levels in plasma of sensitized animals subjected to i.v. challenge were estimated using thiobarbituric acid (Das et al., 1987). Hydrogen peroxide production was estimated as described by Das and Sandhya (1979),. using horse radish peroxidation in yeast stimulated polymorphonuclear cells of sensitized animals. In another lot of the same cells the superoxide production was estimated using nitroblue-tetrazolium (Das et al., 1987).

4. Flavonoid Treatment

Flavonoids were finely suspended in 1% CMC in normal saline. For in-vitro studies on antimediator action and Dale-Schultz reaction, the tissue was pretreated with the suspension for 3 min bgefore spasmogen or antigen was administered. For in-vitro studies on mast cell degranulation, the mesentery was pretreated with for 10 min with the flavonoid suspension before antigen or chemical challenge was given to the mast cells. The in-vitro effects of flavonoids in sensitized animals were studied after systemic administration through specific phases to evaluate the effects on events of restricted occurrence in the developing phase, developed phase, or expression phase in the ontogeny of immune response (i.e. day -1 to +7, day +7 to +14, or 2 h before challenge), and in nonsensitised animals 1 h before study.

Results

Antimediator action

Quercetin inhibited the spasms of guinea pig ileum induced by histamine (Hist), 5-HT and acetylcholine (Ach), its 50% inhibitory concentration (IC-50) values (with 95% confidence limits) being 12.44 (10.35-14.96), 8.28 (7.11-9.63), and 15.31 (12.61-18.57) ug/ml respectively, indicating a nonspecific antagonism. Similarly, when a series of compounds representing some subgroups of flavonoids,k and several substituted flavones

were studied against Hist and Ach induced spasms, similar nonspecific activity was observed with all except one or two compounds which showed some selectivity (Table I).

Inhibition of Dale Schultz reaction

Quercetin in-vitro inhibited the Dale Schultz reaction in both the actively and passively sensitized animals in dose-related manner (Table II), the IC-50 values being 2.82 (95% C.L.=2.50-3.19) μg/ml (b=61.15; r=0.9501, $P<0.001$) and 0.90 (95% C.L.=0.75-1.08) μg/ml (b=36.62; r=0.9643, $P<0.001$), respectively against the active and passive Dale-Schultz response.

Effects on non-sensitized mast cells

In a study with series of simple flavonoidal compounds and their hydroxy (mono/di) derivatives in ring A, 7-OH flavone was found to be most potent inhibitor of carrageenan induced rat paw oedema. In the subsequent study with 7-OH flavone in-vitro/ex-vivo on mast cells, it was found to inhibit the compound 48/80 induced mast cell degranulation in concentration/dose-dependent manner in the lower range, but with the higher range the protective effect against the compound 48/80 induced degranulation was reversed (Table III).

When the mesenteric mast cells were exposed to increasing concentrations/doses of 7-OH flavone, an increasing degree of degranulation was observed with higher range only (Table IV).

In a further study, injection of increasing but minute doses of 7-OH flavone subplantarly into the rat's hind paw, doses up to 100 μg did not produce any inflammatory effect per se while higher doses (200, 400, and 800 μg) produced paw oedema which reached peak at 3 h after the injection, and then declined gradually to near normal in 24h. Effect against the compound 48/80 induced degranulation was reversed (Table III).

Table I: Relative activity of flavonoids on guinea pig ileum in-vitro

Compound	Relative activity	
	Anti-Hist	Anti-Ach
Flavone	1.00	1.82
Flavanone	1.17	1.00
Flavan (7-OH)	1.00	1.48
Chalcone	1.00	1.06
3-OH Flavone (Flavonol)	6.07	1.00
5-OH Flavone	1.91	1.00
7-OH Flavone	3.24	1.00
5,7-di-OH Flavone	3.78	1.00
Fisetin	1.00	19.26
Quercetin	1.22	1.00
Gossypin	1.00	2.30
Gardenin	(poor response, calculation not possible)	

Relative potency ratios estimated on the basis of molar IC-50 values by regression analysis.

Studies on Sensitized Mast Cells

Quercetin in-vitro and ex-vivo (1 h prior orally) inhibited the antigen-induced mast cell degranulation of actively and passively sensitized rats in concentration/dose dependent manner (Table V).

When quercetin was administered phasically (50 mg/kg i.p.), it produced significant inhibition of antigen induced degranulation of mast cells sensitized with horse serum in all the three treatment schedules (Table VI).

Table II: Effect of Quercetin in-vitro on Dale-Schultz reaction in sensitized guinea pigs.

Concentration (μg/ml)	n	% response (mean±SE)	% inhibition (mean±SE)	P value (Dunnett's t)
A. Active Dalte-Schultz				
Control	8	88.60 ± 4.19	-	-
4	4	34.98 ± 5.80	58.02 ± 3.97	<0.01
8	3	18.63 ± 4.64	79.94 ± 5.14	<0.01
16	6	4.86 ± 0.23	94.83 ± 0.31	<0.01
B. Passive Dalte-Schultz				
Control	4	63.18 ± 7.36		-
4	4	16.48 ± 2.84	74.39 ± 1.95	<0.01
16	4	2.54 ± 1.08	96.30 ± 1.64	<0.01

Table III: Effect of 7-OH flavone in-vitro and ex-vivo on compound 48/80 (2.5 μg/ml) induced mast cell degranulation in non-sensitized rats.

Concentration/ dose	n	% Degranulation (mean±SE)	% Protection	P value (Dunnett's t)
Control (48/80)	15	93.5 ± 1.51	-	-
25 μg/ml	12	81.4 ± 1.60	13	<0.01
50 μg/ml	15	57.4 ± 3.10	39	<0.01
100 μg/ml	10	44.3 ± 3.59	53	<0.01
200 μg/ml	8	59.5 ± 4.13	46	<0.01
400 μg/ml	8	85.1 ± 2.47	9	<0.05
25 μg/ml	6	69.0 ± 2.40	26	<0.01
50 μg/ml	6	62.2 ± 2.55	33	<0.01
100 μg/ml	6	86.2 ± 4.04	8	NS

NS = not significant

Table IV: Effect of 7-OH per se in-vitro and in-vivo on the mesenteric mast cells of non-sensitized rats.

Concentration/ dose	n	% Degranulation (mean±SE)	P value (Dunnett's t)
A. In-vitro			
Control (1%CMC)	10	6.8 ± 0.99	-
25 μg/ml	12	9.8 ± 1.18	NS
50 μg/ml	15	13.8 ± 1.41	<0.05
100 μg/ml	10	15.0 ± 1.31	<0.05
200 μg/ml	8	22.6 ± 2.17	<0.01
400 μg/ml	8	69.3 ± 2.47	<0.01
B. In-vivo			
Control (1%CMC)	5	13.2 ± 1.56	-
25 mg/kg	6	17.3 ± 1.69	NS
50 mg/kg	6	21.3 ± 2.33	<0.05
100 mg/kg	6	78.1 ± 3.31	<0.01

Table V: The inhibitory effect of Quercetin on antigen induced mast cell degranulation in rats sensitized with chicken egg albumin.

	Actively sensitized		Passively sensitized
Parameter	ex-vivo (P.O.)	in-vitro	in-vitro
ED 50	29.17 mg/kg	-	-
(95% C.L.)	(25.35-33.57)		
IC 50	-	3.01 μg/ml	1.00 μg/ml
(95% C.L.)		(2.63-3.46)	(0.84-1.19)
b (slope)	68.87	111.65	68.45
r value	0.8816	0.8195	0.8349
(P value)	(<0.001)	(<0.01)	(<0.001)

Effect on Parameters of Oxidant Biochemistry

Quercetin, when administered phasically, significantly decreased in all the three treatment schedules, the serum MDA levels, and superoxide and H_2O_2 production by polymorphonuclear cells in sensitized animals challenged with antigen, horse serum (Table VII) in case of MDA studies, and without challenge for superoxide and H_2O_2 studies.

Discussion

Our studies with flavonoids revealed their influences on several events of the immune response. They appeared to exert an anti-mediator action of nonspecific nature on the target tissues. In an earlier study with amentoflavone (Chakravarthy et al., 1981), similar nonspecificity was observed against three spasmogens. Chau and Haley (1969), using bradykinin, angiotensin and eledoisin, and Capasso et al (1988), using PGE_2 and LTD_4, also reported similar results. Flavonoids are reported to possess a variety of actions that may underlie the nonspecific antagonism of several stimulants of smooth muscles. They uncouple oxidative phosphorylation (Ravanel et al., 1990), inhibit cAMP phosphodiesterase (Beretz et al., 1979), inhibit membrane transport ATPases (Fewtrell and Gomperts, 1977), and have antioxidant activity (Pincemail et al., 1990; Cillard et al., 1990).

Table VI: Effect of phasic treatment with Quercetin (40 mg/kg i.p.) on horse serum - challenge anaphylaxis in albino rats.

Treatment Schedule	Survival Time (in min)	Hematocrit Volume (%)	Mast Cell Degranulation (%)
Schedule 1			
Control	20.25 ± 0.65	61.45 ± 1.96	45.54 ± 2.25
Quercetin	23.45 ± 0.47*	45.25 ± 3.34*	32.12 ± 1.23**
Schedule 2			
Control	20.18 ± 1.10	61.16 ± 2.53	50.00 ± 1.27
Quercetin	26.32 ± 1.27+	47.65 ± 2.21*	32.24 ± 1.42**
Schedule 3			
Control	20.15 ± 1.67	59.95 ± 2.03	50.00 ± 2.54
Quercetin	25.43 ± 1.55	47.27 ± 2.73*	39.80 ± 2.91*

P value: + <0.05; * <0.025; ** <0.01 (Student's t test)
Schedule 1: -1 to +7 days; Schedule 2: +7 to +14 days; Schedule 3: 2 h before challenge. Data (mean ± SE) from 5 animals in each group.

Table VII: Effect of phasic treatment with Quercetin (50 mg/kg i.p.) on plasma MDA levels following horse serum anaphylaxis and on superoxide and H_2O_2 production by yeast stimulated polymorphonuclear cells in sensitized rats.

Treatment Schedule	Plasma MDA (nMol)	Superoxide production (O.D.)	H_2O_2 production (O.D.)
Schedule 1			
Control	2.06 = 0.03	0.10 = 0.01	0.08 = 0.01
Quercetin	1.22 = 0.02**	0.04 = 0.001*	0.03 = 0.002*
Schedule 2			
Control	1.98 = 0.07	0.11 = 0.01	0.09 = 0.002
Quercetin	1.52 = 0.06*	0.05 = 0.003**	0.03 = 0.001**
schedule 3			
Control	1.97 = 0.09	0.09 = 0.02	0.08 = 0.009
Quercetin	1.28 = 0.02*	0.025 = 0.001**	0.045 = 0.005+

P value: + <0.05; * <0.025; ** <0.01 (Student's t test)
Schedules of treatment as in Table VI. Data (mean = SE) from 5 animals in each group.

Flavonoids have been widely reported to inhibit the mast cell degranulation which may contribute to their possible benifical effect in immunologic disorders (Fewtrell and Gomperts, 1977; Middleton et al., 1981; Bronner and Landry, 1985). Our studies with flavonoids on sensitized mast cells and the Dale schultz reaction, which is a consequence of the release of mast cell mediators, reveal similar results when given in vitro as well as in-vivo in all the treatment schedules. Similarly, the studies in nonsensitized tissues also show that flavonoids prevent mast cell degranulation against chemical challenge with compound 48/80. Thus flavonoids tend to suppress the activation of mast cell membrane against a variety of stimuli. The stabilizing effect may result from one or more of several actions reported for the flavonoids, namely inhibition of membrane transport ATPases and Ca^{++} influx (Fewtrell and Gomperts, 1977), cAMp phosphodiesterase inhibition (Beretz et al., 1979), inhibition of lipoxygenases (Busse et al., 1984), and antioxidant activity as referred above.

Apart from the mast cell stabilisation, flavonoids produced effects like hypotension and oedema, and anaphylactic symptoms (Lecmte and Van Cauwenberge, 1974; Lazar et al., 1977). In our studies, several flavonoids showed a reversal of anti-inflammatory activity against carrageenan oedema in rats, when doses were increased. The probability that higher doses were promoting the degranulation of mast cells was proved by our studies which showed that with the increase in the dose in vivo, or the concentration in vitro, of the flavonoids, the protective effect of flavonoids against compound 48/80 induced degranulation was reversed, and when studied directly on the mast cells, a dose/concentration dependent activation of mast cells was observed. The oedema produced by the direct subplantar administration of flavonoids in the rats further substantiated the above observation. Studies with more flavonoidal compounds (Dasgupta and Gambir, 1988) revealed tachyphylaxis with flavonoids and polymyxin. a mast cell degranulator, and cross-tachyphylaxis between the two, using frog isolated atrial stimulant response,

mesenteric mast cells and carrageenan induced rat paw oedema, further substantiating mast cell activation with higher doses of flavonoids. This effect may be related to the report cGMP phosphodiesterase inhibition (Ruckstuhl et al., 1979), inhibition of cyclooxygenases and enhance the PG-mediated down regulation (Moroney and Hoult, 1990) and a pro-oxidant activity (Hodnick et al., 1988).

The phasic treatment with quercetin in sensitized rats showed that the compound could inhibit the anaphylaxis parameters in all the phases of treatment. A wide array of response i.e. initiation (developing phase) as well s the later events (developed phase) wherein formation and control, of antibodies are involved. If affected in general by the flavonoids. such a broad-based action on multiple events would need to be explained on unified molecular mechanism(s) applicable pluralistically to different immune competent cells. Among the various referred activities of the flavonoids antioxidant activity is of particular appeal in the context. Monitoring of oxidant mechanisms revealed inhibitory effect of quercetin on the different parameters, with the effects consistently observed in all the phasic treatment schedules. Although these results alone are not adequate to establish any cause and effect relationship between the antioxidant potential and immuno-modulation activity of flavonoids, they do point to the same, projecting the need for further mechanistic studies to establish such relationship.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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ANTICARCINOGENICITY OF FLAVONOIDS AS STUDIED BY INHIBITION OF LIPID PEROXIDATION, MICROSOMAL DEGRANULATION AND THEIR INTERACTIONS WITH BENZO(A)PYRENE METABOLITES

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Summary

Plant flavonoids have been found to inhibit lipid peroxidation both in rat liver microsomes as well as a number of routinely used frozen meats. Out of the various meats studied, goat's meat shows negligible lipid peroxidation even on storage while fish meat showed maximum lipid peroxidation. Elution profiles of rat liver microsomes over Sepharose 2B columns showed marked reduction in the degree of ribosomal detachment from the microsomes when the incubated mixtures contained various flavonoids like flavone and mint extract containing flavonoids. Studies on benzo(a)pyrene metabolites formed on incubation with homogenates of a number of rat organs employing the techniques of TLC and HPLC demonstrated that its ultimate carcinogen is majorly formed in the incubates of lung and kidney homogenates. The metabolites of benzo(a)pyrene were also isolated employing TLC after incubating this carcinogen in lung or kidney homogenates both in the presence and absence of flavonoids like flavone and catechin as well as β-carotene separately. Our results showed that many of the intermediate metabolites of benzo(a)pyrene are possibly interacting with the flavonoids and β-carotene as they do not appear on the TLC plates after incubations are made in the presence of these plant pigments.

Introduction

Free radicals are known to cause damage to membranes in terms of lipid peroxidation in general and microsomal degranulation in particular (Arstila et al., 1972). We performed a number of experiments to determine whether various meats frozen at -20°C showed any lipid peroxidation and whether it could be inhibited in the presence of flavonoids or not. Carcinogens are now very well known to cause microsomal degranulation in vivo as well as in vitro (Butler, 1966; Gupta and Dani, 1986). We therefore performed a number of experiments to determine whether plant flavonoids could inhibit microsomal degranulation employing Sepharose 2B gel filtration technique. Microsomal degranulation has been found to be inhibited by flavone and a number of other flavonoids.

One of the suggestions made in literature regarding the mode of action of flavonoids as anticarcinogens is that flavonoids may act as free radical scavengers and therefore might react with carcinogenic metabolites before they reach DNA and damage this molecule (Das, 1989). To confirm this possibility, a number of experiments were performed to study the effects of two flavonoids (flavone and catechin) and β-carotene on benzo(a)pyrene metabolites after incubating them in lung and kidney homogenates. Separation of the metabolites employing TLC showed that many of the intermediate metabolites of benzo(a)pyrene possibly interact with flavonoids studied and β-carotene.

Materials and Methods

Inhibition of Lipid Peroxidation by Plant Pigments

Five different types of routinely consumed meats (muscle, liver etc.) were frozen with and without the addition of flavonoids at a concentration of 0.07g/4ml of DMSO and lipid peroxidation was assayed at weekly intervals for 3 weeks. The control samples (without any treatment and DMSP-control) were also run simultaneously. Lipid peroxidation was estimated by the method of Ohkawa et al. (1979).

Inhibition of Microsomal Degranulation by Flavonoids

Male albino rats of Wistar strain (weighing 100 to 150 g) were fed ad libitum and killed by cervical dislocation. Livers were removed, suspended in normal saline, finely chopped in 0.225 M sucrose and 50 mM Tris buffer (pH 7.4) and homogenised in a Potter Elvehjem apparatus at 4°C. Post-mitochondrial supernatant was prepared by centrifuging the homogenate at 9,000 'g' for 20 min at 4°C in a fixed angle Zanetski 6x26 ml rotor. The supernatant was carefully drawn out with a Pasteur pipette. Microsomal degranulation by urethane and its inhibition by flavone was studied by employing Sepharose 2B gel filtration technique (Gupta et al., 1982). For microsomal degranulation, 0.1 ml of urethane in DMSO (0.4 mg/ml) was used. Study on flavonoids was carried out by adding 0.1 ml solution of flavonoid dissolved in DMSO (0.4 mg/ml). Protein contents in various column fractions were assayed by the method of Lowry et al. (1951). RNA estimations were carried out by the method of Munro and Fleck (1966).

Studies on Benzo(a)pyrene Metabolites

Homogenates from five organs (liver, lung, kidney, brain and intestine) of rat were prepared in sucrose-Tris buffer as mentioned above. Ten ml of the homogenate of each organ was incubated with NADPH regenerating system (Sims, 1967) and 10 mg of benzo(a)pyrene dissolved in 5 ml of ethanol at 37°C for 15 min with and without the addition of flavonoid (2mg/ml of DMSO). The metabolites of benzo(a)pyrene were extracted with ethyl acetate and examined by TLC as described by Sims (1967). Chromatograms were examined in U.V. light before and after an exposure to ammonia. Streaks obtained on the TLC plates were scrapped off separately and after elution their contents were rechromatographed by TLC using benzene as the solvent. These streaks were also separately dissolved in methanol for HPLC studies according to the method of Hall and Grover (1987).

Results and Discussion

Results obtained from the experiments carried out to study the effects of storage of routinely consumed meats on non-enzymatic lipid peroxidation and effects of purified

flavonoids like flavone and quercetin showed that lipid peroxidation linearly increased up to 21 days even in frozen tissues like muscles from pork and chicken, livers from these animals and fish. Both quercetin and flavone showed inhibitory effects on lipid peroxidation but the level of inhibition was much more with quercetin. Data pertaining to only intercostal muscle are presented in Table 1. Results from data pertaining to other tissues are only being discussed here for brevity.

Table 1 Lipid peroxidation (nmoles MDA/mg protein) in pork intercostal muscle stored at -20°C for 21 days with and without the addition of flavonoids

Duration of storage (in days)	Control (tissue frozen)	DMSO-control (tissue frozen in DMSO)	Tissue frozen in flavone dissolved in DMO	Tissue frozen in quercetin dissolved in DMSO
0	0.030	-	-	-
7	0.087	0.094 (+7.4*)	0.058 (-27.6)	0.028 (-70)
14	0.149	0.16 (+6.9)	0.115 (-28.0)	0.045 (-72)
21	0.209	0.221 (+5.4)	0.154 (30.3)	0.060 (-73)

* Values in parentheses show % increase (+) or decrease (-) in lipid peroxidation in comparison to their respective controls.

Data on fish were interesting as flavone and quercetin showed much less inhibitory effects as compared to those of other types of tissues studied. Negligible lipid peroxidation was recorded in goat muscle, salami and sausages even on storage for 21 days.

The anticarcinogenicity of flavonoids has also been studied by the inhibition of microsomal degranulation caused by 4-dimethyl-aminoazobenzene as well as urethane. Figure 1. depicts the protection by flavone against microsomal degranulation caused by urethane.

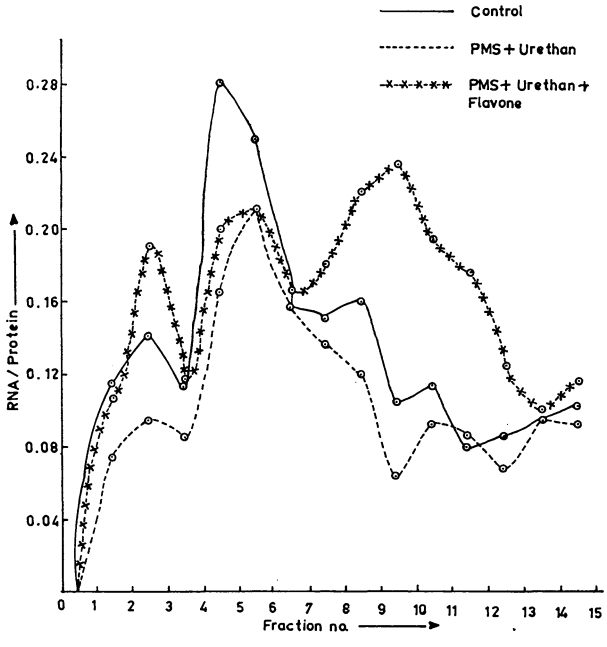

Fig. 1 Inhibition of microsomal degranulation by flavone as studied against the carcinogenicity of urethane.

A number of experiments were performed to study the organ specificity of benzo(a)pyrene. Metabolites of benzo(a)pyrene were isolated by employing the techniques of TLC and HPLC. Both TLC and HPLC data indicated that the ultimate carcinogen of benzo(a)pyrene (7,8, dihydro-7,8 dihydroxybenzo(a)pyrene) is probably being formed only when this carcinogen was incubated with homogenates from lungs and kidneys (Figs. 2 and 3).

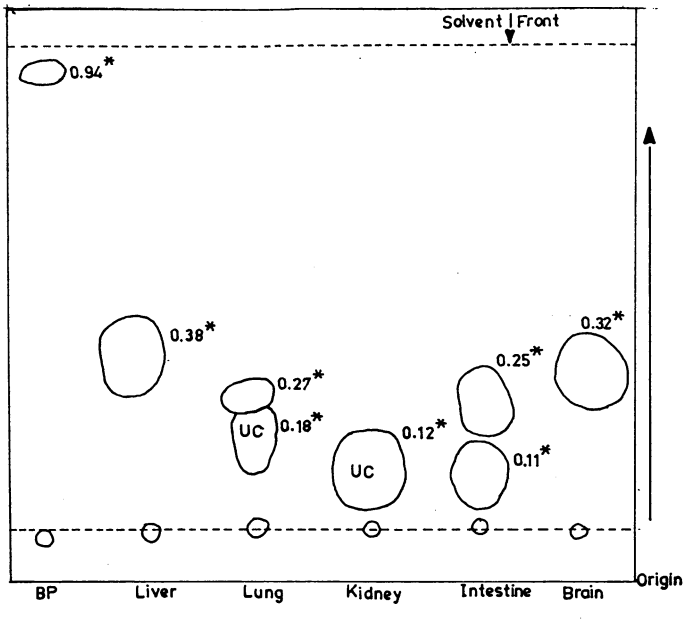

Fig. 2. Thin layer rechromatography of slowest moving fraction separated from a TLC plate to which an incubate of benzo(a)pyrene with homogenates of different organs from rat were applied

UC ; ultimate carcinogen

* ; Represents Rf values

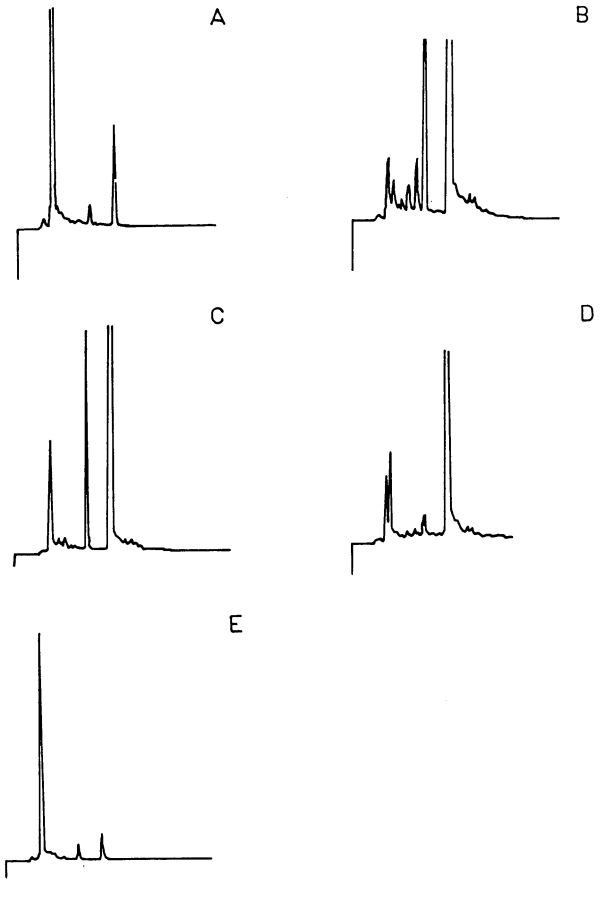

Fig. 3. HPLC elution profiles of the slowest moving fraction separated from thin layer chromatograms of benzo(a)pyrene incubated with various rat tissues : a) liver; b) lung; c) kidney; d) intestine and e) brain.

Detailed studies were further conducted to find out any possible interaction of activated metabolites of benzo(a)pyrene with flavonoids (like flavone and catechin). A very interesting observation has been made as per depicted in schematic chromatograms (Figs. 4 and 5) in the sense that the possible ultimate carcinogenic moieties with almost

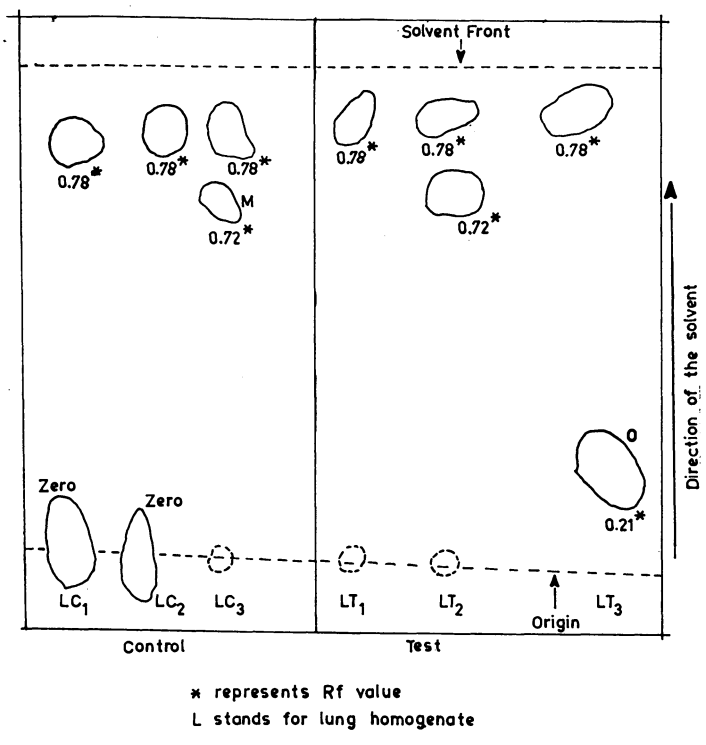

Fig. 4. Schematic chromatogram after thin layer rechromatography of the streaks separated from an incubated mixture of benzo(a)pyrene in lung homogenate with and without the addition of flavone.

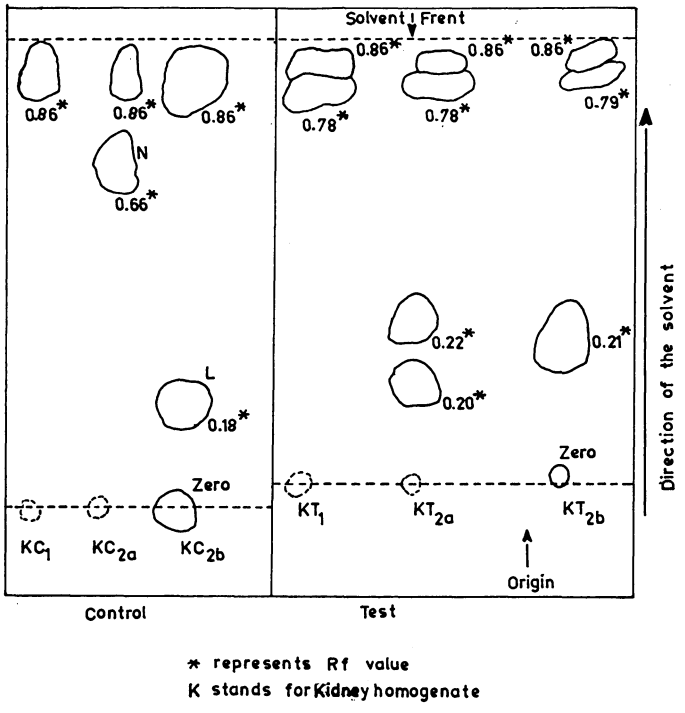

Fig. 5. Schematic chromatogram after thin layer rechromatography of the streaks separated from an incubated mixture of benzo(a)pyrene in kidney homogenate with and without flavone.

zero 'Rf' values completely disappear from the lowermost (LT_1) and middle (LT_2) streaks obtained by incubating benzo(a)pyrene with lung homogenate, and from the uppermost streak (KT_{2b}) in the kidney homogenate, the intensity of the possible ultimte carcinogenic

moiety becomes much less in test. Moreover, another metabolite (M) from the upper streak (LC_3) in case of lung homogenate also disappears on treatment with flavone.

Similarly another intermediate metabolite (N) from the middle layer (KC_{2a}) in case of kidney homogenate also disappears. Another metabolite (L) in kidney homogenate from the topmost layer (KC_{2b}) also clearly disappears on treatment with flavone. In case of kidney homogenate, a number of new differently coloured spots appeared on treatment with flavone which might be some fluorescent complexes of flavone with benzo(a)pyrene metabolites. An orange coloured complex (O) appeared in lung homogenate in (LT_3) sample.

Similar results were obtained with catechin and β-carotene.

The above observations provide convincing evidence that plant flavonoids and antioxidants like β-carotene can arrest benzo(a)pyrene metabolites by interacting with them which might contribute to their anticarcinogenic potentials.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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ROLE OF RETINOIDS IN MODULATING THE MOLECULAR ACTIONS OF ENVIRONMENTAL CARCINOGENS

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Summary

It has been found that carcinogenic growths are associated with the occurrence of low-level retinoids. In the population suffering from malnutrition, vitamin A-deficiency or, as a whole, deficiency of retinoids has been encountered. This is liable to bring about the occurrence of epithelial carcinoma caused by several carcinogens. The experiments relating to the formation of DNA adduct with carcinogen like aflatoxin B_1 (AFB_1) catalysed by microsomal mono-oxygenase of liver could be controlled through a series of retinoid compounds such as vitamin A and carotenoids having pro or non-pro-vitamin A status. A brief review has been illustrated.

Introduction

Considerable importance has been attributed to the relationship of several nutritional factors on the occurrence as well as development of carcinogenic growth. Although several nutritional factors have been examined for the last several decades, yet very little is known about the inhibitory action of these dietary factors on the onset of the process of carcinogenesis. Nutritional deprivation has been shown to decrease host

susceptibility to tumor development (Jose & Good, 1973). Some nutritional factors such as vitamins, trace elements, coumarins, flavonoids, phenolic antioxidant such as butylated hydroxy-anisole, butylated hydroxytoluene etc.inhibit the process of cancerous growth (Rogers & Longnecker, 1989). Retinoids, a class of lipid soluble compounds consisting of vitamin A and carotenoids have been shown to be necessary in the regulation of growth, vision, reproduction and maintenance of epithelial linings (Moore, 1957). The major natural sources of vitamin A in the diet are certain plant pigments, such as β-carotene, cryptoxanthin etc. and the long-chain retinyl esters found in animal tissue. From different studies it has been seen that the consumption of food rich in vitamin A-status or other carotenoids may decrease the risk of the occurrence of malignancy (Goswami, et al., 1988; Kummet & Meyskens, 1983; Lippman et al., 1987; Lotan, 1980; Lotan & Nicolson, 1977; Mathew-Roth, 1986; Olson, 1989; Paganini - Hill et al., 1987). Further experiments dealing with the light supplementation of vitamin A and β-carotene in different communities or areas have also shown a considerable low occurrence of various types of cancer (Rogers & Longnecker, 1989). Although much attention on retinoids and their relation with carcinogenesis has been focused from 1960 onwards, yet its utility and metabolic effects were cautioned long ago by Wolbach & Howe in 1925 by the fact that specific tissue changes follow the deprivation of fat soluble vitamins. n epithelial tissue the prime effects are the formation of stratified keratinized epithelium. The classic report submitted by Wolbach and Howe demonstrated that a vitamin A-deficient diet led to metaplasia. With this keen insight and prophetic finding, further work on the effects of vitamin A and its analogues including the carotenoids having both pro and non-provitamin A activity on cancer was pursued and has received momentum during the last two decades.

In the present report environmental carcinogen such as aflatoxin B_1(AFB_1) were studied in connection with their *in vitro* DNA- adduct formation catalysed by microsomal monooxygenase and modulation with different retinoids.

Material and Methods

Different vitamin A analogues and carotenoids were obtained from F. Hoffman La-Roche, Switzerland, BASF- Germany, Roche Products Limited India, and from Professor S. Liaaen Jenson Norwegian Institute of Technology, University of Trondheim, Trondheim, Norway. Retinoyl glucuronide was obtained from Dr. A. B. Barua, Department of Biochemistry and Biophysics, Iowa State University Ames, Iowa U. S. A.

The sources of chemicals, solvents, isolation of microsome and estimation of protein etc. have been described in our earlier reports (Goswami et al., 1988, 1991). The adduct formation was carried out by reacting DNA (0.5 mg) with 0.5 μCi of [^{3}H] AFB_1 (spec. act. 0.25 Ci/mmol, 2 μM final conc.) in 1 ml of reaction mixture containing 10 mM potassium phosphate buffer, pH 7.4, 5 mM Mg^{2+}, 0.65 mM NADPH and 0.35 mg of microsomal protein. Following incubation at 37°C for 60 min, the DNA was extracted, estimated and counted for radioactivity according to a previous procedure (Bhattacharya et al., 1987). Metabolic activation of AFB_1 was performed in a similar manner but containing 0.1 M Tris HCL, pH 7.2. DNA was omitted and 50 μM AFB_1 replaced [^{3}H] AFB_1. The activated metabolite was separated by bi-phasic extraction and measured using a fluorescent techinque as described earlier (Firozi et al. 1986.). Retinoids to be tested was freshly dissolved in DMSO at the required concentration and added to each of the above reaction mixtures before the addition of the microsomes. Solvent concentration was kept at 1% (v/v) and a solvent control was included with each assay. Each assay was conducted in duplicate and the results were found to be reproducible within $\pm$ 10%.

Result and Discussion

The adduct formation made by AFB_1-DNA catalysed by microsomal protein showed that the carcinogen-DNA adduct formation could be effectively controlled after

addition of retinoid compounds. In Table I the summary of the ID_{50} values of different retinoids for adduct formation and activation has been shown.

Table I

Amount of Retinoids required to inhibit by 50% (ID_{50}) the adduct formation and activation of AFB_1.

Retinoids	ID_{50} (nmol) for Adduct formation	Activation
β-carotene (Natural)	90.0	24.0
8'- apo-o-carotenal	38.5	48.0
10'-apo-p-carotenal	36.0	26.0
Cryptoxanthin	53.5	20.0
Lutein	52.5	16.0
Retinal	20.0	40.0
Retinol	40.0	40.0
Retinoic acid	60.0	75.0
Retinyl acetate	45.0	50.0
Retinyl palmitate	190.0	170.0
Retinoyl-p-glucuronide	54.0	87.0

The amount of each carotenoid required to inhibit adduct formation or activation by 50% (ID_{50}) was determined from the graph of percentage inhibition plotted against the dose of the retinoids. Adduct formation refers to AFB_1 -DNA adduct measured in a microsome catalysed reaction, while activation represents Tris-AFB_1 diol formation, where the adduct formation is preceded by the enzymatic activation of AFB_1. The activated metabolite (AFB_1-8-9 epoxide) is highly reactive as well as unstable. However, measurement of its hydrolysis product AFB_1-8-9-dihydrodiol as Tris-diol was followed as described (Firozi et al., 1986).

Benzo(a)pyrene was also studied in detail by Bhattacharya and his coworkers (Shah, 1986). Their studies showed that some retinoids specially vitamin A compounds could inhibit *in vitro* BP-DNA-adduct formation.

From this analysis it is clear that retinoids act actively and effectively as controlling agents in modulating the carcinogen DNA adduct formation catalysed by microsomal protein. AFB_1 and BP are potent carcinogens and are activated by the microsomal monooxygenase forming the most reactive electrophilic group which binds covalently with the DNA molecules. In case of AFB_1-DNA-adduct, carotenoids like 8' apo-, 10'-apo-carotenals cryptoxanthin, lutein are quite effective. It has also been found that the biopotency of 8'-apo and 10'-apo carotenals showed better results than β-carotene. Further, it may be mentioned that β-carotene metabolised into retinal through terminal oxidation (Ganguly & Shasuy, 1985) result in the formation of 8'-apo-β-, 10'-apo-β-, 12'-apo-p-carotenals. These apocarotenoids have ample scope for further detailed studies to be made. In our recent studies with β-carotene, 8- apo- β-carotenal, 8'-apo-β-carotene methylester and palm oil carotenoids, it has been seen that these carotenoids can significantly control BP- induced fore-gut tumors in mice (Azune et al., 1992). Lutein is a precursor of 3-4-dehydroretinol (Barua & Goswami, 1977; Goswami & Barua, 1981; Goswami & Bhattacharjee, 1982; Goswami & Basumatary, 1988; Goswami & Dutta, 1991; Goswami, 1984a,b.). The metabolism of lutein and cryptoxanthin, in their conversion into 3-4 dehydroretinol, is almost similar (Goswami 1984b). The metabolism of lutein and crytoxanthin with reference to biogenesis of 3-4 dehydroretinol have been studied in detail by the workers mentioned above, both in mammals and in lower vertebrate, like freshwater fish. Both lutein and cryptoxanthin showed an almost similar biopotency in controlling the adducts formed by AFB_1-DNA. We are now examining the effect of 3-4 dehydroretinol, isolated from the fish liver oils and have received some encouraging results in controlling such molecular actions in modulating the actions of environmental carcinogens like AFB_1 & BP.

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With reference to other vitamin A compounds, retinal has shown good response in controlling the adduct in both AFB_1-DNA and BP-DNA adducts (Shah, 1986). Retinoyl glucuronide is a metabolite of retinoic acid and being water soluble in nature it has depicted ample scope in its application (Barua & Olson 1988). The compound with 54 nmole concentration could inhibit 50% (ID_{50} value) of AFB_1-DNA-adduct.

In view of the above results it may be mentioned that retinoids are involved in a direct role with the anti-proliferative activity of carcinogenic growth. Environmental carcinogen like AFB_1, BP etc. are posing a serious threat to tropical regions as well as areas of industrial growth and it has been found that incidence of occurrence of epithelial cancer is increasingly on the rise. The results of earlier epidemiological studies and different *in vitro* and *in vivo* experiments including taking various cell lines, indicate that retinoids play a responsible role in modulating the action of carcinogenic growth.

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FREE RADICALS AND ANTIOXIDANTS IN MALARIA

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Summary

Phagocyte-derived reactive oxygen species have been suggested to be involved in the host response against the intra-erythrocytic malaria parasite. It also has been hypothesized that these oxidants are responsible for some of the tissue damage underlying malaria pathology. These concepts are here critically examined in the light of existing evidence and some new observations.

Introduction

Malaria is a tropical disease of immense human, medical and economic importance. There are some hundreds of millions of cases of malaria each year world-wide and at least two million fatalities. Malaria is caused by parasites of the genus *Plasmodium*, which spend part of their life-cycle in a mosquito and part in an alternate host. Each *Plasmodium* species infects only a small range of alternate hosts. The species

most relevant to human malaria are *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*. The life-cycle of the parasite is complex and will not be discussed here, except to note that the host immune response and the pathological complications of the disease both occur during an asexual intra-erythrocytic cycle in the alternate host. This concordance is probably not coincidental, since there is substantial evidence that some of the pathological complications of the disease are a result of the host immune response against the parasite, as discussed below.

The nature of the host immune response to malaria has been a matter of debate for many years. For some time it was believed that circulating parasitized erythrocytes (PRBC) became coated with antibody against parasite-derived proteins, facilitating phagocytosis by host leukocytes in the micro-circulation of the spleen or liver. However, during the 1970s a number of observations were made which led to some new hypotheses about the nature of the host immune response against the malaria parasite.

Ian Clark, working in London, rediscovered the malaria "crisis forms" (Clark et al., 1976), so named many years earlier by Taliaferro and Taliaferro (1944). These were malaria parasites degenerating within intact, circulating erythrocytes and their existence indicated that the classical view of malaria immunity could not totally explain the observed pattern of response. Clark later (Clark et al., 1981) provided evidence for a central role of macrophages, and more particularly their products, in the anti-malarial response.

J.B.S. Haldane (1949) first proposed that certain deleterious genetic traits might have been preserved in human populations through their ability to protect the carrier against certain tropical diseases, particularly malaria. Evidence both for and against this idea was gathered in epidemiological studies during the 1950s and 1960s. Later, when it became possible to culture *P. falciparum* in human erythrocytes, a number of studies showed that this parasite grew poorly in erythrocytes from people with certain haemoglobinopathies and some types of glucose-6-phosphate dehydrogenase deficiency (reviewed by Nagel & Roth, 1989; Golenser & Chevion, 1989) . At about the same time, Eaton and colleagues (1976) showed that *P. berghei*, a form of malaria that grows in rodents, developed poorly in vitamin E-deficient animals. The underlying theme of these studies was that the parasite itself might exert an oxidative stress on the parasite/erythrocyte unit, that this

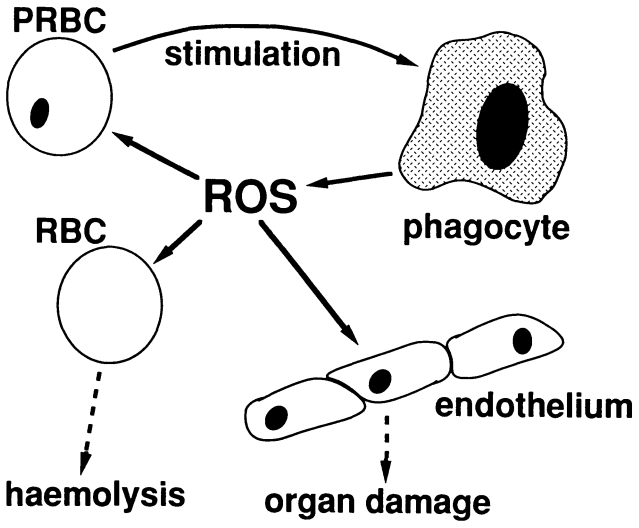

Fig. 1. Possible involvement of reactive oxygen species (ROS) in host immune response against malaria parasites and in the pathology of the disease. Circulating parasitized erythrocytes (PRBC) stimulate host phagocytes to release ROS which damage parasites, PRBC, normal erythrocytes (RBC) leading to haemolysis, and endothelium leading to organ damage.

might be aggravated by the generation of reactive oxygen species (ROS) by unstable haemoglobins, and that reductions in anti-oxidant mechanisms were also deleterious to the parasite.

The 1970s was also a period of rapid increase in our knowledge of the role of ROS in the anti-microbial actions of phagocytes (Babior, 1984). Clark took all these developments into account in formulating the idea that the host anti-malarial response depended, at least in part, on the destruction of intra-erythrocytic parasites by ROS generated by host phagocytes (Clark & Hunt, 1983). Since ROS are non-specific effector

molecules, this idea was further developed to include the concept that the pathology of malarial infection might, in part at least, be the result of damage to "innocent bystander" erythrocytes and endothelial cells by phagocyte-derived ROS generated as part of the host anti-parasite response. These concepts are represented diagrammatically in Figure 1.

ROS and the Anti-Malarial Response

The hypothesis that ROS are involved in the host antimalarial response has been examined by ourselves and others. A number of testable propositions were derived from the central hypothesis, and these will be discussed individually here.

During the Natural Course of Infection, Host Phagocytes and PRBC Should Often be in Close Proximity

Since phagocyte derived ROS have short biological half-lives, they would have to be generated close to PRBC to cause oxidative damage. The role of the spleen in the host response to malaria is well established experimentally (Stevenson & Kraal, 1989; Kumar et al., 1989), and there is ample histological and electron microscopic evidence that PRBC do indeed come into close contact with circulating and tissue-associated phagocytes during their passage through the micro-circulation of that organ.

Evidence of Increased ROS Production in Host Phagocytes during Malaria Infection

Macrophages taken from mice infected with various species of malaria parasite and then exposed to PRBC (Wozencraft et al., 1985; Ockenhouse & Shear, 1984; Makimura et al., 1982) or non-specific stimuli such as PMA (Dockrell et al., 1986; Brinkmann et al., 1984) *in vitro* show an enhanced ability to generate ROS. Similar results have been obtained with mouse (Stocker et al., 1984) and human (Descamps-Latscha et al., 1987) blood and human monocytes (Ockenhouse et al., 1984; Nielsen & Theander, 1989). These observations are therefore consistent with the main hypothesis.

ROS Should Kill Intra-Erythrocytic Malaria Parasites

There have been many studies showing that ROS, or agents that generate ROS, kill malaria parasites *in vivo* and *in vitro*, and these have been reviewed elsewhere (Buffinton et al., 1991; Hunt & Stocker, 1990). In the case of *Plasmodium* species that infect rodents, e.g. *P. vinckei, P. berghei* and *P. yoelii*, agents such as H_2O_2, alloxan, t-butyl hydroperoxide and some 5-hydroxypyrimidines have been shown to kill intra-erythrocytic parasites *in vivo*. In many cases it has been demonstrated that concurrent administration of iron chelators or radical scavengers with the oxidative agents abrogates their anti-malarial activity, indicating that ROS are the effector molecules in this regard. A number of agents that generate ROS have been shown to kill *P. falciparum in vitro*, including t-butyl hydroperoxide, xanthine/xanthine oxidase and amine peroxides. Thus, there is little doubt that intra-erythrocyte malaria parasites are sensitive to oxidative damage.

Evidence for Changes in the Antioxidant Status of PRBC

If PRBC are exposed to oxidative attack of host origin during the natural course of infection, changes would be expected in the antioxidant status of the parasite, the erythrocyte, or both. Such changes might also be expected in the plasma, which contains antioxidants and oxidizable constituents such as low density lipoproteins. A number of studies from our group (Stocker et al., 1985, 1986a, b, c, 1987a) have shown that such changes do occur in P. vinckei infection in mice (Table I).

A notable change in the plasma anti-oxidant status is a substantial increase in the content of bilirubin (Figure 2). As the infection progresses, the percentage parasitaemia (the proportion of erythrocytes containing at least one parasite) increases as the parasite multiplies and infects more erythrocytes. As the parasitaemia increases, the plasma bilirubin concentration rises from low or undetectable levels to concentrations as high as 6 μM. There is extensive haemolysis during *P.vinckei* infection, leading to the release

Table I

Changes in endogenous anti-oxidants in parasitized erythrocytes (PRBC) and plasma during the later stages of P. vinckei infection in mice.

Anti-oxidant	PRBC	Plasma
SOD	⇓	-
Catalase	⇓	N.D.
GSH-Px	⇓	N.D.
GSSG-R	⇓	N.D.
GSH	⇑	N.D.
Ascorbate	⇑	⇑
Uric acid	N.D.	⇑
Bilirubin	N.D.	⇑
α-Toc (w/v)	⇑	⇓
α-Toc (w/lipid)	⇓	⇓

SOD = superoxide dismutase; GSH-PX = glutathione peroxidase; GSSG-R glutathione reductase; GSH reduced glutathione; α-Toc = α-tocopherol; w = weight; v volume; N.D. = not determined. References: Stocker et al., 1985, 1986a, b, c, 1987a.

of intact or partly-degraded haemoglobin which presumably is then metabolized in the liver to bilirubin. This large flux of bilirubin might spill over into the plasma. Since albumin-associated bilirubin is a very efficient antioxidant in some circumstances (Stocker et al., 1987b, c), it may be that an incidental consequence of haemolysis is an increase in the protective ability of the plasma against oxidative attack on the PRBC. However, in human malaria where haemolysis is often of a lesser degree, the overall antioxidant capacity of the plasma seems to be reduced (Thurnham et al., 1990).

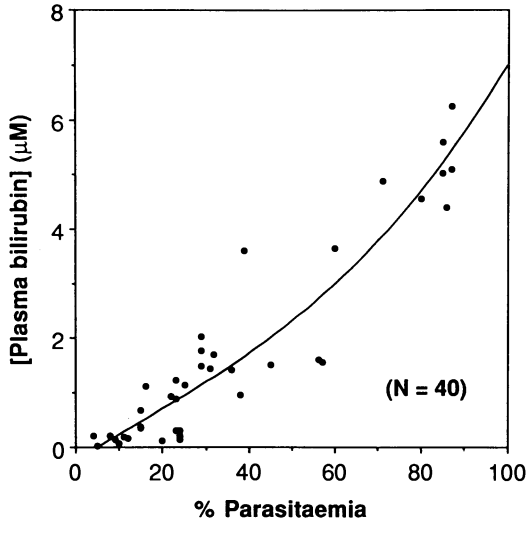

Figure 2. Plasma bilirubin levels during *P. vinckei* infection in mice. At various times after inoculation of the parasite, at various parasitaemias, mice were killed. Plasma was prepared and assayed for unconjugated bilirubin by HPLC as described elsewhere (Stocker et al., 1987b).

The changes in the antioxidant status of the PRBC in murine malaria are complex (Table I). The proteinaceous antioxidants, namely superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, were all decreased in PRBC in *P. vinckei* infection (Stocker et al., 1985). Broadly similar results have been obtained in other experimental malarias (reviewed in Hunt & Stocker, 1990) These enzymes perhaps are pinocytosed from the erythrocyte cytosol along with haemoglobin, from which the parasite obtains amino acids by degradation. The glutathione and ascorbic acid content of the PRBC is substantially increased in *P. vinckei* infection (Stocker et al., 1985, 1986a, b) It might be expected that, if the PRBC were exposed to oxidative stress by the host

immune response, the proportion of ascorbate in its oxidized forms would be increased, but in fact the opposite is the case in *P. vinckei* infection (Stocker et al., 1986b) . It should be noted that although the evidence for an increase in parasite glutathione content in experimental murine malarias is overwhelming (Hunt & Stocker, 1990), the opposite appears to be true in *P. vivax* infection in humans (Bhattacharya & Swarup-Mitra, 1987), as reported elsewhere at the Workshop. The α-tocopherol content of PRBC from *P. vinckei*-infected animals was increased when expressed in terms of packed red cell volume (Stocker et al., 1985), but was decreased when expressed per unit unsaturated lipid (Stocker et al., 1987a).

An overall summary of the antioxidant status of the PRBC, in *P. vinckei* infection at least, is presented in Figure 3. The increased parasite content of glutathione might serve to maintain ascorbate predominantly in its reduced form, and this in turn might serve to preserve erythrocyte plasma membrane α-tocopherol in its reduced form. Although these changes in antioxidant status of the PRBC and plasma are striking, unfortunately they do not throw light on the appropriateness of the main hypothesis.

Evidence for Oxidative Damage to Parasite or Erythrocyte

If the PRBC is exposed to host oxidative attack during the natural course of infection, it would be expected that oxidation products of lipid, protein or DNA might be detectable in the blood of infected animals or humans. The best studied of these parameters is lipid oxidation products.

PRBC isolated from *P. vinckei*-infected mice and incubated in vitro showed a modest level of autoxidation, as indicated by the formation of malonyldialdehyde (measured as thiobarbituric acid reactive material), as shown in Figure 4. The susceptibility of these PRBC to oxidation by t-butyl hydroperoxide was enormously enhanced over that of normal mouse erythrocytes (Figure 4). Injection of t-butyl hydroperoxide into mice infected with *P. vinckei* gives rise to PRBC which subsequently have a greatly enhanced spontaneous autoxidation rate in vitro (Buffinton et al., 1986). However, in none of these circumstances was there evidence for malonyldialdehyde in the blood of *P. vinckei*

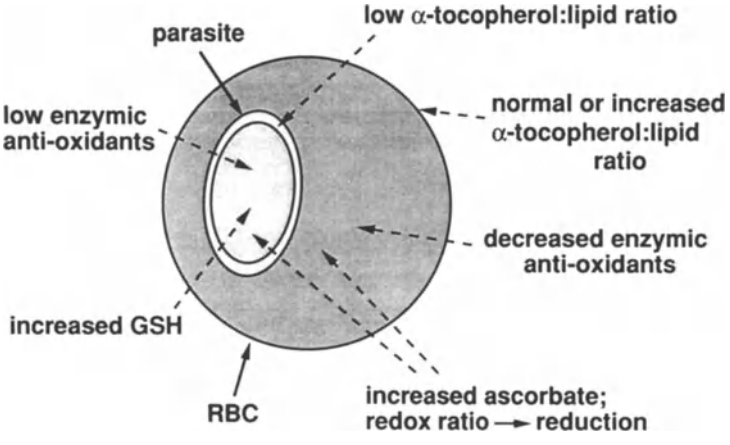

Fig. 3. Summary of changes in the antioxidant status of PRBC in *P. vinckei* infection in mice. Drawn from data published elsewhere (Stocker et al., 1985; Stocker et al., 1986a, b, c; Stocker et al., 1987a). GSH reduced glutathione; RBC = red blood cell.

infected mice during the natural course of infection. Increased plasma levels of malonyldialdehyde have been observed in human *P. falciparum* infection (Das et al., 1988), as reported elsewhere at this Workshop.

PRBC exposed to t-butyl hydroperoxide in vitro generate not only malonyldialdehyde (Figure 4), but also 4-OH-2-nonenal, 2,4-decadienal and hexanal (Buffinton et al., 1988). Since some of the aldehydic products of lipid peroxidation are known to be cytotoxic, we tested their effects against *P. falciparum in vitro* (Clark et al., 1987a). A number of 4-OH-2-alkenals and 2,4-alkadienals were toxic to the parasite, as was a mixture of four aldehydes in appropriate concentrations. These toxic effects were achieved at

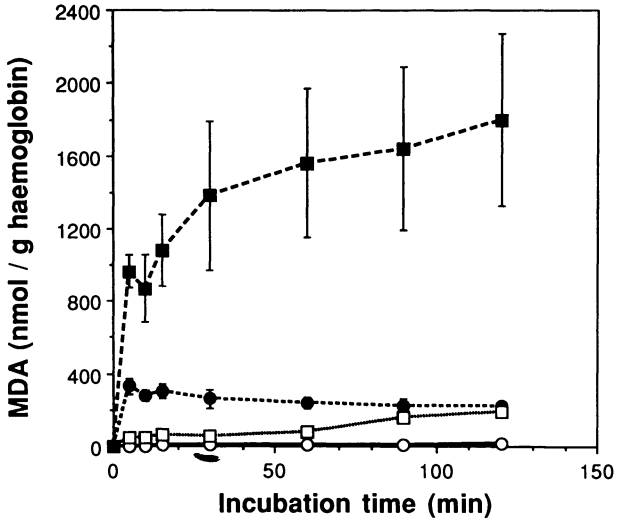

Figure 4. Malonyldialdehyde (MDA) formation in erythrocytes from control, uninfected mice and *P. vinckei*-infected mice in vitro. Washed erythrocytes were prepared, incubated in vitro for various periods of time and analyzed for MDA as described elsewhere (Clark et al., 1984) . ○, control; ●, control + t-butyl hydroperoxide (lmM); □, P. vinckei; ■, P. vinckei + t-butyl hydroperoxide. Values are mean ± SEM (n=5).

concentrations equivalent to those obtained in PRBC exposed to t-butyl hydroperoxide in vitro (Buffinton et al., 1988). It therefore may be that exogenous oxidants exert their action in part by causing the formation within PRBC of aldehydes which then damage the malaria parasite. However, whether this also occurs during the host immune response to the parasite is not clear, since there is no evidence for these cytotoxic lipid peroxidation products in the blood of animals or humans during malaria infection.

The detection of oxidation products of PRBC components in the blood during malaria infection requires further study, since the evidence is currently not strong at this point.

Exogenous Antioxidant Administration Should Protect Malaria Parasites Against the Host Immune Response

If oxidative events are an essential component of the host response against the parasite, it should be possible to intervene in this process by administration of antioxidants. These agents are known to protect PRBC against exogenous oxidative agents, both *in vivo* and *in vitro* (reviewed in Hunt & Stocker, 1990; Buffinton et al., 1991). However, the evidence is not so clear-cut in the case of the natural immune response *in vivo*.

The resonance-stabilized, free radical scavenger butylated hydroxyanisole slightly inhibited the rate of increase of parasitaemia in a resolving *P. chabaudi* infection (Clark et al., 1987b) . In a virulent murine malaria model, *P. berghei*, butylated hydroxyanisole and polyethylene glycol-coupled superoxide dismutase and catalase did not affect the rate of increase of parasitaemia although, as will be discussed later, they protected against malaria pathology in this model (Thumwood et al., 1989).

This crucial test of the main hypothesis has so far not been satisfactorily resolved. It is pertinent to question whether the exogenous antioxidants can gain access to the site of phagocyte/PRBC interaction. However, it could equally be argued that ROS are not important effector molecules in the host anti-malarial response.

Summary and Unresolved Issues

A number of predictions derived from the main hypothesis have been satisfactorily confirmed. However, much better evidence for the existence of oxidation products derived from PRBC during the natural cause of infection should be sought. Our laboratory is currently investigating this. The lack of parasite protection by exogenous antioxidants *in vivo* is also of concern, and we are approaching this problem in a number of ways.

Currently, the balance of evidence is in favor of the idea that ROS are involved in the host immune response against the intra-erythrocytic malaria parasite, but deficiencies in the experimental evidence still leave substantial room for doubt.

The Role of ROS in Malaria Pathology

As discussed earlier, oxidative damage to host erythrocytes and endothelial cells by phagocyte-derived ROS could explain certain aspects of the pathology of malaria infection. If this hypothesis is true, then a number of testable predictions may be derived from it.

Firstly, there should be detectable oxidative damage to organs endothelium or erythrocytes during the natural course of infection. This has not been well studied to date.

Secondly, changes in organ antioxidant status might be expected to occur during malaria infection. There have been few studies on this aspect, though the antioxidant status of the liver in a murine malaria model does exhibit changes, as described elsewhere at this Workshop by Areekul.

Thirdly, it would be predicted that exogenous antioxidants should protect against some aspects of malaria pathology, the best studied form of which is cerebral malaria.

Cerebral Malaria

Human cerebral malaria is a major cause of death from *P. falciparum* infection. It is characterized by convulsions, hemiplegia and coma, and histologically by petechial haemorrhages, monocyte adherence to the endothelium in various organs including the brain, and the plugging of cerebral micro-vessels with PRBC. In order to throw light on the pathogenesis of this condition, animal models have been developed.

In our own studies, we have used CBA mice infected with *P. berghei* ANKA strain (Thumwood et al., 1988, 1989). These mice predictably show convulsions, hemiplegia and coma, leading to death in 95% of cases, some 7 days after inoculation of the parasite. Histologically, the animals show petechial haemorrhages, monocyte adherence to endothelium, oedema, and disruption of the blood-brain barrier. The pathogenesis in this model is T-lymphocyte, and cytokine, dependent (Grau et al., 1986, 1987, 1988, 1989). We therefore reasoned that the monocytes adhering to the endothelium of the cerebral micro-vasculature might cause disruption through the local generation of ROS, thereby leading to the breakdown of the blood-brain barrier (Thumwood et al., 1989).

Administration of antioxidants (butylated hydroxyanisole or polyethylene glycol-coupled superoxide dismutase/catalase) protected these mice against death from cerebral malaria (Thumwood et al., 1989), implicating ROS in the pathogenesis. In our most recent studies, we are examining this phenomenon at the cellular level in the retinae of these animals, since the histological and functional changes there mirror those occurring in the brain. We will look for evidence of oxidative damage and changes to tissue antioxidant defenses in the retina during the course of malaria infection in this model.

Summary

Few studies have been carried out on the role of ROS in the pathology of malaria, but the evidence from the murine cerebral malaria model is consistent with the main hypothesis.

Summary and Closing Remarks

The host immune response to malaria is complex and undoubtedly may differ between species. In some murine malaria models, at least, it appears that activated phagocytes play a major role. The possible processes through which the phagocyte become activated are expressed diagrammatically in Figure 5.

Parasite-derived antigens, either shed from the surface of PRBC or released when, after asexual multiplication, merozoites are released from ruptured erythrocytes, may be processed by antigen-presenting cells. Processed antigen may be presented, in the context of the major histocompatibility complex, to T-lymphocytes, inducing their activation. The activated T-cells are capable of releasing a range of cytokines, including interleukin 4, which promotes antibody release from B-lymphocytes. Antibody against parasite-derived antigens may aid in the phagocytosis of PRBC, free merozoites or debris. Other cytokines, for example γ-interferon, may activate phagocytes, particularly monocytes/macrophages. Another, more simple, route of phagocyte activation for ROS production has been demonstrated *in vitro* (Kharazmi et al., 1987) and seems to involve direct interaction of one or more PRBC products (perhaps antigens) with phagocytes.

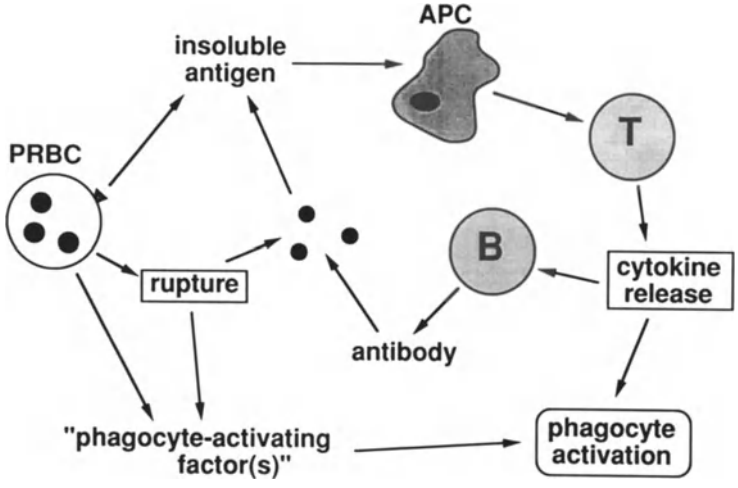

Figure 5. Possible scheme for phagocyte activation in malaria infection. See text for description. PRBC = parasitized erythrocyte; APC = antigen-presenting cell; T = T-lymphocyte; B = B-lymphocyte.

The activated phagocytes, which as discussed earlier are probably located in the micro-circulation of the spleen, release a number of soluble factors that are relevant to the host anti-malarial response (Clark, 1987; Clark et al., 1989), particularly oxygen-derived free radicals and cytokines (Figure 6). Recently, there has been increasing evidence for a role for nitric oxide in host anti-parasite responses (Liew & Cox, 1991) oxygen-derived free radicals potentiate the release of certain cytokines, e.g. tumor necrosis factor (Chaudhri & Clark, 1989), and tumor necrosis factor itself can potentiate the production of ROS from phagocytes (Hoffman & Weinberg, 1987; Wewers et al., 1990). As

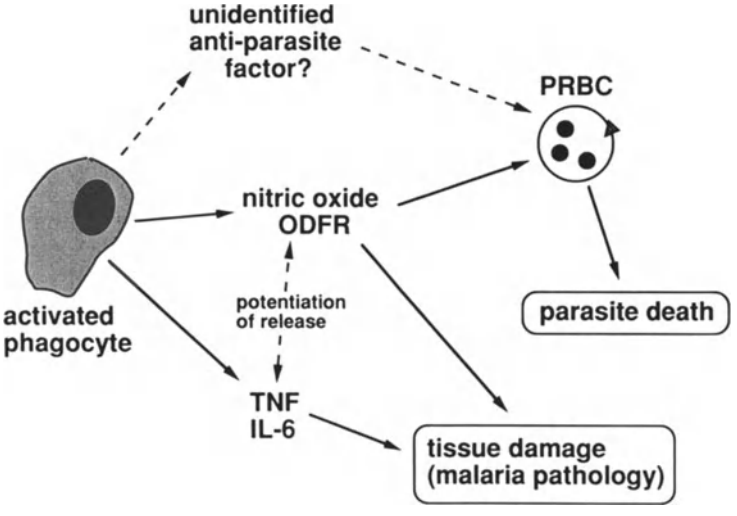

Figure 6. Possible role of activated phagocytes in host response to malaria and the pathology of the disease. See text for description. ODFR = oxygen-derived free radicals; PRBC = parasitized erythrocyte; TNF tumor necrosis factor; IL-6 = interleukin-6.

discussed earlier, there is substantial circumstantial evidence for a role of ROS in the destruction of intra-erythrocytic parasites. However, this is not yet conclusive. If ROS are not the important factor in phagocyte-mediated parasite killing, it would be necessary to postulate the existence of a phagocyte-derived antiparasite factor capable of entering PRBC to destroy the parasite. This is completely speculative.

There is some evidence for a role of ROS in the tissue damage of at least some forms of malaria pathology (Thumwood et al., 1989) and it seems that tumor necrosis factor also plays an essential role in the development of cerebral malaria (Grau et al., 1987).

There is clearly a need to demonstrate definitively whether ROS are involved in both the host anti-malarial response and the pathology of the disease. It certainly seems that there is further scope for investigating the use of antioxidants as intervention therapy in life-threatening complications of malaria, such as cerebral malaria. This has to be balanced against the possibility of such intervention providing extra protection for the intraerythrocytic parasites if the host immune response indeed depends on oxidative mechanisms. However, since there are still a number of effective anti-malarial drugs it seems likely that the possible benefits of antioxidant therapy might outweigh the potential risks.

Another area in which further studies are warranted is the possible association between dietary status, the host immune response against malaria and the pathology of the disease. We have found, in unpublished studies, that even modest dietary restriction can protect mice against experimental cerebral malaria. It is well established that dietary vitamin E deficiency inhibits parasite growth (Eaton et al., 1976) and enhances certain types of experimental anti-malarial therapy (Levander et al., 1989). However, the roles of dietary factors such as the tocopherols and carotenoids have not been well studied in experimental models or human malaria. Such studies may have implications for our understanding of the factors influencing the appearance of the life-threatening complications of malaria.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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OXYGEN FREE RADICALS IN MALARIA

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Summary

Erythrocytes infected with Plasmodium berghei and Plasmodium chabaudi had higher lipid peroxidation than normal erythrocytes. The increase in lipid peroxidation in malaria infected cells may be due to reactive oxygen species. Inhibition of endogeneous superoxide dismutase activity in malaria-infected cells with potassium cyanide increased the rate of lipid peroxidation. Addition of 3-amino-1,2,4-triazole or sodium azide which inhibited endogeneous catalase activity also increase the rate of lipid peroxidation. Furthermore, lipid peroxidation was inhibited by hydroxyl radical scavengers, thiourea and sodium formate and iron chelator, desferrioxamine.

Introduction

A burden in metabolism is caused by toxic intermediates resulting from reduction of oxygen and causes the formation of superoxide anion. Hydrogen peroxide can be produced by superoxide dismutase catalyzed reaction. Hydrogen peroxide reacts with superoxide anion in the presence of iron to generate hydroxyl radicals.

$$O_2 + e^- \text{----------} > O_2^{-\bullet}$$

$$2O_2^{-\bullet} + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2$$

$$O_2^{-\bullet} + H_2O_2 \xrightarrow{\text{Iron}} OH^\bullet + OH^- + O_2$$

It has been shown that the malaria parasite exerts on oxidant stress on infected red blood cells. Injection of alloxan into Plasmodium vinckei-infected mice cause parasite death probably by hydroxyl radical generation (Clark et al., 1983), Hydrogen peroxide was reported to be the main oxygen species in killing of Plasmodium voelii in vitro (Dockrell et al., 1984). Superoxide dismutase protected red blood cells against oxidative damage and was reported to be elevated in Plasmodium berghei-infected erythrocytes (Fairfield et al., 1983).

Lipid peroxidation are used as the indicator of effect of oxidant stress. In the present study, the change in membrane lipid peroxidation of malaria-infected erythrocytes was investigated. We report the possible involvement of superoxide anion and hydrogen peroxide on membrane lipid peroxidation and the combined effect may be potentiated by iron probably through generation of hydroxyl radicals.

Materials and Methods

Plasmodium berahei-infected red cells with 40% parasitemia were separated from leucocytes by passing through a cellulose CF-11 column. The eluted erythrocytes from the column were centrifuged at 800 g for 15 min. The brown top layer of packed red cells contained the enriched infected cells with 85% parasitemia was used in our study as Plasmodium berghei-infected cells. Plasmodium-chabaudi-infected erythrocytes were prepared as described in the above experiment and red blood cells with 85% parasitemia

was used as Plasmodium berghei infected cells. As a measure of lipid peroxidation, spontaneous malonyldialdehyde (MDA) production of red blood cells was measured by color reaction with thiobarbituric acid (Stock et al., 1972).

Result

As shown in Figure 1, spontaneous lipid peroxidation in malaria infected erythrocytes occurred to a greater extent than in normal erythrocytes. Lipid peroxidation in Plasmodium chabaudi infected erythrocytes was higher than Plasmodium berghei-infected erythrocytes and lipid peroxidation increased as the incubation time increased. These results demonstrated that red blood cells from malaria-infected mice had increased lipid peroxidation and the degree of lipid peroxidation in erythrocytes infected by parasites of different strains was different.

In order to find out whether the increase in lipid peroxidation is dependent on oxygen, normal and parasitized cells were incubated in the absence and presence of oxygen and assayed for MDA production. Lipid peroxidation of malaria-infected erythrocytes was increased under aerobic condition. Incubation of normal erythrocytes under aerobic condition did not change their susceptibility to lipid peroxidation (Figure 2).

In human red blood cells there are enzyme superoxide dismutase and catalase. Superoxide dismutase catalyzed the conversion of superoxide anion to hydrogen peroxide and oxygen. Fairfield reported that Plasmodium berghei infected mice derive superoxide dismutase from host cell cytoplasm. Catalase enzyme decomposed hydrogen peroxide to water.

The role of superoxide dismutase and catalase inhibitors on host cell membrane lipid peroxidation was studied (Figure 3). Inhibition of endogeneous superoxide dismutase activity in malaria-infected cells with 0.5 mM potassium cyanide increase the rate of lipid peroxidation. Addition of 5 mM sodium azide or 50 mM 3-amino-1,2,4 triazole which inhibited endogeneous catalase activity also increase the rate of lipid peroxidation.

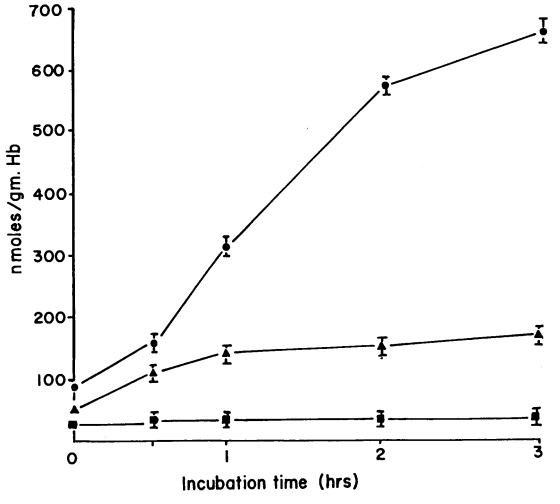

Figure 1: MDA formation of erythrocytes from normal and malaria-infected mice, normal:(■); P. berghei:(▲) and P. chabaudi:(●) infected erythrocytes.

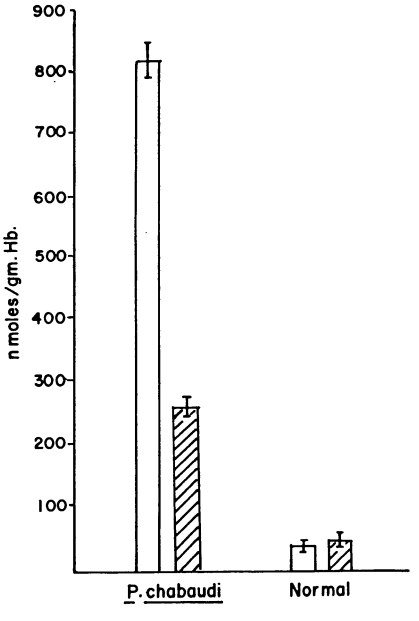

Figure 2 Lipid peroxidation of normal and P. chabaudi infected erythrocytes in aerobic (□) and anaerobic (■) conditions.

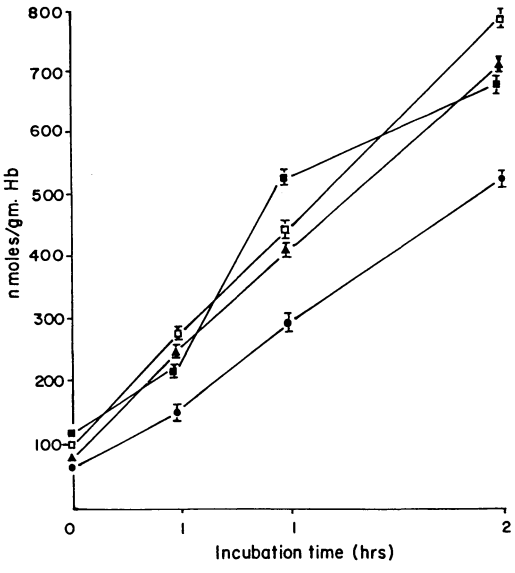

Figure 3 Effects of superoxide dismutase and catalase inhibitors on lipid peroxidation, control (●); potassium cyanide (▲); sodium azide (□) and 3-amino-1,2,4 triazole (■).

Hydrogen peroxide has been reported to react with superoxide anion in the presence of iron to generate hydroxyl radicals. The involvement of iron was tested by addition of $FeCl_2$, $FeCl_3$ or by the addition of iron chelator. Addition of 10 μM $FeCl_2$ or 10 μM $FeCl_3$ increase the rate of lipid peroxidation whereas 10 mM EDTA or 10 μM desferrioxamine inhibited lipid peroxidation (Figure 4).

The specific hydroxyl radical scavenger urea, thiourea or sodium formate inhibited lipid peroxidation (Figure 5).

Discussions

The present study indicates that lipid peroxidation in malaria infected erythrocytes is higher than normal cells. Red cells parasitized with Plasmodium vinckei exhibit

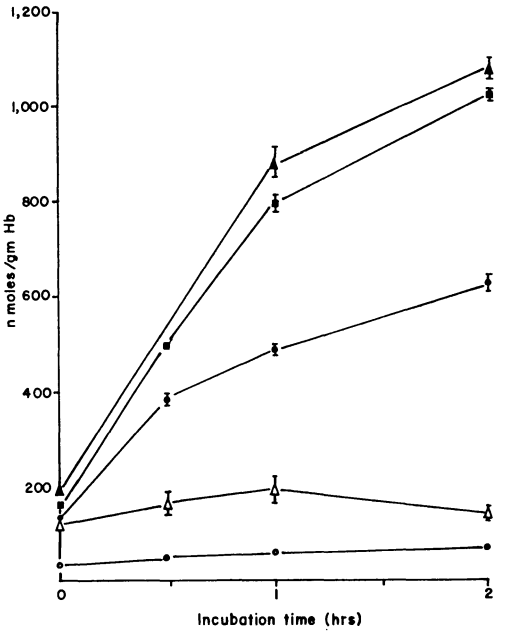

Figure 4: Effects of iron and chelators on lipid peroxide, control (●); $FeCl_3$ (▲); $FeCl_2$ (■); EDTA (Δ); and Desferrioxamine (○).

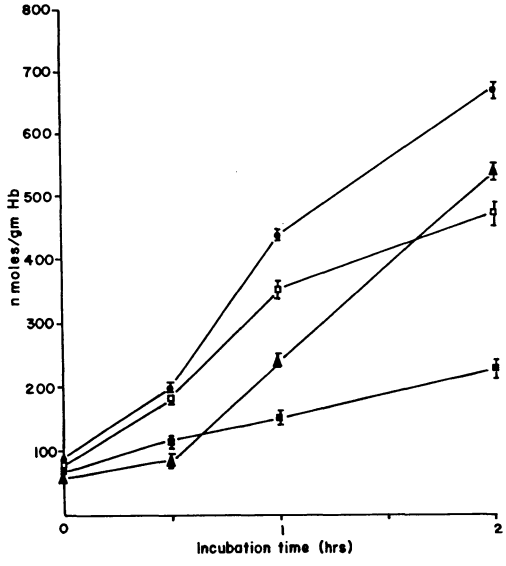

Figure 5: Inhibition of lipid peroxidation by hydroxyl radicals scavengers control (●), urea (▲), sodium format (□) and thiourea (■).

increased lipid peroxidation (Buffinton, et al., 1988). The increased oxidation of host erythrocyte membranes may be due to impairment, by the parasite, of normal erythrocyte oxidant defense and repair pathway. Plasmodium berghei infected erythrocytes tend to become quantitatively deficient in the activity of superoxide dismutase (Fairfield et al., 1983; Fairfield et al., 1986). This deficiency arises through the adoption of host cell superoxide dismutase by the malaria parasite, which internalizes the mammalian enzyme and may use it in its own oxidant defense. An increase in parasite load is also accompanied by decreased activities of the red cell enzymes catalase, glutathione peroxidase and NADH-methemoglobin reductase (Nair et al., 1984; Stocker et al., 1985).

When lipid peroxidation of erythrocytes infected with different strains of plasmodium was compared, lipid peroxidation is elevated in Plasmodium chabaudi-infected cells compared with Plasmodium berghei-infected cells. This can be explained by the fact that the former gives synchronous cells of matured trophozoites and schizonts while the later gives asynchronous cells of ring stages and mature parasites. The increase in lipid peroxidation in parasitized cells may be due to reactive oxygen species generated by the living parasites (Nakornchai et al., unpublished data). One possibility is that some of the oxidants are produced secondary to catabolism of host cell hemoglobin. Superoxide anion can be produced when oxyhemoglobin becomes methemoglobin (Misra and Fridovich, 1972; Carrell et al., 1975) or is digested and transformed by the parasite into hemozoin, conversion of Fe(II) in heme into Fe(III) in hemin involved here. Methemoglobin concentration was increased in Plasmodium berghei-infected cells and the increase correlates closely with the severity of infection (Etkin and Eaton, 1975). The metabolism of the malaria parasite may produce a certain amount of activated oxygen. Plasmodia has enzyme dihydroorotate dehydrogenase (Gutteridge et al., 1979) and xanthine oxidase (Sharma et al., 1978) which will reduce oxygen to superoxide anion (Rajogopalan et al., 1962).

Conclusion

Erythrocytes infected with Plasmodium berghei and Plasmodium chabaudi had higher lipid peroxidation than normal erythrocytes. The increase in lipid peroxidation in malaria-infected cells may be due to reactive oxygen species. Superoxide anion and hydrogen peroxide may play a role in lipid peroxidation and the combined effect is potentiated by iron probably through generation of hydroxyl radicals.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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THE MECHANISM OF ANTIMALARIAL ACTION OF ARTEMISININ (QINGHAOSU)

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Summary

A marked antagonism in antimalarial (Plasmodium falciparum) activity was found for combination of artemisinin or arteether with desferrioxamine. The result suggests a possible involvement of iron in the generation of reactive oxygen intermediates initiated by artemisinin or its derivative. The role of intracellular antioxidant mechanism in parasite defence against the drugs was also studied. Buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthase, an enzyme involved in glutathione synthesis was used in combination with arteether and artesunate. A synergistic effect was observed with artesunate at all concentrations, while for arteether a synergistic effect was observed at the concentrations where the inhibitor alone had little effect on the malaria parasite, while an antagonistic effect was found at higher concentrations. Diethyldithiocarbamate, an inhibitor of superoxide dismutase, also showed a similar biphasic effect in combination with artemisinin. These results suggest that intracellular antioxidant mechanism may be important in countering the antimalarial effect of artemisinin and its derivatives. They further imply that artemisinin and its derivatives exerted antimalarial action through the oxidant mode.

Introduction

Artemisinin or qinghaosu is a promising new antimalarial drug isolated from a Chinese traditional herb Artemisia annua L. since 1972. It has the structure of a sesquiterpenoid lactone with an endoperoxide linkage required for its antimalarial activity

(Brossi, et al., 1988; Klayman, 1985). The necessity of the endoperoxide function leads to the hypothesis that artemisinin exerts its antimalarial action through an oxidant mode. Many lines of indirect evidence have been gathered to support this hypothesis. Artemisinin and its derivatives have been found to cause hemolysis (Gu & Warhurst, 1986; Scott et al., 1989), premature lysis of infected red cells (Gu & Inselburg, 1989), lipid peroxidation of red cell membrane (Meshnick et al., 1989), and decrease of red cell deformability (Scott et al., 1989). The antimalarial activity of artemisinin can be enhanced by increasing oxygen tension, by addition of oxidant generating drug, doxorubicin, (Krungkrai & Yuthavong, 1987), and also potential catalytic oxidant catechol flavone, castecin and artemitin (Elford et al., 1987). α-Tocopherol, catalase, dithiothreitol (Krungkrai & Yuthavong, 1987), ascorbate and reduced glutathione (Meshnick et al., 1989) were found to antagonize the antimalarial effect of artemisinin. Recently, artemisinin was found to react with hemin and cause oxidation of protein thiols (Meshnick et al., 1991).

In this paper, we report the evidence of possible involvement of iron in antimalarial action of artemisinin and derivative, and the role of intracellular antioxidant system in countering the antimalarial action of artemisinin and derivatives, arteether and artesunate. The results support the hypothesis that artemisinin and derivatives work in conjunction with intracellular iron to produce an oxidant stress on the parasite.

Materials and Methods

Culture of P. falciparum and Drug Testing

P. falciparum K1 strain was cultured in vitro by the candle jar technique (Trager & Jensen, 1976). Antimalarial activity of artemisinin and its derivatives (artesunate and arteether) in combination with iron chelator desferrioxamine (DFO), or enzyme inhibitors (buthionine sulfoximine, BSO, and diethyldithiocarbamate, DDC) was tested by using the ^{3}H-hypoxanthine incorporation method (Desjardins et al. 1979). Stock artemisinin and derivatives were dissolved in dimethylsulfoxide; other agents were dissolved in water. The

drugs were diluted with culture medium (RPMI 1640 medium, supplemented with HEPES 25 Mm, $NaHCO_3$ 0.2%, gentamicin 40 μg/ml and human serum 10%, Ph 7.4). The final organic solvent concentration was 0.001% and had no effect on parasite growth.

25 μL of medium containing drugs (individually or in combination) and 200 μL of 1.5% cell suspension (1-2% parasitemia) were placed in 96 well plastic plates and cultured in the candle jar, at 37°C for 24 hours. 25 μL of 0.5 μCi ^{3}H-hypoxanthine was added to each well and cultured in the same condition for additional 18 hours. The radioactivity incorporated was determined and the EC_{50} (the drug concentrations required to reduce radioactivity incorporated to 50% of control without drug) of the drug concentrations between artemisinin or derivatives and the others were constructed as isobolograms.

Results

Figure 1 shows the marked antagonistic effect of the iron chelator DFO with artemisinin (Figure 1A) and arteether (Figure 1B). To ensure that the antagonistic effect was due to the iron chelation property of DFO, iron-saturated DFO was prepared (Asbeck et al., 1984) and the combination with arteether tested. The result is also shown in Figure 1B. The chelator presaturated with iron did not show any antimalarial activity at up to 6 times the concentration of its EC_{50} without iron added, and did not cause any change in EC_{50} of arteether. Therefore, we conclude that antagonistic effect of iron chelator on artemisinin and arteether is due to chelation of intracellular iron, making it unavailable to augment the activity of these two drugs.

Figure 2 shows the effect of combination of BSO, an inhibitor of glutathione synthesis, with artesunate and arteether. A mild synergistic effect was observed for artesunate/BSO combination (Figure 2A). By contrast, the combination effect observed for arteether was biphasic (Figure 2B). At low BSO concentrations, the effect was synergistic while at high concentrations, the effect was antagonistic. A similar biphasic effect was also obtained from DDC, an SOD inhibitor, in combination with artemisinin, Figure 3.

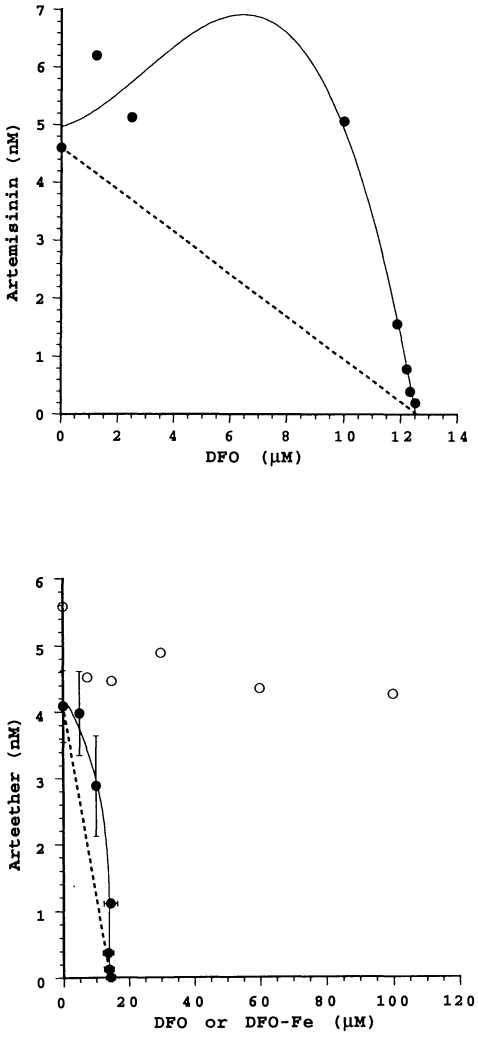

Fig. 1. Isobolograms of artemisinin (1A) and arteether (1B) in combination with desferrioxamine, DFO (●), or iron saturated DFO, DFO-Fe (○). The curves are fitted with the third order polynomial equation. The line for additive effect is also shown (---).

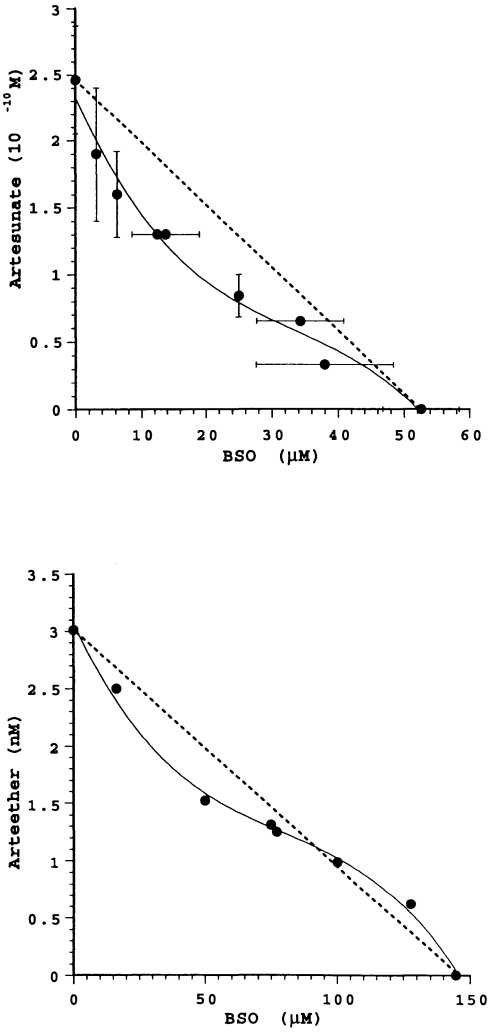

Fig. 2. Isobolograms of artesunate (2A) and arteether (2B) in combination with buthionine sulfoximine (BSO). The curves are fitted with the third order polynomial equation. The line for additive effect is also shown (---).

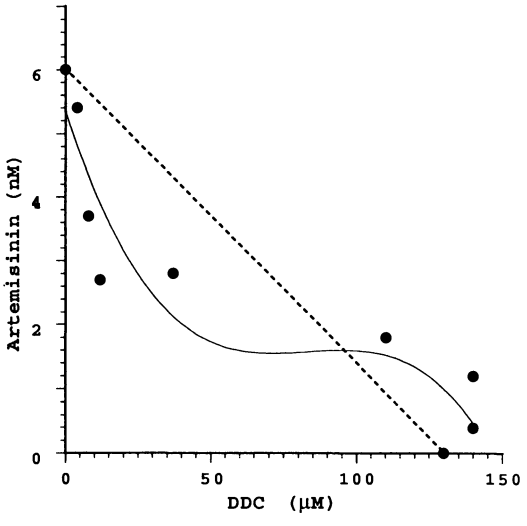

Fig. 3. Isobologram of artemisinin and diethyldithiocarbamate(DDC). The curve is fitted with third order polynomial equation. The line for additive effect is also shown.

Discussion

The mechanism of action of artemisinin and its derivatives has been studied with respect to possible involvement of iron by using the iron chelator DFO, which has antimalarial activity in itself (Fritsch et al., 1985; Raventos-Suarez et al., 1982). DFO showed a marked antagonistic effect in combination with artemisinin and arteether, while iron-saturated DFO showed no antimalarial activity and did not change the EC_{50} of

arteether. The absence of antimalarial activity of iron-saturated DFO had been observed before (Fritsch et al., 1987a). Therefore, we can conclude that DFO, which works as an antimalarial by chelating with intracellular iron is antagonistic with artemisinin and arteether because it takes away iron which participates in the action of these drugs. Furthermore, DFO has been used widely as a specific in vitro and in vivo probe for iron-dependent radical reaction (Gutteridge et al., 1979; Halliwell 1985; Hershko & Weatherall, 1988). Hence, the antagonistic effect of DFO on artemisinin and arteether action provides good evidence both for artemisinin and derivatives as radical generator, and for the involvement of iron in radical generation processes. In the presence of iron, artemisinin and arteether may undergo the so-called Fenton-type reactions (Halliwell & Gutteridge, 1986; Saltman, 1989), providing hydroxyl radical or other radicals derived from them. Other reactive intermediates (e.g. superoxide anion, hydrogen peroxide) may also be produced. These may serve as mediators in killing the malaria parasite, which is known to be susceptible to oxidant stress (Clark & Hunt, 1983; Friedman, 1979; Golenser & Chevion, 1989; Kamchonwongpaisan et al., 1989; Ockenhouse et al., 1984; Wozencraft et al., 1984). This antagonism is therefore similar to previously observed prevention of alloxan-induced killing of P. vinckei by DFO (Clark & Hunt, 1983). The antagonistic action is not confined only to DFO. We have found marked antagonism between arteether and other iron chelators of different types, namely, pyridoxal benzoylhydrazone and 1,2-dimethyl-3-pyrid-4-one (data not shown).

If artemisinin and derivatives act through generation of oxidant stress, it is possible that the glutathione status of the parasite is important in the mechanism of action. Glutathione is the substrate for hydrogen peroxide detoxification by glutathione peroxidase. Buthionine sulfoximine is an inhibitor of enzyme γ-glutamylcysteine synthase, a crucial enzyme in biosynthesis of glutathione (Griffith & Meister, 1979). The observed synergistic effect of BSO with artesunate and of low BSO concentration with arteether (Fig.2) suggests that the level of glutathione is indeed important for the parasite for detoxification of the drugs. This result can be compared with a previous observation on decreased efficacy of artemisinin by addition of reduced glutathione (Meshnick et al.,

1989). In the previous report, however, the glutathione was added to the medium, and its effect must have an extracellular origin. Our present result also supports the observation that added dithiothreitol, α-tocopherol and catalase also reduced the efficacy of artemisinin (Krungkrai & Yuthavong, 1987). The actual role of intracellular glutathione is, however, not yet clear. Depletion of selenium, which is a part of the active site of glutathione peroxidase, in P. berghei infected mice did not affect antimalarial activity of artemisinin showing that the Se-dependent glutathione peroxidase may not be the enzyme responsible to detoxify the effect of artemisinin (Levander et al., 1989) . However, malaria parasites also possess a Se-independent glutathione peroxidase activity (Fritsch et al., 1987b). Its role in artemisinin action has not been elucidated, as also the role of other glutathione-utilizing enzymes. Our results are somewhat complicated by the fact that at high concentrations of BSO, an antagonistic effect with arteether is observed. However this observation may be explained by assuming that, although the biosynthesis of new glutathione is blocked by BSO, the already existing glutathione may be adequate for countering the effect of arteether at low concentrations.

The function of antioxidant enzymes may also be important if artemisinin acts through an oxidant generating mode. Superoxide dismutase (SOD) is an antioxidant enzyme for detoxification of superoxide anion, one of the oxygen intermediates believed to be produced in the killing action of artemisinin. P. falciparum has been found to contain mostly the adopted host copper-zinc SOD with a minor amount of endogenous SOD isozyme (Fairfield, et al., 1988; Ranz & Meshnick, 1989). A Cu-Zn SOD inhibitor, diethyldithiocarbamate (DDC) (Heikkila, et al., 1976) has been found to have an antimalarial activity (Meshnick, et al., 1990; Scheibel, et al., 1979). In the combination of artemisinin with DDC, a synergistic effect was observed at high artemisinin concentrations, although there was antagonistic effect at low artemisinin concentrations. The result suggests an important role of SOD in countering the killing effect of artemisinin.

Conclusion

Artemisinin and derivatives exert the antimalarial action through oxidant stress with the involvement of intracellular iron. The roles of intracellular iron in enhancing the antimalarial activity, and of reduced glutathione and superoxide dismutase in reducing the activity, as concluded from the effects of DFO, BSO and DDC are summarized in Figure 4.

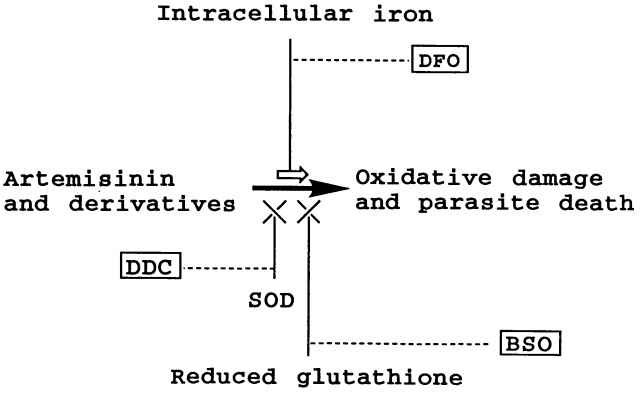

Fig. 4. Proposed roles of intracellular iron, SOD and reduced glutathione in modifying the antimalarial activity of artemisinin and derivatives. Iron enhances, while SOD and reduced glutathione reduce the oxidative damage by the drugs. By chelating with iron, DFO reduces the activity, while by inhibiting SOD and synthesis of reduced glutathione respectively, DDC and BSO enhance the activity of artemisinin and derivatives.

Acknowledgements

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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ERYTHROCYTIC GSH LEVEL AND STABILITY IN PLASMODIUM VIVAX MALARIA

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Summary

The existence of haemolytic anaemia in malaria indicates disturbances in red cell stability due to physical as well as metabolic stress attributable to the malarial parasite. Impact of Plasmodium vivax infection on the oxidative metabolism of host red cells was studied by evaluating the parameters, erythrocytic enzymes glucose-6-phosphate dehydrogenase (G-6-PD), glutathione-reductase (GR), glutathione-peroxidase (GSH-Px) activity, methaemoglobin (Met Hb) level, erythrocytic reduced glutathione (GSH) level as well as its stability in forty P.vivax infected patients both before and after therapy with chloroquine. Forty (40) normal subjects, age and sex matched, were simultaneously studied as controls. The role of parasitaemia on these parameters was also studied by analyzing the changes at different levels of parasitaemia. During infection there was a significant decrease in GSH level and its stability ($P < 0.05$) along with depression in the activity of GR ($P < 0.05$). There was also a positive correlation between GSH level and stability with GR activity. On the other hand Met Hb level increased significantly ($P < 0.05$) having a negative correlation with GR activity and GSH level. Activity of G-6-PD and GSH-Px remained unaltered. Studies of these parameters at different levels of parasitaemia showed a steady decrease in of GSH level and stability, GR activity and an increase in of Met Hb level with increasing parasitaemia. All parameters were normalized after successful therapy and cure of malaria. This composite picture indicates that P.vivax was responsible, directly or indirectly, for reduction of GR activity with subsequent decrease in GSH level and stability favoring oxidation of Hb to Met Hb and thus affecting the oxidative metabolism of host red cells by disturbing red cell integrity. This in turn could predispose to haemolysis, thereby leading to anaemia in P.vivax malaria.

Introduction

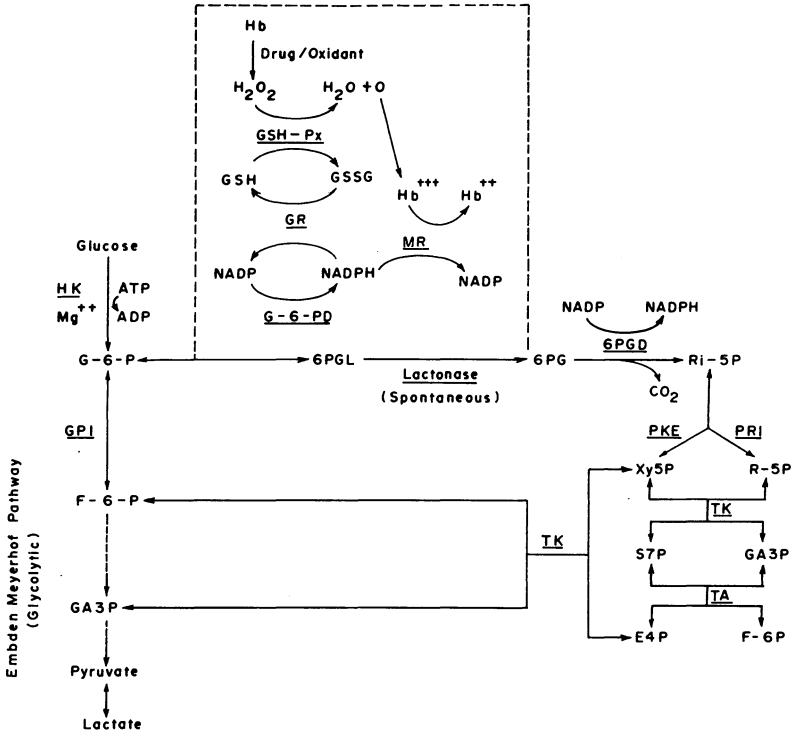
Malaria, a tropical 'blood disease' and a great killer of mankind, is still a serious global public health problem of the day. Haemolytic anaemia is one of the hallmarks of malaria. The enzymes involved in maintaining the integrity of red cells[5] under stress[8] are those of the oxidative metabolism of red cells[5]: viz. glucose-6-phosphate dehydrogenase[2]

(G-6-PD), glutathione reductase[9] (GR) and glutathione-peroxidase (GSH-Px) Another important factor is erythrocytic reduced glutathione[3] (GSH) level and its stability. A continuous supply of NADPH (reduced nicotinamide adenine dinucleotide phosphate) is necessary to maintain an adequate level of GSH and thus the competence of the red cell to combat oxidants, since GSH facilitates the action of the erythrocytic enzymes GR and GSH-Px. NADPH supply is mediated by the action of the enzyme G-6-PD. (Fig.1).

Welt et al. (1971) have emphasized that G-6-PD-deficient red cells[5] are handicapped in generating NADPH at a rate sufficient to facilitate the destruction of the peroxides produced by drugs or infection and are consequently susceptible to haemolysis. Besides G-6-PD, any deficiency of GR or GSH-PX under oxidative stress also may cause haemolysis by hampering GSH metabolism. GSH plays an important role in the protective mechanism of red cell[5]. It is a reductant that maintains free-sulfhydryl groups and keeps haemoglobin (Hb) in a functionally active form. There is also evidence that the malarial parasite grows to a lesser extent in G-6-PD deficient red cells (Luzzatto et al., 1969).

Various theories have been advanced to explain the mechanism by which malaria causes excessive anaemia[6]. One of them is altered red cell[5] metabolism because of metabolic and oxidant stress during Plasmodial infection. The relationship between the decreased red cell[5] survival and increased oxidant stress[8] is not well understood; although the parasite is said to generate hydrogen peroxide (Etkin & Eaton, 1975), very little is known about whether this leads to oxidative damage to RBC. It is likely that the host cell metabolism will be affected by the intracellular parasite for its growth and development.

Studies on different species of animals have shown the involvement of red cell[5] metabolism in malaria. Fletcher & Maegraith (1962) showed an increase in G-6-PD and 6-P-GD levels in rhesus monkey erythrocytes during P. knowlesi infection, while mouse and hamster erythrocytes infected with P. berghei and P. vinckei respectively, did not show any increase in G-6-PD activity, though 6-P-GD activity increased. P. berghei-infected mouse erythrocytes and P. lophurae-infected duck erythrocytes showed



HK=Hexokinase, ADP=Adenosine diphosphate, ATP=Adenosine triphosphate, NADP=Nicotinamide adenine dinucleotide phosphate, NADPH=Reduced NADP, G-6-PD=Glucose-6-phosphate dehydrogenase, 6-P-GD=6-phosphogluconate dehydrogenase, H_2O_2=Hydrogen peroxide, GSH=Reduced glutathione, GSH-Px=Glutathione peroxidase, GSSG=Oxidized glutathione, Mg=Magnesium, MR=Methaemoglobin reductase, G-6-P=Glucose-6-phosphate, F-6-P=Fructose-6-phosphate, GA3P=Glyceraldehyde-3-phosphate, GPI=Glucose phosphate isomerase, 6PGL=6-phosphogulonolactone, R-5P=Ribose-5-phosphate, S-7P=Sedoheptulose-7-phosphate, E-4P=Erythrose-4-phosphate GR=Glutathione reductase, TK=Transketolase, PRI=Phosphoribose isomerase, PKE=Xylulose phosphate epimerase, Xy-5P=Xylose-5-phosphate, TA=Trans aldolase, 6PGL=6-phospho gulonolactone, 6GP=6-phospho gluconate, Ri-5P=Ribose-5-phosphate CO_2=Carbon dioxide.

Figure 1. Pentose Phosphate Pathway(Aerobic) of Glucose Metabolism in Red Cell. Barricaded lines denote effect of oxidant on erythrocytic GSH and related enzymes

no increase in G6-PD activity with parasitaemias ranging from 28-78%, though above this level there was a decrease in enzyme activity (Sherman, 1965). This evidence suggests that there is some involvement of red cell metabolism in malaria. Erythrocytic GSH plays an important role in maintaining the integrity of red cells (Fegler, 1952), particularly when exposed to oxidative stress due to drugs or infection (Allen & Jandl, 1961). As there is a close relation between the erythrocytic enzyme G-6-PD and GSH metabolism, it seems useful to follow any parallel changes in these two parameter. Working with P.knowlesi malaria in monkeys and P. berghei malaria in mice several workers (Fulton & Grant 1956; Fletcher & Maegraith, 1970; and Eckman & Eaton, 1979) showed substantial changes GSH metabolism as consequence of the metabolic demands of the malaria parasite. On the other hand work on rodents showed no change in erythrocytic GSH level during P. berghei infection. Work on P. lophurae in duck erythrocytes also showed no change in GSH level (Sherman, 1965). These reports suggest that there is a species-related difference with regard to metabolism of GSH in malaria.

Anaemia[6], the common feature of malaria is caused by the premature destruction of red cells[5] (Woodruff et al., 1979) following invasion by the parasite. Severe anaemia is observed in approximately 20% of patients with P. falciparum malaria having normal erythrocyte G-6-PD activity (Caulfield, 1967). Eaton et al. (1976) showed that the malarial parasite exerted "Oxidative stress" to the host red cell metabolism.

Methaemoglobin[10], (Met Hb) is formed when the ferrous porphyrin complex of haemoglobin (Hb) is oxidized to the ferric form. A reversible oxidation of oxyhaemoglobin to Met Hb occurs continuously within red cells. Under normal conditions the rate of Met Hb accumulation is very slow due to the action of Met Hb reductases (Gibson, 1948), but when red cells are exposed to stress or drugs with high redox potential Hb metabolism is altered (Allen & Jandl, 1961). As malaria acts as a stressor for the red cell (host) metabolism, an enquiring into the status of Met Hb in patients of malaria seems pertinent.

There is little information on the metabolic interrelationship between the human malarial parasite and host red cell. A study therefore was undertaken on some aspects of the oxidative metabolism[7] of host red cells[5] to identify the changes, if any, brought about by the malarial parasite P. vivax, which is prevalent in India, particularly in West Bengal (Hati and Mukhopadhyay, 1980). The parameters studied were G-6-PD, GR, GSH-Px activities, level and stability of GSH and Methaemoglobin (Met Hb) level of host red cells. The effect of chloroquine therapy on these parameters was also studied.

Materials and Methods

Forty confirmed cases of P. vivax infection with normal activity of erythrocytic glucose-6-phosphate dehydrogenase[3] (G-6-PD), admitted to Carmichael Hospital for Tropical Disease, Calcutta, were investigated before administration of antimalarial drugs. G-6-PD deficient patients were excluded from this study. The patients were aged between 8 and 50 years and comprised 24 males and 16 females. Forty (40) age and sex-matched uninfected subjects were studied simultaneously as controls.

Routine clinical and haematological examinations were carried out according to standard techniques (Dacie & Lewis, 1975). Malaria was confirmed by clinical symptoms as well as microscopical examination of blood smears. Erythrocytic GSH level was determined by the method of Beutler et al. (1963) and GSH stability was determined by Beutler's technique (1957) known as the "glutathione stability test" based on incubation of erythrocytes with acetyl phenylhydrazine (APH). The erythrocytic enzyme GR activity was determined by the technique of Racker (1955). Erythrocytic GSH-Px activity was determined by the method of Gross et al. (1967). The G-6-PD screening test was performed by the method of Berstein (1962). Activity of erythrocytic G-6-PD was assayed by the method of Kornberg and Horekar (1955) as modified by Marks (1958). Erythrocytic met Hb level was estimated by the method of Evelyn and Malloy (1988).

The above parameters of host red cell[5], these were evaluated at different stages of parasitaemia. The parasites per 100 leucocytes were counted and values per cubic millimeter of blood were obtained via the white cell count (Pinder, 1973; Seshadri et al., 1983).

A repeat study was carried out in 30 patients 8-10 days after conclusion of therapy with chloroquine phosphate which consisted of a loading dose of 1 g of the drug followed by 500 mg after six hours and 500 mg daily for the next two days. By this time the patients were afebrile, their blood was free of parasites and patients were cured of malaria.

For the statistical analysis of the data obtained in this study the ANOVA (Analysis of Variance) technique was applied (Snedecor & Cochrane, 1967).

Results

Haemotological Parameters

Results of haematological investigations of 40 malaria patients studied both before and after therapy are shown in Table I, along with 40 normal controls. There was a decrease in Hb level in the malaria patients before therapy. Anaemia[6] was present in 34 cases, where the Hb level was below 12 g/100ml. In the remaining 6 cases the Hb level was within normal limits. Their PCV (packed cell volume) and MCHC (mean corpuscular haemoglobin concentration) values were uniformly low, but reticulocyte count was increased in most of the cases as compared to controls. There was also some monocytosis; leucopenia was found in 9 cases and lymphopenia was found in 11 patients.

All the other parameters studied both before and after therapy, in malaria patients, are shown in Table II along with normal controls.

TABLE II Effect of Plasmodium vivax on the Oxidative Metabolism of Host Red Cell

	G-6-PD units/ml RBC/min	GR units/100 ml RBC/min	GSH-PX in K/g Hb/min	GSH in mg/100 ml RBC		Met Hb (%)
				Preincubation	Post-incubation	
Control (C)						
Mean ± S.E.	5.10 ± 0.19	4.40 ± 0.19	36.66 ± 0.66	63.97 ± 2.22	55.30 ± 2.21	1.41 ± 0.07
Range	3.60 - 8.00	3.50 - 6.60	32.80 - 49.30	36.20 - 88.90	32.10 - 83.10	0.81 - 1.80
No. of Subjects	40	25	25	40	40	20
Before Therapy (B.T.)						
Mean ± S.E.	5.04 ± 0.25	2.20 ± 0.17	35.92 ± 0.94	41.08 ± 2.16	35.50 ± 2.02	5.80 ± 0.54
Range	3.50 - 9.80	0.80 - 4.30	31.70 - 48.35	19.30 - 66.40	15.20 - 61.10	3.00 - 12.40
No. of Subjects	40	25	25	40 *9	40 *17	20
After Therapy (A.T.)						
Mean ± S.E.	4.41 ± 0.16	4.00 ± 0.22	34.02 ± 0.82	57.90 ± 2.78	47.83 ± 2.89	2.20 ± 0.22
Range	3.40 - 7.00	2.80 - 7.30	30.50 - 45.80	32.00 - 80.90	24.80 - 76.80	1.50 - 4.00
No. of Subjects	30	20	20	30	30	15
Comparison Between Groups						
C. vs. B.T.	NS(P>0.05)	S(P<0.05)	NS(P>0.05)	S(P<0.05)	S(P<0.05)	S(P<0.05)
C. vs. A.T.	NS(P>0.05)	NS(P>0.05)	NS(P>0.05)	NS(P>0.05)	NS(P>0.05)	NS(P>0.05)
B.T. vs. A.T.	NS(P>0.05)	S(P<0.05)	NS(P>0.05)	S(P<0.05)	S(P<0.05)	S(P<0.05)

S = Significant NS = Not Significant n = No. of Subjects Studied; * indicates no. of cases showing GSH below 30 mg/100 ml

GSH Level and Stability

Erythrocytic GSH level in malaria patients during infection was significantly lower than that in controls ($P < 0.05$). In 12 cases the values were 40 mg/100ml and in 9 they were less than 30 mg/100ml (Table II). In normal subjects instability of erythrocytic GSH after incubation with APH was not found. In malaria patients before therapy the GSH level dropped below 30 mg/100ml erythrocytes (RBC) after APH incubation in 17 cases, indicating GSH instability. These cases include the 9 subjects having GSH values below 30 mg/100ml before APH incubation, whose values dropped by a further 10-25% on incubation. The mean value for GSH stability was significantly lower than that of controls ($P < 0.05$; Fig. 2). Hence, P. vivax exerts a significant effect on GSH level and stability.

G-6-PD Activity

The activity of G-6-PD during infection was not significantly different from normal ($P > 0.05$).

GR and GSH-PX Level

There was a significant depression in the activity of GR in malaria patients during infection ($P < 0.05$). In 6 cases the enzyme activity was less than 1.5 units/ml RBC/min (Table II and Fig.3). GSH-PX activity was not significantly different in malaria patients as compared to normal controls. During P.vivax infection there was a significant increase in Met Hb level as compared to in controls ($P < 0.05$; Table II and Fig. 4).

There were positive correlations between GSH level and GR activity (co-efficient of correlation, $r=0.91$; Fig. 5) and between GR activity and GSH stability ($r= 0.92$; Fig. 6). On the other hand, with the Met Hb level and GR activity a negative correlation existed ($r=-0.70$; Fig. 8).

To study the influence of parasitaemia on these parameters the values were analyzed at different levels of parasitaemia (Table III and Fig. 9).

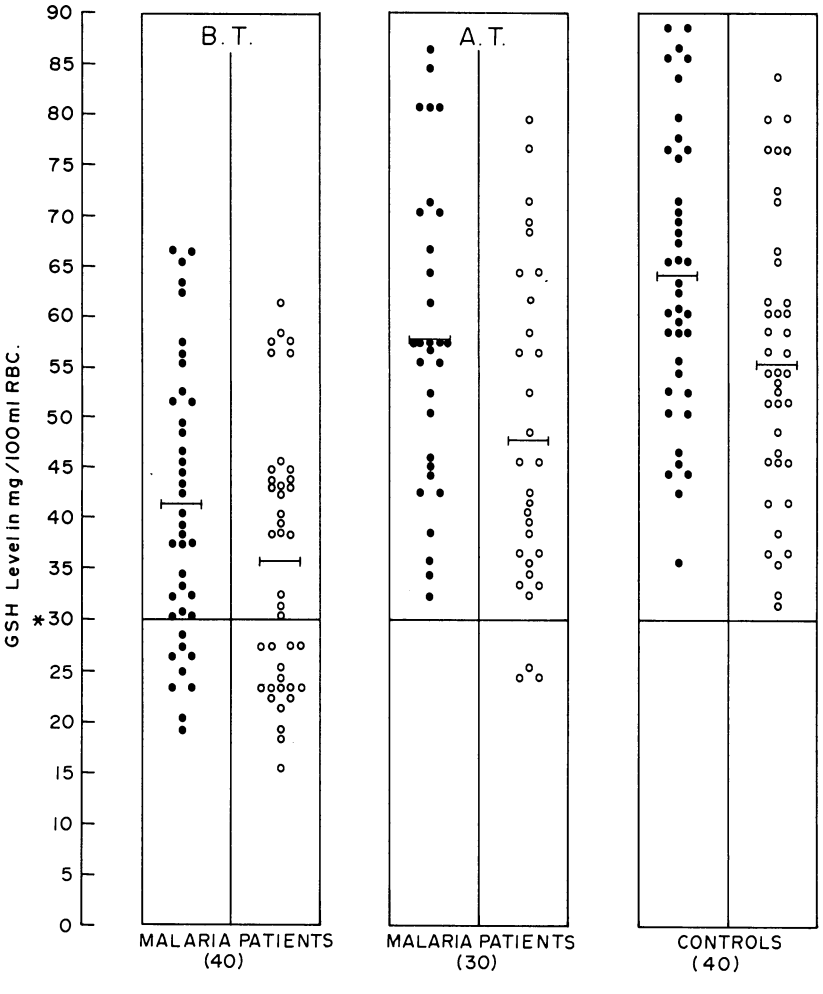
TABLE I Haematological parameter of malaria patients and normal controls

Parameters	Malaria Patients B.T. (40)		Malaria Patients A.T. (30)		Normal(40)	
	Mean ± S.E.	Range	Mean ± S.E.	Range	Mean ± S.E.	Range
Hb g/100ml	10.75 ± 0.33	5.20 - 14.50	11.75 ± 0.30	7.00 - 14.00	12.95 ± 0.15	11.50 - 15.70
PCV %	35.80 ± 0.99	18.00 - 45.00	38.90 ± 0.99	24.00 - 48.00	42.00 ± 0.54	36.00 - 48.00
Ret %	1.90 ± 0.13	1.00 - 5.00	1.46 ± 0.09	0.20 - 2.60	0.91 ± 0.05	0.20 - 1.50
MCHC %	29.22 ± 0.26	24.70 - 34.00	30.10 ± 0.18	28.88 - 34.00	30.64 ± 0.19	27.30 - 34.52
WBC $10^3/mm^3$	6.27 ± 0.15	3.05 - 11.80	7.77 ± 0.44	3.10 - 12.30	7.24 ± 0.28	4.20 - 11.90
Neutro $10^3/mm^3$	3.74 ± 0.26	1.61 - 8.02	4.32 ± 0.28	1.71 - 7.35	4.09 ± 0.17	2.16 - 6.17
Lympho $10^3/mm^3$	1.84 ± 0.12	0.71 - 3.33	2.68 ± 0.17	1.05 - 4.70	2.42 ± 0.14	1.18 - 4.65
Mono $10^3/mm^3$	0.44 ± 0.04	0.09 - 1.25	0.34 ± 0.04	0.09 - 1.23	0.22 ± 0.03	0.05 - 0.95
Eosino $10^3/mm^3$	0.23 ± 0.03	0.05 - 0.77	0.42 ± 0.05	0.04 - 0.99	0.32 ± 0.04	0.06 - 0.76
Baso $10^3/mm^3$	0.01	0.00 - 0.12	0.01	0.00 - 0.42	0.03	0.00 - 0.19

Figures within parentheses indicate number of subjects studied ; B.T. = Before Therapy, A.T. = After Therapy ; SE = Standard error.
Hb = Haemoglobin ; PCV = Packed Cell Volume ; Ret = Reticulocytes ; MCHC = Mean Corpuscular Haemoglobin Concentration;
WBC = White Blood Cell ; Neutro = Neutrophil ; Baso = Basophil ; Mono = Monocytes ; Eosino = Eosinophil ;
Lympho = Lymphocytes.

TABLE III G-6-PD, GR, GSH-PX Activity, GSH Level and Stability and Met Hb Level at Different Levels of Parasitaemia

Parameter		Controls	Parasitaemia/mm^3 Blood				
			100-500	501-1000	1001-2000	2001-3000	3001-4000
GSH (Preincubation) in mg/100 ml RBC	Mean ± S.E.	63.97 ± 2.22	61.65 ± 1.40	46.47 ± 1.89	36.31 ± 1.96	32.70 ± 1.50	24.20± 1.35
	Range	36.20 - 88.90	55.80 - 65.40	33.20 - 51.80	26.08 - 49.30	29.90 - 39.10	19.30 - 30.90
	No. of Subjects	40	8	9	11	5	7
GSH (Preincubation) in mg/100 ml RBC	Mean ± S.E.	55.30 ± 2.21	51.06 ± 3.32	40.82 ± 3.39	32.07 ± 2.04	27.52 ± 3.73	21.97 ± 1.32
	Range	32.10 - 83.10	39.30 - 61.10	22.70 - 86.80	19.60 - 42.20	22.73 - 44.19	15.20 - 27.80
	No. of Subjects	40	8	9	11	5	7
G-6-PD in Units/ml RBC/min	Mean ± S.E.	5.10 ±0.19	4.41 ± 0.37	4.92 ± 0.45	5.10 ± 0.55	5.34 ± 0.61	5.50 ± 0.71
	Range	3.60 - 8.00	3.40 - 6.80	3.40 - 7.60	3.40 - 8.10	3.50 - 7.30	4.00 - 9.80
	No. of Subjects	40	8	9	11	5	7
GR in Units/100 ml RBC/min	Mean ± S.E.	4.40 ± 0.19	3.54 ± 0.29	2.70 ± 0.16	2.32 ± 0.12	1.97 ± 0.12	1.16 ± 0.12
	Range	3.50 - 6.60	3.00 - 4.30	2.30 - 3.00	2.00 - 2.80	1.80 - 2.30	0.85 - 1.80
	No. of Subjects	25	4	4	6	4	7
GSH-PX in K/gm of Hb/min	Mean ± S.E.	36.66 ± 0.66	39.73 ± 3.78	34.83 ± 0.08	34.63 ± 1.12	35.41 ± 1.35	33.84 ± 0.91
	Range	32.80 - 49.30	32.70 - 48.35	34.60 - 35.01	31.70 - 38.30	32.60 - 39.12	31.97 - 38.68
	No. of Subjects	25	4	4	6	4	7
Met Hb Level	Mean ± S.E.	1.41 ± 0.07	3.23 ± 0.14	3.93 ± 0.08	4.07 ± 0.12	4.70 ± 0.43	8.81 ± 0.83
	Range	0.81 - 1.80	3.00 - 3.50	3.80 - 4.00	4.30 - 5.00	5.20 - 6.60	7.10 - 12.40
	No. of Subjects	20	3	3	5	3	6



● Pre-incubation
○ Post-incubation

B.T. = Before therapy
A.T. = After therapy

* Values below 30mg/100ml RBC represent an unstable pattern of GSH. Mean indicated by bar.

Figure 2. Scatter diagram showing level of erythrocytic GSH and its stability before and after specific therapy in malaria patients and in normal controls. Stability of GSH was tested by incubation with APH

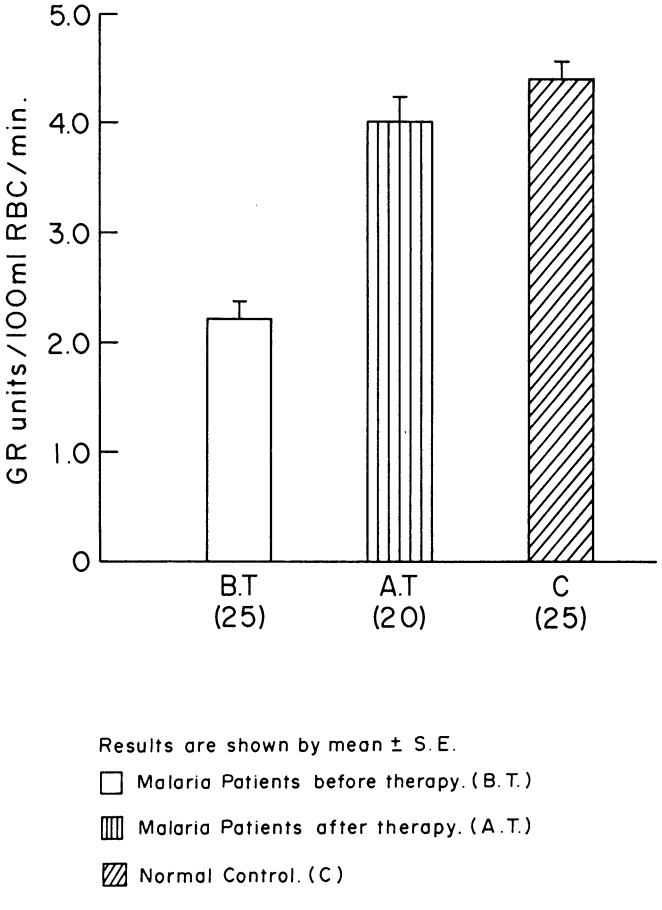


Figure 3. Activity of the enzyme GR in malaria patients before and after therapy.

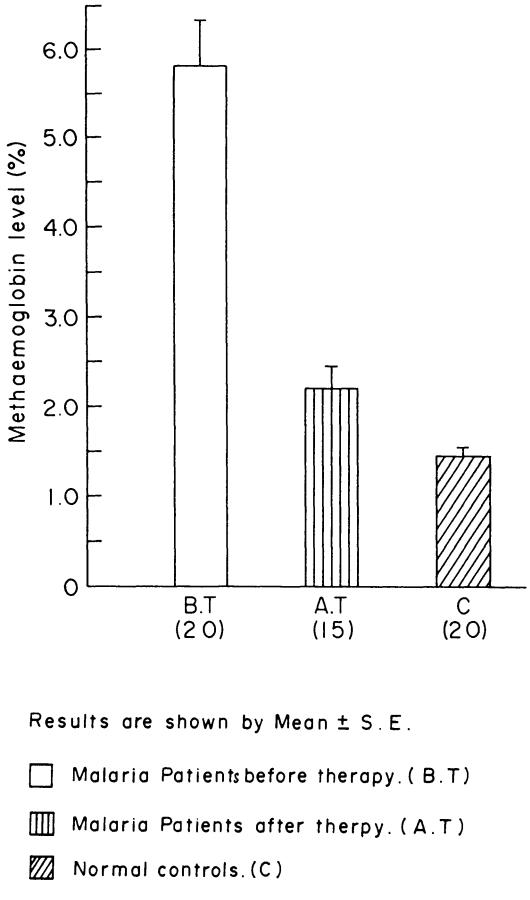


Figure 4. Effect of Malaria on methaeglobin level. Results are shown by Mean ± S.E.

GSH Level and Parasitaemia

There was a progressive decrease in GSH level as well as its stability with increasing parasitaemia (Table III). The mean values of GSH (before and after incubation with APH) for parasitaemias of 501-1000/mm^3 blood, differed significantly

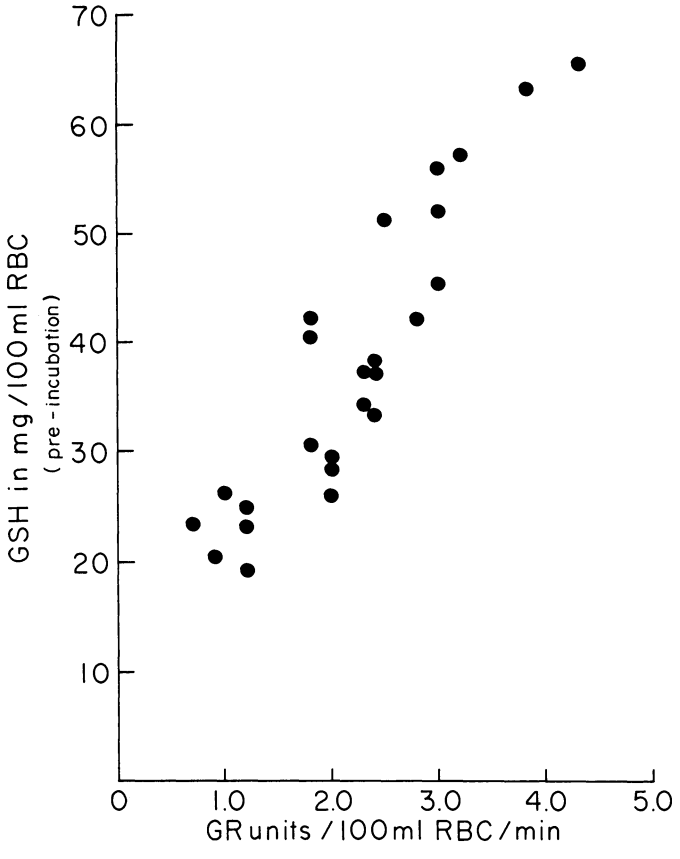


Figure 5. Scatter diagram to show the relationship between erythrocytic GSH level and GR activity in P. vivax infection of host Red Cell. The co-efficient of correlation r=0.9102.

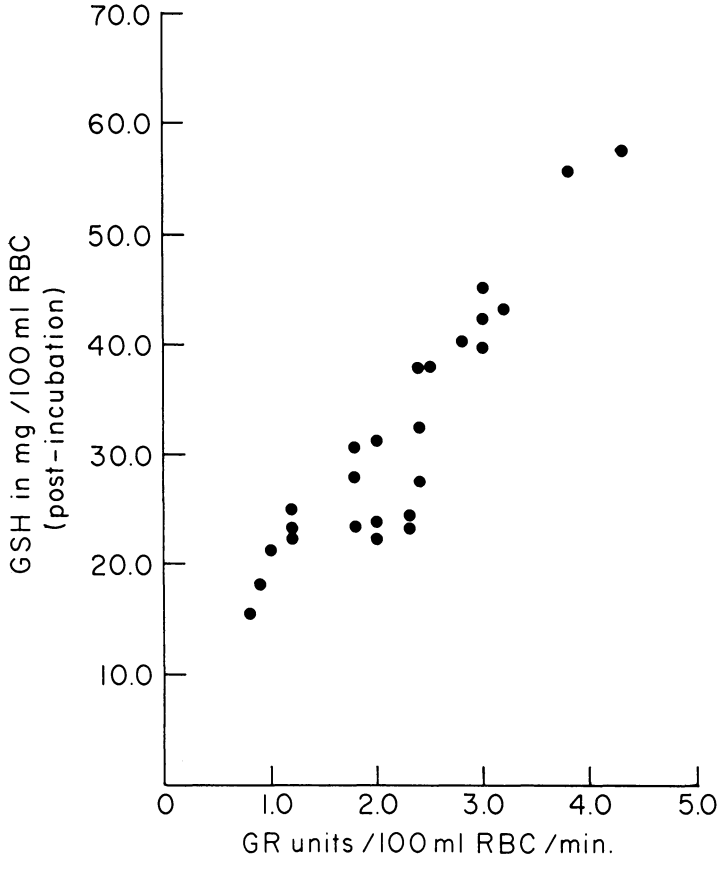


Figure 6. Scatter diagram to show the relationship between erythrocytic GSH stability and GR activity in P. vivax infection of host Red Cell. The co-efficient of correlation r=0.9218.

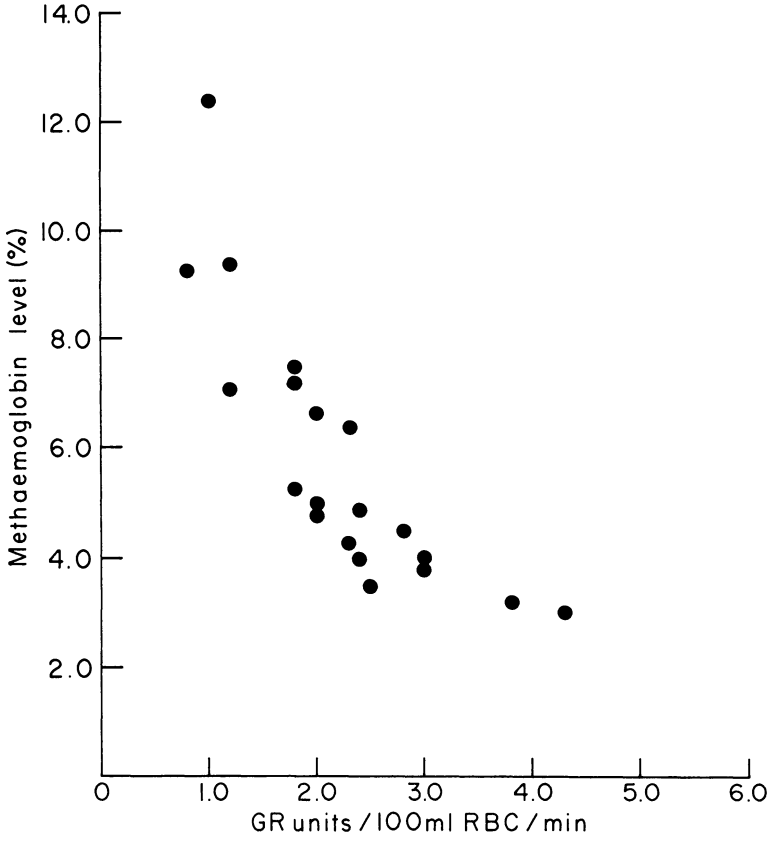


Figure 7. Scatter diagram to show the relationship between erythrocytic GR activity and methaemoglobin level during P. vivax infection of host Red Cell. The co-efficient of correlation r=-0.7940.

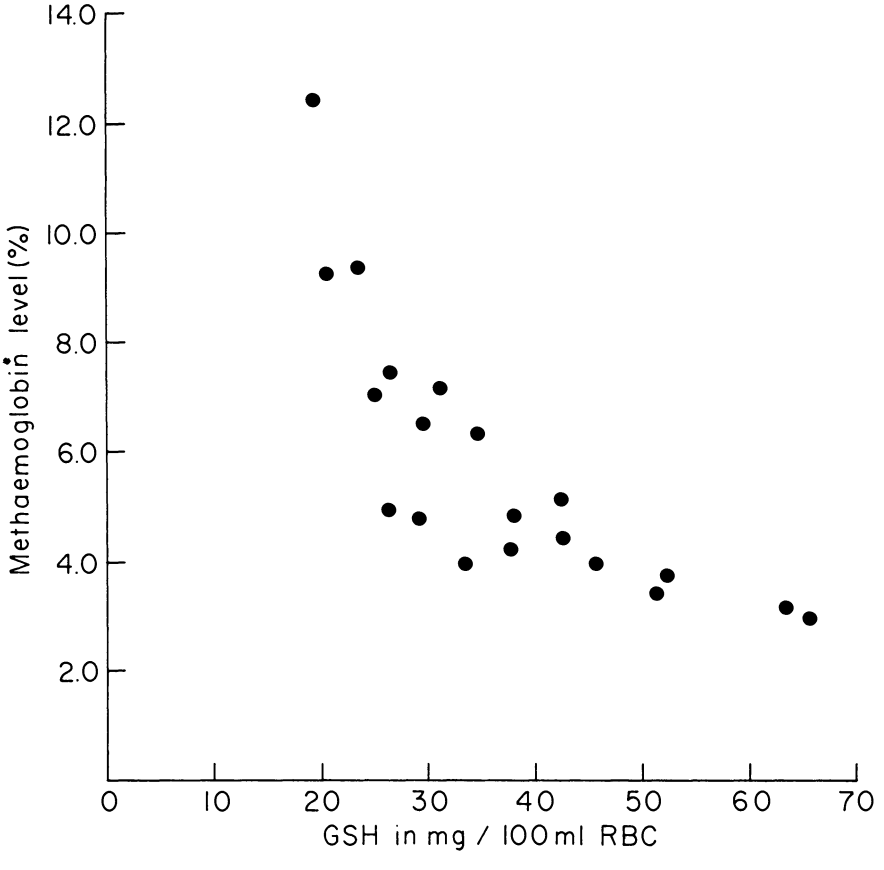


Figure 8. Scatter diagram to show the relationship between erythrocytic GSH level and methaemoglobin level during P. vivax infection of host Red Cell. The co-efficient of correlation r=-0.7049.

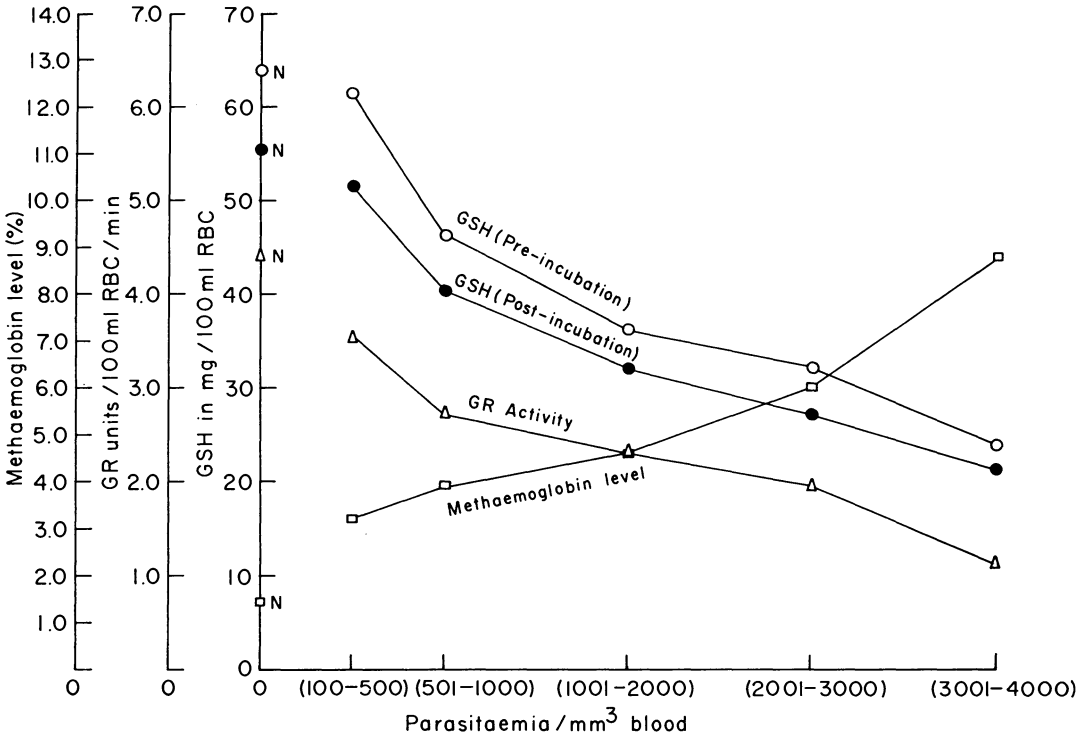


Figure 9. Parasitaemia vs GSH level and stability, GR activity and Met Hb level. Mean value of each parameter was taken in the respective group of parasitaemia. N = Mean value of normal controls of each parameter.

from those for parasitaemias of 100-500/mm^3 ($P < 0.05$). The higher levels of parasitaemia, above 501-1000/mm^3 blood, also differed with significantly with respect to GSH level and its stability from the values for lower parasitaemia ($P < 0.05$). In mild infections (parasitaemia 100 to 500/mm^3 blood), the changes in host cell GSH level and stability were not significantly different from that of controls ($P < 0.05$). In heavy infection (3001 to 4000 parasites/mm^3), 6 out of 7 patients had a GSH level below 30 mg/100 ml and all showed GSH instability (Table III). These findings confirm that the changes in host red cell GSH status in malaria patients were due to the presence of P. vivax.

G-6-PD Level and Parasitaemia

Table III indicates that there was no significant relation between G-6-PD activity and parasitaemia.

GR Level and Parasitaemia

The mean value of GR activity in the parasitaemias of 501-1000/mm^3 blood and above differed significantly from that of mild infection (100-500/mm^3 blood; $P < 0.05$). GR activity showed a steady decrease with increasing parasitaemia (Table III), indicating that P. vivax was responsible for the decreased GR activity in the host red cell.

GSH-Px Level and Parasitaemia

There were no significant differences in the activity of GSH-Px at different levels of parasitaemia.

Met Hb Level and Parasitaemia

There was a steady increase in Met Hb level with increasing parasitaemia. The Met Hb level at parasitaemias of 501-1000/mm^3 blood and above was significantly higher than that in mild infection, i.e. 100-500/mm^3 blood ($P<0.05$). This could be due to oxidant stress exerted by the malaria parasite on the host red cell, resulting in the oxidation of Hb to Met Hb (Fig.1).

Effect of Therapy

After specific therapy and cure of malaria the haematological parameters studied on 30 patients showed a return of the Hb level, PCV values, reticulocyte count, WBC level and other haematological parameters to near normal values (Table I).

GSH level was higher compared with pre-therapy values ($P < 0.05$) and did not differ significantly from the control group indicating a normalization of the GSH level after cure of malaria. GSH instability was noticed, in only 3 cases and the mean value was significantly higher as compared to pre-therapy value ($P < 0.05$) (Table II and Fig.I). G-6-PD and GSH-Px activity showed no significant change (Table II).

There was an improvement of GR values after therapy (A.T.) when values were restored to control levels ($P > 0.05$) (Table II and Fig.3). Hence P.vivax exerts a significant inhibitory effect on the enzyme GR of the host red cell[5]. Met Hb level also decreased significantly from their pre-therapy values to near normal level values (Table II and Fig.4).

Discussion

The data presented show that human malaria (P.vivax) affected the oxidative metabolism[7] of host red cells[5] leading to lower erythrocytic GSH and stability. Erythrocytic GR activity was also depressed. On the other hand the activities of the enzyme G-6-PD and GSH-Px remained unaffected. There was also a significant increase in methaemoglobin level during P. vivax infection.

This composite picture suggests that in P.vivax malaria red cells failed to maintain a normal level of erythrocytic GSH in spite of normal activity of G-6-PD. GSH is known to play an important role in maintaining integrity of the red cell[5], particularly when exposed to oxidative stress (Benesch & Benesch, 1954; Fegler, 1952). During malarial infection, a continuous supply of GSH is necessary. An adequate activity of the enzymes G-6-PD, GR and GSH-Px is required to maintain adequate levels of GSH and also to combat the oxidative stress.

Fulton and Grant (1956) showed that in monkeys, P.knowlesi required sulphur-containing amino acids for its growth and development and that these amino acids were obtained from GSH of red cells. No information in this regard is available in man though it is known that G-6-PD deficient children are more susceptible to infection in general. The human malaria[1] parasite P.falciparum does not grow as well in G-6-PD deficient red cells[5] as in normals (Luzzatto et al., 1969). However no documented information is available on the effect of malaria in enzymatically normal subjects. In view of the normal activity of G-6-PD in patients with P.vivax infection it can be presumed that this enzyme was not responsible for the changes in the level of GSH which were probably brought about by a reduction in erythrocytic GR activity together with an increase in demand due to oxidative stress.

The increase in methaemoglobin may be related to decreased GSH level found in the present study. SH groups are important in the prevention of Met Hb accumulation (Sokoleviskii and Pavlovs, 1961). There are two aspect to the accumulation of Met Hb: (1) interference in the mechanism which protects Hb from oxidation and (2) interference in the mechanism which reduces Met Hb back to Hb. During infection the decreased GR activity may causes a reduction in GSH level as GR is involved cleavage of glutathione-Hb complex formed under stress, (Shrivastava & Beutler 1970). Met Hb accumulation is prevented by a NADPH-dependent MetHb reductase (Gutmann et al., 1947). Under physiological conditions the conversion of methaemoglobin to oxyhaemoglobin is so rapid that only minute quantities of Met Hb are detected in normal blood. But when the red cell undergoes stress, H_2O_2 released under oxidant stress can convert oxyhaemoglobin to methaemoglobin[10] at an increased rate.

The present study shows that P. vivax infection exerts an inhibitory effect on the enzymes GR and MR and ultimately GSH level and stability of host red cells[5], favoring oxidation of Hb to Met Hb and thus affecting the oxidative metabolism[7] resulting in predisposition of red cells to haemolysis and contributing to anaemia[6] in P.vivax malaria.

Conclusion

From the results of this study it appears that during Plasmodium vivax[4] infection in man the following significant changes take place in the host red cell[5].

1. Decrease in erythrocytic GSH level and stability and this decrease is directly proportional to the increase in parasitaemia.
2. Decrease in the activity of host red cell GR, inversely proportional to the parasitaemia.
3. Steady increase in Met Hb level with increase in parasitaemia.
4. Activity of the erythrocytic enzymes G-6-PD and GSH-Px remains unaffected during infection.
5. This composite picture suggests that host red cells fail to maintain normal level of erythrocytic GSH in spite of normal activity of G-6-PD in P.vivax infection.
6. P.vivax may affect the oxidative metabolism[7] of the host red cell[5] by inhibiting the enzyme GR.
7. These conclusions gain further support in normalization of all these changes after specific therapy and cure of malaria.
8. Lastly, it can be said that P.vivax itself, or some of its metabolites, exert an inhibitory effect on GR activity of host red cell which ultimately affects GSH level and stability resulting in disturbances of red cell integrity with subsequent predisposition to haemolysis, this being perhaps one of the causes of anaemia[6] in P.vivax malaria.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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PLASMA LIPID PEROXIDATION IN P. FALCIPARUM MALARIA

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Summary

Augmented generation of reactive oxygen species (ROS) and the consequent enhanced tissue and plasma lipid peroxidation are crucial events in P. Falciparum malaria. Release of lytic products including haemoglobin from the ruptured erythrocytes possibly magnify the process by acting as prooxidants. The concentration of plasma antioxidants are altered due to acute phase response and consumption by oxidant stress. Simultaneous presence of ROS as well as haemoglobin and loosely bound iron overwhelms antioxidant defence system giving scope to a vicious cycle that perpetuates the disease process.

Introduction

Generation of reactive oxygen species (ROS) in malaria has been considered as a host defence mechanism for killing of intraerythrocytic parasites, exaggerated pursuance of which possibly is responsible for most of the complications (Clark and Hunt 1983, Wozencraft et al., 1984). Peripheral blood phagocytes have been shown to be activated by plasmodium components in vitro to generate ROS (Kharazmi et al., 1987). Increased production of ROS in the whole blood was observed during P.Vinkei infection in mice (Stoker et al., 1984) and in patients with acute falciparum malaria (Descamps-Latscha et. al. 1987). Circulating plasma lipoproteins, being in close contact with components of host pathogen interaction are therefore easy substrates for ensuing lipid peroxidation. Plasma

lipid peroxides measured as TBA reactive material was found to be increased in P. falciparum malaria, the increase being proportional to the severity of the disease (Das et al., 1988, Thurnham et al., 1990, Das et al., 1990).

On the other hand, being rich in both preventive and chain breaking antioxidants, human plasma is not easily vulnerable to oxidant stress (Halliwell and Gutteridge, 1985, Wayner et al., 1987). Blood level of some of these antioxidants is dependent on the nutritional status of the individual and on the acute changes brought about by infection thus altering its impact on development of lipid peroxidation.

One of the early pathological events in malaria is invasion by the malaria parasite into erythrocytes for its growth and multiplication. This leads to the rupture of the erythrocyte releasing merozoites, haemoglobin and other lytic products. Malaria is different from other infections and ROS induced diseases in that it produces both ROS and catalysts for lipid peroxidation in large quantities. In the following pages the changes observed in the level of plasma antioxidants, possible presence of prooxidants in malaria infection and their influence on plasma lipid peroxidation is discussed.

Prooxidants

Plasma of malaria patients has been suspected to contain prooxidants. Thurnham et al., (1988), by measuring total radical trapping capacity (TRAP) in plasma of malaria patients observed lower experimental TRAP values than the values calculated from individual antioxidants present. This was attributed to the presence of some prooxidants in the plasma of malaria patients. Mixtures of linoleic acid and malaria plasma spontaneously initiated oxygen uptake even in the absence of water soluble free radical initiator. Haemoglobin can be one of the prooxidants as it is known to catalyze decomposition of lipid hydroperoxides and enhance the chain reaction (Halliwell & Gutteridge 1985). Mixtures of haemoglobin and hydrogen peroxide are capable of

accelerating lipid peroxidation (Gutteridge 1987). Moreover haemoglobin in the presence of excess of hydrogen peroxide can cause haem breakdown to release iron ions (Pupo and Halliwell 1988). The presence of bleomycin-detectable iron in plasma of P.vinckei infected mice lends credence to this (Buffinton et al., 1986). Free haemoglobin in plasma binds with the haemoglobin binding protein, haptoglobin, whose blood level normally increases in diseases as a part of the acute phase response (Fleck and Myers, 1985). In malaria endemic areas hypohaptoglobinaemia and even ahaptoglobinaemia have been reported (Rougemont et al., 1988, Trape and Fribourg-Blanc 1988); low blood levels are attributed to exhaustion brought about by repeated attacks of malaria and haemolysis. This will allow free haemoglobin to be present in the circulation even when there is only marginal haemolysis. Extensive haemolysis occurs in severe falciparum malaria. In one of our recent studies (Das et al., unpublished observations) considerable reduction in haemoglobin, with concomitant elevation of unconjugated bilirubin indicating haemolysis was observed in severe malaria patients. Plasma MDA correlated inversely with haemoglobin and directly with bilirubin in these cases. Therefore haemoglobin and possibly other lytic products of blood might be playing a crucial role in generating plasma lipid peroxides by acting as prooxidants in malaria infection.

Antioxidants

Plasma contains preventive antioxidants like transferrin and ceruloplasmin and chain breaking antioxidants like ascorbate, urate, protein sulphydryl, tocopherol etc. Wayner et al., (1987) had reported that the first line of defence for lipid peroxidation is provided by the plasma sulfhydryl groups. However Frei et al., (1988) found plasma ascorbate to be much more effective in protecting lipids from peroxidative damage by aqueous oxidants than all the other endogenous antioxidants. The impact of malaria infection on some of the chain breaking antioxidants as observed in the studies from Nigeria (Thurnham et al.,1988), Thailand (Thurnham et al., 1990) and India (Das et al., unpublished results)

is summarized in Table I. In Indian patients only plasma ascorbate was found to correlate well with MDA in both mild and severe malaria. Ascorbate has multiple antioxidant properties. It can scavenge superoxide, singlet oxygen, hydroxyl radical, water soluble peroxyl radical, hypochlorous acid (HOCl), etc (Bendich et al., 1986). In vivo it also helps in regeneration of tocopherol from tocopheryl radical thus renewing a lipid soluble antioxidant (Packer et al., 1979). Ascorbate can inhibit lipid peroxidation induced by mixtures of hydrogen peroxide and haemoglobin (Kanner and Harel 1985) which are released into the plasma in abundance in malaria. Ascorbate is considered to be the most important water soluble antioxidant present in the plasma. Intracellularly

TABLE I Plasma antioxidants in malaria infection

Place of study	Ascorbate (μMol/L)	Urate (μMol/L)	Sulphydryl (μMol/L)	Tocopherol (μMol/L)
Nigeria				
Control	17.0(6-44)	*210(150-287)	551(419-971)	13.7(3.4-47.6)
Patients	21.4(10-62)	240(65-538)	585(346-868)	11.1(5.3-17.1)
Thailand				
Control (R)	43.9(17-118)	312(113-586)	589(348-895)	12.7(8.8-17.9)
Patients(R)	32.6(3-66)	265(172-476)	500(369-1015)	9.8(3.0-17.2)
Control (U)		387(339-619)	625(539-1548)	18.6(15.7-54)
Patients(U)		321(143-587)	632(376-1306)	13.3(9.0-23.5)
India				
Control	48.3(23.6)	**228(60.3)	42.5(3.4)#	
Mild-Malaria	42.2(22.6)	246(55.3)	35.9(4.5)	
Severe-Malaria	31.8(19.7)	377(213.3)	29.1(7.8)	

R-rural, Urban, * median and range,** mean ± SD, # albumin(G/L)

semidehydroascorbate and dehydroascorbate, the oxidation products of ascorbate, are reduced to ascorbate by the enzymes NADH-semidehydroascorbate reductase and GSH dehydroascorbate reductase respectively. But in plasma and other extracellular fluids, in conditions of oxidant stress, ascorbate is likely to be depleted very rapidly to produce oxidation products such as oxalate and threonate as shown in the following reaction (Hallivell B. 1990).

ascorbate ------> semidehydroascorbate

2 semidehydroascorbate ------> ascorbate + dehydroascorbate

dehydroascorbate ------> oxalate, threonate, etc.

Plasma ascorbate has been reported to be low in malaria patients from Thailand, and India (Table I). In contrast plasma ascorbate was found to be higher in infected specimens than in the controls in mice (Stoker et al., 1986) and in Nigerian children (Thurnham et al., 1988). However, both control and malaria patients from Nigeria had values far below that of Caucasians. An enhanced dehydroascorbate-reducing capacity within the erythrocytes for regeneration of ascorbate may account for the finding in mice. However the reason for the finding in Nigerian children is unclear. Nutritional deficiency and repeated infection could be contributory factors but the possibility of deterioration of the sample during storage and transportation before estimation could not be ruled out.

Urate is considered to share many of the antioxidant properties of ascorbate (Ames et al., 1981). Urate can function both as chain breaking as well as preventive antioxidant. The preventive antioxidant role of urate derives from its metal binding property (Davies et al., 1986). Urate, when acted upon by hydroxyl radical is converted to urate radical which can damage certain proteins like antiproteinase but the radical can be effectively scavenged by ascorbate (Aruoma and Hallivell 1989). It therefore acts more as a preventive antioxidant than as a chain breaking antioxidant. Plasma urate level was observed to be higher in Indian patients with severe malaria; but in Thai patients it was slightly increased or unaltered (Table I). The discrepancy could have been due to difference in the severity of the disease among these groups. P.vinckei infected mice also had high plasma urate values (Stoker et al.,1986). The rise could be due to increased

production as a result of breakdown of nucleic acids and ineffective clearance by the kidney due to associated renal impairment. In fact the studies from India have demonstrated that when acute renal failure cases were excluded from the severe malaria group, the mean urate level fell significantly. Nonetheless it was marginally but significantly higher than the control population. The rise in plasma urate level in malaria may be considered as a defence mechanism against the oxidant stress.

Antioxidant properties of plasma proteins are reported to be due to presence of sulphydryl groups (Wayner et al., 1987). Though not very efficient antioxidants the plasma proteins, form an effective antioxidant defence by virtue of their overwhelming presence. The role of albumin is significant. It protects vital targets like erythrocytes or vascular endothelial cells from the copper ion induced fenton type reaction (Halliwell 1988) by binding copper ion to its active site, during which it becomes damaged (Gutteridge and Wilkins, 1983). Plasma albumin level decreases in infections and following injury (Fleck et al., 1985). Plasma albumin level may fall in malaria as a consequence of increased transcapillary leakage (Areekul 1988) and due to its degradation by the peroxyl radicals (Wolff & Dean 1986). The variance in by albumin level with respect to MDA in the Indian study was found to be very small (6%), and hence its decrease might have been due to the acute phase response. Whatever may be the mechanism of decrease, low albumin values in malaria deprives plasma of an effective antioxidant.

Lipid soluble antioxidants like tocopherol, retinol and carotenes provide protection to plasma lipoproteins. Plasma retinol and carotenes decrease in malaria infection (Thurnham and Singkamani 1991). However their contribution towards antioxidant capacity in plasma appear to be insignificant because of their low plasma concentration (Ames et al., 1981) and because of their effectiveness only at low oxygen tension (Burton and Ingold, 1984). They can function better as intracellularly antioxidants than as plasma antioxidants. Tocopherol serves as an excellent antioxidant in the lipid phase. Plasma tocopherol level decreases but the ratio between cholesterol and tocopherol, regarded as a better indicator of tocopherol status, was not altered significantly (Thurnham et al., 1990). Plasma lipid pattern is altered in severe malaria. Plasma cholesterol level falls,

triglycerides and non-esterified fatty acid levels rise (Angus et al., 1971, Nilsson-Ehle and Nilsson-Ehle, 1990). In one of our recent studies from India (Mohanty et al., Manuscript submitted for publication) plasma HDL and LDL cholesterol was found to be significantly lower and triglycerides higher in severe malaria. LDL bound tocopherol could be lost along with the cholesterol-rich LDL keeping the ratio of cholesterol to tocopherol unaltered. Logically it seems, the cholesterol tocopherol ratio, may not reflect its efficiency as an antioxidant in protecting all the classes of lipoproteins. Normally tocopherol levels are adjusted according to the level of circulating lipids in each individual. However the capacity of tocopherol to adjust to the changes in lipid pattern brought about abruptly in malaria is not known.

The two important preventive antioxidants present in plasma are transferrin and ceruloplasmin. Transferrin binds to iron thus making it unavailable for its catalytic function in lipid peroxidation. Transferrin, like albumin, decrease in conditions of chronic infection and injury (Fleck and Myers, 1985). If there is reduction in transferrin content, any iron released may remain unbound, thus becoming a potential catalyst. However there is one report from Africa indicating increase in transferrin level following malaria infection (Aremu 1989). The level of ceruloplasmin increases as a consequence of the acute phase response (Fleck and Myers 1985). By its action as ferroxidase it converts iron(II) to iron(III) and thus prevents the formation of catalytically more active iron. Ceruloplasmin concentration in plasma is found to be increased in malaria (Thurnham et al., 1990, Das et al., unpublished results) but its precise impact in influencing plasma lipid peroxidation is not known.

Pathological Significance

What is the significance of increased plasma lipid peroxidation in malaria? First it reflects tissue lipid peroxidation which spills into the circulation, and therefore the extent

of plasma lipid peroxidation can be assumed to reflect disease severity, albeit indirectly (Yagi 1987). Second, it does add an external oxidant stress on the erythrocytes. In malaria extensive haemolysis takes place and in most instances, the extent of haemolysis is out of proportion to the parasitaemia indicating breakdown of non-parasitised erythrocytes. It is probable that erythrocytes with inadequate protection due to low level of membrane bound antioxidants are more vulnerable to the oxidant stress and to haemolysis.

It therefore seems increasingly plausible that in malaria infection, enhanced plasma lipid peroxidation is brought about by a two pronged mechanism of increased ROS production and availability of increased prooxidants in the form of haemoglobin and possibly free iron. Haemolysis and lipid peroxidation form a vicious cycle. The role of antioxidants has remained inconclusive. Ascorbic acid is consumed during oxidative stress but the role of other antioxidants in severe falciparum malaria needs further scrutiny.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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PRESENCE, FORMATION AND FUNCTION OF UBIQUINONES Q_6 AND Q_8 IN FILARIAL PARASITES

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Summary

Setaria digitata, a cattle filarial parasite reported to be very similar to parasites causing human filariasis shows cyanide insensitive oxygen uptake with the resultant production of H_2O_2. The production of the latter is associated with the generation of ATP. It is known to be a ubiquinone mediated process. Setaria has two quinones Q_6 and Q_8 in place of one higher quinone in host systems, thus opening up a very vulnerable target in the parasite.

Introduction

The parasitic helminth species so far studied use oxygen when it is available and it has been shown that the electron transport chain of these parasites differs from mammals in having multiple terminal oxidases (Barrett, 1981, Cheah, 1975a, 1975b and 1976). Instead of the common ubiquinone as electron carrier, many helminths have been reported to possess rhodoquinone (Kohler 1985).

Setaria digitata, a cattle filarial parasite reported to be very similar to the parasites causing human filariasis (Hawking 1978) showed oxygen uptake which was not affected by respiratory poisons such as, cyanide (Kaleysa Raj et al.,, 1988). Though typical

cytochromes could not be detected, H_2O_2 generation which is known to be a quinone mediated process was observed.

Soon it became clear that the Setaria system does not possess a complete TCA cycle but instead has a phosphoenol pyruvate-succinate-glyoxylate pathway (Rafi and Kaleysa Raj, 1991). The study forms part of an effort in identifying targets in parasites which are different from that of the host for developing drugs for parasite control.

Materials and Methods

Parasites

S. digitata used for the study was collected from local abattoir freed of extraneous host materials by repeated washings with Tyrode solution and kept in the same solution at 37°C until use (Tyrode solution : NaCl 0.8%, Kcl 0.02%, $CaCl_2$ 0.02%, $MgCl_2$ 0.01%, Na_2HCO_3 0.015%, Na_2HPO_4 0.05% and glucose 0.50%).

Isolation of Ubiquinones

The non-saponifiable lipids were extracted (Crane and Barr, 1977) and separated into fractions by column chromatography using 4% deactivated alumina (Joshi and Ramasarma 1966). The 5% ether in petroleum ether eluate was purified by TLC using silica gel. The spots were identified using leucomethylene blue spray (Linn et al., 1959). Spectral comparison with standard quinones was made in a UV 240 Schimadzu spectrophotometer (Kaleysa Raj et al., 1988). Incorporation study was carried out using ^{14}C acetate. Santhamma and Kaleysa Raj, 1990).

Cell Fractionation

The weighed live worms were suspended in 0.25 M Sucrose containing 0.1% bovine serum albumin and 5 Mm EDTA (10ml. solution per gm. weight of the worm) and the mixture was homogenized and fractionated using a refrigerated centrifuge. The pellet

Table 1. H_2O_2 generation in MLP fractions

Substrate	Concn. (m moles)	P mole of H2O2 generated min/mg Protein*
Succinate	6	950 ± +84
Fumarate	4	594 ± 62
Malate	2	235 ± 18
a Glycerophosphate	1	118 ± 12

* Average of eight experiments.

Table 2. Inhibition of H_2O_2 production by the different inhibitors

Inhibitors	Conc./mg protein	Percentage of inhibition Succinate	Fumerate	Malate	Glycerophosphate
Rotenone	0.03n moles	25	100	50-80	NIL
Antimycin A	0.5ug	35	100	30	NIL
TTFA	0.15mm	65	50	25	NIL
SHAM	2.5 g moles	65	70	80-90	90-100
OHD	15 g moles	30	30	30	30

Average of eight experiments.

collected between 800 and 1200g was used as the mitochondrial preparation (Kaleysa Raj et al., 1988).

Assays

Protein was estimated using Folins reagent (Lowry et al., 1951). Oxygen uptake studies were carried out using the Warburg manometer (Umbreit et al., 1972). Activities involving oxygen uptake were estimated using a Gilson oxygraph with Clark type oxygen electrode having a cell capacity of 1.4 ml. and those involving dehydrogenase activities were studied using UV 240 spectrophotometer. The decrease in fluorescence of scopolitin at 360 nm (excitation) and 465 Nm (emission) was measured in a Hitachi spectrofluorimeter for the estimation of H_2O_2 generated in presence and absence of different substrates and inhibitors. (Swaroop and Ramasarma 1981; Santhamma and Kaleysa Raj, 1991a). Mitochondrial preparations were scanned in a spectrophotometer from 650 to 550 nm for the detection of cytochromes (Riesket 1967). ATP generated was estimated using different substrates and inhibitors (Williamson and Corhey, 1969; Santhamma and Kaleysa Raj, 1991b).

Results

Oxygen Uptake

The oxygen uptake in the presence and absence of glucose as well as in the presence of cyanide is given in Fig.1.

Cytochrome Scan

S.digitata did not give a characteristic cytochrome scan when scanned between the range 650 to 550 nm, in the presence and absence of dithionate.

H_2O_2 Generation

Generation of H_2O_2 with different substrates as well as the effect of known inhibitors on its production are given in Tables 1 and 2.

Table 3. Inhibition of ATP formation by the different inhibitors

Inhibition	Concn/mg protein	% of inhibition			
		Succinate	Fumarate	Malate	Glycerophosphate
Rotenone	0.03 nmoles	70	100	100	40
Antimycin A	0.5 μg	35	75	25	35
OHD	15 g moles	30	75	30	45
SHAM	2.5g moles	25	25	25	50
TTFA	0.15mM	70	35	20	20
DNP	2.4mM	100	100	100	100

Table 4. Rf values of the 5% fractions from alumina column separated by TLC

S.digitata (Parasite)	Cattle liver (host)	Standards	R. value
S_1*	**	Q_6	0.29
S_2*	L1	**	0.39
**	L2	Q_{10}	0.52
**	L3	**	0.68
S_3	**	**	0.78

* Spots which yielded characteristic quinone spectra.

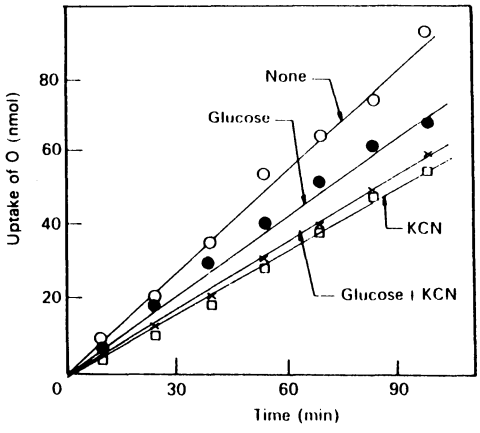


Fig. 1. Oxygen uptake by whole worms

The details of the medium and incubation are given in Materials and Methods section. Where mentioned, concentrations of glucose and KCN were 0.1 % and 30 Nm, respectively. Oxygen uptake was measured in a Warburg apparatus.

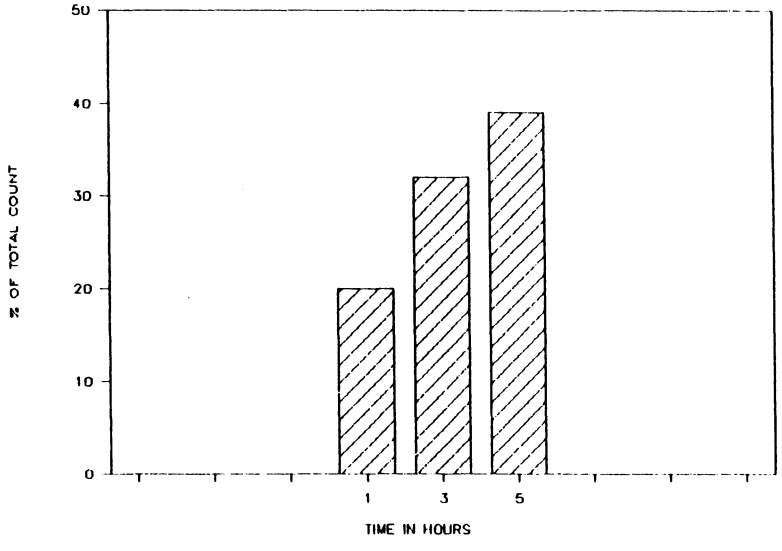
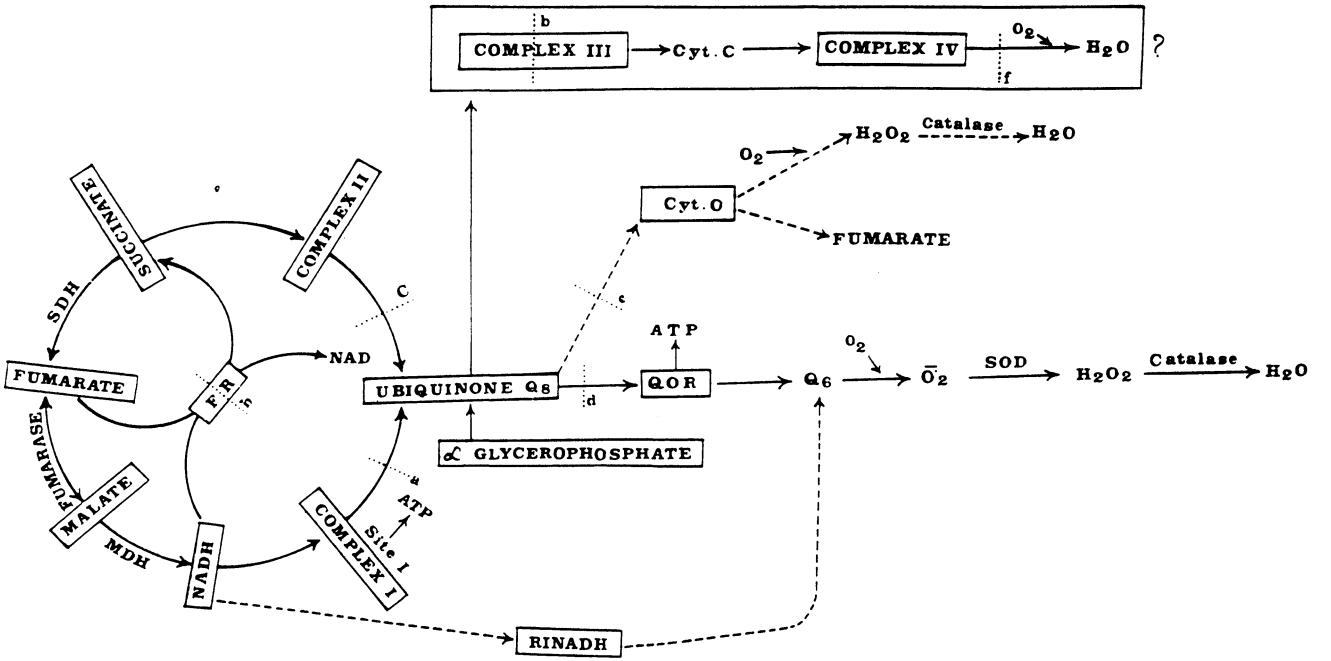


Figure 2. Histogram showing the percentage changes in incorporation of [^{14}C] acetate in the 5% fraction with respect to time of incubation.

Figure 3

Proposed Oxidative Electron Transport System in S. digitata



——— major pathway ; ------ minor pathway; ····· inhibition ; a- rotenone ; b - antimycin A ; C - TTFA ; d - SHAM ; e - OHD ; f - cyanide ; QOR - quinol oxidoreductase ; FR - fumarate reductase ; RINADH - rotenone insensitive NADH dehydrogenase SOD - superoxide dismutase

ATP Generation

Generation of ATP has been detected during the production of H_2O_2 with different substrates and the effect of inhibitors on its production are given in Table 3,

Identification of Ubiquinones

TLC separation and identification of the ubiquinones is clear from Table 4. The time course of ^{14}C acetate incorporation study on the quinol fraction is given Fig 2.

Discussion

The Cattle filarial parasite S. digitata, which is reported to be similar to the human parasites causing filariasis, shows cyanide insensitive oxygen uptake, production of H_2O_2 and associated generation of ATP. Cyanide insensitive respiration has been known even in rat liver mitochondria under certain conditions such as presence of menadione (Meera Rau and Ramasarma, 1979). Such reactions would be expected to yield H_2O_2 as the product of the reduction of oxygen and is a quinone mediated process. In Setaria, ATP generation has been shown to be associated with the production of H_2O_2. These have been confirmed by studies using different substrates and inhibitors as is clear from the Tables 1-3.

As Setaria did not yield typical cytochrome spectrum between the usual range of 650 to 550 nm, the role of quinone system in the production of H_2O_2 and associated generation of ATP assume prominence. The fact that two quinones Q_8 and Q_8 are present in Setaria, makes it unique and different from the host, thus exposing the site as a very vulnerable target. The fact that both the quinones are rapidly synthesized as is evidenced by the incorporation study, further suggest the significance of these materials in the metabolism of the parasite.

Unpublished results from this laboratory have shown the activities of both rotenone sensitive and insensitive NADH dehydrogenase (Complex I) in Setaria (Sivan and Kaleysa

Raj, under publication). Activities of succinate dehydrogenase and fumarate reductase (Complex II) have also been shown in Setaria (Unnikrishnan and Kaleysa Raj, under publication). Highlighting the role of the two quinones and incorporating the effect of inhibitors a tentative scheme for the transport of electrons in Setaria mitochondria has been proposed in Scheme I. The reducing equivalent from Q_8 can pass on to quinol oxido-reductase sensitive to SHAM (alternate oxidase inhibitor) or to a cytochrome-O like system reported from parasites such as Ascaris lumbricoides (Bryant and Behm 1989) which are sensitive to OHD. Quinones with shorter side chains are autoxidizable (Ramasarma and Lester, 1960) and hence Q_6 can act as an acceptor in S. digitata as is suggested in the proposed scheme I.

Conclusive proof for the significance of the quinones in the case of S. digitata is further evidenced by recent *in vitro* studies using structural analogues of ubiquinone (Santhamma and Kaleysa Raj, under publication) which produced concentration dependent effect on the parasite.

Conclusions

Cyanide insensitive respiration is detected in S. digitata. The absence of typical cytochromes and the production of H_2O_2 and associated generation of ATP were also observed and these assign a major role for quinones in the parasite. The fact that Setaria has two lower quinones (Q_6 and Q_8) in place of one higher quinone (Q_{10}) in the host makes the quinone system a very susceptible target of attack for the effective control of filarial parasites.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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CATALASE ACTIVITY IN RED CELL AND LIVER OF MICE INFECTED WITH PLASMODIUM BERGHEI

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Summary

Catalase activity was studied in red cells and liver of mice infected with P. berghei. The catalase activity in infected red cells was significantly lower than that of normal red cells. An inverse relationship between catalase activity and parasitemia was demonstrated. Crude parasite lysate contained no catalase activity and liver catalase content in the infected mice was also significantly lower than that of the normal mice.

Introduction

Accumulated evidence suggests that asexual forms of malaria parasites in erythrocytes are sensitive to oxidant stress. Hydrogen peroxide (H_2O_2) has been implicated as a possible mechanism of malaria parasite killing. Injection of H_2O_2 into mice infected with P.chabaudi or P.yoelii rapidly reduced the parasitemia (Dockrell and Playfair, 1983). Both P.yoelii and P.falciparum were found to be highly susceptible to H_2O_2 produced by the interaction of the glucose/glucose oxidase system in vitro (Wozencraft et al., 1984). This effect could be reversed by the addition of catalase, implying that H_2O_2 is the chief effector molecule. H_2O_2 was also generated with

P.berghei-infected mouse red cells, which could further result in the alteration of catalase activity (Etkin and Eaton, 1975). As the status of the host catalase has not been studied in this species of malaria infection, the objective of the present study was, therefore, to determine catalase activity in red cells and liver of mice infected with p.berghei.

Materials and Methods

The study was performed in both normal and P.berghei-infected mice of both sexes weighing 30 - 40 g. P.berghei-infected blood was injected intraperitoneally into each mouse of the infected group. About 3 - 5 mice were used for each experiment and the blood or liver specimens were pooled into one sample.

Blood samples

Blood was withdrawn into heparin solution by cardiac puncture. White blood cells and platelets were eliminated by passage through the Whatman CF 11 cellulose packed in a siliconized glass chromatograph column (Richards and Williams, 1973). Washed red cell samples in isotonic saline solution were lysed by the addition of 4 parts by volume of distilled water. A 1:500 dilution of the lysate with phosphate buffer was prepared immediately before being assayed for catalase activity by the method described by Aebi (1974).

Crude parasite lysates

After passing the infected blood through the cellulose column erythrocytes were washed 3 times in phosphate buffer solution (PBS) after which parasites were isolated by saponin lysis (Zuckerman et al., 1967). Parasites were sedimented by centrifugation at 4,500 g for 5 minutes and washed 4 times in PBS. The initial parasite-free supernatant was used to represent the host red blood cell cytoplasm. The precipitate was frozen and thawed and Triton X-100 was added to 0.05% before catalase and hemoglobin determination were done. This sample represented the crude parasite lysates.

Liver samples

Mice were killed by decapitation and livers were removed quickly, rinsed with ice-cold saline and homogenized in 0.02 M phosphate buffer, Ph 7.0. An aliquot of the homogenate was used for protein estimation. The rest of the homogenate and was treated with Triton X-100 (0.1% s/v) for 30 minutes and centrifuged at 14,000 g for 60 minutes. The supernatant was used for estimation of catalase by the method described by Aebi (1974).

Catalase activity in red cells was expressed as either K or moles (E) per g Hb, 10^{10} RBC or ml red cells. E was calculated from K/K_1, where $K_1 = 3.4 \times 10^7\ M^{-1}\ sec^{-1}$.

Hemoglobin (Hb) was determined by the cyanmethemoglobin method and protein was estimated by the method of Lowry et al., (1951).

Results

Results of catalase activity in red cells of the normal and P.berghei-infected mice are shown in Table 1. It is apparent that the catalase activity, whether expressed as K or mole per g Hb, per ml or 10^{10} red cells is significantly lower ($p < 0.01$) in the infected group than in the normal group. There was an inverse relationship between the parasitemia and the catalase activity as illustrated in Fig. 1.

Catalase activity in infected blood before and after separation into red cell cytoplasm and crude isolated parasite are shown in Table 2.

Red cell cytoplasm catalase action was comparable with that of normal red cells. The isolated parasites contained negligible amount of catalase, which was possibly due to contamination by haemoglobin in the process of preparation.

Table 3 shows the catalase activity in the liver of both groups of mice. Catalase activity, expressed as unit/g liver and as unit/mg protein, in the infected group are significantly lower than in the normal groups ($p < 0.05$).

Table 1:
Catalase activities in red cells of normal (n = 20) and P.berghei-infected (n=17) mice. The results are expressed as mean ± S.D.

Catalase activity	Normal red cells	Infected red cells
K/g Hb	79.7 ± 13.7	62.1 ± 16.6
K/ml red cells	21.7 ± 3.8	15.3 ± 5.3
$K/10^{10}$ RBC	15.2 ± 2.92	11.6 ± 3.6
E/ml x 10^{-8}	0.64 ± 0.11	0.45 ± 0.16
E/g Hb x 10^{-8}	2.34 ± 0.41	1.82 ± 0.50

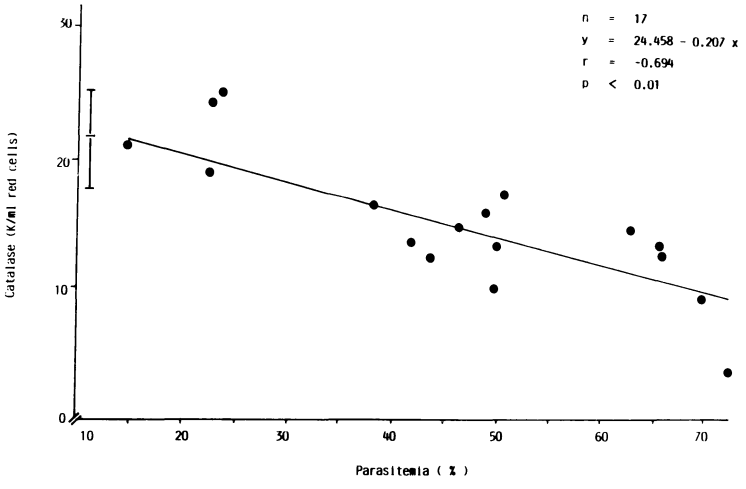

Fig. 1. Relationship between % parasitemia and catalase activity (K/ml red cells) in the infected mice. The mean and S.C. of catalase activity (I) in the normal mice is also shown for comparison.

Table 2. Catalase activities of infected red cells, red cell cytoplasm and isolated P.berghei. Results are expressed as mean ± S.C. from 5 experiments.

Samples	Catalase activity	
	K/g Hb	K/ml red cells
Infected red cells	54.8 ± 14.5	13.8 ± 4.0
Red cell cytoplasm	93.2 ± 19.9	23.8 ± 3.9
Isolated parasites*	-	0.31 ± 0.15

*Hb contamination in isolated parasite lysates was 0.04 ± 0.03 g % and the measured catalase activity in malaria parasites was due to this Hb contamination.

Table 3. Catalase activity in liver of normal mice and mice infected with P.berghei.

	No	Liver Protein (mg/g)	Catalase activity	
			Unit/g liver X 10^3	Unit/mg protein
Normal mice	8	333 ± 38	10.35 ± 1.28	31.49 ± 5.52
Infected mice	7	324 ± 26	7.95 ± 1.43	24.59 ± 4.26

t-test : Normal vs infected mice, unit/g liver, $p<0.01$ and unit/mg protein, $p<0.05$.

Discussion

Results of the present study showed that red cell catalase was significantly lower ($p<0.01$) in the infected group and the decreased catalase activities showed an inverse relationship with the degree of parasitemia. These findings indicated that p.berghei was responsible for the reduced catalase activity in the infected red cells. A significantly decreased erythrocyte catalase has been reported previously in patients with P.vivax and

in mice with P.vinckei was accompanied by a decrease in the catalase activity in infected mouse red cell lysates. The observation that P.berghei parasites contained no catalase activity indicates that they could take up some host catalase, resulting in the low catalase activity in the infected red cells. D-amino oxidase has been reported to be present in P.berghei, but there is no evidence to indicate that this parasite possesses catalase (Gutteridge and Coombs, 1977).

Hepatic catalase activity was found to be significantly reduced by 23% in the infected mice. This figure was in the same order of magnitude as that reported in rats infected with P.berghei(29%) and mice infected with P.vinckei(25%) (Kulkarni et al., 1981; Pickard Maureau et al., 1975). It has been suggested that the reduced activity of catalase observed in the liver may account for the elevated level of H_2O_2 and may enhance lipid peroxidation in the liver of these infected mice (Kulkarni et al., 1981).

It has been shown that there is an increased phagocytic activity during malaria infection (Mc Gregor et al., 1969; Areekul et al., 1973). This would result in an increased release of a considerable amount of H_2O_2 from monocytes and neutrophils. As the malaria-infected red cells squeeze between the fixed macrophages of the spleen and liver, they trigger oxidative bursts and are exposed to oxygen products released by these cells. The large amount of release H_2O_2 would result in the exhaustion of endogenous catalase activity which normally protects cells against oxidative stress generated extracellularly. We believe that our finding of reduced catalase activity in red cells and livers of mice infected with P.berghei can be explained by this mechanism.

P.berghei is quite different from other murine malaria in that it generates H_2O_2 and yet resists high levels of this toxic radical (Etkin and Eaton, 1975). Injection of H_2O_2 into mice infected with P.chabaudi, P.voelii or P.berghei rapidly reduced the parasitemia in all cases except P.berghei (Dockrell and Playfair, 1983). P.berghei-infected mice also accumulate met-haemoglobin, which would result in the parallel loss of catalase activity (Etkin and Eaton, 1975). Experiments with red cells containing methemoglobin indicated that once met-haemoglobin is formed, it protects the red cells from further loss of the catalase activity (Sullivan and Stern, 1980). It is still not known how P.berghei resists

high levels of H_2O_2, but it is possible that this decreased catalase enzyme still can decompose H_2O_2 efficiently in this species of malaria infection.

Conclusion

Hydrogen peroxide (H_2O_2) has been incriminated in the oxidative killing of malaria parasites. As P.berghei-infected mouse red cells generate H_2O_2 in vivo, this would result in the alteration of catalase status of the host. The present study was undertaken to determine catalase activity in red cells and liver of mice infected with P.berghei.

The studies were performed in 17 samples of infected red cells as well as 20 samples of the normal red cells. Results showed that the catalase activity in red cells of the infected group was significantly lower ($p < 0.01$) than that of the normal group. There was an inverse relationship between catalase activity and parasitemia. Crude parasite lysates possessed no catalase activity. Liver catalase content in the infected group was also found to be significantly lower ($p < 0.05$) than that of the control group. All these findings indicate that P.berghei-infected mice caused a depressed catalase activity in red cells and liver. This is postulated to be the result of exhaustion of the enzyme in the process of detoxifying the increased amounts of H_2O_2 .

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

THE HYPOLIPIDAEMIC EFFECT OF DIFFERENT DIETS

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Summary

Saturated fatty acids are still accepted as stimulating and polyunsaturated fatty acids (PUFAs) as reducing total cholesterol and low density lipoprotein cholesterol levels; these changes seem to be mediated by modulating LDL receptor reactivity and therefore the uptake of cholesterol. Confusion remains on the role of individual fatty acids with stearic and medium chain fatty acids considered ineffective in raising cholesterol levels. Lauric and myristic acids dominate with palmitic acid traditionally linked to high cholesterol levels. New studies suggest a modest effect of palmitic but studies with formula feeding are probably flawed because of the absence of dietary fibre; these polysaccharides increase cholesterol catabolism, reduce its absorption and may promote the hypolipidaemic effects of PUFAs. The impact of hyperlipidaemia on coronary heart disease may be particularly dependent on the genetically controlled apoproteins in the lipoproteins but dietary effects on the turnover and reactivity of these proteins is unresolved; these proteins may have fibrinolytic and thrombotic effects.

Introduction

It is now nearly 35 years since Keys and his colleagues reported the impact of different dietary components on serum cholesterol. Since then there has been a plethora of research into cholesterol and lipoprotein metabolism but only now is the role of specific lipids being reconsidered in mechanistic terms and at the more sophisticated levels of nutrient-gene interactions and of changes in receptor activities. There have been many

recent reviews of the field so the purpose of this analysis is to highlight some of the issues which still need to be remembered when linking diet to the development of coronary heart disease.

In 1957 Keys et al., (1959) compared 30 different diets and showed the quantitative effects of various classes of fatty acids on total serum cholesterol. Then in 1965 Hegsted et al., (1965) reported on the effects of 36 diets. Both groups used regression analysis of the change in serum cholesterol expressed in mg/dl in relation to changes in saturated, mono- and polyunsaturated fatty acids. These fatty acid intakes were expressed as a percentage of calories but Hegsted also included dietary cholesterol in his calculations. Although their quantitative results differed, they agreed that dietary saturated fatty acids (SAFA) raised serum cholesterol, polyunsaturated fatty acids (PUFA) decreased it while monounsaturated fatty acids (MUFA) had no effect. They also agreed that the SAFA effect was two or three times greater than the PUFA effect. These findings were the basis for the early recommendations (e.g. by the American Heart Association) to reduce consumption of saturated fat. Keys et al., (1956b) later accepted that dietary cholesterol had a very modest effect on serum cholesterol.

Hegsted (1991) has recently reviewed data on 227 changes in dietary fat to assess their effects on serum cholesterol. He excludes studies using formula diets and manipulated oils because he concluded that they behave very differently from natural diets (see below). He then looked separately at data for metabolic studies which are usually conducted on small numbers of volunteers but in a well-controlled manner. Field trials which involved large numbers of subjects under less controlled conditions were dealt with separately. Regression analysis of all these studies suggested that 75% of the variance in cholesterol changes could be accounted for by changes in the SAFA intakes. Dietary cholesterol also raised total serum cholesterol concentrations but again the MUFAs seemed to have no effect. Recent analyses have now shown statistically significant interactions between SAFAs intakes and changes in both dietary cholesterol and PUFAs, i.e. they produce a combined effect not predictable from the individual changes.

Hegsted (1991) still considers the proposal by Keys et al., (1957, 1965a) that SAFAs have twice the impact of PUFAs on serum cholesterol as a reasonable estimate, but because of other confounding dietary factors one cannot always predict quantitatively the effects of dietary change. Also any modest interactions can rarely be convincingly demonstrated in small studies. The best predictive equation accounts for 83% of the total variance, but the SE is large and amounts to 12 mg/dl. This means that apparently similar diets may produce a change in serum cholesterol which differs by 50 mg/dl (2 SEs either side of line). Explanations for these differences could include the fact that other factors affect serum cholesterol, or that the mean value for serum cholesterol is not always a reliable or reproducible estimate.

Individual Responsiveness

It has been well understood by all investigators that people differ in their cholesterol response to dietary change but there continues to be considerable confusion about what this might mean and about its practical implications. If the well documented but rare homozygous and heterozygous cases of hypercholesterolaemia are excluded, it is still apparent that hypercholesterolaemia runs in families. The susceptibility to increases in cholesterol has been described as polygenically inherited and far more interest is now being taken in the inheritance of different plasma concentrations of apoproteins, e.g. apo(a) and $apoB_{100}$ (Utermann, 1990; Scott, 1990) . What some investigators find hard to encompass, however, are the concepts of nutrient-gene interactions. These interactions are fundamental to many biological phenomena. Thus we should not be arguing about whether a high blood cholesterol level in individuals reflects either their genetics or their diet, but which components of the diet are particularly hazardous in individuals with a particular genetic make-up. It is the response to the stimulus of a dietary change which reveals differences in individuals' gene expression.

Keys, in his classic studies, understood well the link between constitutive processes determining cholesterol levels and the impact of diet. His equations dealt with the average for the group's response but he went on to demonstrate that a chart could be developed

based on the cholesterol level of an individual on a standard diet (Keys et al., 1965c). Thus an individual with a high plasma cholesterol on a standard diet had a predictably larger increase in cholesterol to an increment in dietary SAFAs than normal. In essence, he was arguing for what we could call a "proportionality" phenomenon which is apparent when looking at other biological responses, e.g. the response in faecal bulking to an increase in the intake of non-starch polysaccharides, the response in plasma triglycerides to intakes of n-3 fatty acids or the hypotensive response to dieting in obese patients with or without hypertension. This proportionality effect applies to a huge variety of biological indices and it seems reasonable to suppose that the ranking of an individual's serum cholesterol, serum triglyceride, blood pressure or colonic transit time is markedly affected by constitutive factors which reflect predominantly genetic determinants.

Bouchard (1990) has emphasized that the variance in a biological feature, e.g. in cholesterol or in body fatness, is the sum of the variances from the isolated effects of genes and the environment together with a third variance which is that due to the interaction between the two. Thus we know that in homozygous familial hypercholesterolaemia there is an unremittingly high serum cholesterol and diet has little impact. The environmental effect in normal adults can be shown by the changing average levels of total cholesterol on changing dietary fatty acid intake. These grouped data reflect the variance ascribable to diet alone. Under normal conditions the saturated fatty acid intake may have a range of perhaps $\pm$ 5% of dietary energy on a population average intake of 15% energy. Thus the dietary effect on cholesterol may imply a difference in cholesterol of about 0.7 mmol/l between those on 10% and those on 20% SAFA intakes. Yet individuals on these diets show two-fold differences in cholesterol levels on a 10% diet with a doubling of the variance on the 20% diet. Thus a 4 mmol/l difference within a group reflects the importance of the inter-individual variability in responsiveness and shows the dominance of the genetic dietary interaction over the dietary effect alone.

The Basis for Genetically Controlled Response

What is now emerging as an issue is whether we can identify the gene locus and the mechanisms which affect these variances. Whereas the inheritance of the lipoprotein Lp(a)

accounts for striking differences in its circulating concentrations between people, the concentrations are very consistent within individuals (Utermann, 1990). MBewu & Durrington (1990) have recently emphasized the potential role of the lipoprotein(a) in modulating both thrombosis and atherosclerosis. The Lp(a) is composed of apolipoprotein B, which contains the amino acid sequence responsible for binding to the LDL receptor and, complexed together with apoB chain is the water soluble apolipoprotein(a) which is structurally remarkably similar to the natural inducer of fibrinolysis, plasminogen. Apo(a) concentrations are in some individuals in excess of circulating plasminogen activity and therefore promote thrombosis. In healthy adults with a parental history of premature heart disease apo(a) concentrations (but not apoB or apoA levels) are three to four times higher than controls (MBewu & Durrington, 1990).

Lipoprotein(a) also binds to both fibronectin in early atherosclerotic lesions and to the glycosaminoglycans of the arterial wall. This may enhance the susceptibility of the apoB to oxidative damage as the protein comes into closer conjunction with metabolically active and free radical generating tissues. This in turn will promote the development of atherosclerosis as noted elsewhere in this symposium.

Whether the catabolism of Lp(a) can be modulated by dietary induced changes in the LDL receptor is unclear; there is a doubling of Lp(a) in familial heterozygous hypercholesterolaemia (F_H) patients where an abnormality of LDL receptor uptake is well recognized. This produces a two to three fold increase in LDL plasma concentrations (Goldstein & Brown, 1989) The Lp (a) concentration seems to be a key factor in the development of CHD in F_H together with an elevated LDL and apolipoprotein B concentration. The apoB acts as the ligand for the LDL receptor which is down regulated when saturated fat in the diet is eaten (see below). Thus the LDL concentration in the blood rises because there is a reduced uptake of LDL by the liver. When the structure of apoB is altered, e.g. by free radical induced damage, then this leads to the ready uptake of the LDL through the "scavenger" pathway in the endothelial macrophage. It is this which leads to the onset and development of the atherosclerotic process. The conjunction of high apo(a) concentrations and elevated LDL and apoB levels seems to identify a high

proportion of individuals with a strong family history of premature coronary heart disease who are also prone to heart disease themselves.

Consistency of Response

The studies on the genetic and dietary factors affecting lipoprotein metabolism and plasma cholesterol concentrations all tend to presuppose that there is a real consistency in the response of individuals when studied repeatedly. Katan et al., (1986) investigated this in terms of dietary cholesterol and found that individuals were indeed consistent in their serum cholesterol response: they might increase their serum cholesterol markedly or poorly but this distinction was retained on repeated testing. More recently Katan et al., (1988) have considered whether the hyper-responders are coherent when faced with two different stimuli, viz. an increase in first dietary cholesterol and then in SAFA. A modest link between the responses was observed accounting for about 35-40% of the variance in responses. This is understandable if dietary cholesterol enriches first the pre-existing chylomicrons and VLDL and then their remnants so that there is an enhanced LDL cholesterol concentration. The enriched remnants on being taken up by the liver not only down regulate endogenous cholesterol synthesis but also the LDL receptor. This last effect is similar in action to SAFA. The LDL half-life will therefore tend to become prolonged with both cholesterol and SAFA feeding. The smaller intestinal form of apoB, $apoB_{48}$, may be responsible for delivering dietary cholesterol rapidly to the liver with a cycle time of 3 hours whereas $apoB_{100}$ is responsible for hepatic export of cholesterol and lipids in VLDL with a cycle time of 36 hours. Thus down regulation of LDL receptors may in itself produce somewhat different effects on blood cholesterol turnover given the different cycles of the apoB proteins to which dietary cholesterol and saturated fatty acids relate. So a complete uniformity of response to the two feeding stimuli should not be expected in individuals with a particular LDL receptor reactivity.

Formula Diets: A Fibre Depletion Problem?

Hegsted (1991) in his recent extensive review of the literature chose to ignore all those studies, e.g. by Ahrens and his colleagues, where lipoprotein metabolism had been

studied by the use of formula diets. This was because Hegsted's meticulous re-analysis of a large number of formula feeding studies showed that no further lowering of serum cholesterol could be achieved on formula diets if a polyunsaturated oil was substituted for olive oil. Yet safflower oil, for example, is accepted by everybody as having a greater lipid lowering effect than olive oil when incorporated into normal diets. Hegsted did not offer an explanation for this observation but considered that there could be some factor missing from the formula feed which modified the response. The obvious candidates are the various dietary fibre components, best considered scientifically as the non-starch polysaccharides. The non-cellulosic water soluble components such as the pectins and gums are well recognized hypocholesterolaemic agents with a presumed ability to reduce cholesterol absorption from both the diet and from the biliary pool of cholesterol. If PUFAs enhance hepatic cholesterol uptake by up-regulating LDL receptors (see below) then dietary pectins might promote its biliary and faecal excretion by interrupting cholesterol recycling.

Although the cellulosic polysaccharides found in abundance in cereal fibre fractions do not lead to a fall in serum cholesterol concentrations, the ingestion of this type of fibre markedly enhances bile acid excretion by a number of processes, including the physical sequestration and chemical binding of the bile acids to the residual plant cell walls and their associated bacteria within the colon. The colonic recycling of the secondary bile acid, deoxycholate, is reduced and this enhances cholesterol catabolism to bile acids. Supplementing a diet with wheat bran cereal fibre alone does not lower serum cholesterol levels, presumably because there is a reasonably good feedback regulation between cholesterol catabolism and de novo synthesis in the hepatocyte. This may be a more important regulatory system than that derived from the inflow of LDL derived cholesterol. Some cereal fibres, e.g. oat bran and rice bran, do have an effect however, but this is probably dependent on such non-cellulosic polysaccharides on the B glucans whose mechanism of action may be small intestinal. Oat fibre produced both a fall in total cholesterol and in LDL cholesterol, with a rise in HDL and in the ratio of apolipoprotein AI to B (Kestin et al., 1990) . It is unclear, however, what the effect of having a high cereal fibre intake and then changing the PUFA intake might have on cholesterol turnover.

Some of these dietary inter-relationships are speculative, but given the multiplicity of intestinal effects of a fibre-rich diet, it is not surprising that there is an increase in the faecal excretion of both acid and neutral stools on high fibre feeding. Lewis and his colleagues (1981) found, in carefully conducted trials, strongly additive effects of fat modification and fibre supplementation on LDL cholesterol levels; these fell markedly by 35%. Furthermore, the short-term reduction in HDL_2 cholesterol was less when a high intake of dietary fibre was included in the dietary regimes. Thus we may conclude that Hegsted (1991) was correct to be cautious in considering the formula feeding regime as physiologically meaningful, painful though it may be to set aside decades of meticulous research on formula diets.

Saturated Fatty Acids

Both Keys and Hegsted were concerned with predicting the effects of the dietary changes on serum cholesterol in their carefully controlled metabolic studies. They fed different diets containing different oils and from this inferred that the changes observed were attributable to the chain length and degree of saturation of the fatty acids. Since this analysis was all based on regression analysis there are considerable problems if associated factors in the dietary oils modulate the response.

Originally Keys and Hegsted implied that all the saturated fatty acids in the diet were equivalent in their effects with palmitic acid dominating the composition of many SAFA rich diets. However, Keys et al., (1965d) continued their studies to explore whether there were differences between the individual saturated fatty acids. Fatty acids with fewer than 12 carbons are polar and less hydrophobic and we now recognize that these medium chain triglycerides do indeed have a different route of absorption in the free fatty acid form via the portal vein and are then preferentially oxidized by the liver.

Fig. 1 taken from the US National Research Council (1989) review illustrates the different views of the Keys and Hegsted schools. As Hegsted (1991) has noted, these assessments are all based on regression analyses of different diets. Hegsted found a greater effect of the C_{14} and C_{16} fatty acids than Keys, but Keys did not at first attempt

to discriminate between the SAFA. Later he and others assessed the effects of cocoa butter because 35% of its fatty acid content is in the stearic acid (C_{18}) form. Beef fat also contains substantial amounts (20%) as does mutton fat (30%), milk fat (10%) and lard (13%) . On testing cocoa-butter had little effect on cholesterol levels so Keys et al., concluded that lauric (C_{12}) I myristic (C_{14}) and palmitic (C_{16}) were the main promoters of hypercholesterolaemia. These conclusions seemed to be confirmed by Grundy (Bonanome & Grundy, 1988) in formula feeding studies but their diet had a specially prepared re-esterified oil as the test substance. Hegsted et al., (1965) in their original detailed estimates (Table I and Fig. 1) suggested that each saturated fatty acid had an individual tendency to increase blood cholesterol and in their assessment they estimated that myristic was more hypercholesterolaemic than palmitic, with stearic having if anything a depressant effect. Recently Hayes et al., (1991) assessed the effect of purified diets containing blends of four different oils: coconut, palm, high oleic acid safflower and soy bean oils in three species of monkey (rhesus, cebus and squirrel) . Hegsted and Keys' regression equations provided a good fit for the data but the predicted total cholesterol response was perfectly matched if the palmitic acid was considered to be neutral in its effects. The 16:0 intake seemed only slightly more cholesterolaemic than the linoleic acid content of these oils.

Numerous reviewers since then have therefore assumed that palm oil, which has palmitic acid as its predominant SAFA, would be expected to increase cholesterol levels. This assessment neglects the substantial unsaturated component in palm oil as well as the other components which might affect both its health impact and its effect on lipid metabolism. Furthermore, as Hornstra (1988) has stressed, experimentally palm oil does not promote atherosclerosis and the fall in cholesterol concentrations may relate to the presence of α-tocotrienols in the oil. These tocotrienols may also account for the reduced synthesis of pro-thrombotic factors. Recently Qureshi et al., (1991) have observed a lowering of total and LDL cholesterol, a fall in apoB and thromboxane in subjects given a tocotrienol enriched fraction of palm oil so the role of these additional factors is of increasing interest. Curiously, few formalized studies have been undertaken and this has obviously been of concern to the industries concerned.

Table I

The equations for predicting changes (Δ Ch) in total serum cholesterol concentration (mg/dl) induced by alterations in the diet.

Keys:	Δ Ch = 2.7ΔS - 1.35ΔP + 1.5ΔC where C is the square root of the dietary cholesterol intake in mg/1000 kcal.
Hegsted:	Δ Ch = 2.16ΔS - 1.65ΔP + 0.008ΔC The total SAFA intake (S) and PUFA intake (P) is expressed as % calories whereas Hegsted's cholesterol value is in mg/day.

Extended Hegsted formula:

$$\Delta Ch = 0.66\ S_{10} + 1.03\ S_{12} + 4.98\ S_{14} + 3.76\ S_{16} - 0.49\ S_{18} - 0.24\ M - 1.89\ P + 5.70\ C - 9.44$$

where S_n is the change in % dietary energy from SAFA of chain length n, M is the change in % energy from MUFAs and C is the change in dietary cholesterol in mg/100g.

In a recent symposium (Kritchevsky, 1991) a number of studies are reported which question some of the original assumptions. For example, Ng et al., (1991) noted that in all five previous human studies conducted in North America or Europe, palm oil maintained cholesterol levels at a higher concentration than PUFA based oils, e. g. corn, safflower seed, soy bean or sunflower seed oils. Nevertheless, the palm oil led to a 7-38% lower total cholesterol than when the subjects entered the trial. Ng et al., undertook a feeding trial on 83 subjects with natural diets adjusted to have either coconut oil, corn oil or palm oil in as the dietary component providing 75% of the fat calories. These studies led to similar effects to those noted previously, i.e. differences between the oils but a reduced cholesterol level when subjects changed from their normal diet to a palm oil diet. The precise role of palm oil therefore remains unclear.

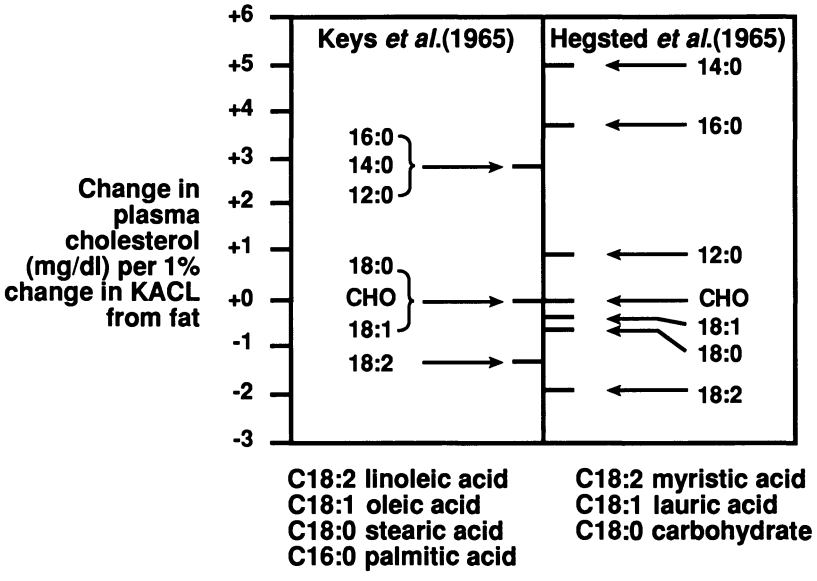

Figure 1

Of greater potential significance was McGandy's finding of little difference between the fatty acids when they were re-esterified with either lauric, myristic, palmitic or stearic acid (McGandy et al., 1970). However, as Hegsted (1991) has noted, the use of re-esterified oils may be flawed because the stearic acid residues will be randomly linked to the glycerol backbone of the triglyceride. It is widely recognized that the absorption and metabolism of the 2-monoglyceride fatty acid is different from that of the fatty acids on the 1 and 3 positions. Many of the assumptions about the health importance of specific chain lengths on the cholesterol elevating effect of saturated fatty acids may be incorrect but for the moment the US National Research Council (1989) has concluded that stearic acid has no effect on serum cholesterol levels whereas myristic and palmitic acids dominate in their effects of SAFA.

Mechanisms of Action of Dietary Fatty Acid

Studies on primates suggest that SAFA increase LDL cholesterol concentrations by slowing the turnover of the LDL particles. This effect seems to depend on a reduced rate of uptake of the LDL by the liver and a change in the fractional catabolic rate of LDL apoB has been observed in volunteers (Shepherd et al., 1980). It seems not unreasonable to consider changes in membrane stability as SAFA are incorporated into the membranes as a potential way whereby the LDL receptor uptake is reduced. However, Fox et al., (1987) found a fall in the mRNA for the LDL receptor protein in primate studies so the SAFAs are in some way affecting the expression of this protein. These and other animal experiments support the idea that the rate of uptake at the LDL receptor site is the principal mechanism whereby dietary fatty acids alter total and LDL cholesterol levels in the blood.

Polyunsaturated Fatty Acids

The cholesterol lowering effect of PUFAs has been ascribed not only to changes in LDL uptake but also to increased excretion of cholesterol in the bile. Of concern, however, has been the repeated finding of a fall in HDL levels, a change which is increasingly seen as disadvantageous (Stampfer et al., 1991). Brinton et al., (1990) have recently studied the fractional catabolic rate (FCR) and the transport rate (TR) of the main HDL apoprotein apoA1. This is the apoprotein recently shown by Stampfer et al., to predict a lower risk of coronary heart disease. Volunteers were studied on a low fat, high carbohydrate diet and on a high fat diet also. When the diet was low in fat a decline in HDL levels occurred but this depended on increases in TR and not on a change in FCR. The individual difference in prevailing HDL concentrations depended, however, on the FCR of the individual. Thus the intrinsic differences between people may depend on a different mechanism from that which responds to dietary change. This study has not been extended as yet to an analysis of the effects of substituting PUFAs for SAFAs but the need to distinguish short from long-term effects is becoming clear. A change in the dietary fat/carbohydrate ratio has long been known to alter triglyceride levels and it takes

several weeks to readjust (Antonis & Berson, 1962). Now Hegsted (1991) is also suggesting that we need to consider the potential long-term adjustments to diet and to the level of fat feeding. He points out that a cholesterol-lowering effect of PUFAs cannot be expected in diets which are already below 15% fat. Gibney (1991) has also recently re-analyzed all the dietary trials published in the last 10 years to assess the impact of PUFAs on HDL and concluded that only in those with very high P:S ratios, e.g. over 3.5 or with studies lasting less than 6 weeks, is there a fall in HDL cholesterol concentrations. Those studies which continue longer do not show any clear decline in HDL levels provided the P:S ratio is below about 1.5. These more modest changes may be the physiologically relevant ones.

Monounsaturated Fatty Acids

These have been of great interest recently since they are considered to sustain HDL concentrations when they are substituted for SAFAs (Grundy, 1986) . The LDL cholesterol decline seems primarily dependent on the fall in SAFA as the dietary substitution occurs. The increase in carbohydrate with a switch from SAFA is accompanied normally by a fall in HDL. These may be a short-term effect because populations on a habitual high carbohydrate/low fat diet have high HDL levels. However, these populations differ in other ways, e.g. by being more active; exercise is known to raise HDL levels.

Isomeric Fatty Acids

The process of hydrogenation can alter the cholesterol effects of a number of PUFA rich oils. The generation of SAFAs under conditions of complete hydrogenation seems to lead to the type of response expected from studies with the natural fats which are rich in SAFAs. It is often assumed that hydrogenated products necessarily induce hypercholesterolaemia but this is not so. In practice a substantial proportion of the unsaturated fatty acids are hydrogenated to stearic and oleic acids which are considered poor inducers of hypercholesterolaemia. Recently Katan (Mensink & Katan, 1990) has

found that large doses of the 18:1 isomers of elaidic acid can affect HDL cholesterol levels adversely and increase LDL cholesterol but the relevance of this to habitual Western diets is uncertain.

Trans fatty acids are a normal component of the diet because ruminant animals' fat depots contain trans fatty acids generated by the microbes in the animals' rumen. From 2-7% of beef and mutton fat are *trans* isomers but this figure can rise to 10-30% in some margarines, hardened fats and salad oils. There is increasing concern about the potential effects of these isomers and attempts are now being made to limit any further increase in the consumption of isomeric fatty acids. Under conditions of partial hydrogenation a huge variety of unusual cis and trans isomers form. These isomers have individual effects on essential fatty acid metabolism which are not readily predictable; they also affect blood clotting mechanisms when studied *in vitro*. Thomas and his colleagues (1983) undertook a series of studies which showed that the concentrations of *trans* fatty acids in the fat depots of patients dying from coronary heart disease were higher than those of patients dying from other conditions. This implied that these isomeric fatty acids might be an important risk factor. No further studies are available using more refined techniques for measuring the individual isomeric fatty acids.

An emphasis on thrombosis and essential fatty acid metabolism is appropriate because for too long the assessment of the significance of fats for health has been judged simply on whether they increased LDL or reduced HDL cholesterol concentrations. Peanut oil illustrates this problem because in animal studies it has been shown to be markedly atherogenic whilst being rich in MUFAs and PUFAs and therefore capable of maintaining reasonably healthy levels of the cholesterol components in the blood.

N-3 Polyunsaturated Fatty Acids

These n-3 PUFAs are abundant in many fish and are now being studied for their effects on blood lipids, thrombosis, atherogenesis and longevity. There is now substantial evidence that fish consumption is linked to a reduced incidence of coronary heart disease (Kromhout et al., 1985) . These dietary fatty acids are considered to act via a number of

mechanisms (Kinsella et al., 1990) . These fatty acids have a marked depressive effect on plasma triglycerides by inhibiting triglyceride and possibly apoprotein synthesis. They replace arachidonic acid in phospholipid pools with eicosapentaenoic acid (EPA) and dicosa-hexaenoic acid (DHA). These two metabolites of α-linolenic acid inhibit cyclo-oxygenase and lipo-oxygenase, thereby reducing the synthesis of thromboxane (TXA_4) and leukotriene B_4 (LB_4) by both platelets and macrophages. Thus they can affect both platelet clotting and potentially the progression of atherosclerosis. TXA_2 is a pro-aggregatory factor so the fall of TXA_2 and a rise in an anti-aggregatory prostaglandin, PGI_3, derived from EPA shifts the whole clotting mechanism. Eicosanoid metabolic changes in platelets, monocytes and macrophages are one potential mechanism for limiting atherosclerosis. These oils also seem to moderate the prevalence of hypertension by mechanisms which remain obscure.

N-3 PUFAs are known to reduce intestinal synthesis of triglycerides and reduce chylomicron secretion. This response and the dampening of both apoB and very low density lipoprotein secretion from the liver seem to explain the often startling effect of fish oil supplements on hypertriglyceridaemia. Experimentally fish oils alter apoB pool sizes but detailed studies of their effects on properly measured apo(a) are awaited. The effects on LDL cholesterol also need careful appraisal because, as is often the case in lipid studies, the provision of fish oils may be as a substitute for SAFA; the interpretation of the data then becomes suspect (Harris, 1989). When n-3 fatty acids are added to a diet with a constant supply of SAFA then LDL cholesterol does not alter.

Other dietary factors

Mention should be made of alcohol which is recognized to increase HDL cholesterol levels; moderate alcohol consumption also seems to be associated with a lower cardiovascular risk (Rimm et al., 1991). Alcohol also seems to inhibit the synthesis of apo (a) because alcoholics are recognized to have low levels. Nevertheless, in those individuals who are prone to hypercholesterolaemia, alcohol may lead to a marked increase in VLDL triglyceride synthesis by the liver. The role of animal protein has also

been investigated but the significance of a substitution of vegetable for animal protein does not seem great (Wiebe et al., 1984).

Conclusions

It is all too easy to become overwhelmed by all the studies which have been conducted on the hypolipidaemic effect of different diets. It is well to remember, however, that the principal concern in these studies is to assess the role of diet in the development of cardiovascular disease. It is becoming apparent that a dietary item such as palm oil or sunflower oil may have many different components in the oil other than the individual fatty acids on which there has been so much emphasis. These other factors may well prove crucial in modulating the pathophysiology of arterial disease.

As the biology of intestinal damage and thrombosis is revealed, it is evident that we need to re-appraise the relative importance of the specific amounts of cholesterol or other lipids in different circulating lipoprotein components. Not only is the turnover of these components important but the role of the lipoprotein structures and their reactivity needs to be considered separately from the lipid fractions. Far more emphasis has been given to cholesterol turnover because plasma cholesterol was the first crude index of risk to be identified. We now need to understand the effects of diets on the synthesis, biological reactivity and turnover of the highly interactive and exchangeable proteins which provide the framework for the lipoproteins of different densities. Then we can move from the somewhat crude analysis of the effects of diet on heart disease and specify with increasing precision the nutrient-gene interactions which are likely to be fundamental to the real explanation of coronary heart disease.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

EPIDEMIOLOGICAL CORRELATIONS BETWEEN POOR PLASMA LEVELS OF ESSENTIAL ANTIOXIDANTS AND THE RISK OF CORONARY HEART DISEASE AND CANCER

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Summary

Many dietary surveys all over the world have shown that vegetable-rich diets can be related to higher life expectancy. Thus, previous dietary guidelines have given general recommendations to lower dietary fat (particularly of mammalian origin) and to increase the percentage of vegetables/fruits and suitable vegetable oils which are known to contain a series of natural antioxidants.

Various types of epidemiological studies in westernized countries have revealed an increased risk for cancer and Coronary Heart Disease respectively of a poor plasma status of essential antioxidants such as vitamins C and E as well as carotene and vitamin A. In consequence, a more specifically updated prudent diet should aim for optimal intake of β-carotene as well as of vitamins E and C in order to use their presumable potentials regarding the prevention of cancer and/or Coronary Heart Disease.

Introduction

Since aggressive oxygen species (free radicals such as superoxide anion radical, hydroxyl radical, hydrogen peroxide and singlet molecular oxygen) can damage DNA, proteins, carbohydrates and unsaturated lipids, they have been implicated in the etiology

of many diseases (Sies 1985, 1991). Unfortunately because of an extremely short half-life most aggressive species cannot easily be trapped under physiological conditions in vivo and thus mostly escape a direct investigation. But indirect information on the impact of free radicals may be obtained by comparisons of the antioxidant potential since any pathogenic onset of radicals depends also widely on the efficacy of the body's multilevel defense system against radicals. The defense lines consist of enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase), non-essential endogenous radical scavengers (glutathione, proteins, uric acid, ubiquinol-10, etc.) and essential radical scavengers, i.e. the antioxidant vitamins C (ascorbic acid) and E (α-tocopherol) as well as the singlet oxygen-quenching carotenoids (e.g. β-carotene which is a potential vitamin A precursor but may have other specific effects, or the non-vitamin A precursor lycopene) and finally vitamin A (retinol) (DiMascio et al., 1991; Sies 1985, 1991). The level of essential antioxidants, however, is in contrast to that of other antioxidative defense lines mainly determined by their dietary supply. In consequence, if free radicals were indeed involved in pathological mechanisms, an optimum status of essential antioxidants should reduce the risk of disease and thus be a prerequisite of "optimum health" as defined by WHO. This working hypothesis is based on a series of observations (ref. cit. Gey 1992). Briefly, at first, many epidemiological studies all over the world have led to the general assumption that vegetable-rich diets are associated with a higher life expectancy. Secondly, the calculated dietary intake of essential antioxidants has been found to be inversely related to the risk of the major causes of death in westernized societies, i.e. of cancer (Ziegler 1991; Block 1991) and of Coronary Heart Disease (CHD) (ref. cit. Gey 1992). Thirdly, more reliable indicators of the actual antioxidant status such as the measurement of plasma levels of essential antioxidants (reviewed below) also reveal inverse associations with these major causes of death.

Cancer

Prospective/Longitudinal Surveys

A series of prospective, epidemiological studies (with analysis of cancer cases in comparison to surviving controls) have revealed a significant correlation of low levels of

β-carotene, in part also of vitamins E, C and A, with subsequent cancer in follow-up periods up to 12 years (Table 1). Whereas prospective studies have mostly a "blood-bank" design (with storage of serum for years which unfortunately causes some antioxidant loss even in the deep-freezer) the Basel Prospective Study was devoid of such inherent weakness and was also unique in measuring simultaneously all principal plasma antioxidants (Stahelin et al., 1991) . The sequential evaluations of the Basel Study have consistently revealed a statistically significant predictive power of a poor carotene status for most cancers (Figures 1, 2) in accordance with a series of other study sites (Table 1). In addition, the Basel Study has established a statistically significant increase of the risk of gastrointestinal cancers at low plasma concentrations of vitamin C and at low levels of lipid-standardized vitamin A (Figures 1, 2) . And finally the Basel Study has indirectly supported results from other study sites demonstrating an increased cancer risk at a truly poor vitamin E status (Table 1) . Thus vitamin E levels in Basel lacked (at most follow-up's) a significant correlation to subsequent cancer but the vitamin E levels of the Basel population were unusually and uniformly high (Figures 1, 2) and very probably above any level of risk. Clearly, an increased risk of a poor antioxidant level can only be detected if the latter occurs in a statistically appropriate percentage of study subjects, which happened in the Basel Study for the above mentioned antioxidants except vitamin E (Stahelin et al., 1989). Taking together the available prospective data the potential cancer-preventive properties of essential antioxidants are likely to have the following rank order for

- bronchus cancer: carotene (β-, possibly also α-carotene) > lipid-standardized vitamin A, vitamin E;
- gastrointestinal cancer: vitamin C > carotene (as above) = vitamin E =vitamin A;
- all cancers: carotene > vitamin A = vitamin C.

The combination of low levels of several antioxidants, e.g. of carotene and vitamin A, increased the risk additively (Stahelin et al., 1991) or in part presumably synergistically (Gey et al., 1989).

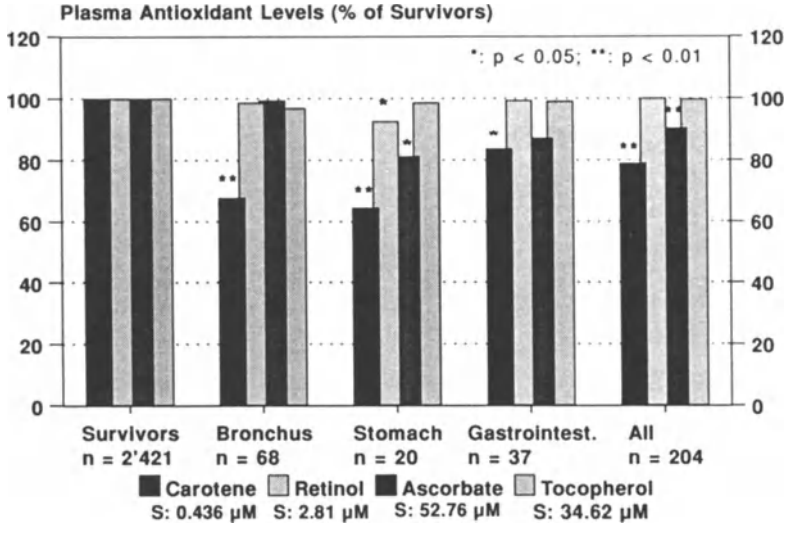
TABLE 1.

Prospective Studies: Poor Plasma Antioxidant Status predicts Subsequent Cancer Mortality

Antioxidant	Cancer (in 5-12 vs.)	Reference
Carotene	Lung	1-10
	Gastrointestinal	9
	All (in males)	4-9
Vitamin A	Lung	4-6,9
	Gastrointestinal	4-6,9,11
	All (in males)	9
Vitamin C	Gastrointestinal	4-6,9
	All (in males)	4-6,9
Vitamin E	Lung	3-6,12
	Gastrointestinal	13
	Urinary Organs	13
	All (in males)	12-14,15
	Breast	13,16
	Fem.Reproduct.	13,17
	All (in females)	13,17

References: [1]Stahelin et al., 1984, [2]Nomura et al., 1985, [3]Menkes et al., 1986, [4]Stahelin et al., 1987, [5,6]Gey et al., 1987, 1989, [7]Wald et al., 1988, [8]Comstock et al., 1991, [9]Stahelin et al., 1991a,b, [10]Connett et al., 1989, [11]Willett et al., 1985, [12]Kok et al., 1985, [13]Knekt et al., 1988a, 1991, [14]Salonen et al.,1985, [15]Knekt et al., 1988b, [16]Wald et al., 1984, [17]Knekt 1988.

Figure 1: Mean base-line values of plasma antioxidants (vitamins A and E lipid-standardized) in cases of subsequent cancer in comparison to survivors: 12-year follow-up of the Prospective Basel Study. The absolute plasma values of survivors are indicated at the bottom (Stä et al., 1991).



Current Intervention Trials

At present more than 20 intervention studies in randomized subjects with high cancer risk (e.g. in smokers or in asbestos-exposed workers) and mostly sponsored by the US National Cancer Institute, NIH, Bethesda, Maryland, are under way in order conclusively to test the cancer-preventive potentials of specific supplements of β-carotene and/or of vitamins A, C and E (Malone 1991) . Results can be expected within a few years. Future research will be needed regarding the optimum dosage of essential antioxidants, particularly in case of combinations because of conceivable synergistic effects of antioxidants (Kagan et al.,1992; Negre-Salvayre et al.,1991; Niki et al.,1991; Packer 1992; Stocker et al., 1992). Further potentials remain also to be explored for hitherto widely neglected plant antioxidants, e.g. special carotenoids such as lycopene (DiMascio et al., 1991; Sies 1991; Comstock et al., 1991) or the large fraction of phenolic bioflavonoids (Das 1992; Dani 1992; Osawa 1992; Stavric & Matula, 1992) , at least for the gastrointestinal tract.

Figure 2: Relative risk of low plasma levels of essential antioxidants (at base-line) for subsequent cancer death (first 2 years excluded) after adjustment for age, smoking and lipids, with 95% confidence intervals: 12 year follow-up of Prospective Basel Study. Vertical scale and figures to the right respectively: Relative risk of quartile 1 (threshold levels at the right edge) in comparison to quintiles 2 to 5 (with higher antioxidant levels) in Cox proportional hazard regression model (Stähelin et al., 1991).

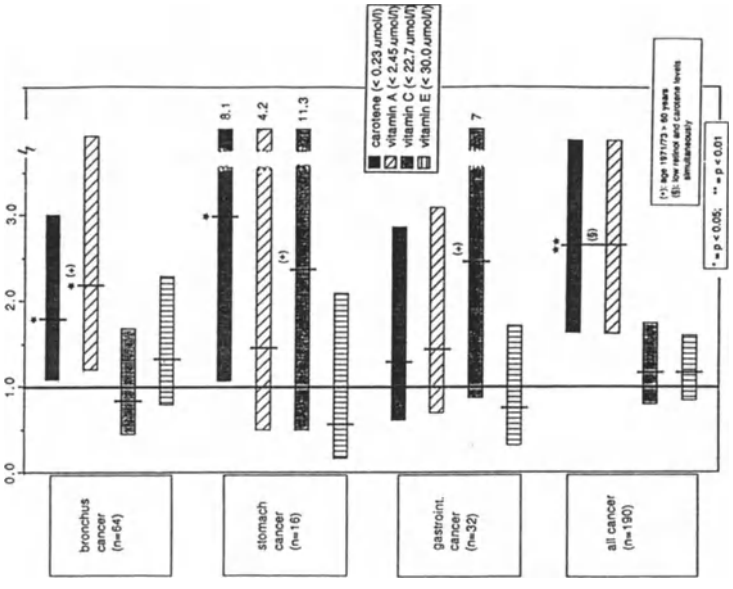
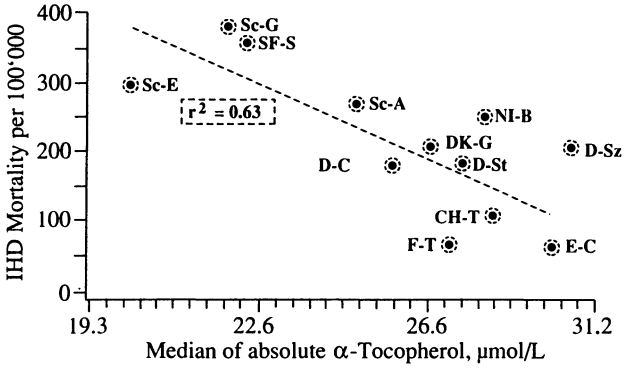
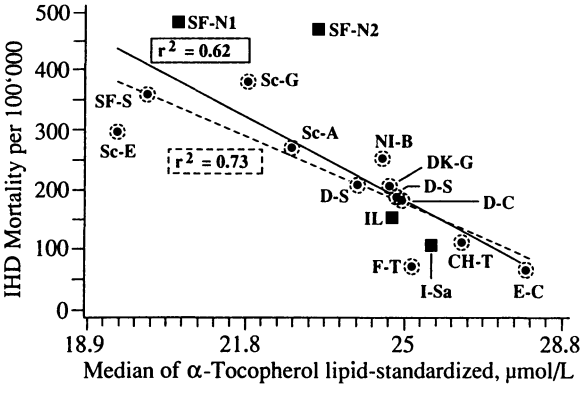


Figure 3:Inverse correlation between age (40-59 Years)-specific CHD mortality and medians of (logarithms of) plasma vitamin E: cross-cultural comparisons of European study populations (n=16) by Vitamin Substudy of WHO/Monica Project (Gey et al., 1991).
Upper graph: absolute values of plasma vitamin E (α-tocopherol) in study population with common total cholesterol and regular blood pressure (n=12).
Lower graph: lipid-standardized plasma vitamin E in the same study sites as above (again dotted regression line) and in all study populations, i.e. after inclusion of population with extreme cholesterol levels (solid regression curve).





Conceivable Mechanisms of Action

Although the essential antioxidants have well-established functions as radical scavengers, the mechanisms by which they counteract mutagenic actions and may consequently prevent cancer in mammals in vivo is poorly understood, and this is particularly true for the highly intriguing carotenoids (Krinsky 1991). Since radicals can damage DNA (Ames 1983) and may independently act as tumor promoters (Cerutti 1985), the radical scavengers conceivably reduce tumor initiation and/or promotion, the latter presumably by modulating the expression of proto- and anti-oncogenes (Bertram et al., 1987; Birt 1986). Indeed, in the human vitamin A as well as β-carotene reverses the precancerous leukoplakia and reduces the occurrence of pathological micronuclei even on continuous exposure to mutagens from betel nuts and/or tobacco chewing (Stich et al., 1991). But all essential antioxidants might also, at least in part, act more directly through special mechanisms, e.g. by improvements of immunoresponses (Bendich 1990) or inhibition of adenylate cyclase (Hazuka et al., 1990). Protection of the gastrointestinal tract by vitamins C and E (Correa 1984) may have special mechanism such as inhibition of nitrosamine formation (Tannenbaum et al., 1991) and diminution of fecal mutagens (Dion et al., 1982).

Coronary Heart Disease

Evidence for associations of a poor plasma status of essential antioxidants and an increased risk of CHD has emerged from several types of studies, i.e. first from cross-cultural comparisons in Europe where CHD mortality varies up to six-fold (similar to world-wide differences) and where the antioxidant levels differ up to about twofold, secondly from comparisons of individuals with and without early angina pectoris (i.e. in early CHD) in Edinburgh/Scotland where plasma antioxidants vary from sufficient/fair levels to biologically poor levels, and finally from complementary observational data in individuals with myocardial infarct.

Cross-Cultural Epidemiology

The Vitamin Substudy of the WHO/MONICA Project (a standardized and by far the largest trial for monitoring determinants and trends of Cardiovascular disease) compared in randomly selected representatives of 16 European study populations the plasma antioxidant status with the concurrent age-specific CHD mortality. Thereby, in the majority, i.e. in 12 study populations the classical risk factors, plasma cholesterol, blood pressure and smoking did not differ significantly and could thus not sufficiently explain the up to six-fold differences in CHD mortality. In contrast, inverse correlation existed between the age-specific CHD mortality and the plasma status of essential antioxidants with the following rank order: vitamin E > > vitamins C and A (Gey 1986, Gey et al., 1991). The impressively strong inverse correlation of vitamin E (r^2 >0.6; Figure 3) occurred for both absolute vitamin E (in populations with comparable plasma lipoproteins) and lipid-standardized vitamin E (in all study populations). Clearly in these European study populations vitamin E was a stronger predictor of CHD mortality than the classical risk factor hypercholesterolemia and hypertension. By combination in multivariate analysis of vitamins E and A with the above mentioned classical risk factors

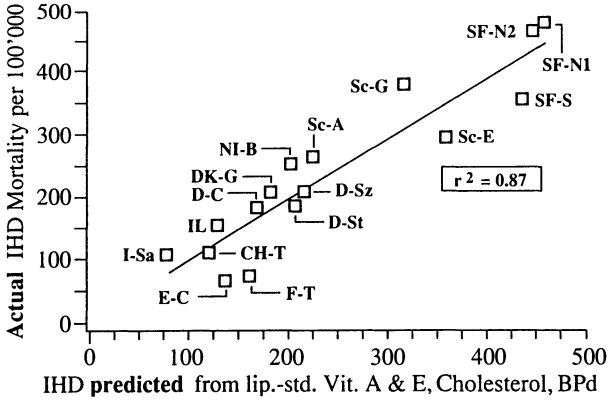


Figure 4: Correlation between age-specific CHD mortality in middle-aged males of 16 European study populations of the Vitamin substudy of the MONICA/WHO Project (Gey et al., 1991)
Ordinate: Actual mortality rates: Abscissa: prediction in multivariate analysis by the combination of lipid standardized vitamin E and A, total cholesterol and systolic blood pressure.

the existing CHD mortality could be predicted to 87% (Figure 4), and after inclusion of vitamin C to about 90% (Gey et al., 1991).

Case-control Study in Early Angina Pectoris

In Edinburgh/Scotland, i.e. a community with a traditionally low consumption of fresh fruits and vegetables, a screening of inhabitants identified a considerable percentage of men with angina pectoris which had previously neither been diagnosed nor led to any intentional change of life style. Low plasma levels (quintile 1) of vitamin E, of vitamin C and of carotene were associated with an up to 2.6-fold higher risk of this early stage of CHD as compared to high antioxidant levels (Riemersma et al., 1991). Whereas the increased risk of low vitamin C and carotene were confounded by (and most likely mainly due to) cigarette smoking, the statistically significant linear risk attributable to low vitamin E was independent of classical risk factors (Figure 5). Thus in these Scottish individuals essential antioxidants seemed again to have a rank order similar to that in European study populations, i.e. vitamin E $>>$ vitamin A = vitamin C = carotene. As in the cross-cultural comparisons the threshold of "safe", i.e. presumably "optimum" plasma levels of (lipid-standardized) vitamin E may be above 28-30 μmol/l (Gey et al.,1992).

Prospective Data

According to a recent preliminary evaluation of the 8-year follow-up of the US Nurses Study (by the Harvard Medical School; Manson et al., 1991) the risk of CHD is significantly lower at the highest intake of either vitamin E or A or carotene which was presumably mostly due to self-supplementation (unfortunately information on confounding of variables as well as on dietary vitamin C are missing). Correspondingly, a preliminary report on the US Physicians Study states that supplements of β-carotene, at least when given alternately with aspirin, can reduce the CHD risk (Gaziano et al., 1990). In further agreement, in the above mentioned Basel Prospective Study the lowest quartile of lipid-standardized plasma carotene or of vitamin A showed (after adjustment for age, cholesterol and smoking) a statistically significant association with an increased coronary

mortality within the 12-year follow-up (Gey et al., 1992). Vitamin E in the Basel Study failed to reveal any correlation to subsequent CHD as had to be expected from the (already above mentioned) uniformly high vitamin E levels in this study population (mean 35 μmol/l, i.e. presumably above the critical threshold). Thus this finding may indirectly support the working hypothesis of CHD-protective potentials of a high plasma status of vitamin E.

Forthcoming intervention trials

Any CHD-Preventive potentials of essential antioxidants remain conclusively to be tested by specific supplementation in randomized subjects of high CHD risk and poor antioxidant status - in analogy to current intervention studies testing cancer-preventing potentials of antioxidants (above). The first logical step may be to test a combination of vitamins E, C and β-carotene (Steinberg 1992) - according to the principle of multirisk factor intervention in the multifactorial multistage process of arteriosclerosis.

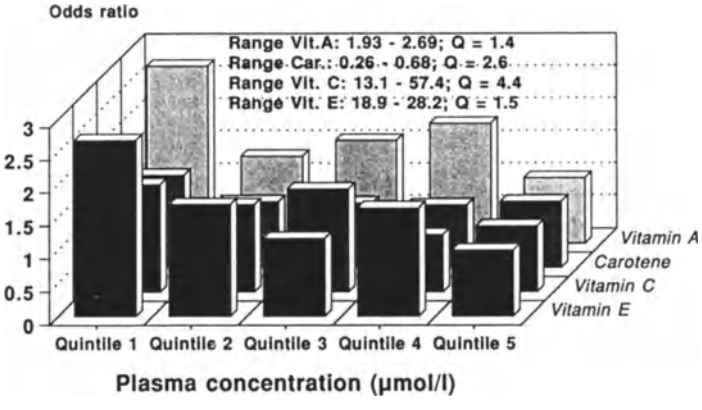


Figure 5: Relative risk (odds ratio) for angina pectoris in middle-aged males in Edinburgh/Scotland due to different plasma levels of essential antioxidants.
Quintile 1: poor status; Quintile 5: highest status, with absolute values indicated on top.
Q = quotient of levels of quintile 5/quintile 1, i.e. quotient of antioxidant variation in the study population with previously undiagnosed and yet untreated angina pectoris (Riemersma et al., 1991).

Conceivable Mechanisms of Action

Free radical oxidation of low density lipoproteins (LDL) is presumably involved in atherogenesis (Steinberg et al., 1989) although its primary role in vivo in the critical subendothelial space of arteries has not yet been proven (Steinberg 1991, 1992). According to a recent abstract isolated LDL of patients with CHD seems to be more vulnerable to oxidative modification than that of apparently healthy controls (Davies et al., 1991). In the aqueous phase of plasma the first and major line of antioxidative defense consists of vitamin C (Frei et al., 1989, Jialal et al., 1990) whereas within LDL vitamin E has been generally accepted as the principal radical scavenger. After dietary manipulation of the vitamin E level in LDL the latter is responsible for at least half the antioxidative resistance of isolated LDL (Dieber-Rotheneder et al., 1991). Thus also in vivo vitamin E-enriched LDL might be able to resist longer against oxidative modification by free radicals which can be released after transient anoxia ("reperfusion injury") as well as from activated macrophages ("respiratory burst") or from arterial cells, e.g. endothelial cells. Besides the antioxidant protection of LDL by vitamin E other beneficial effects of essential antioxidants might also counteract atherogenesis (lit. cit. Gey 1992):

- in the endothelium, e.g. inhibition of lipoxygenase(s), prevention of cellular transitions, reduction of monocyte adhesion (Hayes 1992),
- in macrophages, e.g. modulation of their migration into the subintimal space, improvement of the catabolism of modified LDL by resident macrophages resulting in diminished foam cell formation, reduced production of crucial cytokines such as interleukin-1,
- in smooth muscle cells, e.g. inhibition of proliferation and of the signal transducing protein kinase C (Azzi et al., 1992),
- in blood platelets, e.g. inhibition of adhesion, or
- by immunomodulation (Bendich 1990) .

Concluding Remarks

Essential antioxidants can under certain experimental conditions inhibit experimental carcinogenesis in animals, and vitamin A deficiency results in leukoplakia, i.e. in precancerous lesions. Arteriosclerosis-like lesions have been observed during chronic marginal deficiency of vitamins C and E in several animal species (lit. cit. Gey 1992). In the human a series of observational data (reviewed above) corroborates the working hypothesis that a suboptimal status of essential antioxidants (although with different rank orders) is involved in both carcinogenesis and atherogenesis. In consequence the presently available information seems well to justify the current and forthcoming intervention trials which intend to verify the actual preventive potentials of the various essential antioxidants. Whereas previous dietary guidelines have given general recommendations to lower dietary fat (particularly of mammalian origin) and to increase the percentage of vegetables/fruits and suitable vegetable oils (which are known to contain a series of natural antioxidants) an updated prudent diet should more specifically aim for optimal intake of β-carotene as well as of vitamins E and C in order fully to use their presumable potentials for the prevention of cancer and/or CHD. A prudent intake of vitamin A may be in the range of 1 mg daily (1 RDA), of vitamin C in the range of 60-250 mg (1-3 times the present RDA) whereas for vitamin E a markedly higher dietary intake, i.e. in the range of at least 60-100 IU daily, and for β-carotene about 15 mg may be advisable to achieve "optimum health" (Gey et al., 1987; Diplock 1989).

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

EFFECT OF DIFFERENT ANTIOXIDANTS IN EXPERIMENTAL MYOCARDIAL INFARCTION

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Summary

Changes in metabolism of lipidperoxides was observed in rats in which myocardial infarction was induced by isoproterenol. The mortality rate in isoproterenol treated animals were high (35% to 40%). Histopathological examination of the heart tissues showed extensive confluent necrosis of the myocardium with interfascicular spaces of edema. Degeneration of muscle fibers resulting in disintegration of sarcoplasm and nuclear fragmentation. Confluent lesions were observed in the apex and papillary muscles with focal lesions in other areas of the ventricle. Biochemical studies showed that there was an increase in the levels of lipidperoxide, free fatty acids, glutathione, taurine and decrease in activity of superoxide dismutase and catalase, during experimental myocardial infarction. The effect of administration of Vitamin A, Vitamin C, Vitamin E, and a fatty acid transmembrane transport molecule carnitine on the severity of myocardial infarction was also studied in rats. Judging from the mortality and histopathological studies, it was observed that the order of protection offered to the myocardium from experimental myocardial infarction is as follows - carnitine, Vitamin A, Vitamin C and Vitamin E offered the least protection.

Introduction

Myocardial ischemia is a major cause of death in humans and it has reached alarming proportion in recent years. Myocardial ischemia is the result of necrotic change taking place in the cardiac muscle resulting in decreased supply of blood to a portion of the myocardium below the critical level necessary for its viability and proper function. Under normal conditions the cardiac muscle generally utilizes fatty acids as its major source of energy. Of the total oxygen consumed, 60-90% is utilised to oxidise fatty acids under aerobic conditions (Neely and Morgan, 1974; Opie, 1969). During episodes of myocardial ischeamia, as anoxia sets in the cardiac muscle is not in a position to oxidise the available fatty acids to energy which therefore results in an increase in their levels and also long chain acyl CoA derivative (Wittmer et al., 1978). These changes in the fatty acid and its derivatives have been attributed to cause an alteration in the membrane structure and function. The increased levels of fatty acid and its derivatives may result the activation of microsomal lipid peroxidation.

Lipid peroxidation has been implicated in the pathogenesis of a number of lesions, associated with ethanol and carbon tetrachloride poisoning (Di Luzio, 1973) radiation exposure (Myers, 1973) and exposure to ozone (Goldstein et al., 1967) nitrogen dioxide and to other air pollutants (Thomas et al., 1968). Lipid peroxides have also been observed to be involved in atherosclerosis (Dormandy et al., 1973) aging (Takeuchi et al., 1976) carcinogenesis (Cheesman et al., 1986) etc. It has been postulated that lipid peroxidation may be associated with myocardial infarction (Kumari and Menon, 1987).

Number of substances have been tried for their possible protective action against the induction of myocardial infarction (MI). Since it has been postulated that lipid peroxides may be associated with MI, we studied the changes taking place in the metabolism of lipid peroxide in the myocardium in animals with experimental MI. We also studied the change in animal pretreated with antioxidants like Vitamin A, Vitamin C and Vitamin E on

experimental MI. Further since the substrate for lipid peroxidation is fatty acids we have also studied the effect of carnitine a transmembrane molecule which helps in the transport of fatty acids from the cytoplasm into mitochondria for energy production on the intensity of experimental MI.

Materials and Methods

The animals used were virgin male albino rats of Sprague Dowley strain with body weight in the range 130-170 g. The different substances were administered as shown in the table given below:

Substance	Concentration	Mode	Duration
Vitamin A	2000 IU/100g	oral (oil)	15 days
Vitamin C	150 mg/100g	oral (water)	15 days
Vitamin E	6 mg/100g	oral (oil)	1 month
Carnitine	10 mg/100g	Subcutaneous(water)	10 days

Myocardial infarction was induced by subcutaneous injection of isoproterenol (35 mg/100g) . All the chemicals used were of analytical grade and obtained from Sigma (USA). After the first injection of isoproterenol some of the surviving rats were sacrificed at time intervals of 5, 17 and 24 hr. To the remaining surviving animals a second dose of isoproterenol was administered (24 hour after first injection). The animals surviving the second injection were sacrificed at 1½, 2, 3½ and 7½ days after the first injection. This was done to study the changes taking place in MI at different stages on isoproterenol administration.

The animals administered isoproterenol exhibited signs of shock, tachycardia, dyspnea and rapid respiration.

Heart tissue was removed to ice cold containers. The extraction and estimation of malondialdehyde, (Nichans and Sammuelsson, 1968), Glutathione, (Patterson and Lazarow, 1955), Free fatty acid (Itaya, 1977) and Taurine (Parker, 1980) were carried out. Catalase (Machly and Chance, 1954), and superoxide dismutase (Kakker et al, 1984) activities were also quantified. For histopathological study the heart was fixed in 10% buffered formalin and the sections were stained with Haematoxylin and eosin.

Results

I. Effect of isoproterenol induced myocardial infarction.

Mortality rate: The rate of survival of rats given isoproterenol alone was 60-65% when compared to normal.

Fatty acids: The results are given in Fig.1. The concentration of free fatty acids registered an increase in both the heart and serum on administration of isoproterenol to rats. In the case of serum the concentration of free fatty acids increased immediately (5 hr) while in the heart the increase was observed only after 12 hrs. During the recovery phase they slowly returned to normal levels.

Lipid peroxide: On induction of myocardial infarction by isoproterenol the concentration of malondialdehyde (an index of the extent of lipid peroxidation), increased in the heart (12 hr) and serum (5 hr). on administration of the second injection of isoproterenol, the concentration increased at the peak period (11 days) and then slowly returned to normal during the recovery phase (Fig.2).

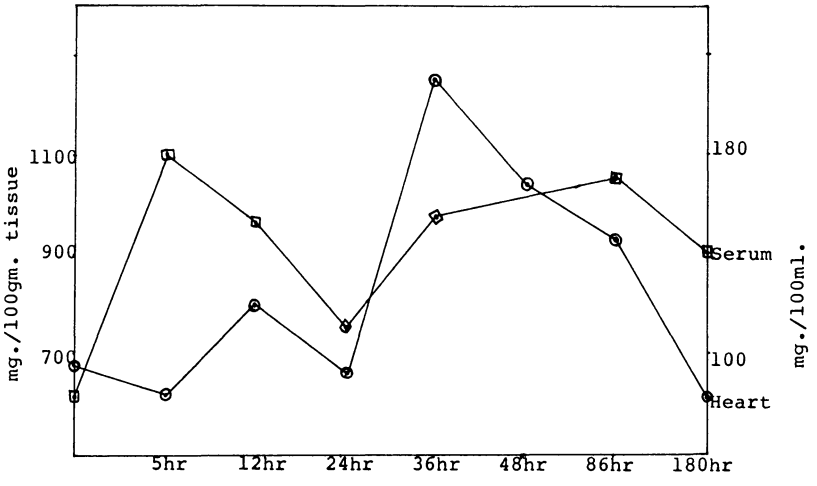

Fig.1. Level of free fatty acids in the serum and heart.

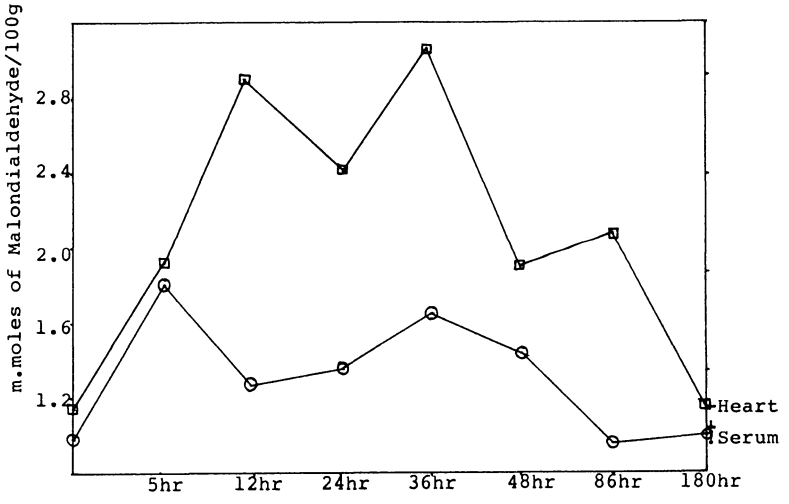

Fig. 2 Levels of lipidperoxides in the heart and serum

Superoxide dismutase and catalase: The results are given in the Fig.3 superoxide dismutase a key enzyme in scavenging the superoxide radical showed decreased activity in the heart at peak period of infarction, ie 12 hr and 1½ days after the first injection of

isoproterenol. On initiation of myocardial infarction (5 hr) the activity of superoxide dismutase increased in the heart.

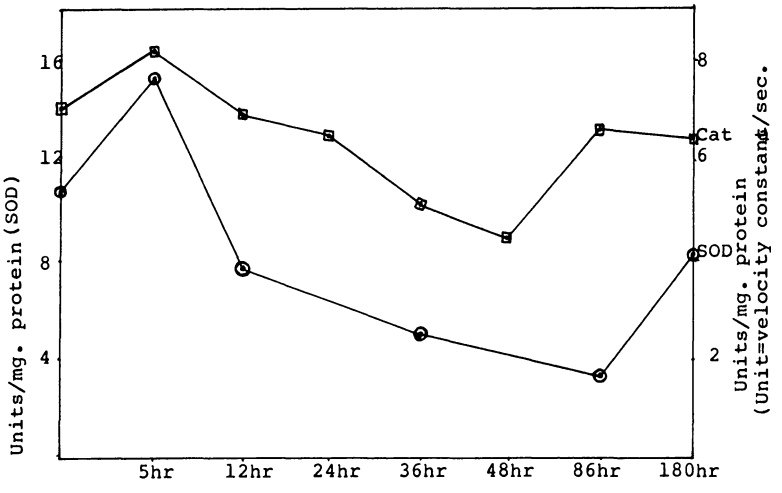

Fig.3. Activity of superoxide dismutase and catalase

Catalase, a key enzyme which helps in clearing the hydrogen peroxide formed during incomplete oxidation showed a decrease in its activity at peak period of infarction (1½ days) and slowly returned to normal during recovery phase.

Glutathione and Taurine: The levels of glutathione increased in the heart on administration of isoproterenol when compared to normal rats. The levels of taurine was significantly high at period of peak infarction (36 hr) and then decreased at levels below normal after 8 days in rats given isoproterenol (Fig.4).

Histopathy: Histopathological examination of the heart at peak period of infarction showed that rats treated with isoproterenol and extensive confluent necrosis of the myocardium at 1½ days. Interfascicular spaces of edema were observed with degeneration of muscle fibres resulting in disintegration of sarcoplasm and nuclear fragmentation. Confluent

lesions were observed in the apex and papillary muscles with focal lesions in the other cases of the ventricle. Vacuolation of the cell was also observed.(Fig.5)

II. Effect of Administration of Antioxidants on Isoproterenol Induced Myocardial Infarction

The percentage survival of rats after the second injection of isoproterenol in the groups administered Vitamin A, Vitamin E and ascorbic acid was similar to that in the animals treated with isoproterenol alone (60-65%).

Substance	Mortality
Vitamin A	40%
Vitamin C	40%
Vitamin E	40%

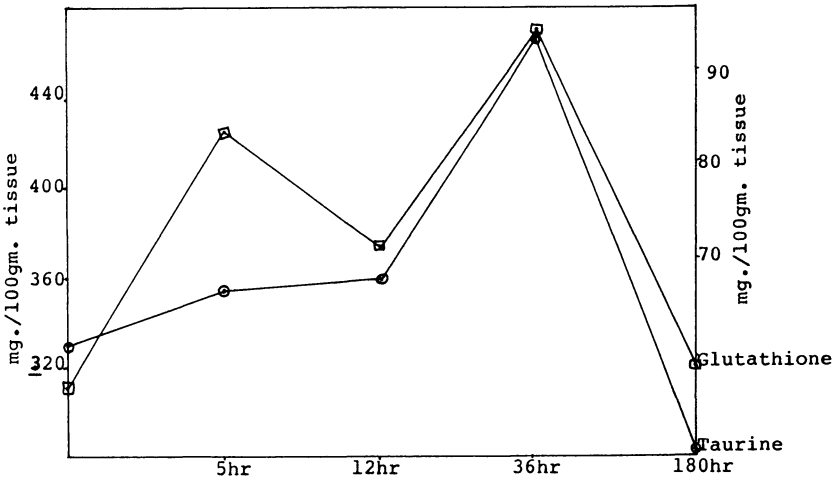

Fig. 4. Levels of glutathione and taurine in heart.

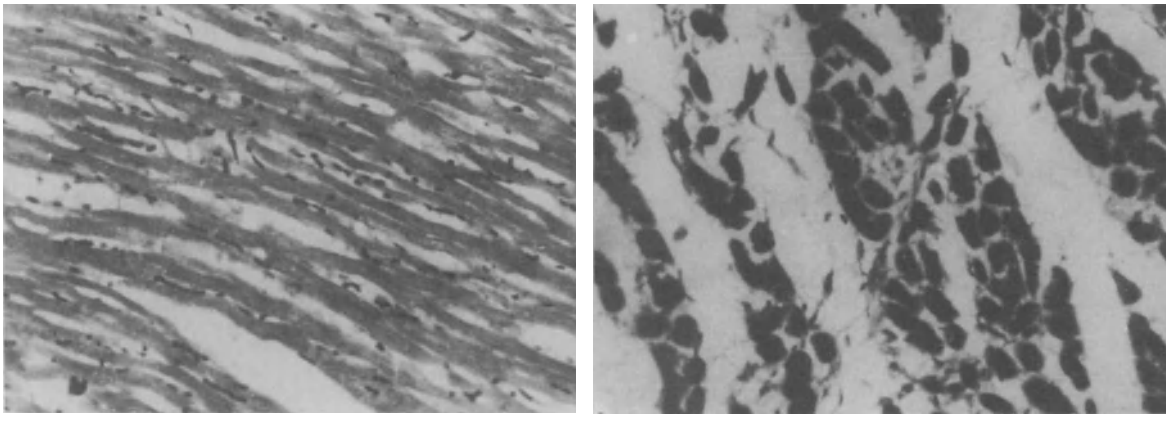

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Fig. 5. Section of heart from the apical region stained with hematoxylin and eosin (magn x 450) 1. Normal control, 2. Normal + Isoproterenol

Histopathological examination of the heart in the peak period of infarction in the animals treated with Vitamin A, Vitamin C and Vitamin E showed extensive confluent necrosis in myocardium with interfascicular species of edema and muscle fibre disintegration. Unlike focal lesions which were observed in isoproterenol treated rats, rats treated with Vitamin A, Vitamin C and Vitamin E had diffused necrosis. Among these three Vitamins the histopathological study shows the degree of necrosis as Vitamin E > Vitamin C > Vitamin A. (Fig. 6) .

III. Effect of Carnitine

The rate of survival in rats pretreated with carnitine and given isoproterenol was 80-85%. Histopathological examination of the heart in animals pretreated with carnitine before the administration of isoproterenol showed little change in the myocardium. Small areas of focal degeneration was observed which was insignificant when compared to control rats, which had extensive necrosis.(Fig.6).

Discussion

Myocardial ischemia has been found to trigger a number of pathological and biochemical events. Mortality studies show that during experimental myocardial infarction about 60-65% animals survive. Histopathological examination shows extensive necrosis of the myocardium. Biochemical studies reveal increased lipid peroxidation at peak period of infarction. Biological membranes are rich in unsaturated fatty acids which are easily susceptible to microsomal lipid peroxidative attack. This increased lipid peroxidation can result in multiple membrane damage and disturbances to cardiac metabolism, structure and function. In this context Meerson (1980) has observed that emotional and painful stress is usually accompanied by high plasma level of glucocorticoids and catecholamines resulting in membrane damage and disturbance to cardiac metabolism. Gudbjarnesan et al., (1983) postulated that the lipid peroxidation may be associated with myocardial infarction, but he failed to observe increase in levels of malondialdehyde. This may probably be due to low dose of isoproterenol which he used in the experiment.

The increased levels of lipid peroxidation is associated with an increase in concentration of free fatty acid which are the substrate for microsomal lipid peroxidation. Associated with these changes is the observation that two key enzymes superoxide dismutase (SOD) and catalase (CAT) must play important roles in scavenging toxic intermediates of incomplete oxidation decreased at peak period of infarction. A decrease in activity of these enzymes can result in disruption of cardiac membrane. It has been observed that isolated perfused rabbit intraventricular septum pretreated with SOD can withstand ischaemia (1 hour) with little structural damage when compare to control septum (Burton, 1984).

Glutathione (GSH) and Taurine increase in the heart at peak period of infarction. Increased level of taurine has been associated with hypertrophy of myocardium (Chubb and Huxtable, 1978). Taurine has also been implicated to increase the influx of calcium into cardiac cell (Dolara et al., 1978). The increased level of GSH associated with high level of lipid peroxidation suggests its decreased utilisation. GSH is an essential substrate for the enzyme glutathione peroxidase which finds wide distribution in the myocardium

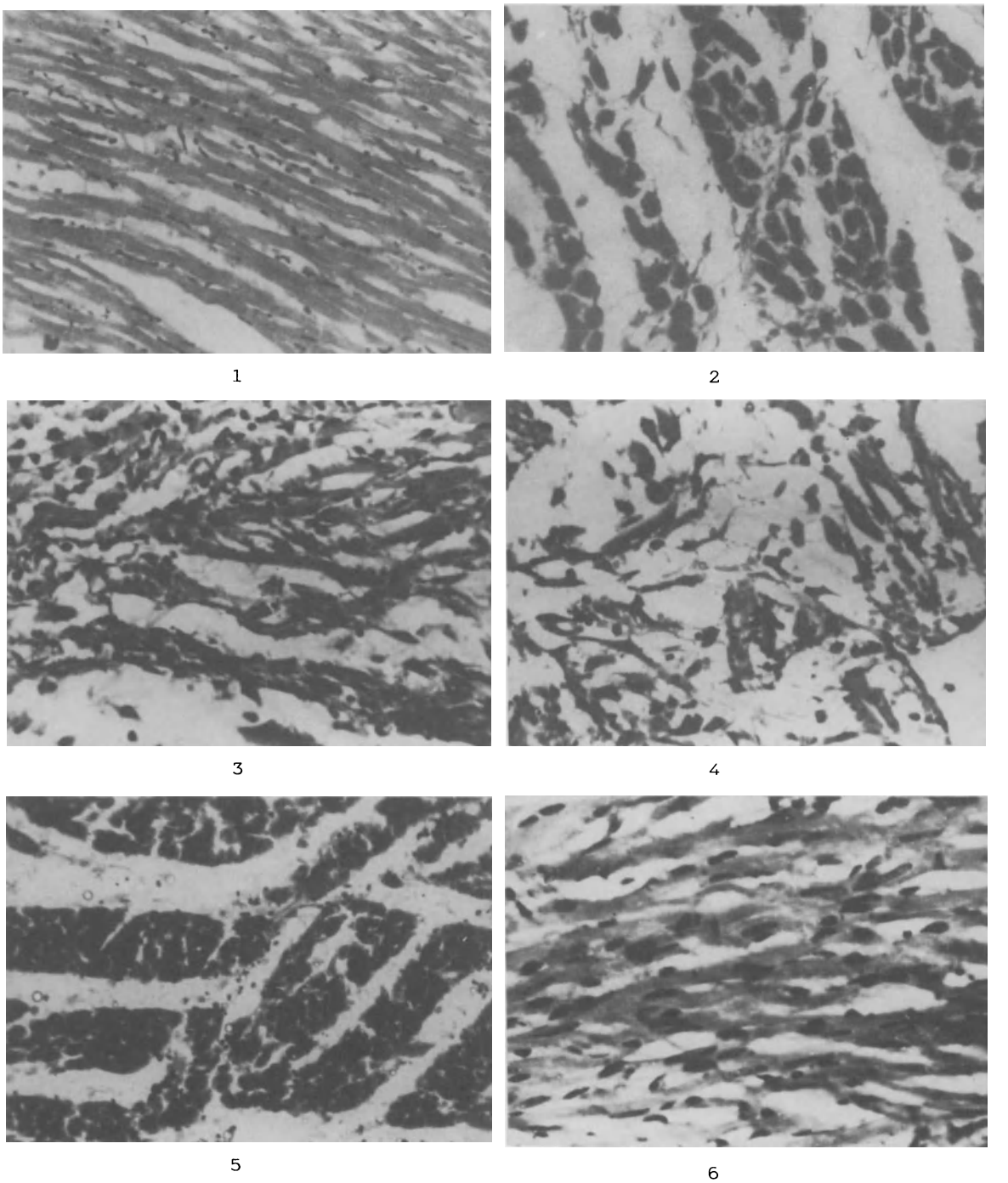

Fig. 6. Section of the heart from the apical region stained with hematoxylin and eosin (magn x 450). 1. Normal, 2. Normal + Isoproterenol, 3. Vitamin A + Isoproterenol, 4. Vitamin C + Isoproterenol, 5. Vitamin e + Isoproterenol and 6. Carnitine + Isoproterenol

(Xia et al., 1985). The increased level of GSH may therefore probably be due to the decrease in activity of glutathione peroxidase. In this context it has been observed that an addition of GSH result in high amplitude swelling of rat liver mitochondria (Lehninger and Schaneider, 1959).

Our results show that Vitamin A, Vitamin C and Vitamin E offered very little protection to experimental MI. Mortality rate in animals pretreated with these antioxidants are high, while histopathological studies show extensive area of necrosis and fibrosis in the myocardium. The increased degree of necrosis in the animals pretreated with these antioxidants may probably be due to the decreased absorption of these substances by the myocardium.

We have also seen that carnitine offers protection to the myocardium from experimental MI. This is evident from the mortality studies (80-85%) and histopathological observations wherein only a small area of focal degeneration was observed which was insignificant when compared to the control rats given isoproterenol. This decrease in the intensity of MI has been postulated to be due to i the decreased formation of lipid peroxides in the myocardium in rats pretreated with carnitine (Kumari and Menon, 1988). This is achieved by the administered carnitine which helps in transporting the fatty acids into the mitochondria for oxidation by the available oxygen. This results in its oxygen availability by the microsomal system for the formation of lipid peroxides.

Conclusion

Thus the study shows that during myocardial ischemia the pathological changes are closely associated with biochemical alterations. Increased levels of lipid peroxidation and taurine causes increased necrotic changes in the myocardium. Antioxidants like Vitamin A, Vitamin C and Vitamin E offer very little protection to experimental MI while a fatty acid transmembrane transport molecule-carnitine offers very good protection to the myocardium from experimental myocardial infarction.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

LIPID-SOLUBLE PLANT PHENOLS AS ANTIOXIDANTS AND ANTI-MUTAGENS

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Summary

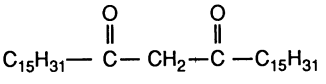
Lipid-soluble lignens were isolated from sesame seeds as the new type of lipid-soluble plant phenols, in particular sesaminol was identified as the main antioxidative component. Sesaminol is the effective antioxidant when evaluated by both in vitro and *in vivo* systems, and also shown to have a strong inhibitory effect on mutagenicity induced by indirect mutagens such as heterocyclic amines. By an approach to obtain a novel type of antioxidative compound, tetrahydrocurcumin, strong antioxidant which has both phenolic hydroxy and β-diketone groups in the same structure, was formed from hydrogenation of curcumin.

Introduction

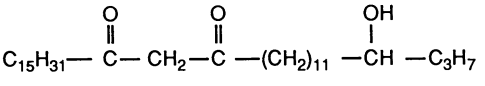
Recently, an intensive search for novel type of antioxidants has been carried out from numerous plant materials, including those used as foods. Our objective was based upon the hypothesis that endogenous antioxidants in plants must play an important role in antioxidative defence systems against oxidative damages (Osawa et al., 1990). From this background, we have isolated and identified a number of lipid-soluble plant antioxidants such as β-diketones (n-tritriacontane-16,18-dione and 4-hydroxy-tritriacontane-16,18-dione) in the *Eucalyptus* leaf wax (Osawa & Namiki, 1981 & 1985), and

novel tocopherol conjugates, prunusols A and B, in the *Prunus* leaf wax as shown in Fig. 1 (Osawa et al., 1991). Sesame seeds are also good source of lipid-soluble lignans (Fukuda et al., 1986a), which have a strong antioxidative activity in vitro as well as *in vivo* systems (Osawa, 1991).

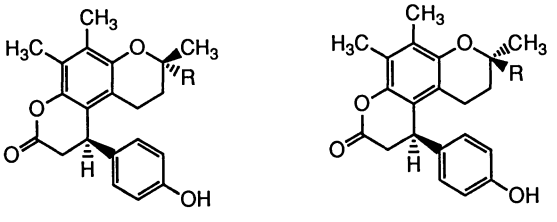
This background prompted us to investigate whether these lipid-soluble plant antioxidants are effective in protection from oxidative damages during lipid peroxidation induced by the free-radical chain reaction of cell membrane
lipids and also act as an effective inhibitor of mutagenicity induced by the food related mutagens such as protein pyrolysates.



***n* - tritriacontane -16,18- dione**



4-hydroxytritriacontane-16,18-dione



R:$(CH_2)_3CH$-$(CH_3)(CH_2)_3CH$-$(CH_3)(CH_2)_3CH$-(CH_3)

prunusol A **prunusol B**

Fig. 1 Structures of lipid-soluble antioxidants isolated from plant leaf wax.

Lignin Type Antioxidants in Sesame Seeds

Sesame seeds are very common, in particular sesame oils are widely used in Chinese and Japanese dishes, and are evaluated as being highly antioxidative. However, the quantity of the known antioxidants, sesamol and γ-tocopherol, was not sufficient to exhibit the strong antioxidative activity in sesame oils (Fukuda et al., 1986a). This prompted us to investigate other antioxidative components in the sesame seeds, because we speculated that antioxidative components in sesame seeds must be important for antioxidative defenses, and we have made a large-scale isolation and identification of antioxidative components in sesame seeds. As shown in Fig. 2, two novel lignin type antioxidants, sesamolinol (Osawa et al.,1985) and sesaminol (Nagata et al., 1987), were

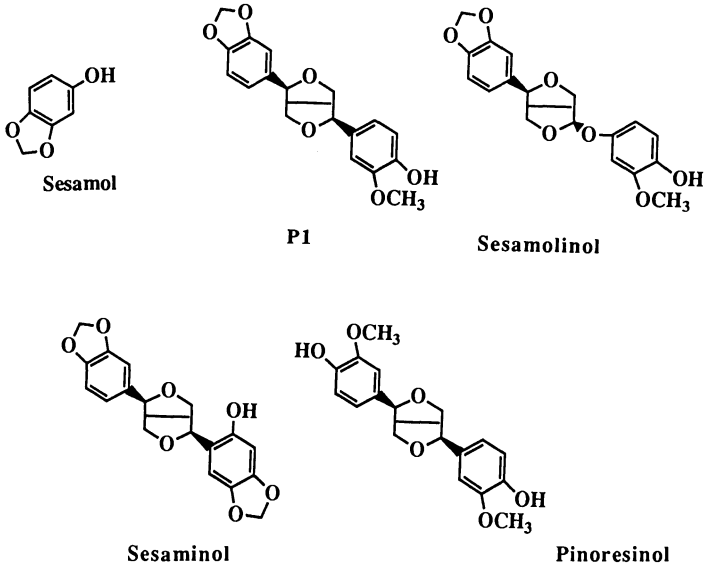


Fig. 2 Structures of lipid-soluble lignans isolated from sesame seeds.

found to be present together with two other lipid-soluble lignin derivatives, Pl and pinoresinol (Fukuda et al., 1986a).

Usually, sesame oils are manufactured in two different ways, as roasted oil and salad oil (unroasted sesame oil). Salad oil is extracted by an expeller and refined by alkaline treatment, water washing, bleaching with acid clay, and a deodorizing process. By quantification using HPLC, the content of sesaminol was found to be dramatically increased during the manufacturing process, in particular, the bleaching process (Fukuda et al., 1986b).

These results suggested that sesaminol has been chemically transformed during the bleaching process, and it was found that sesaminol has been produced by intermolecular transformation from sesamolin (Fig. 3) . When the amounts of sesaminol were quantified by HPLC in commercially available sesame oils, the total amounts of sesaminol isomers were about four times that of , γ-tocopherol in most commercially available sesame seed oils. Therefore, it is concluded that sesaminol is the main antioxidative component present in sesame oils.

Antioxidant Activity of Sesaminol

We have evaluated the antioxidative activity, using several *in vitro* lipid peroxidation systems, in particular the rabbit erythrocyte ghost and rat microsome systems. t-butylhydroperoxide was used to induce autoxidation of membrane lipids of erythrocyte ghost. However, enzymatic lipid peroxidation of rat microsome was induced by ADP-Fe^{+++}/NADPH and ADP-Fe^{+++}/NADPH/EDTA-Fe^{+++}.

All lignans effectively inhibited lipid peroxidation induced in the rat liver microsomes and the erythrocyte ghost membranes (Table I) . Although other lignin-type components also have comparable antioxidant activity, the role of sesaminol is very important because sesaminol is the main antioxidative component in sesame oil and has the superior heat stability.

The protective role of sesaminol against oxidative damage has also been reported using cultured human diploid fibro-blasts at various *in vitro* age. The constitutive level

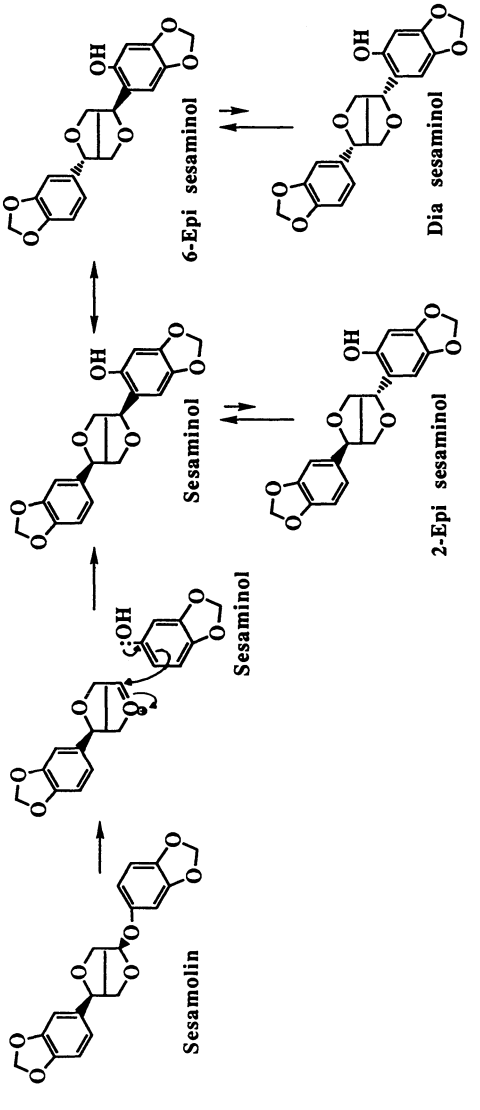


Fig. 3 Scheme for the mechanism of formation of sesaminol from sesamolin.

Table I.

Antioxidant activity of sesame lignans using rat liver microsome and erythrocyte ghost systems.

	ADP/NADPH/Fe	ADP-Fe3+/EDTA-Fe3+/NADPH	Erythrocyte
Control	100	100	100
Pl	14.9	13.2	20.2
Sesamolinol	4.6	6.3	24.7
Sesaminol	8.6	10.3	22.8
Pinoresinol	17.2	14.4	18.4
Sesamol	24.1	19.0	19.0
α-Tocopherol	9.2	19.0	24.7

of lipid peroxidation was higher in cells at late passages (75% of lifespan) than those at early passages (26% of life span) . Lipid peroxidation in response to the addition of t-butylhydroperoxide was more marked in cells at late passages; however, the presence of sesaminol effectively reduced peroxidation stimuli by a peroxidation initiator (Shima, 1988).

Recently, Yamashita et al., (1990) reported that sesaminol inhibited the oxidative damage caused by carbon tetrachloride using SAM (senescent accelerated mouse) mice as the *in vivo* system, although the long term feeding experiment of sesame seeds has been carried out.

Inhibitory Effects of Antioxidative Components on Mutagenesis

There are many indications that lipid peroxidation play an important role in carcinogenesis, although there is no definite evidence. However, the peroxidative breakdown of the membrane polyunsaturated fatty acids is known to produce a complex mixture of many mutagenic secondary products (Marnett et al., 1985) such as malondialdehyde (Basu et al., 1984), 2-hexenal, 4-hydroxypentenal, 2,4-hexandienal and 4-hydroxynonenal (Cajelli et al., 1988). Recently, the authors observed that mutagenic lipid peroxidation products can be formed in the erythrocyte ghost membranes although the structure of mutagenic product was not identified. Because oxygen radical scavengers such as superoxide dismutase, catalase, and mannitol were not effective, it was supposed that oxygen radical species such as superoxide anion, hydroxy radical and hydrogen peroxide are assumed not to induce both lipid peroxidation and mutagenicity. Both lipid-soluble antioxidants such as α-tocopherol and sesaminol inhibited lipid peroxidation and mutagenicity effectively (Osawa et al., 1990) By detailed analysis, it was shown that sesaminol and tocopherol inhibited the formation of mutagenic lipid peroxidation products, therefore, they showed strong inhibition of mutagenicity induced during the lipid peroxidation of erythrocyte membranes. However, they had no inhibitory effects against the mutagenicity of t-butylhydroperoxide.

On the other hand, thermal processing and cooking of protein foods are important methods to produce safe and desirable food products, but it can also form mutagens, in particular protein pyrolysates in cooked foods (Sugimura et al., 1983). However, these heat-induced mutagens can be inactivated by the modulators in the diets (Miller, 1985). This background prompted us to investigate the role of sesaminol, which has characteristic heat stable property, in the inactivation of heat-induced mutagens. Sesaminol inhibited effectively on mutagenicity of these heterocyclic amines such as Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, TQ, MeIQ and MeIQx (unpublished data). As shown in fig. 4, sesaminol strongly inhibited the mutagenicity of Trp-P-2 although α-tocopherol did not show any inhibition of mutagenicity induced by Trp-P-2. Absence of antimutagenicity in

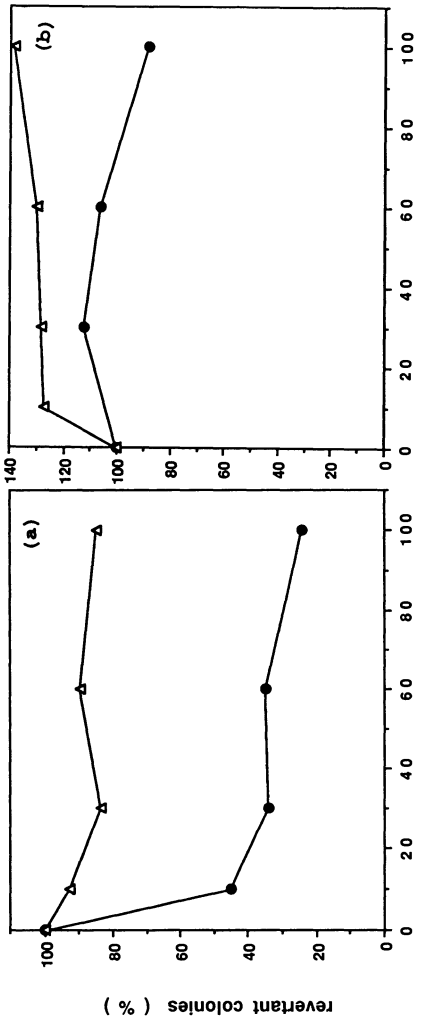


Fig. 4 Inhibitory effects of sesaminol and α-tocopherol on mutagenicity induced by Trp-P-2 and activated form of Trp-P-2 (N-hydroxy-Trp-P-2) using *S.typhymurium* TA98. (a) Trp-P-2 with S9. (b) N-hydroxy-Trp-P-2 without S9.

Δ: α-tocopherol, ●: sesaminol

α-tocopherol toward indirect mutagens suggests that antimutagenicity of sesaminol is due to a mechanism of action other than radical scavenging (Osawa et al., 1991).

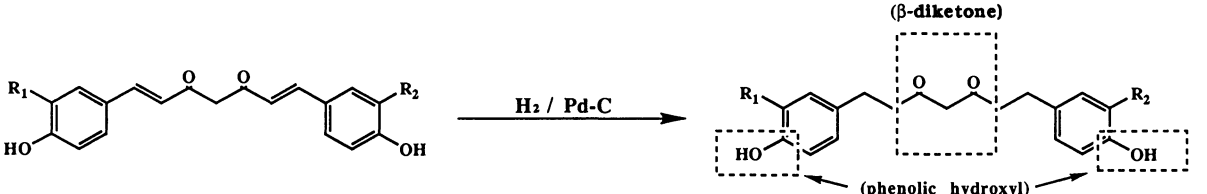
However, sesaminol did not inhibit the mutagenicity induced by activated form of Trp-P-2 (N-hydroxy-Trp-P-2). This result supports the conclusion that sesaminol inhibits the metabolic activation step from Trp-P-2 to N-hydroxy-Trp-P-2 by hepatic microsomal oxidation systems-in particular, P-448 cytochrome species-which is also a common activation step for other heterocyclic amines although specificity of the inhibitory effect of sesaminol on metabolic activation is not known (Saito et al., 1983).

Approach to Obtain a Novel Type of Lipid-Soluble Antioxidants

In the course of our investigation to find a novel type of antioxidative substances in the plant materials, most natural lipid-soluble antioxidants can be classified into two different types: phenolic and β-diketone type antioxidants. However, there have not been found the antioxidative substances which have both functional groups; phenolic and β-diketone groups in the same molecule. This background prompted us to develop a new type of antioxidative compound which has both phenolic and β-diketone moiety in the same molecule.

Curcumin (Ul), main yellow pigments of *Curcuma longa* (turmeric), was extracted with diethyl ether together with two minor yellow pigments, U2 and U3. Ul has been used widely and for a long time in the treatment of sprain and inflammation in indigenous medicine, however, there is some limitation to utilize Ul for food and medicinal purposes because of its yellow color. Recently, we succeeded in obtaining a strong lipid-soluble antioxidant, tetrahydrocurcumin (TeHUl), by hydrogenation using Pd-C (or Raney-nickel) as the catalyst as shown in Fig. 5 (Osawa et al., 1991). U2 and U3 are also converted to TeHU2 and TeHU3, respectively, by hydrogenation.

Antioxidative activity of curcuminoids was evaluated by *in* vitro system using rabbit erythrocyte ghost membrane by determining TBARS formation after induction of lipid peroxidation by t-butylhydroperoxide (Fig. 6). TeHU1 showed the strongest antioxidative



U1; $R_1=R_2=OCH_3$

U2; $R_1=OCH_3$, $R_2=H$

U3; $R_1=R_2=H$

TeHU1; $R_1=R_2=OCH_3$

TeHU2; $R_1=OCH_3$, $R_2=H$

TeHU3; $R_1=R_2=H$

Fig. 5 Scheme for hydrogenation of curcuminoids to tetrahydro curcuminoids.

and other two tetrahydro curcuminoids (TeHU2 and TeHU3) exhibited much more activity than original curcuminoids (U2 and U3)

Ul was reported to inhibit the microsome-mediated mutagenicity of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (Nagabhushan et al., 1987), and it was also reported that curcumin acts as a strong inhibitor of tumor promotion, and this effect can be explained to roughly parallel the relative antioxidant activity (Huang et al., 1988). More recently, Hirose et al., (1991) reported that n-tritriacontan-16,18-dione inhibited hepatic and pancreatic carcinogenesis effectively. Therefore, it is expected that the novel type of antioxidants which have two functional groups, tetrahydro-curcuminoids, may have antimutagenicity as well as anti-tumor promoter activity although the detailed experiment is now in progress.

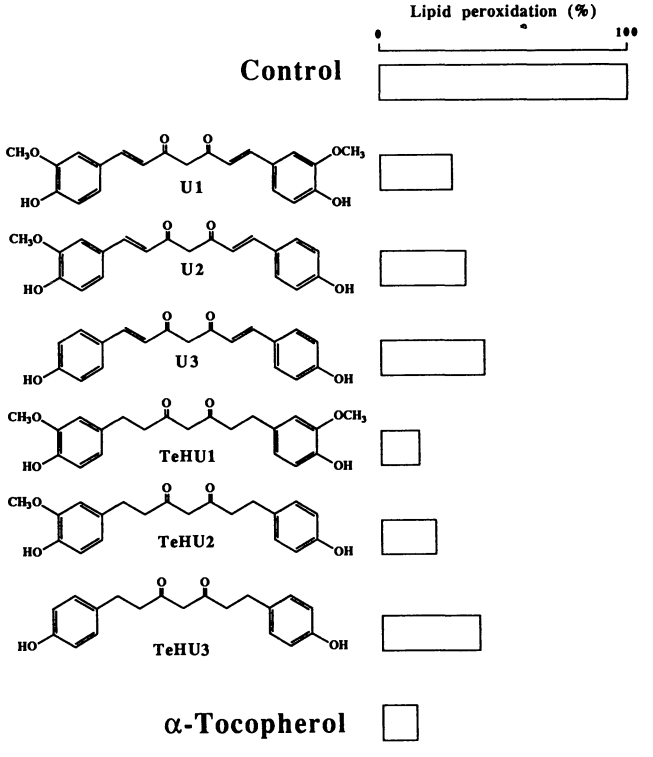


Fig. 6 Antioxidative activity of curcuminoids derivatives using rabbit erythrocyte ghost system.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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ANTI-CANCER EFFECTS OF CIS-UNSATURATED FATTY ACIDS BOTH IN VITRO AND IN VIVO

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Summary

Cis-unsaturated fatty acids (c-UFAs) such as gamma-linolenic acid (GLA), arachidonic acid (AA) and eicosapentenoic acid (EPA) were found to be toxic to human breast tumor cells (ZR-75-1) but not to normal human fibroblasts (41-SK) in vitro. This selective tumoricidal action of GLA, AA and EPA was blocked by anti-oxidants but not by prostaglandin and leukotriene inhibitors suggesting a role of free radicals and lipid peroxidation. On the other hand, LA (linoleic acid), GLA, dihomo GLA (DGLA), EPA and docosahexaenoic acid (DHA) showed cytotoxicity towards human cervical carcinoma cells (HeLa) in vitro. The fatty acid-induced cytotoxic action of HeLa cells could be blocked by antioxidants, calmodulin antagonists, and cyclo- and lipoxygenase inhibitors. These results are in contrast to those observed with ZR-75-1 cells, indicates that though, free radicals are the mediators of the tumoricidal action of fatty acids, the mechanism of their production may be different in different types of tumor cells. In addition intra-tumoral GLA therapy induced substantial decrease in the size of the human gliomas as evidenced by serial CT scan examination. Further, both in vitro and in vivo studies showed that GLA and DGLA can prevent genetic damage induced by radiation and chemicals. These results suggest that specific fatty acids have anti-cancer actions.

Introduction

It is now believed that loss of immune surveillance, chromosomal aberrations -leading to oncogene(s) activation and the consequent cellular events predispose to the development of cancer. On the other hand, cancer therapy aims to achieve selective

elimination of tumor calls. Currently available anti-cancer therapeutic modalities have several side-effects. If efforts are made to identify chemicals either natural or synthetic which have selective tumoricidal action and at the same time posses anti-mutagenic action then their use may form a new approach in the management of cancer. Cis-unsaturated fatty acids, which form precursors to eicosanoids, such as gamma-linolenic acid (GLA, 18:3 n-6), dihomo-gamma-linolenic acid (DGLA, 20:3,n-6), arachidonic acid (AA, 20:4, n-6), eicosapentanoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) seem to have some of these properties, Here we report the effect of various cis-unsaturated fatty acids (c-UPAs) on the survival of normal and tumor cells in vitro and the mechanism of their tumoricidal action. In addition, we have also studied the effect of c-UFAs on the mutagenicity of benzo(a)pyrenes (BP), gamma-radiation and cis-platinum. Some of these studies have already, been published (Begin et al., 1985; Begin et al., 1986a and 1986b; Das et al., 1987a, 1987b, 1986a, 1986b, 1985a, 1985b, 1987c, 1989; Das, 1989a; Das, 1991) and here I have presented an over review of these studies.

Materials and Methods

Cells and Culture Conditions

Normal monkey kidney (CV-1), normal human fibroblasts (CCD-41-sk), human prolmyelocytic leukemia (HL60), human breast cancer (ZR-75-1), human cervical carcinoma (HeLa) cells were used for these studies. The cells were grown either in bicarbonate buffered Dulbecco's modified Eagle's medium or RPMI medium containing 10% heat inactivated human AB serum or foetal calf serum, streptomycin and penicillin. The cells were seeded at 5 x 10^4 or 1 x 10^4 cells per plate or well respectively depending on the experimental protocol and as described earlier (Das, 1991; Das et al., 1987a; Das, 1990). In all the experiments, cell viability was tested by trypan blue dye exclusion method. The cells were checked for possible mycoplasma contamination and confirmed that there was no contamination during the period of the study. The cells were grown in 0.5 ml or 2.0 ml, in 24 well tissue culture plates or petri dishes respectively of medium with or without the added fatty acids at 37°C in 5% CO_2 humidified incubator as

described earlier (Begin et al., 1985; Das, 1991). The fatty acid ester(s) were initially dissolved in 95% ethanol and the final concentration of ethanol was not more than 0.2% in all control and fatty acid supplemented cultures.

Chemicals

All the fatty acids, culture medium, and other fine chemicals used in the study were obtained from Sigma Chemical CO., USA.

Studies with Antioxidants, Metals and Calmodulin Antagonists

These studies were performed in 24- well tissue culture plates where in the cells were seeded at 1 x 10^4 cells/well/ml. One day after seeding, 10, 20 or 40 μg/ml of GLA was added to the cells depending on the experimental protocol and the type of cell line used. To study the effect of possible enhancers or inhibitors of tumor cell death by GLA, the following chemicals were added simultaneously to the cultures with GLA: vitamin A, vitamin E, uric acid, superoxide dismutase, glutathione peroxidase, catalase, heat-inactivated catalase, $FeCl_2$, $FeCl_3$ (ferrous and ferric chloride respectively) and copper sulfate ($CuSO_4$). All these chemicals were obtained from Sigma Chemical Co., USA and were of the highest grade of purity available. For studies performed with ZR-75-1 (human breast cancer) and 41-SK (human fibroblasts) cells, the cells were treated with 10 or 20 μg/ml of GLA depending on the type of experiment where as when experiments were done with HeLa cells 40 μg/ml of GLA was used. These doses were arrived at after performing dose-response and time-course studies. These preliminary results suggest that different cell types differ in their sensitivity to various fatty acids.

Appropriate controls without fatty acids were also performed. The cultures were observed every day and cell viability was determined every day until day 7 of supplementation of GLA and the various additives. In the case of HeLa cells the experiments were performed till day 3.

NBT Reduction

The superoxide anion can reduce nitroblue tetrazolium (NBT) to the insoluble formazan. It is generally accepted as a reliable and simple method of assaying superoxide anion and possibly, other free radicals (Das et al., 1987a; Das et al., 1987b). The ability to reduce NBT was assayed by incubating HeLa cells (with/without fatty acid treatment) for 2 hours with 0.1% NBT dissolved in the PBS at the end of 24, 48 and 72 hours. Termination of the assay was done by adding 0.6 ml of glacial acetic acid into which the reduced NBT dye was extracted and the extract was read at 560 nm. This NBT reduction assay in our system was found to be SOD inhibitable suggesting that we were measuring superoxide anion.

TBA Reaction

For these studies, cell cultures seeded with 5 x 10^4 cells/petri dish (35 mm) were used. ZR-75-1, CV-1 and HeLa cells were used in this study. Cell cultures were supplemented with GLA, AA, EPA and DHA in the case of ZR-75-1 and CV-1 cells and with GLA only in the case of HeLa cells. The cells were grown in 2.0 ml medium with or without added fatty acids at 37°C in a 5% CO_2 humidified incubator. Similar to the experiments with antioxidants and metals, one day after seeding, the cells were supplemented with various fatty acids. At the end of 7 days, medium and cells were harvested separately, and assayed for TBA reaction as a measure of lipid peroxidation. TBA reaction was performed at the end of 7 days in the case-of ZR-75-1 and CV-1 cells whereas in experiments performed with HeLa cells the reaction was done on day 1, 2 and 3. The amount of lipid peroxides (TBA reaction) formed in ZR-75-1 and CV-1 cells was performed in the presence of GLA, AA, EPA and DHA whereas with HeLa cells the assay was done only with GLA. TBA reaction was performed as described earlier (Gavino et al., 1981; Sangeetha et al., 1990). The absorbance of the reaction was measured at 532 nm with growth medium or PBS as the controls. The absorbance values were converted to pmoles of MDA-equivalent (MDA-eq) from a standard curve obtained with 1,1,3,3 -tetramethoxy propane.

Thymidine Incorporation Studies

The effect of various concentrations of different fatty acids and its modulation by cyclo-oxygenase (CO) and lipoxygenase (LO) inhibitors and calmodulin antagonists on the growth of HeLa cells was assessed by their ability to incorporate radio-labelled thymidine as a measure of DNA synthesis. Labelled thymidine (0.5 μCi, specific activity 18500 mCi/m mole) was added 6 hours before harvesting the cells. At the end of the incubation period, cells were washed at least thrice in PBS (pH 7.4), trypsinized, extracted for DNA and counted in a liquid scintillation counter on day 1, 2 and 3 to assess DNA synthesis.

Experiments with Labelled Thymidine Cells

For these studies, CV-1, CCD-41-SK, HL-60, and ZR-75-1 cells were used. The cells were seeded at 1 x 10^4/ml in 24-well culture plates. 24 hours after seeding, cells were labelled with thymidine and then were supplemented with 20 μg/ml GLA for 24 hours. At the end of the incubation period, the supernatant was collected and counted in a liquid scintillation counter to measure the amount of labelled thymidine released by the cells as a measure of damage to DNA.

Studies on Genetic Damage

Swiss male mice aged 7-8 weeks and weighing 24-27 gm were used for the study. One hour before and after giving the genotoxic agents such as gamma-radiation, benzo-(a)pyrene (BP), or cis-platinum, GLA or other fatty acids was given intraperitoneally in saline. 5 to 6 animals were used in each group. All the animals were killed 24 to 36 hours after the treatment schedule. The bone marrow smears were prepared, stained with May-Grunwald-Geimsa and mounted in Euparol as described earlier (Das et al., 1985a). The presence of micronuclei in polychromatic erythrocytes (PCE) is an indication of genetic damage (Das et al., 1985a). The ratio of polychromatic to normochromatic erythrocytes (P/N) was used to estimate the effect of the treatments on the bone marrow cells of mice. P/N ratio of 1 is normal. An increase in the number of normochromatic erythrocytes is considered as an indication of bone marrow suppression.

Clinical Studies

Gliomas

Patients with malignant gliomas or who had relapse after taking radiotherapy, surgery and chemotherapy were selected for the study. After obtaining the consent of the patients, they received 1 mg of GLA intra-tumorally every day for 10 days. Computerised axial tomography scans (CT scans) of the brain were done both before and treatment with GLA. Response to treatment was scored as $<40\%$ reduction, 40 to 60% reduction or $>60\%$ reduction in the tumor size arbitrarily.

Results

Fatty Acids and Tumor Cells: In Vitro Studies

The effect of 20 μg/ml of various fatty acids on the survival of human breast (ZR-75-1), normal monkey kidney (CV-1), and normal human fibroblast (CCD-41-SX) cells are given in figure 1. It is clear from these results that GLA, AA and EPA have tumoricidal action, whereas DHA is without any significant action. Thus, GLA = AA > EPA > DHA in terms of their cytotoxic action on tumor cells. Both LA and ALA (linoleic acid, 18:2, n-6 and alpha-linolenic acid, 18:3, n-3 respectively) were also found to have cytotoxic action on ZR-75-1 cells (data not shown). Lymphoma, leukemia, and breast tumor (ZR-75-1) cells were killed by almost all the c-UFAs (LA, ALA, GLA, AA, DGLA, EPA) except by DHA, where as other cell lines such as colon cancer cells were found to be sensitive only to GLA, AA and EPA (data not shown). Generally, the extent of cytotoxicity by c-UFAs was found to be influenced by the cell number, cell density (whether calls were confluent or not) the type of cell line and fatty acid tested (Begin et al., 1985 and Begin et al 1986a, Das, 1990). It is also clear from these results that the maximum tumoricidal action of c-UPAs is seen by day 6 or day 7 for ZR-75-1 cells (fig.1).

The results shown in fig.2 suggest that both AA and EPA but not GLA are toxic to normal cells (CCD-41-SK) at doses 2 to 3 times the dose effective in killing the tumor cells. This is especially so when normal human fibroblasts (41-SK) were supplemented with 60 μg/mi of AA or EPA. This indicates that GLA has more selective tumoricidal action compared to AA and EPA when CCD-41-SK and ZR-75-1 cells are compared in their response to the fatty acids. Thus, GLA > AA > EPA when their capacity to kill tumor cells selectively and least cytotoxicity to normal cells are taken into account.

On the other hand, the results shown in fig.3 and 4 reveal that various fatty acids can kill HeLa cells (human cervical carcinoma) in vitro in a dose dependent manner. The most effective fatty acids in order of their potency is as follows: DHA > EPA > GLA = DGLA > LA > AA > ALA. In contrast to the results shown in fig.1, where GLA, AA and EPA were the most effective cytotoxic agents on ZR-75-1 calls, DHA and EPA were found to be the most potent cytotoxic fatty acids on HeLa cells (fig 3 and 4). Similarly, EPA was the most effective cytotoxic fatty acid to colon carcinoma cells compared to AA and GLA (data not shown). These results suggest that the response of different types of tumor cells to various fatty acids are variable. Though all c-UFAs seem to possess tumoricidal actions, their potency and effectiveness may vary depending on the type and nature of the tumor cell line under investigation.

Effect of Antioxidants and Metals on the Cytotoxic Action of GLA

The effect of various inhibitors or augmenters of the tumoricidal action of GLA on human breast tumor (ZR-75-1) cells are given in table 1 and 2. For testing the action of antioxidants, a concentration of 20 μg/ml of GLA was used since, earlier studies and the results shown in fig 1 and 2 showed that this is the optimum concentration to produce maximum cytotoxicity to tumor cells with least cytotoxicity to normal cells (Begin at al, 1985; Begin et al., 1986a; Das, 1991; Das 1990a). On the other hand to test the action of possible enhancers of cytotoxicity a sub-optimal dose of GLA, which is 10 μg/ml was used. It is clear from the results shown in table 1, that anti-oxidants and superoxide anion quencher, superoxide dismutase (SOD), can effectively block the tumoricidal action of

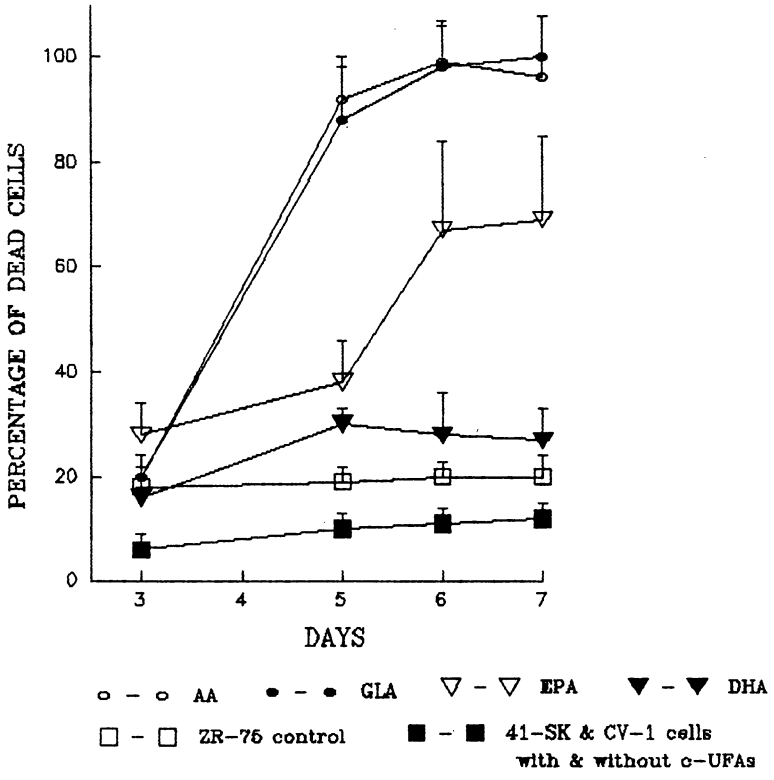


Fig. 1. Effect of 20 μg/ml of c-UFCs on the survival of ZR-75-1, CV-1 and CCD-41 SK cells in vitro

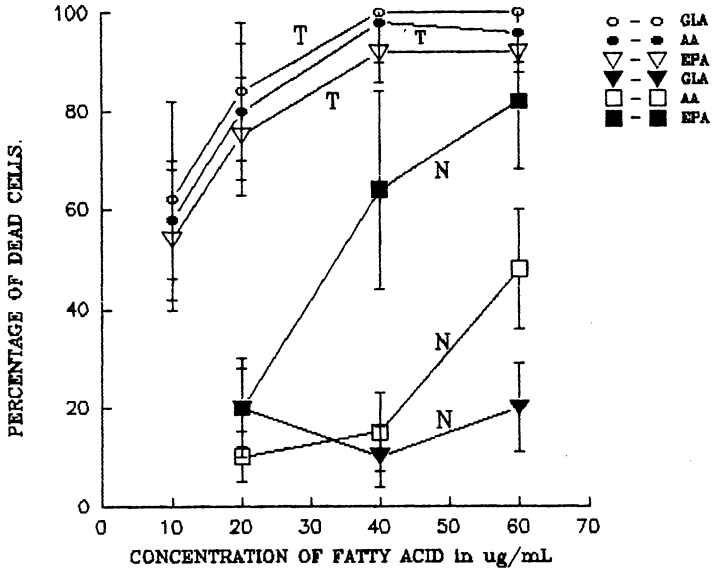


Fig. 2. Effect of GLA, AA and EPA on the survival of normal (41-SK) and tumor cells (ZR-75-1) in vitro.

T = Tumor cells ZR-75-1 = Human breast tumor cells.
N = Normal cells 41-SK = Human noormal fibroblast.

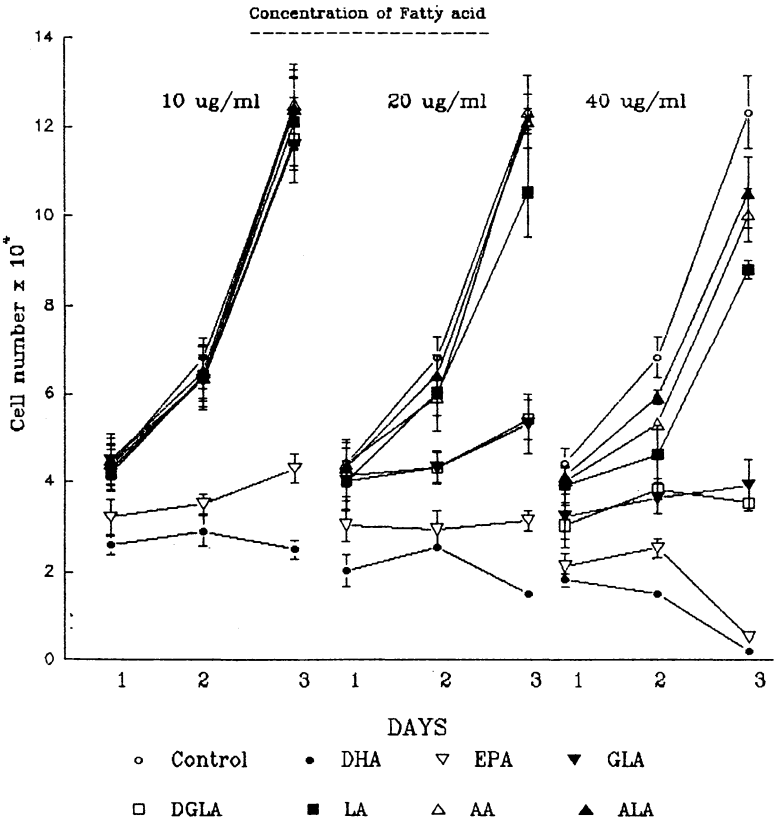


Fig. 3. Effect of various PUFAs on survival of HeLa cell in vitro.

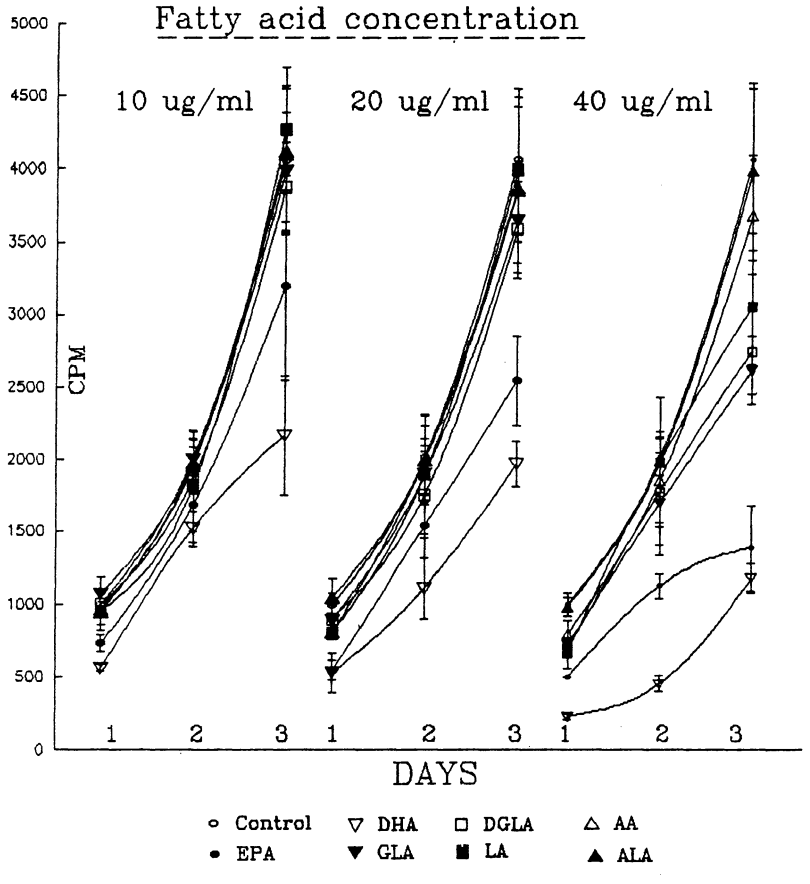
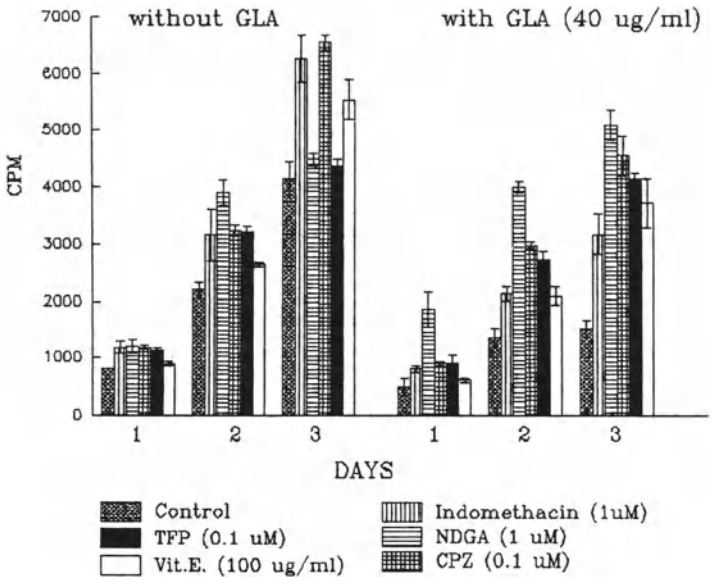


Fig. 4. Effect of various PUFAs on growth of HeLa cells in vitro.



*P < 0.05 compared to GLA control with all the inhibitors

Fig. 5. Effect of various inhibitors on the GLA induced cytoxicity in HeLa cells.

GLA. Similar results were obtained with AA and EPA (data not shown). The inhibitory action shown by SOD suggests that possibly, superoxide radical has a role in the tumoricidal action of GLA and other fatty acids. Both vitamin A and E, known anti-oxidants and inhibitors of lipid peroxidation, could also block the cytotoxic action of GLA. Similarly, uric acid is an anti-oxidant.

Both catalase and heat-inactivated catalase enhanced the cytotoxic action of GLA at the doses tested as shown in table-2. This suggests that hydrogen peroxide radical does not participate the tumoricidal action of c-UPAs. Both $FeCl_2$ (when used at 20 and 40 μg/ml) enhanced the cytotoxic action of GLA. Since these metals are known to augment lipid peroxidation process and free radical generation (especially that of superoxide anion, Halliwell and Gutterridge, 1986) their enhancing action further supports the concept that free radicals and lipid peroxidation process participate in the cytotoxic action of c-UPAs. Catalase is rich in copper and this may explain why heat-inactivated catalase enhanced the cytotoxic action of GLA on ZR-75-1 cells in vitro (table 2). Similar studies were also performed with HeLa calls. Results shown in fig.5 demonstrate that indomethacin, a cyclo-oxygenase, nordihydrocguaretic acid (NDGA) a lipoxygenase inhibitor and vitamin E an anti-oxidant can completely block the cytotoxic action of GLA on HeLa cells in vitro. In addition, it was also observed that both TFP (trifluoperazine) and CPZ (chlorpromazine) known calmodulin antagonists, can also prevent the cytotoxic action of GLA on HeLa cells (fig. 5). To verify whether prostaglandins (PGs) themselves are involved in the cytotoxic action of fatty acids, the effect of PGE_1, PGE_2 and PGI_2, on the growth of HeLa cells was tested. These results showed that contrary to the expectation, all the three PGs tested in fact, stimulated the growth of HeLa cells (data not shown) suggesting that PGs themselves are not involved in fatty acid induced tumoricidal action. Similar to the results obtained with GLA, both AA and EPA induced tumoricidal action was also blocked by indomethacin, NDGA, vitamin El CPZ and TFP (data not shown). From these results, it can be suggested that free radicals and lipid peroxidation process play a significant role in the tumoricidal action of c-UFAs on HeLa cells. It is possible that this free radical-generation is a cyclo-oxygenase (CO) and lipoxygenase (LO) process. Since both CO and LO inhibitors can inhibit the tumoricidal action of GLA, AA and EPA but,PGs themselves are not cytotoxic to HeLa calls. Since, calmodulin antagonists also

Table 1.

Effect of antioxidants and other chemicals on the Cytotoxicity of GLA on ZR-75-1 (human breast cancer) cells in vitro.

No.	Chemical/Enzyme	Dose(s) used	%Dead cells
1.	Control	-	20.0±5.0
2.	GLA	20 μg/ml	95.0±5.0
3.	Vitamin A	10 μg/ml	30.0±7.0
	Vitamin A	100 μg/ml	32.0±9.0
4.	Vitamin E	10 μg/ml	10.0±5.0
	Vitamin E	100 μg/ml	10.0±7.0
5.	Trans-retinoic acid	$1x10^{-5}$ M/ml	35.0±8.0
6.	Uric acid	$1x10^{-4}$ M/ml	40.0±10.0
	Uric acid	$1x10^{-5}$ M/ml	47.0±8.0
	Uric acid	$1x10^{-6}$ M/ml	51.0±7.0
7.	Cis-retinoic acid	$1x10^{-5}$ M/ml	65.0±18.0
8.	Superoxide dismutase	$1x10^{-6}$ M/ml	15.0±5.0
	SOD	$3x10^{-6}$ M/ml	20.0±7.0
	SOD	$3x10^{-7}$ M/ml	22.0±7.0
	SOD	$3x10^{-8}$ M/ml	30.0±10.0
9.	ATP	200 μM/ml	22.0±9.0
10.	Glutathione peroxidase	0.1 μg/ml	21.0±12.0
	Glutathione peroxidase	0.01 μg/ml	28.0±8.0

All chemicals 4 were tested along with 20 ug/ml of GLA on ZR-75-1 cells (1 x 10^4 cells/ml). All cultures were harvested on day 7 of the addition of GLA with and without the inhibitors. Percentage of dead cells with various concentrations of vitamin A, El retinoic acid,l uric acid, SOD, ATP and glutathione peroxidase alone is approximately 14.0 ± 7.0. All values are expressed as Mean±SD.

TABLE 2.

Effect of iron and copper salts and catalase on the cytotoxicity induced by GLA on human breast tumor calls.

No.	Chemical	Dose tested	% Dead cells
1.	Control	-	20.0±5.0
2.	GLA	10μg/ml	60.0±15.0*
3.	Catalase	500 U/ml	75.0±10.0*
	Catalase	1000 U/ml	80.0±12.0**
	Catalase	3000 U/ml	81.0±8.0**
4.	Heat-inactivated catalase	1000 U/ml	77.0±12.0
	Heat-inactivated catalase	3000 U/ml	73.0±8.0*
5.	$FeCl_2$	1 μg/ml	61.0±12.0
		4 μg/ml	65.0±9.0
		20 μg/ml	98.0±2.0**
		40 μg/ml	98.0±2.0**
6.	$FeCl_3$	1 μg/ml	65.0±10.0
		4 μg/ml	68.0±8.0
		20μg/ml	95.0±5.0*
7.	$CuSo_4$	1 μg/ml	57.0±11.0
		4 μg/ml	78.0±14.0**

All chemicals were tested along with 10 μg/ml of GAL on ZR-75-1 cells (1 x 10^4 cells/ml). All the cultures were harvested on day 7 of addition of GLA with and without other chemicals. Percentage of dead cells with catalase, inactivated catalase, $FeCl_3$, $FeCl_3$ and $CuSO_4$ alone at various concentrations used was approximately 18±5. All values are expressed as Mean±SD.

*$P < 0.001$ compared to control
**$P < 0.05$ compared to control

could block the cytotoxic action of GLA, AA and EPA (data on AA and EPA not shown as they are similar to that GLA, fig.5), it is likely that fatty acid-induced free radical generation is a calmodulin dependent process. This is supported by the observation that fatty acid-induced free radical generation in human neutrophils is a calmodulin dependent process (Sangeetha et al., 1990 b; Das et al., 1990b).

Both copper and iron salts ($CuSO_4$, $FeCl_2$ and $FeSO_4$) augmented the cytotoxic action of GLA, AA, EPA and DHA on HeLa cells in vitro (data not shown). These results were similar to those seen with GLA plus iron and copper salts on ZR-75-1 cells as shown in Table 2.

Effect of Fatty Acids on Free Radical Generation and Lipid Peroxidation

Further support to the idea that free radicals and lipid peroxidation process are involved in the tumoricidal action of fatty acids is derived from the results shown in table 3 and fig.6. MDA-eq detected in the medium and human breast cancer cells and normal monkey kidney cells (CV-1) on day 7 after supplementation with 20 μg/ml of various

TABLE 3.

Effect of various c-UFAs on MDA-eq formation in normal and tumor cells on day 7.

Cell line	Treatment	pmol of MDA-eq	
		In the calls	In-the medium
CV-1	Control	0.5±0.3	0.3±0.1
(Normal monkey	GLA	6.2±1.1	1.1±0.3
kidney cells)	AA	7.1±0.4	2.8±1.0
	EPA	15.0±2.7	3.3±0.5
	DHA	11.4±3.0	10.1±0.3
ZR-75-1	Control	0.2±0.3	0.9±0.2
(Human breast	GLA	26.5±2.7	10.6±2.4
cancer cells)	AA	35.9±10.4	3.5±1.6
	EPA	16.7±1.7	10.2±0.6
	DHA	11.3±3.9	14.4±0.4

All the values are expressed as mean±SD of three separate estimations.

c-UFAs is given in table 3. Both AA and GLA supplemented tumor cells (ZR-75-1) produced large amounts of MDA-eq (approximately 4-fold increase) compared with normal cells (CV-1) tested and controls. On the other hand, EPA supplemented normal cells produced significantly increased amounts of MDA-eq as compared to controls. This suggests, at least in part why 41-SK and CV-1 cells were more susceptible to the cytotoxic action of EPA compared with that of GLA and AA (see fig 2).

Results shown in fig.6 indicate that GLA can augment superoxide generation and the formation of lipid peroxides in HeLa calls in vitro. Similar results were obtained with EPA, DHA and AA (data not shown). The NBT reduction reaction studies in these cells induced by fatty acids could be completely inhibited by SOD suggesting that under, the conditions employed what we were measuring, in fact was the superoxide radical. Both superoxide radical generation and lipid peroxidation process in HeLa cells induced by GLA could be completely inhibited by indomethacin, NDGA, vitamin El CPZ and TFP (fig.6). These results are similar to the inhibitory action of these compounds on the cytotoxicity of GLA on HeLa cells. This suggests that the inhibitory action of indomethacin, NDGA, vitamin E, CPZ and TFP on the tumoricidal action of GLA and other fatty acids with respect to HeLa cells can be attributed to their action on free radical generation and lipid peroxidation process.

Studies with Thymidine Labelled Cells

The results given in table 4 indicate that GLA-treated tumor cells but not normal cells released significant amounts of thymidine compared to controls. This suggests that GLA can, possibly, damage DNA of tumor cells and thus, bring about its tumoricidal action. It is well known that free radicals and lipid peroxides can damage DNA. Since, c-UFAs augment free radical generation and lipid peroxidation, it is likely that fatty acids damage DNA and thus, destroy the tumor cells.

Radiation, Benzo(a)Pyrene and Cis-Platinum-Induced Genetic Damage and c-UPAs

Our earlier studies have shown that prostaglandins can modify genetic damage-induced by gamma-radiation and benzo(a)pyrene to the bone marrow cells of mice

(Das et al., 1985a, Das et al., 1985b; Das at all 1989b). Some eicosanoids are capable of inhibiting the proliferation of some types of tumor cells (Todo et al 1984a; Tanaka et al 1984; Sasaki at al, 1984; Kobayashi et al 1985), where as we observed that PGE_1, E_2 and I_2 can augment proliferation of tumor cells (HeLa cells) in vitro (unpublished data). since, GLA and other c-UFAs showed significant tumoricidal action, it is interesting to study the

Table 4.

Effect of 20μg/ml Of GLA on the Release of Thymidine from radiolabelled Normal and Tumor Cells in vitro.

S.NO.	Cell Line	Control	GLA treatment
1.	CV-1	887.6±82.5	916.5±68.6
2.	CCD-41-SK	546.2±41.2	564.7±59.4
3.	HL-60	2903.5±396.5	3446.3±302.6*
4.	ZR-75-1	1579.4±211.6	1995.2±291.1*

*P < 0.05 compared to the control value. All values are in cpm.

effect of c-UFAs on normal cells in the presence of known mutagens and carcinogens as mutagenesis predisposes to the development of cancer. In view of this, we studied the effect of c-UFAs on genetic damage induced by gamma-radiation, benzo(a)pyrene (BP), and cis-platinum (cis-DDP) to the bone marrow cells of mice. We selected GLA as a representative of c-UFAs, for this study.

The results of this study given in fig.7 and table 5 show that GLA is capable of preventing genetic damage induced by gamma radiation, BP and cis-DDP to the bone marrow cells of mice. This genoprotective action of GLA was not due to a delay or arrest

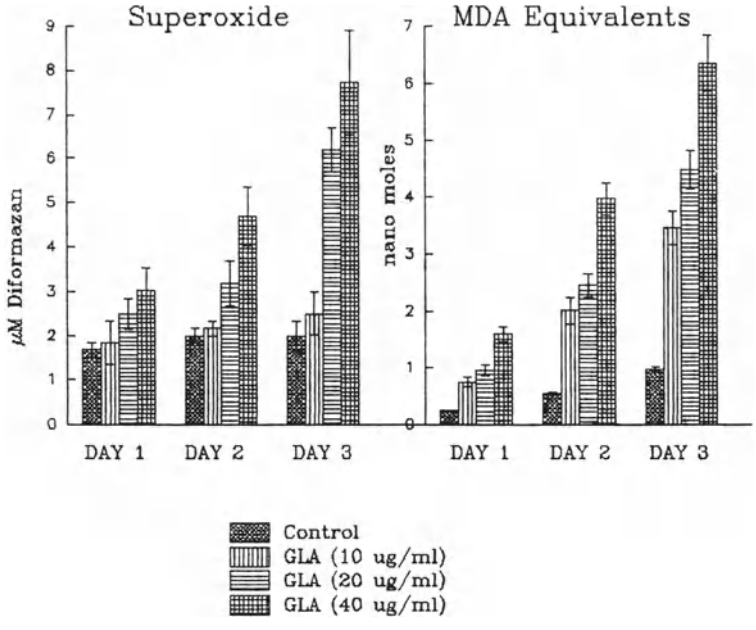


Fig. 6. Effect of various inhibitors on the GLA induced cytoxicity in HeLa cells.

of mitosis of the bone marrow cells of mice (data not shown) and at the same time GLA did not interfere with the tumoricidal action of cis-DDP (data not shown).

Clinical Study

Gliomas

Patients who had histological and radiological evidence of a malignant glioma clinical and/or radiological evidence of tumor progression at least one month after the patient's last course of chemotherapy or radiotherapy and a tumor of sufficient size to warrant further therapy were selected for the study. Patients then underwent a neurosurgical procedure as indicated by their tumor. Patients were treated with a craniotomy for removal of the tumor (to the extent possible) followed by the placement of a catheter into the post-operative tumor cavity. Two weeks after surgery, a CT scan was obtained which was compared with subsequent studies. Each patient received 1 mg of GLA in saline on alternative days/or daily to a maximum of 10 mg or 20 mg. The next day of the last injection, repeat CT scan was performed.

So far 6 patients have been treated by this regiment. All 6 have shown considerable regression in the size of the tumor and 2 typical cases are given in figure 8. There were no side effects during the therapy.

Discussion

The results presented here and elsewhere support and extend the earlier results that c-UFAs, especially GLA, AA, EPA and DHA can kill tumor cells through a free radical and lipid peroxidation dependent process (Das et al., 1987a; Das et al 1987b; Das, 1991; Das, 1990a; Zhu et al., 1985; Gabor et al., 1986; Booyens et al., 1984; Seigel et al., 1987; Tolnai and Morgan, 1962). The enhancing effect of copper and iron salts, the inhibitory action of anti-oxidants and superoxide dismutase levels support to this idea. Increase in the intracellular content of MDA-eq in tumor cells supplemented with c-UPAs adds strength to this contention. The increased amounts of MDA-eq observed in normal cells supplemented with EPA suggests that accumulation of large amounts of lipid peroxides in the cells beyond a limit can be toxic and may ultimately cause cell death

TABLE 5.

Effect of GLA and AA on radiation and BP-induced genetic damage to the bone marrow cells on mice.

No.	Group	Micronuclei in polychromatic RBC	Micronuclei in normochromatic RBC	P/N ratio
1.	saline control	32/6003 (0.53%)	10/6057 (0.16%)	6003/6057 (0.99)
2.	Gamma-radiation	692/6253 (11.07%,**)	50/10288 (0.49%)	6253/10288 (0.61)
3.	GLA	25/6339 (0.39%)	6/6352 (0.09%)	6339/6352 (0.09)
4.	GLA + Radiation	346/6260 (5.53%,***)	28/7448 (0.38%)	6260/7448 (0.84)
5.	AA	16/6108 (0.26%)	4/6087 (0.07%)	6108/6087 (1.00)
6.	AA + Radiation	627/6252 (10.03%)	18/7365 (0.24%)	6252/7365 (0.85%)
7.	BP	154/11175 (1.38%)	33/11106 (0.29%)	11175/11106 (1.01)
8.	BP + GLA	41/6088 (0.67%)	11/5337 (0.21%)	6008/5337 (1.14)
9.	BP + AA	97/6237 (1.56%**)	18/6831 (0.26%)	6237/6381 (0.91)

*P < 0.001 compared to control

**P < 0.0001 compared to control

***P < 0.001 compared to control

since, EPA is toxic to 41-SK (normal human fibroblasts) cells at higher concentrations (fig.2). The inability of DHA to kill ZR-75-1 (Human breast cancer) cells and its potent toxicity to HeLa (Human cervical carcinoma) cells indicates that the sensitivity of different types of tumor cells to various c-UPAs can be variable. In view of this, one has to exercise caution in interpreting results obtained with one or two fatty acids on one, two or few tumor cells lines before concluding with regard to the potency or ability of a particular fatty acid about its (their) tumoricidal action. But, the mechanism of the tumoricidal action of

fatty acids seem to be a free radical process. Inhibition of CO and LO enzymes did not prevent the tumoricidal action (data not shown), but anti-oxidants did, of c-UFAs on ZR-75-1 cells (table 1) where as in the case of HeLa cells both CO and LO inhibitors and antioxidants prevented the cytotoxicity of c-UFAs (figs.3 and 4). This suggests that the mechanism of production of free radicals in response to c-UFAs by different cells can be different. At least in HeLa cells the production of free radicals and lipid peroxidation seem to be a calmodulin dependent process (see fig. 6).

It is known that free radicals can damage DNA, and/or cause induction of protein and nucleic acid cross-linking by lipid degradation products. The increase in the release of labelled thymidine by GLA-treated tumor cells but not normal cells (table 4) indicates that there could be damage to DNA. The observed selective cytotoxicity of c-UFAs to tumor cells (see fig.2) is not just a laboratory curiosity. The results of the clinical studies reported here (fig.8) and else where (Singh et al., 1987, Naidu et al., 1991) prompts us to believe that c-UFAs can kill tumor cells even in an in vivo situation. These limited clinical studies are particularly interesting since, no side-effects were observed. As the patients selected for the study reported here had advanced malignancy and already took radiotherapy and/or chemotherapy but, still had a tumor of sufficient size to warrant further therapy and still showed significant regression to the GLA therapy, their response can be considered as remarkable. It is likely that if GLA is administered at a much early stage of the disease perhaps, the response may be more dramatic.

Further, GLA was found to be capable of preventing genetic damage induced by radiation, BP or cis-DDP (figs. 7 and table 5) in vivo. A simple dose of GLA could prevent genetic damage induced by cis-DDP up to 48 to 72 hours after its administration

(data not shown). In addition, GLA seems to be able to protect the bone marrow cells from the damaging action of radiation and prevent bone marrow suppression. This suggests that GLA may be tried as a bone marrow protective agent in patients receiving chemotherapy and radiation.

Recent studies have shown that interleukin-1 (IL-1) plays a key role in the regulation of hemoproisis and is useful in protecting rodents and primates from the dose-limiting myelosuppression (Morrissey et al., 1988) and neutropenia (Stork et al., 1988; Morre and Warren, 1987) that accompany the use of ionizing radiation or chemotherapeutic drugs for cancer treatment. It is now known that IL-1 can activate phospholipase A_2 (PLA_2) and

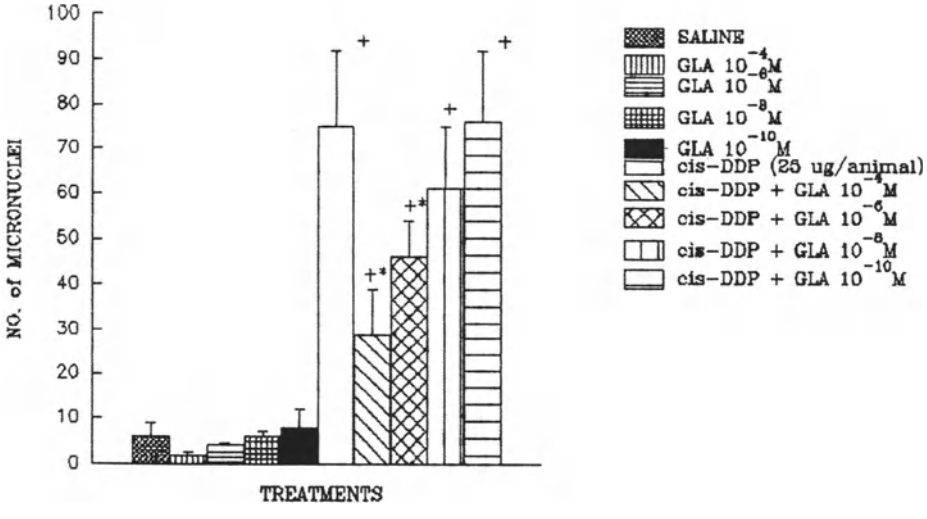


Fig. 7. Effect of Gamma Linolenic Acid (GLA) on cis-Platinum (cis-DDP) induced DNA damage to the bone marrow cells of mice.

$^{+}P < 0.0005$ Compared to Saline Group.
$^{*\wedge}P < 0.005$ Compared to cis-Platinum Group.

induce the release of c-UFAs from the cell membrane lipid pool (reviewed in Das, 1990c). Since, both GLA and AA have bone-marrow protective action (table 5) against radiation similar to IL-1, it is suggested here that the protective action of IL-1 may be due to its ability to induce the release of GLA and AA from the membrane lipid pool.

The bone marrow protective action of either IL-1 or GLA would be futile, if these agents interfere with the tumoricidal action of chemotherapeutic drugs. This is a redundant question for 2 reasons: first because GLA itself has tumoricidal action, and second since, we observed that GLA can potentiate the cytotoxic action of cis-platinum and other anti-cancer drugs on tumor cells in vitro (unpublished data). Thus, it looks as though GLA is not only a selective tumoricidal agent but that it also has the ability to prevent bone marrow suppression induced by radiation and anti-cancer drugs in addition to its genoprotective property. Thus it can be suggested that the ability of GLA and EPA to prevent tumorigenesis by various chemicals in experimental animals (El-Ela et al., 1987; Nelson et al 1988; Minoura at al, 1988;) can at least in part be due to their ability to prevent mutagenesis. This is supported by the observation that EPA can indeed prevent genetic damage (Renner and Delince, 1988) similar to GLA (Das et al.,1985a; Das et al 1987c; Das et al 1989b). The various results presented here are interesting since, it is known that several anti-cancer drugs and lymphokines have the ability to enhance free radical generation and lipid peroxidation in the cells (Das et al 1980b; Sangeetha at al, 1990a) and in patients following chemotherapy (Sangeetha et al., 1990a). This indicates that free radicals and free radical-dependent lipid peroxidation process may be a common pathway by which tumor cells are eliminated both by the natural products of the immune cells and synthetic drugs. Since, GLA is effective in regressing human gliomas further studies are needed to exploit the anti-cancer actions of c-UFAs.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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CLINICAL STUDIES ON POLYUNSATURATED FATTY ACIDS AND ANTIOXIDANTS IN AFRICAN CHILDREN

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Summary

Increased oxidative stress may play an important role in the pathophysiology of human malnutrition. However, deficiencies of antioxidative acting substances like tocopherols and selenium are not consistently found in African children suffering from various clinical forms of malnutrition. Comparative studies investigating clinically well defined groups of patients in different agrogeographical regions are necessary to elucidate the clinical significance of antioxidants in malnutrition.

Introduction

The importance of fatty acids (FA) in human nutrition is mainly related to their function as structural components of cell membranes and their role as precursors of biologically highly active substances, i.e. eicosanoids. The FA composition of human lipids is dependent on dietary intake, FA metabolism, specific incorporation of FA in some lipid fractions, and the protection of polyunsaturated fatty acids (PUFA) against uncontrolled oxidation.

Several aspects of PUFA and antioxidant nutrition may be of clinical importance in cases of severe protein-energy malnutrition (PEM): is PEM associated with a deficiency of essential fatty acids; is the synthesis of eicosanoid precursors affected in PEM; is PEM in general or certain forms of PEM like the kwashiorkor-syndrome, associated with increased oxidative stress, as proposed by Golden and coworkers (Golden & Ramdath, 1987) ?

We focused our nutritional studies in Africa on these questions and compared groups of normally nourished and malnourished children from different agrogeographical regions. Our investigations were performed in West Africa (People's Republic of Congo and Nigeria) and Northeast Africa (Sudan). Although the ecology and nutritional habits of the populations differ widely between these two regions, PEM is a major problem of pediatrics in both parts of Africa. The kwashiorkor type of the disease, however, is more frequent in the humid climate of West Africa, than it is in the dry and hot climate of the Sudan.

Methods

All children investigated in our studies were initially carefully examined by experienced pediatricians, anthropometrically measured and clinically monitored during the study period. Anthropometrical measurements were related to international reference values and grouped according to the Waterlow (Waterlow, 1973) and/or Wellcome (Wellcome Working Party, 1970) classifications. The study protocols were acknowledged by the Ethical Committees of the respective participating universities.

The FA-compositions of plasma cholesteryl esters and phospholipids were determined by capillary gas chromatography and, in cases of unidentified substances, subsequent mass-spectrometry (Leichsenring et al., 1988b). Plasma vitamin A and E were determined by HPLC (Laryea et al., 1990) using a modification of the method by Bieri and coworkers (1979). The selenium content of plasma and hair was measured with

atomic absorption spectrophotometry (Lombeck et al., 1987). The estimation of plasma glutathione peroxidase activity was performed according to the method of Flohé & Günzler (1984).

Results

The plasma FA-composition of normally nourished children from the People's Republic of Congo was, in comparison to children from industrialised countries (Holman et al., 1979), characterized by very low levels of PUFA, like linoleic acid [18:2(n-6), LA], dihomo-gamma-linolenic acid [20:3(n-6), DHLA], arachidonic acid [20:4(n-6), AA] and docosahexaenoic acid [22:6(n-3), DHA]. However, no increase of eicosatrienoic acid [20:3(n-9), ETA], supposed to be present in essential fatty acid deficiency (Holman 1968), was found.

A study on the FA-metabolism in malnourished children, carried out in the Sudan (Leichsenring et al., 1992), revealed that, in comparison to normally nourished Sudanese children, the levels of 20:3(n-6) and 20:4(n-6) in children with PEM ($n=18$) were significantly decreased (mean $\pm$ SD; 1.72 % $\pm$ 0.92 for 20:3(n-6) and 10.92 % $\pm$ 2.24 for 20:4(n-6), respectively). However, no differences were found for 18:2(n-6), 20:3(n-9) and 22:6(n-3). There was highly significant ($p<0.001$) increase in the ratio 18:2(n-6)/20:3(n-6) in patients (15.0 $\pm$ 8.68; mean $\pm$ SD) vs. local controls (5.72 $\pm$ 1.55; mean $\pm$ SD). Differences in the FA-status among children with marasmus, marasmic kwashiorkor, or kwashiorkor were not significant.

The plasma tocopherol of 52 Congolese children (Laryea et al., 1990) showed low values of total tocopherols in all children (351.11 μg/dl $\pm$ 59.38; mean $\pm$ SD). The minimum value was 230.83 μg/dl, the maximum 498.57 μg/dl. This means that all values determined were lower than 500 μg/dl, a value which has been regarded as the lower limit of normal for a long time (Nitowsky et al., 1956). However, when total tocopherols were related to the total plasma lipids the lowest value was 0.77 mg/g (0.99 mg/g $\pm$ 0.17; mean $\pm$ SD), which is above the lowest values found in normal American children

(Farrell et al., 1978). These results were similar with respect to α-tocopherol.

The vitamin E status was also investigated in 30 Sudanese children with severe malnutrition (Ahmed et al., 1990). The tocopherol values and the tocopherol/lipid-ratios in several of these children were higher than in the Congolese group with a normal nutritional status. Although the values scattered widely, the ratio of total tocopherols/total lipids was decreased in only one child. Comparing the three types of malnutrition, according to the Wellcome classification (Wellcome Working Party 1970) no differences in the vitamin E status were found.

Plasma selenium content and glutathione peroxidase activity (GPX) in malnourished Sudanese children were lower than in healthy children from the same area and lower than in reference groups from Germany (Ahmed et al., 1989). In the study group there was a strong correlation between selenium and GPX which usually can not be demonstrated in adequate selenium states (Steiner et al., 1982). Patients with edematous malnutrition (marasmic kwashiorkor and kwashiorkor) showed a significantly lower plasma selenium content than patients with marasmus (median 42 μg/l vs. 57 μg/l; $p < 0.01$). However, in some of the patients with marasmus, marasmic kwashiorkor or kwashiorkor the values were as high as in healthy German children.

Discussion

Some of the results reviewed in this paper refer to "normally nourished" African children. This term is used because the nutritional status - as assessed by anthropometrical measurements - of unselected groups of African children is different from groups in industrialised countries. Retarded growth (stunting), which is commonly regarded as a sign of chronic malnutrition (Waterlow 1973), may be present in a considerable high percentage of the children. Additionally, a high rate of acute and chronic infections

(i.e. malaria), often associated with diarrhoea, may be present. Thus many of the children in unselected groups cannot be defined as being "well nourished" or "healthy". However, most often they appear to be active, without additional clinical signs of nutritional deficiencies (Leichsenring et al., 1988a), and they develop normally. The comparison of biochemical findings in these children with findings in children with acute and severe malnutrition may reveal important information about pathological alterations in PEM.

However, differences in the nutrition of populations living in areas with apparently different ecology and agriculture must be taken into account. It has been stated that PEM is associated with a deficiency of essential fatty acids (Holman et al., 1981, Wolff et al., 1984, Koletzko et al., 1986). This may not be true in all populations. The low levels of 20:4(n-6) found in previous studies were not observed in our study group in Sudan. Also the values of 18:2(n-6) in plasma phospholipids were not lower than in reference groups. The obvious impairment of the PUFA-metabolism in PEM, which can be inferred from the increased ratio 18:2(n-6)/20:3(n-6), led to lower levels of 20:3(n-6) and 20:4(n-6), when malnourished children were compared to normally nourished local controls. However, these levels were still higher than in normally nourished children in the Congo (Leichsenring et al., 1988b), Nigeria (Koletzko et al., 1986), and other African countries (Knuiman et al., 1980). This leads to the conclusion that PEM is associated with disturbances of the PUFA-metabolism and diminished plasma values of eicosanoid precursors, but it is not necessarily associated with a deficiency of essential fatty acids or other PUFA.

Among the clinical forms of PEM the pathogenesis of kwashiorkor remains unclear. It has been proposed that an increased oxidative cell damage, due to impaired protection by antioxidants and increased generation of free radicals by concomitant infections, may be an important factor in the etiology of the disease (Golden & Ramdath 1987). In our study no deficiency of vitamin E was found in malnourished Sudanese children . Plasma tocopherol levels of children with kwashiorkor were not lower than in other malnourished children. This is congruent with results from earlier studies in Nigeria

(Laditan & Ette 1982). Also our results on the selenium status do not support the hypothesis by Golden & coworkers. Although the mean plasma selenium contents in children with kwashiorkor and marasmic-kwashiorkor were decreased, the values scattered so widely that even normal values were found in these children. However, the role of free radicals and antioxidants in the etiology of kwashiorkor requires further clarification. Two major points must be considered when results from studies on this topic are to be evaluated.

First, antioxidative protection in vivo is maintained by different substances in parallel. The interaction and interdependence of these substances in vivo and in vitro is well documented (Jagaadesan & Reddy 1978, Wefers & Sies 1988). Neither do normal levels of one of the protective substances exclude an overall impaired protection nor do low levels of one nutrient provide evidence for a collapse of the antioxidative defense system. Thus, in studies on PEM it is necessary to gain information about the levels of several of the most important antioxidants in parallel, and to establish a model which characterises the antioxidative defense system in vivo. Clinical studies so far have concentrated on single nutrients. An integrated approach may lead to a better understanding of the existing, and partially contradictory, results.

Second, the clinical picture of kwashiorkor is not uniform. The original description of the disease by Williams (1933) included skin lesions, hair changes, mental alterations, fatty liver and oedema. Today, the Wellcome classification (Wellcome Working Party 1970) is widely used. Its definition of kwashiorkor is only based on the presence of reduced weight and oedema; skin lesions and other symptoms may or may not be present. Thus, the symptoms and the severity of the disease may be totally different in children all diagnosed as suffering from kwashiorkor. Consequently the groups investigated in various studies on this topic are most often inhomogeneous and not at all comparable.

The classical picture of the disease, as described by Williams (1933), is rarely seen in Central Sudan. Therefore, we also used the Wellcome classification in our studies. However, in a preliminary study in Nigeria, where kwashiorkor often presents with all classical symptoms, the analyses of plasma tocopherols revealed another picture than in Sudan. (unpublished data; Fig.1).

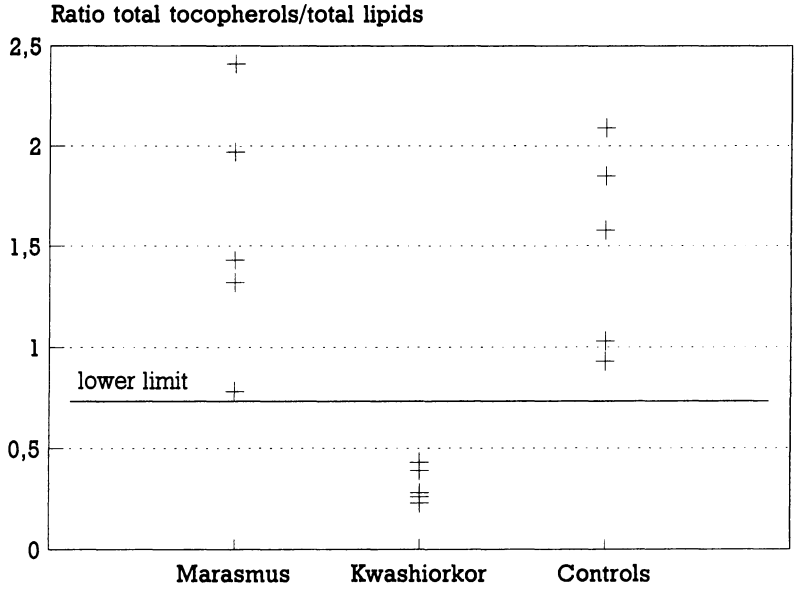

Fig.1:Plasma tocopherol/lipid ratio in Nigerian children with severe protein-energy malnutrition

All five patients with severe kwashiorkor had plasma levels of less than 0.5 mg/g total tocopherols/total lipids, indicating a vitamin E deficiency. All children with marasmus (n=5) and all controls (n=5) had a normal plasma ratio of more than 0.7 mg/g.

Although these results refer to only a small number of children they may show that the exact clinical description of symptoms may be more useful in this kind of clinical research than are, more or less arbitrary, classifications. Only this will allow a comparison of findings in different regions and a better understanding of the complex metabolic alterations in PEM.

Conclusions

Further research should consider regional differences in the nutritional habits, the interaction of antioxidative acting substances, and the broad clinical spectrum of diseases caused by PEM.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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DIHYDROLIPOIC ACID IS PROTECTIVE AGAINST REPERFUSION INJURY

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Summary

Hypoxia/ischemia and reoxygenation/reperfusion were studied in rat hearts and rat hind limbs. Free radicals are known to be generated through these events and to cause complications. In order to reduce hypoxic/ischemic and especially reoxygenation/reperfusion injury we ameliorated (re)perfusion conditions including the treatment with dihydrolipoic acid (DHL).

1. In isolated working rat hearts DHL, if added into the perfusion buffer at 0.3μM concentration, keeps the pH higher (7.15) during hypoxia as compared to controls (6.98). The compound accelerates the recovery of the aortic flow and stabilizes it during reoxygenation. With DHL, ATPase activity is reduced, ATP synthesis is increased and phosphocreatine contents are higher than in controls. Creatine kinase activity is maintained during reoxygenation in the DHL treated series.
2. Isolated rat hind limbs were stored for 4h in a moist chamber at 18°C. Controls were perfumed for 30 min. with a modified Krebs-Henseleit buffer at 60 mm Hg followed by 30 min. Krebs-Henseleit perfusion at 100 mm Hg. The DHL group contained 8 μM DHL in the modified reperfusate. With DHL, recovery of the contractile function was 49% (va. 34% in controls) and muscle flexibility was maintained whereas it decreased by 15% in the controls. Release of creatine kinase was significantly lower with DHL treatment.

DHL is efficient in reducing reoxygenation injury in isolated working rat hearts and reperfusion damage in isolated ischemic rat hind limbs.

Introduction

The monothiol antioxidant 2-mercaptopropionylglycine (MPG) was previously found to improve rat liver mitochondrial function (Zimmer et al., 1978a) and to influence oligomycin-sensitive ATPase from beef heart mitochondria (Zimmer et al., 1978b). MPG ameliorates function in isolated working rat hearts during reoxygenation (Fuchs et al., 1985a; Zimmer and Evers, 1988) and improves canine hearts after cardioplegic arrest (Beyersdorf et al., 1989a). In addition, modification of the initial reperfusion reduces the post-ischemic development of edema in exarticulated rat hindlimbs and restitute ATP contents and contractile function (Beyersdorf et al., 1989b).

Protection of low density lipoproteins (LDL) by the dithiol antioxidant dihydrolipoic acid (DHL) against the attack of peroxyl radicals was shown recently (Freisleben et al., 1991). Reactive oxygen species and peroxyl radicals are intimately involved in ischemia and reperfusion events, and they obviously occur and react in the blood if the circulation alters. However, radicals may not only be generated in the blood but also at a variety of cellular and subcellular sites. Mitochondria may be one of the locations of generation of radicals via impaired electron transport and the mitochondrial ATPsynthase might primarily be attacked (Hyslop et al., 1988).

In this study, we investigate the effects of DHL in order to decrease reoxygenation injury after hypoxia in working rat hearts and reperfusion injury after extended limb ischemia.

Reperfusion injury is a challenging problem in cardiac and vascular surgery. Thus, we studied modifications of reperfusion in two rat models in order to develop clinically applicable methods for use in humans. Exarticulated rat hindlimbs provide a model for ischemic extremities, whereas the working rat heart is a model for hypoxia in hearts. Hence, in the former case the terms "ischemia" and "reperfusion" are used and in the latter case "hypoxia" and "reoxygenation'.

Materials and Methods

The hearts excised from male Wistar rats (Ross, 1972) were cannulated to the perfusion apparatus (Neely et al., 1967; Neely and Rovetto, 1975; Taegtmeyer et al., 1980; Fuchs et al., 1985b). Modified Krebe-Henseleit buffer (Krebs and Henseleit, 1932) was used as perfusion medium. The perfusate (pH 7.4 at 37°C) was gassed with 95% N_2/5% $C0_2$ during normoxia and reoxygenation and with 95% N_2/5% $C0_2$ during hypoxia.

For ^{31}P NMR measurements the working rat hearts had to be inserted into the bore of the magnet inside a 20 mm tube. Connection lines to and from the supply apparatus must be much longer in this case as compared to hearts directly cannulated at the apparatus in traditional working rat heart experiments. Furthermore, several other modifications had to be accomplished (i.e. replacement of metal cannulae by glass and of all other metal parts by non-magnetic material, special insulation of the long connection tubes to achieve temperature constancy etc.) in order to obtain results comparable to those obtained with hearts outside the magnet. These modifications will be described in a separate paper (Assadnazari et al., submitted). NMR spectra were recorded on a Bruker AM-360 spectrometer at 145.7 MHz for ^{31}P. Saturation transfer experiments (Alger and Shulman, 1984) were performed by continuous wave irradiations with a BSV-3BX synthesizer and were recorded in series alternating with nonsaturation transfer. DUL "as added to the perfusion medium at 0.3 μM concentration in the hypoxic period, 15 min. prior to reoxygenation.

Mitochondria from rat heart were prepared according to Mela and Seitz (1979), omitting nagarse. ATP synthesis was measured by means of a LKB Wallace luninometer 1250 (Spielann et al., 1981). Thiols were determined spectrophotometrically with dithionitrobenzoate (DTNB) (Ellman, 1959) or fluorimetrically with bromobinanes (Kosower et al., 1979).

The ATPase complex from beef heart mitochondria was prepared according to Serrano et al., (1976) or according to Freisleben and Zimmer (1989) using deoxy BIG CHAP as detergent. ATPase activity was measured as described by Pullman (1967).

TABLE I

Substance	Modified Krebs-Henseleit buffer mmol/L	Controlled Reperfusate mmol/L
KCl	2.1	3.0
$CaCl_2$	1.7	0.4
KH_2PO_4	0.8	1.2
$MgSO_4$	0.8	1.2
$NaHCO_3$	16.2	107.1
Glucose	5.1	30.3
Aspartate	0.0	13.0
Glutamate	0.0	13.0
NaCl	118.4	118.4
HES 450/0.7	0.1	0.1
Diltiazem	0.0	0.0005
Plasma Substitute ("Plasmasteril")	500ml	500ml
pH	7.4	7.8
Osmolarity	340 mosmol/L	380 mosmol/L
Colloid osmotic pressure	26 mm Hg	26 mm Hg
Dihydrolipoic acid in the treatment group		0.00833mmol/L

Table II

The time course of reperfusion	
I.	controls: 60 min. perfusion without ischemia
II.	controlled reperfusion: 4h ischemia; 30 min. controlled reperfusion; 30 min. reperfusion with Krebs-Henselait buffer
III.	controlled reperfusion plus DHL: 4h ischemia; 30 min. controlled reperfusion containing 8.3μM DHL; 30 min. non-controlled reperfusion

In anesthetized rats, both hindlimbs (weight 20-25g) were exarticulated from the hipjoint, and the skin was carefully removed. The right limb of each animal served as a control, the left one was used for treatment. Femoral arteries and veins were cannulated and the 15 isolated rat hindlimbs were stored for 4h in a moist chamber at 18°C. Thereafter, in controls (n-7) reperfusion was carried out for 30 min with a controlled reperfusate at 60 mm Hg perfusion pressure, followed by 30 min modified Krebs-Henseleit reperfusion at 100 mm Hg (Tables I, II). In the treated group (n=8) 8.3μM dihydrolipoic acid (DHL) was added to the controlled reperfusate.

For determination of the maximal contraction the hindlimbs were fixed in a special device and a weight of 1.5 gm was connected to the ankle. The upper thigh muscles were directly stimulated by impulses from an electric stimulator (frequency lHz; voltage 8V; four times for lmsec). The amplitude of the isotonic contractions was recorded by means of a Kipp & Zonen BD 40 plotter and expressed as percent of pre-ischemic control contractions.

Flexibility was determined as maximal bending of the ankle, i.e. between foot and lower thigh by means of an anatomic goniometer.

Results

The Working Rat Heart

Cardiac parameters precipitously decline during hypoxia, and suddenly increase in reoxygenation. They approximately reach the initial values (Fig.1-3) except for the aortic flow which recovers only by 50% (Fig.4). Figs. 1-4 depict the results from traditional working rat heart experiments (Freisleben et al., 1991). Aortic flow appears to be more sensitive to hypoxia and reoxygenation than other cardiac parameters and was further used for evaluation in NMR studies.

Fig.5 shows the typical course of the aortic flow of a heart beating inside the magnet bore (control). 0.3μM DHL, added in the middle of the hypoxic phase accelerates recovery and stabilizes the aortic flow during reoxygenation.

As a link to mitochondria we looked into the phosphate status of the heart by means of ^{31}P NMR spectroscopy (Fig.6).

^{31}P NMR spectra of the control in late hypoxia exhibit a broad and shifted phosphate peak as compared to the DHL-treated sample, where the phosphocreatine peak is higher than in the control (Fig.6). The part of the phosphate peak representing internal phosphate and its shift can be used for calculation of the intracellular pH. With DHL the pH is 7.15 and in the controls it is 6.98.

The phosphocreatine (PCr) contents were also observed to be higher in the DHL-treated hearts during the subsequent reoxygenation phase.

Furthermore, saturation transfer NMR technique (Alger and Shulman 1984; Brindle 1988) was used, suppressing the γ-ATP peak and observing the decrease of the PCr peak indicating flux of phosphate from PCr-->γ-ATP.

Figure 7 depicts saturation transfer experiments carried on during the course of normoxia-hypoxia-reoxygenation. The graphs show the ratios of PCr without and with saturation as determined in alternating series. The ratio declines in the controls with the start of reoxygenation. In the DHL-treated group this decline in early reoxygenation does not occur. However, there is a transient decrease also in DHL-treated hearts between 40 and 60 min. of reoxygenation which is immediately compensated.

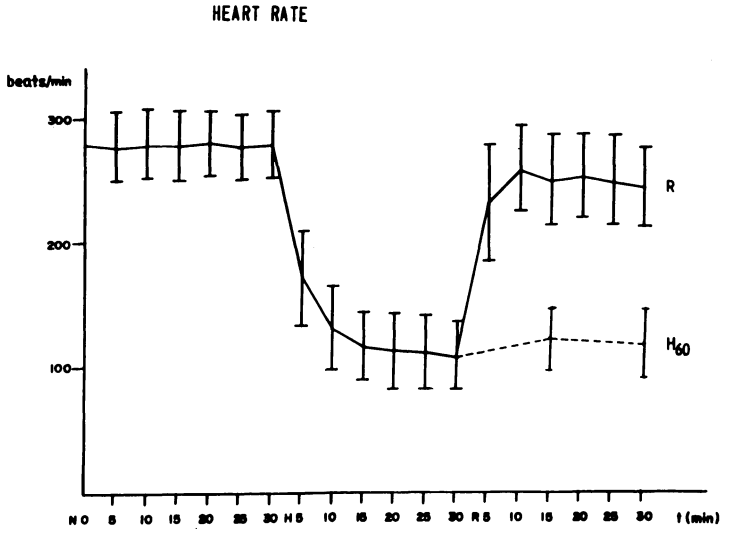

Fig. 1. Heart rate (beats/min) during normoxia-hypoxia-reoxygenation. Dashed lines:hypoxia carried on up to 60 min (H_{60}) as a control to reoxygenation (R) [from:Friesleben et al., (1991), Arzneim. Forsch./Drug Res. 41, 81-88]

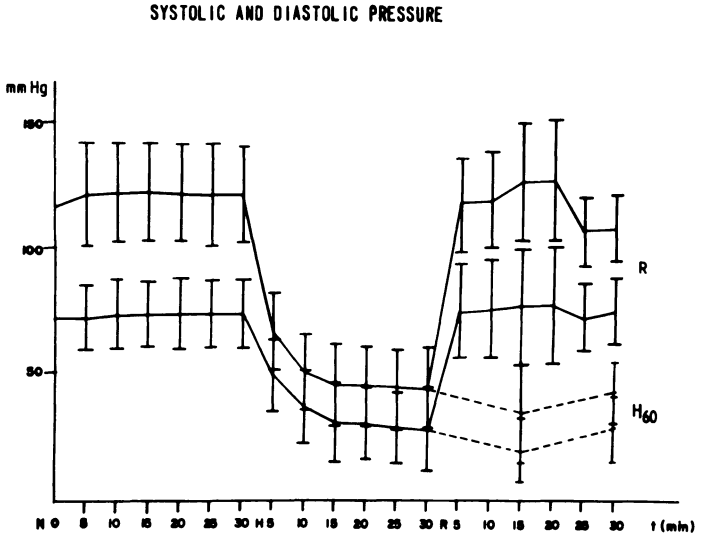

Fig. 2. Systolic and diastolic pressure during normoxia-hypoxia-reoxygenation. Dashed lines: hypoxia carried to 60 min (H_{60}) as control to reoxygenation (R) [from: Freisleben et al., (1991), Arzneim. Forsch./Drug Res. 41, 81-88]

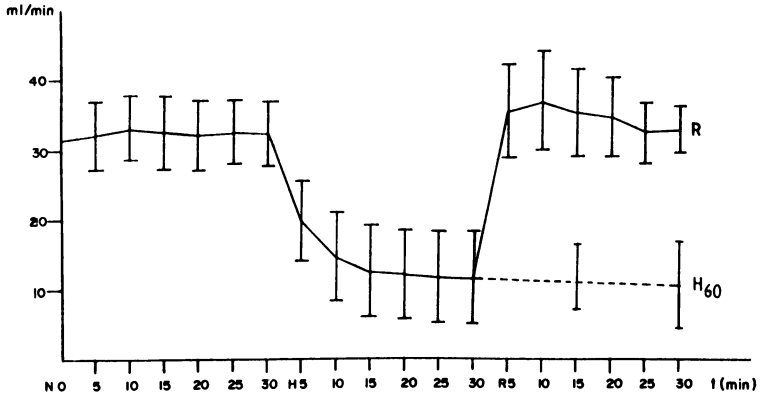

Fig. 3. Coronary flow during normoxia-hypoxia-reoxygenation. Dashed line: hypoxia carried on up to 60 min (H_{60}) as a control to reoxygenation (R) [from Freisleben et al., (1991), Arzneim. Forsch./Drug Res. 41, 81-88]

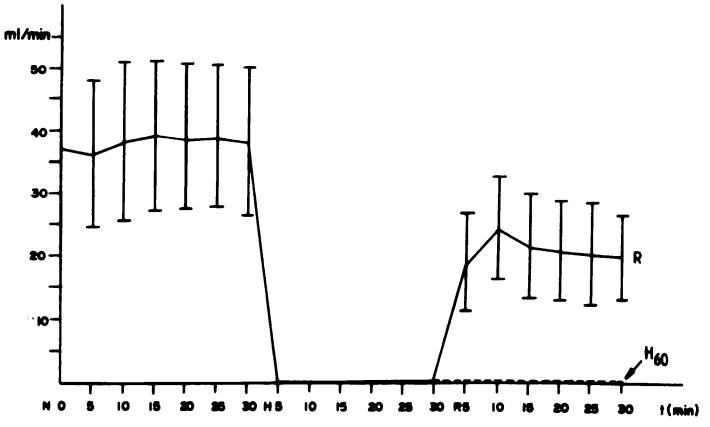

Fig4. Aortic flow during normoxia-hypoxia-reoxygenation. Dashed lines: hypoxia carried on up to 60 min (H_{60}) as control to reoxygenation (R) [from: Freisleben et al., (1991), Arzneim. Forsch./Drug Res. 41, 81-88]

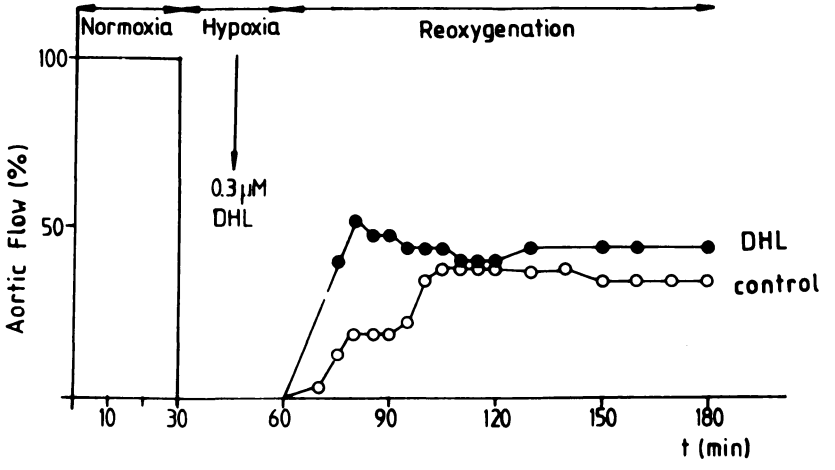

Fig. 5. Aortic flow during representative NMR experiments. 0.3μM DHL was added into the buffer 15 minutes before reoxygenation.

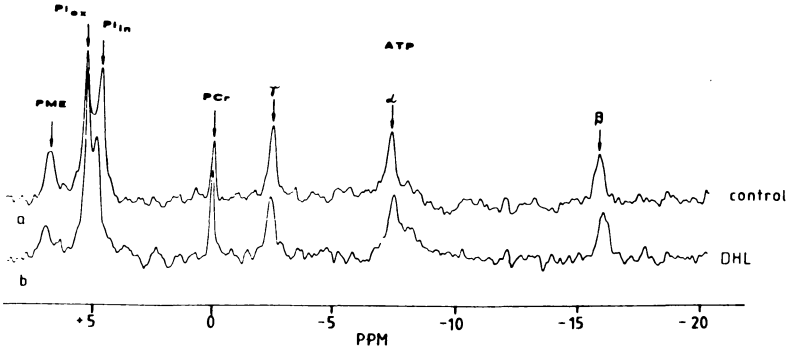

Fig. 6. ^{31}P-NMR spectra of working rat hearts in late hypoxia. Upper trace: control. Lower trace: treated with 0.3 Mm DHL 15 minutes before reoxygenation. Abbreviations: PNE = sugar phosphate monoesters; $P_i$ex = external phosphate; $P_i$in = internal phosphate; PCr = phosphocreatine; α,β,γ - ATP peaks.

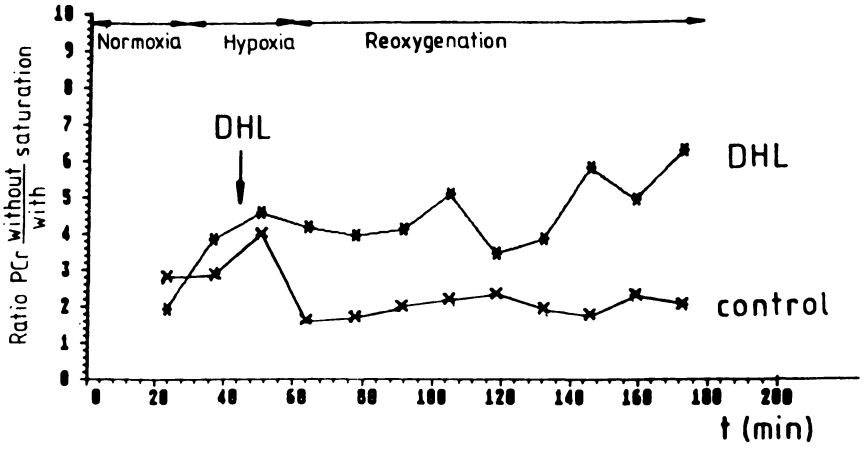

Fig. 7. Ratios PCr without/with maturation of γ-ATP obtained during the whole experimental course. In this type of experimentation, saturation and non-saturation acquisition times were used in sequence. 15 minutes before reoxygenation, 0.3 μM DHL was added into the buffer.

Titration experiments of creatine kinase activity with varying phosphocreatine saturation of the enzyme and increasing DHL concentrations exert a general stimulation of the enzyme by DHL (not shown).

Mitochondria and ATPase/Synthase Complex

DHL treatment of isolated mitochondria at 1-5 nM concentrations per mg of protein shows an activation of oligomycin sensitive thiols (Fig.8A), a decrease in ATPase activity, and a concomitant increase in ATP synthesis (Fig.8B).

In isolated ATPase complexes from beef heart mitochondria (Serrano et al., 1976) incubated with 3.5-7 nM concentrations of HPG or with 1-2 nM concentrations of DHL, ATP hydrolysis was reduced and oligomycin sensitivity and ATP-P_1, exchange were

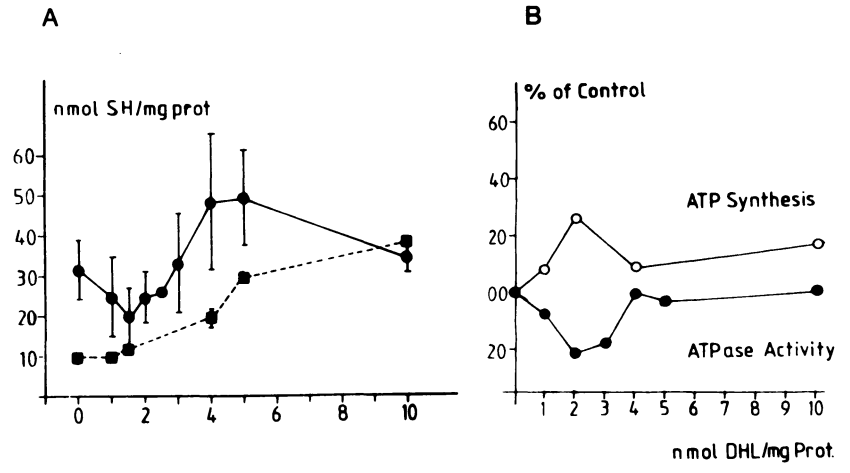

Fig. 8. A: Titration of mitochondrial -SB groups and oligomycin sensitivity ●without oligomycin; ■in presence of 10 μg oligomycin/mg protein (left part); B: ATP synthesis and ATPase activity (right hand side) vs. increasing concentrations of DHL. [modified from: Zimmer, G., Mainka, L. and Krüger, E. (1991) Arch. Biochem. Biophys.).

increased as compared to untreated complexes. In all experiments three-fold higher concentrations of MPG were necessary to reach similar effects as compared to DHL.

Rat Hindlimbs

Contractile function recovered in controls by 34.14% ± 9.1, in the treated group by 49.24% ± 16.7 ($p \leq 0.05$). Muscle flexibility immediately after exarticulation was 116° ± 4 in both groups, at the end of the experiment 99° ± 7 (controls) vs. 111° ± 4 (treated) ($p \leq 0.01$). Release of creatine kinase was measured in the buffer after 15 min of reperfusion (units in controls vs. units in the DHL-treated group) 68 ± 53 vs. 18 ± 14 ($p \leq O.05$), after 30 min 65 ± 30 vs. 31 ± 15 ($p \leq O.02$) (Table IV).

TABLE III

MPG and DHL influences on the isolated ATPase complex:

Incubation:	FCCP	FCCP/MPG	MPG
MPG conc. per mq prot:		58nmol	3.5-7nmol
		FCCP/DHL	DHL
DHL conc. per mg prot.:		20nmol	1-2nmol
Comparison vs. Control=Unmodified ATPase Complex:			
F_0. protein bands in SDS-PAGE:	decreased	compens.	increased
ATPase activity	increased	compens.	decreased
ATP-PA exchange	decreased	n.d.	increased

Table IV

Efficacy of DHL in controlled reperfusate of rat hindlimbs:

		i	c	t	p
contractile function (%CC)		16.7	34.1	49.2	≤0.05
max. banding (116°)			99°	111°	≤0.01
CK release	15min		68U	18U	≤0.05
	30min		65U	31U	≤0.02

i=after 4h ischemia; c=control without DHL; t=treated with DHL; p=significance of DHL-treatment versus controls. %CC refers to contractile function before ischemia (= 100%).

The experiments were carried out with and without pre-incubation with 0.2μM concentration of the uncoupler FCCP (carbonylcyanidep-trifluoromethoxyphenylhydrazone; compens. means compensation to control values.

Discussion

Lipoic acid (lipoamide) in either its reduced (DHL) or oxidized forms, is a physiological cofactor of mitochondrial dehydrogenases. It was shown by Bunik et al., (1990) that DHL is highly effective in reducing thiols in α-ketoglutarate dehydrogenase which are essential for the cooperativity of this enzyme complex. Apart from its physiological role, DHL (or lipoic acid) is of interest in pathophysiological and therapeutic respects which latter activities are considered here.

DHL cannot be used as a drug because it in unstable and rapidly oxidized. However, administration of the oxidized form (lipoic acid or thioctic acid) to the body sufficiently provides the reduced form (DHL) via enzymic and non-enzymic equilibrium mechanisms. In in vitro experiments DHL is the reactive principle, this appears to hold true in vivo also for administration of lipoic acid, because it is a recycling agent and is easily recycled by itself. Compounds involved in recycling processes may be superior to antioxidants or radical scavengers which are not recycled, because the latter may in turn propagate radical chain reactions.

Thioctic acid protects in vivo against the generation of free radicals in early intestinal reperfusion in rats as detected by chemiluminescence (Roldan et al., 1989).

The pK values of the -SB groups of DHL are high, approximately 10.7 (Gascoigne and Radda, 1967). Hence, the superior activity of DHL as compared to MPG (Tables III, V) and other thiols does not appear to be based on the dissociation of the -SH groups.

Similar to other vicinal dithiols, DHL is more easily oxidized than monothiols (Jocelyn 1972). On the other hand, the tension of the oxidized pentacycle (3-6 kcal/mol) explains the tendency for reductive splitting due to environmental conditions (Jocelyn 1972). The redox potential is very low (-0.29 to -0.32 Eo'V) (Searls and Sanadi, 1980), lower than that of the oxidized/reduced glutathione couple (-0.24 Eo'V) which is reduced by DHL.

The mitochondrion is probably a site of action of DHL. The compound is sufficiently hydrophobic to permeate biological membranes at high rates. The lipophilicity of DHL may furthermore be a part of its pharmacologic efficacy, not only in term of pharmacokinetics and distribution, but also in the site of interaction. In the mitochondrial membrane it was shown, that thiols in hydrophobic environment are more easily oxidized to V) than those in polar regions or may become otherwise shielded under comparable uncoupling conditions (Freisleben et al., 1988). Sensitive thiols of membrane proteins in hydrophobic environment may especially be protected by DHL via -SB/-S-Sinterchange. Hence, we expect an influence on the reactivity of mitochondrial thiols, on ATPase activity and ATP synthesis in rat heart mitochondria, and on creatine kinase.

Mitochondrial parameters do not exert such a clear course during hypoxia and reoxygenation as cardiac parameters. They are impaired in a more complex way with reduced function in hypoxia as well as in the reoxygenation period (Freisleben et al., 1991). The course of ischemia/hypoxia and reperfusion/ reoxygenation is characterized by

a) a loss of mitochondrial -SH groups due to oxidative stress,

b) an activation of mitochondrial ATP hydrolysis and

c) a decrease of mitochondrial ATP synthesis due to impaired coupling of oxidative phosphorylation.

DHL exhibits a concentration dependent counteracting efficacy in all three cases (a-c). Between 1.5 and 4-5nnol DHL/mq protein an increase of oligomycin sensitive -SH groups in detected and then a decline again to control values at 10nmol DHL ocith a loss of oligomycin sensitivity. This agrees perfectly with earlier results using the monothiol 2-mercaptopropionylglycine (HPG) where oligomycin-sensitive -SH groups were determined between 5 and 9 nmol per mg mitochondrial protein (Zimmer 1977).

Furthermore, we found an increase in ATP synthesis in rat heart mitoplasts with a peak at 2-3nmol DHL per mg protein. Concomitantly, ATP hydrolysis is decreased at the same amount of DHL.

Isolated Mitochondrial ATPsynthase Complex

ATPase/synthase complexes were isolated from beef heart mitochondria. Previously, we had compared different preparation methods (Freisleben and Zimmer 1986), and also developed a new one (Freisleben and Zimmer 1989).

As reported by Lippe et al., (1988) diamide, a thiol oxidizing reagent (Kosower et al., 1972) obviously impairs the interaction between F_1 and F_0 resulting in loss of oligomycin sensitivity of the ATPase. Concomitantly, they observed a decrease in the 25kDa subunit of the F_0 part of the ATPsynthase.

Preincubation of the complex with 3nmol DHL per mq protein increases the peaks at 25/27 and 3lkDa. This increase is completely inhibited by oligomycin.

Oligomycin sensitivity depends on the proper binding and orientation of the F_1 moiety to the hydrophobic membrane integrated F_0 part of the complex. This is particularly necessary for ATP synthesis.

During hypoxia and reoxygenation, coupling of oxidative phosphorylation is impaired which may be due to either uncoupling between the respiratory chain and ATPsynthase, or improper connection of F_1 with F_0 (Zimmer et al., 1985; Freisleben et al., 1991), or both. Our experiments reveal that the process of uncoupling at least partially concerns the F_0 moiety of the ATPase/synthase complex (Table III). HPG and DHL are able to compensate the uncoupling effect of FCCP in the ATPase/synthase complex, possibly via ameliorated orientation and proper binding of the subunits. Concomitantly, ATPase activity is decreased and ATP-P_1 exchange is increased, confirming an improved coupling of the complex. DHL isothree times more effective in these experiments than MPG (Table III).

Besides mitochondrial ATP synthesis, the creatine kinase system is of vital interest. At very low concentrations of DHL and, moreover, at low, non-saturating concentrations of the substrate phosphocreatine, the activity of mitochondrial CK is increased and remains at a higher level also at increasing amounts of DHL under all experimental conditions.

Discerning mitochondrial and cytoplasmic creatine kinase, we mainly investigated the mitochondrial enzyme. In fact, it is the mitochondrially localized type of creatine kinase which becomes particularly activated in growing organism and especially at higher work load (Perry et al., 1988). Furthermore, nonequilibrium conditions for heart creatine kinase have repeatedly been found (Matthews et al., 1982; Bittl et al., 1985; Koretsky et al., 1986).

With DHL, we found an increase of the fluxes PCr-->γ-ATP and increase of the activity of creatine kinase especially under conditions non-saturated with substrate. This may apply to the early reoxygenation phase where phosphate fluxes abruptly decline in the controls (Fig.7) indicating either a decrease in flux of phosphate PCr-->γ-ATP or a loss of creatine kinase. This loss (of flux or of enzyme) is not replenished within 2h of reoxygenation. DHL either maintains the flux of phosphate or impedes the loss of enzyme. However, also in DHL-treated hearts a transient decline between 40 and 60 min. of reoxygenation may appear, which is immediately repaired (Fig.7).

In NMR experiments, we could also measure the intracellular pH by shifts of the internal phosphate peak. In the controls a pH of 6.98 was determined vs. pH 7.15 in the DHL-treated hearts. This is important because creatine kinase preferentially works in the direction of ATP formation at lower pH values and towards PCr synthesis at higher pH (Saks et al., 1975; Lawson and Veech 1979; Wyss et al., 1990). On the other hand, also loss of creatine kinase from the cells must be considered, as we detected an increase in CK activity in the reperfusate in the rat hindlimbs. However, this latter activity may rather result from a loss of cytoplasmic enzyme from the cells than from the mitochondrially localized enzyme.

Concerning the most sensitive cardiac parameter (Figs. 1-4), two observations are apparent: DHL accelerates recovery and stabilizes the aortic flow during reoxygenation at 0.3mm concentration in the perfusate. This concentration should be equivalent to about 5nmol DHL per mg mitochondrial protein, assumed that the substance is completely taken up into mitochondria. Stabilization of the aortic flew could well be explained by an increase in disposal of high energy phosphate.

Reperfusion injury as detected in rat hindlimbs can be classified into - development of edona, - loss of high energy phosphates and of creatine kinase, calcium overload of the tissue, - no (or low) reflow - endothelial swelling,- reduced oxygen uptake, - myoglobin release, - loss of muscle contractibility and flexibility (Beyersdorf et al., 1989b).

In more than 100 rats conditions for controlled reperfusion had been optimized. The content of calcium and osmolarity had proven to be of central importance. Furthermore, the reperfusion pressure was reduced from 100 to 60 mm Hg. The optimal calcium concentration in the reperfusate was 300μM, higher and lower concentrations exerted phenomena of calcium overload or "calcium paradox". At this concentration (300μM Ca^{++}) also the optimum condition for additional calcium channel blocking with 0.21mg/L diltiazem was obtained (Hanselmann et al., 1990). The optimal osmolarity was found to be 380 mosmol/L. Further improvement of the controlled reperfusate could be achieved by addition of 1.5 mM MPG to the reperfusate. This concentration should be compared to that of DHL (8.3 μM) in the buffer. However, the MPG concentration was taken from the experiences with perfusion of working rat hearts without further control and experimentation in order to reduce the concentration necessary for efficacy in the rat limbs. Nevertheless, if the concentrations of MPG and DHL are compared it is evident that DHL is considerably more effective than MPG.

Recently, the lowest effective concentration of DHL was measured in another system by means of fluorescence polarization. 20pmol per mg of mitochondrial protein were needed for significant increase in fluidity of the mitochondrial membrane during reoxygenation (Scheer et al., in preparation).

Table V

Comparison of the efficacy of MPG and DHL

model	concentration in perfusion buffer	
	MPG	DHL
working rat heart	0.2-1mM	0.31μM
rat hind limbs	1.5mm	8.3μM

Conclusion

Hypoxia and non-controlled reoxygenation impair cardiac function because of insufficiently provided high energy phosphates. At the start of reoxygenation, intracellular pH, ATP synthesis and mitochondrial creatine kinase activity decrease, along with an increase in ATP hydrolysis. Dihydrolipoic acid, at low (nN) concentrations, raises the intracellular pH to normal values, reactivates oligomycin-sensitive mitochondrial thiols, increases mitochondrial ATP synthesis, decreases ATP hydrolysis, and maintains phosphate fluxes within the creatine kinase system. Hence, the aortic flow is improved and stabilized during reoxygenation under DHL treatment. The physiological antioxidant DHL further proven highly efficient in reducing reperfusion damage in 4h ischemic rat hindlimbs. Loss of creatine kinase from the cells is decreased, and contractile function and muscle flexibility are restored under DHL treatment. These effects may partially be ascribed to the low redox potential of DHL and to its antioxidant and/or to -SH/-S-S- exchange activity as well an to its free radical scavenging activity.

The experiences from the perfusion buffer systems as described in this paper are now transferred to controlled reperfusion in vivo. The benefits of the monothiol MPG as a free radical scavenger have already been shown in canine hearts (Mitsos et al., 1986; Myers et al., 1986; Beyersdorf et al., 1989a). At this time, experiments are accomplished in Frankfurt with controlled reperfusion of pig hindlimbs after extended ischemia. Models have been developed for controlled reperfusion in human surgery and on the basis of our investigations, the cyclic dithiol lipoic acid will hopefully be introduced into this new therapeutic strategy.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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EFFECT OF JAPANESE HERBAL MEDICINE, SHO-SAIKO-TO-GO-KEISHI-KASHAKUYAKU-TO (TJ-960) ON AGING

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Summary

Japanese herbal medicine, Sho-saiko-to-go-keishi-kashakuyaku-to (TJ-960), scavenged hydroxyl radicals, superoxide and 1,1-diphenyl-2-picrylhydrazyl radicals. It also diminished carbon centered radicals generated by oxygen stress in the rat cortex homogenate and quenched alpha-tocopheroxyl radicals in rat mitochondria and microsomes of vitamin E supplemented rats. The carbon centered radicals and thiobarbituric acid reactive substances (TBARS) levels decreased in the aged rat brain after 3-week oral administration of 5% TJ-960 solution. TJ-960 elevated superoxide dismutase (SOD) activity in cytosol fraction of hippocampus and hypothalamus of aged animals. It decreased norepinephrine and 5-hydroxytryptamine (5-HT) levels in hypothalamus and increased 5-HT level in cerebellum. TJ-960 treatment increased choline acetyltransferase activity in aged rats. As herbal medicines do not generally have harmful side effects, TJ-960 appears as a suitable prophylactic agent against some neuronal symptoms of aging.

Introduction

Much reports have been accumulated on subjects related to free radicals and aging. The levels of antioxidants such as carotenoids, α-tocopherol and uric acid in plasma, serum or liver have been reported to increase with aging, (Cutler, 1991). However, finding other excellent antioxidants against aging remains an important concern.

Sho-saiko-to-go-keishi-ka-shakuyaku-to (TJ-960,Tsumura & Co., Tokyo), a Japanese herbal medicine made up of a vacuum-concentrated extract of nine herbs, was first found to have an excellent scavenging activity on oxygen free radicals and other free radicals. Subsequently, its effect on lipid peroxidation and superoxide dismutase (SOD) activity in the aged rat brain was examined. The neurotransmitter levels and choline acetyltransferase (CAT) activity, which is a marker of cholinergic neuronal function, were also studied to clarify whether TJ-960 is a suitable prophylactive agent against aging.

Scavenging Activity of TJ-960 on Free Radicals

1.TJ-960

TJ-960 is a vacuum-concentrated extract of nine herbs in the following ratio : 7.0 Bupleuri radix *(Bupleurum falcatum* L.); 5.0 Pinelliae tuber *(Pinellia ternata* Breitenbach); 3.0 Scutellariae radix*(Scutellaria baicalensis* Georgi); 4.0 Zizyphifructus *(Zizyphus Vulgaris* Lamarck var. *inermis* Bunge); 3.0 Ginseng radix *(Panax ginseng* C.A. Meyer); 2.0 Glychyrrhizae radix *(Glycyrrhiza glabra* L. var. glandulifera Regel et Herder, *Glycyrrhiza uralensis* Fisher); 1.0 Ziniberis rhizoma (*Zingiber officinale* Roscoe); 6.0 Paeoniae radix *(Paeonia albiflora* Pallas var. *trichocarpa* Bunge) ; and 4. 0 Cinnamomi cortex *(Cinnamomum cassia* Blume).

2 . Lipid Soluble Radicals

1.1-Diphenyl-2-picrylhydrazyl (DPPH) radicals are stable lipid soluble radicals. Analysis of DPPH radicals was carried out as follows: One hundred microliters of 30 μM DPPH ethanol solution and 100 μM of TJ-960 solution were mixed for 10 sec and transferred to a special flat cell for the estimation of the amount of DPPH radicals by electron spin resonance spectrometer (JEOL JES-FElXG, Tokyo) 60 sec after addition of DPPH solution. The signal intensities were evaluated by the peak height of the second of the five peak signals of the DPPH radicals (Fig.1). It was then found that TJ-960 in the

range of 125 μg/ml to 5 mg/ml scavenged 30 μM DPPH radicals dose dependently (Fig.2) (Hiramatsu et al., 1988).

3. Water Soluble Radicals

Superoxide radicals are generated in water phase by hypoxanthine and xanthine oxidase system. These radicals were measured using ESR spectrometry as follows: fifty microliters of 2 mM hypoxanthine, 35 μl of 10.98 mM diethylenetriaminepentaacetic acid (DETAPAC), 50 μl of sample, 15 μl of dimethyl-l-pyrroline-l-oxide (DMPO) and 50 μl of xanthineoxidase were placed in a test tube and mixed. The mixture was then transferred to a flat cell and the levels of DMPO spin adducts of superoxide were quantified 60 sec after addition of xanthine oxidase. The signal intensities were evaluated by the peak height of the first of the twelve 12 signals of DMPO spin adducts (Fig. 3) . The spin parameters of ^{a}N, $^{a}H\beta$ and $^{a}H\gamma$ were 1.425, 1.145 and 0.125 mT, respectively. It was shown using this method that TJ-960 scavenged superoxide dose-dependently in the range of 12.5 μg/ml to 12.5 mg/ml (Fig. 4) (Hiramatsu et al.,1988).

The generation and analysis of hydroxyl radicals were as follows: One hundred μl of 80 mM α-guanidinoglutaric acid, 100 μl of TJ-960 solution and 20 μl of DMPO were mixed and the sample solution was placed into a flat cell. Carbon centered radicals were

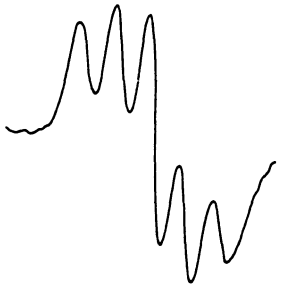

Fig.1 Electron spin resonance spectrum of 1,1-diphenyl-2-picrylhydrazyl radicals.

also generated in this solution. The concentration of DMPO spin adducts of hydroxyl and carbon centered radicals formed was estimated exactly 60 sec after the addition of DMPO. The signal intensities were evaluated by the peak height of the second signal of the quartet of DMPO-OH spin adducts and of the first signal of the hexad of DMPO-C spin adducts (Fig.5). The spin parameters of both ^{a}N and $^{a}H\beta$ in DMPO spin adducts were 1.49 mT and ^{a}N and $^{a}H\beta$ of DMPO-C spin adducts were 1.584 and 2.34 mT, respectively. Five mg/ml of TJ-960 exhibited significantly to quench hydroxyl and carbon centered radicals about 45%, respectively (Fig.6).

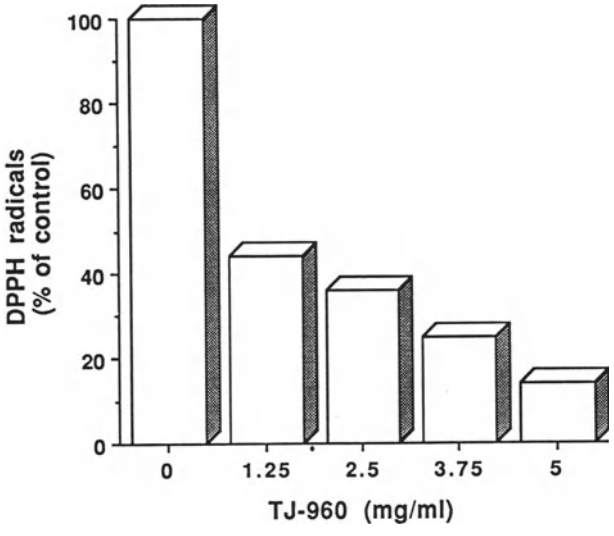

Fig. 2 Effect of TJ-960 on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Each value was obtained from 5～6 determinations.

Fig. 3 Electron spin resonance spectrum of superoxide as spin adducts of dimethyl-l-pyrroline-1 oxide induced by hypoxanthine and xanthine oxidase system.

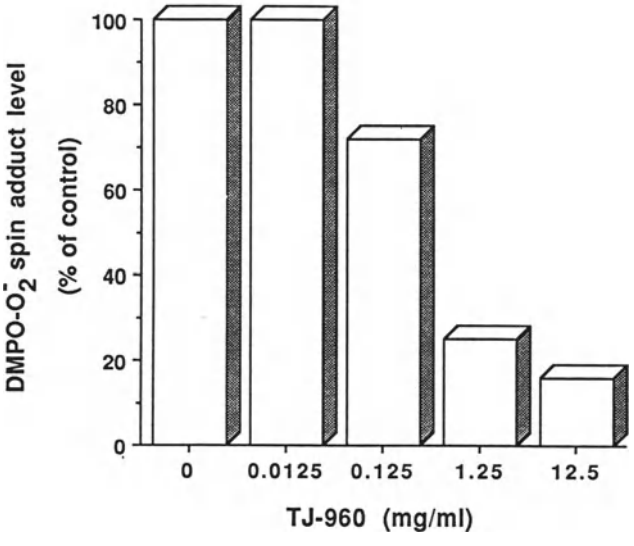

Fig. 4 Effect of TJ-960 on superoxide radicals. Each value was obtained from 5～6 determinations.

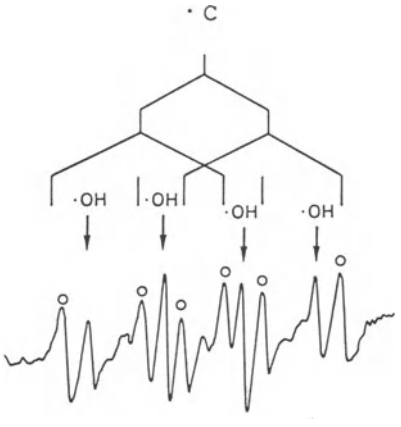

Fig. 5 Electron spin resonance spectrum of hydroxyl and carbon centered radicals ($^{\bullet}C$ and $^{\bullet}OH$) as spin adducts of dimethyl-1-pyrroline-1-oxide generated by α-guanidinoglutaric acid.

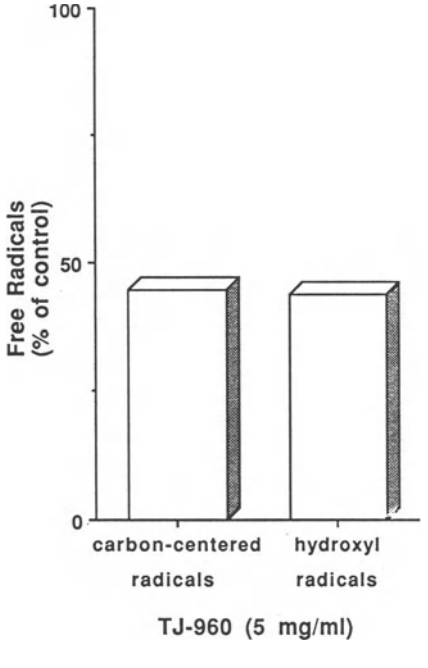

Fig.6 Effect of TJ-960 on hydroxyl and carbon centered radicals generated by a-guanidinoglutaric acid. Each value was obtained from 5-6 determinations.

4. Carbon Centered Radicals Induced by Ascorbic Acid and $FeCl_2$ in the Brain Homogenate

The effect of TJ-960 on carbon-centered radicals in mouse brain homogenate was examined as follows: To 0.5 ml of ddY mouse brain homogenate, 0.4 ml of TJ-960 solution, 0. 1 ml of 20 μM $FeCl_2$ with 50 μl of ascorbic acid, and 0. 4 ml of O.1 M KCl in 0.03M Tris-HCl buffer, pH 7.4 was added. The mixture was incubated at 37°C for 15 min, after which 20 μl of DMPO was added.. The mixture was placed into a flat cell and the carbon centered radicals were measured as DMPO spin adducts (DMPO-C). TJ960 solution of 22.7 mg/ml completely quenched the carbon centered radicals and other free radicals. (Fig.7) (Hiramatsu et al., 1988).

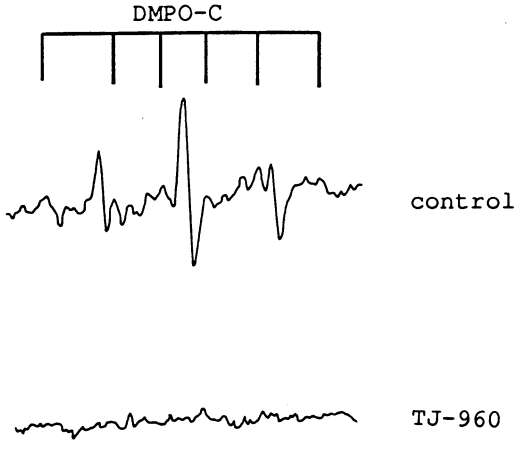

Fig. 7 Effect of carbon centered radicals generated by $FeSO_4$ and ascorbic acid in mouse brain homogenates.

The conditions of ESR spectrometry used in measuring DPPH, hydroxyl and carbon centered radicals were as follows : magnetic field, 335±5 mT; power 8 mW; response, 0.3 s; modulation, 0.2 mT; room temperature; amplitude, 3.2xl,000; sweep time, 0.2 min. For measurement of superoxide, magnetic field, response and sweep time were 335±10 mT, O.ls and 2 min, respectively. Manganese oxide was used as the internal standard.

5. α-Tocopheroxyl Radicals in Vitamin E Rich Membranes of Rats

α-Tocopheroxyl radicals could not be detected in microsomes and subparticle membranes (SPM) of rat liver. Whereas, lipoxygenase and arachidonic acid generated these radicals in both membranes of rat liver after the provision of vitamin E supplemented diet. TJ-960 scavenged α-tocopheroxyl radicals in microsomes and SPM of rat liver and it further decreased the loss of α-tocopherol level by lipoxygenase and arachidonic acid (Fig. 8 & 9).(Hiramatsu et al., 1990)

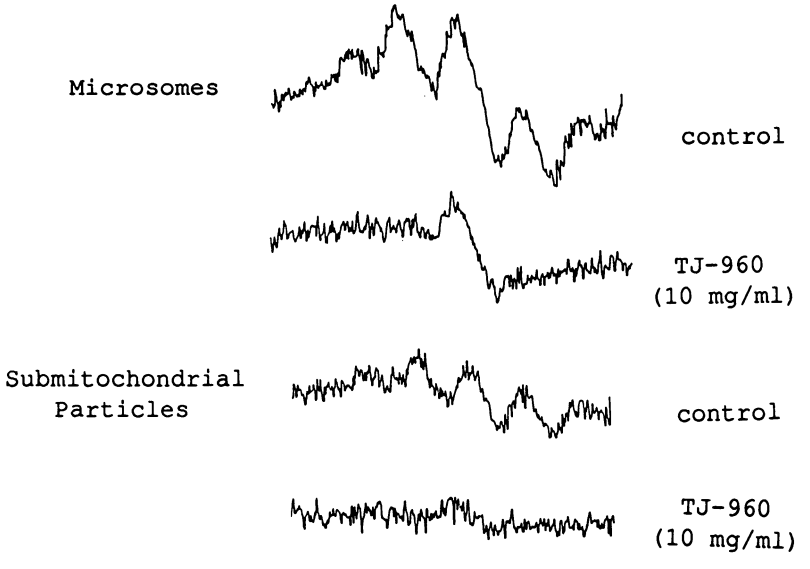

Fig.8 Effect of TJ-960 on the tocopheroxyl radicals in vitamin E enriched rat liver microsomes and submitochondrial particles.

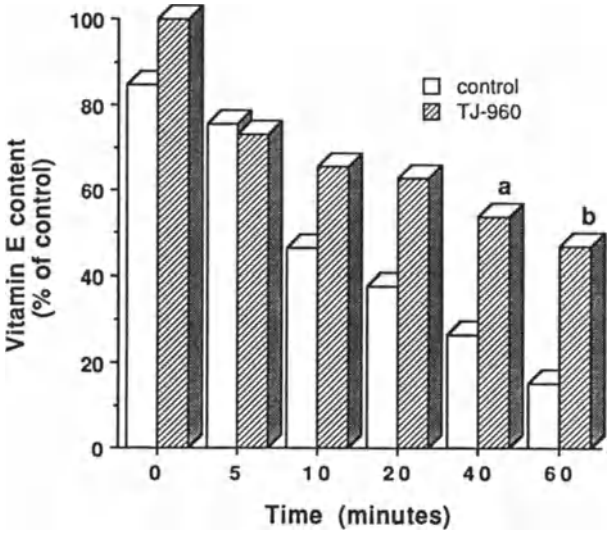

Fig. 9 Effect of TJ-960 on the vitamin E level in vitamin E enriched microsomes of rat liver with oxidation system. Each value was obtained from 3 determinations. Significant differences of $^{a}p < 0.05$ and $^{b}p < O.Ol$ were found compared with control using student's t-test.

In summary, the above in vitro experiments showed that TJ-960 has potent scavenging activities on hydroxyl radicals, superoxide and DPPH radicals. It was further shown that TJ-960 has a quenching action on α-tocopheroxyl radicals and carbon centered radicals in rat liver membranes and mouse brain homogenates, respectively. These findings suggest that TJ-960 is an excellent antioxidant against oxygen free radicals and other free radicals.

Neurochemical Study of TJ-960 in Aged Rat Brain

1. Animals

As excellent scavenging activity on free radicals was found in TJ-960, neurochemical study with TJ-960 was carried out using aged rats. Male Wistar rats aged 3.5 and 24 months were divided into two groups. One group was administered a 5% TJ-960 aqueous solution orally for 3 months and the other group was given water as a control. After 3 months, i.e., at 6.5th and 27th months respectively, animals were sacrificed by decapitation and the cerebrum was rapidly taken out. The cortex, striatum, hippocampus, hypothalamus, midbrain, pons-medulla oblongata and cerebellum were then dissected on ice plate following the method of Glowinski and Iversen (1966). All samples were kept at -80°C until analysis.

2. Thiobarbituric Acid Reactive Substances (TBARS) and Carbon Centered Radicals in the Aged Rat Brain

TBARS was measured using the method of Ohishi (1978) with thiobarbituric acid. While brain lipid peroxide and superoxide radical formation have been reported to increase with age (Sawada & Carlson, 1987), the chronic treatment of TJ-960 decreased the level of carbon centered radicals and TBARS formation in the cerebrum of aged rat brain compared with the control group. (Fig.10).

3. SOD Activity in the Aged Rat Brain

Some papers have reported on total SOD activity, Cu, Zn-SOD activity and MN-SOD activity in rodent aged brain in comparison with those of young adult brain and have found no coincidence in the change of brain SOD activity with aging (Kellogg et al., 1976; Mavelli et al., 1978; Geremia et al., 1990; Danh et al., 1983; De Quiroga, 1990) SOD activity was measured using our method with ESR spectrometry (Hiramatsu et al., 1987) . Our experiments showed that SOD activity in the mitochondrial fraction from the cortex, hippocampus, striatum, hypothalamus, midbrain, pons-medulla oblongata and

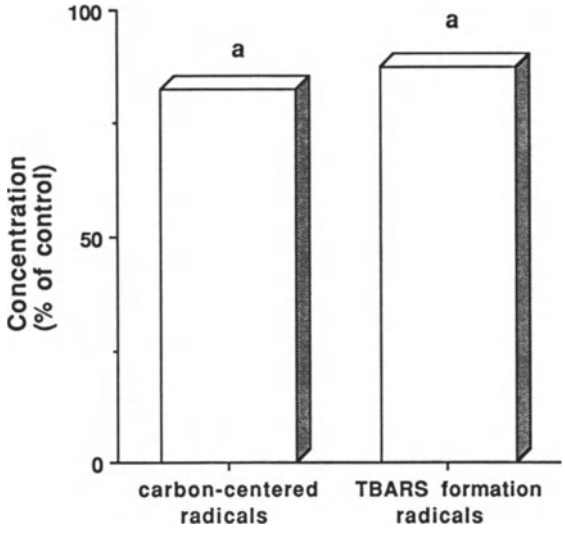

Fig. 10 Effect of TJ-960 on carbon centered radicals and thiobarbituric acid reactive substance (TBARS) formations in aged rat cerebrum. Significant difference of $^{a}p<0.05$ was found compared with control. Each value was obtained from 9～13 animals.

cerebellum were markedly increased in aged rats compared with adult rats. Similarly , SOD activity in the cytosol fraction was elevated in all parts except the hypothalamus in aged rats (Hiramatsu et al., in press). However, the administration of TJ-960 to aged rats resulted in a decrease in SOD activity in the mitochondrial fraction of the striatum, and an increase in the hippocampus and hypothalamus (Fig.11). In adult rats, TJ-960 had no effect except to increase SOD activity in the cytosol fraction of the cortex.

4. Neurotransmitter Levels in Aged Rat Brain

In aged rats and mice, dopamine and norepinephrine levels are decreased (Hirschorn et al., 1982; Estes et al., 1980; Estes et al., 1984; Osterburg et al., 1981; Simpkins et al., 1977). The hypothalamic norepinephrine level is low in aging animals (Estes et

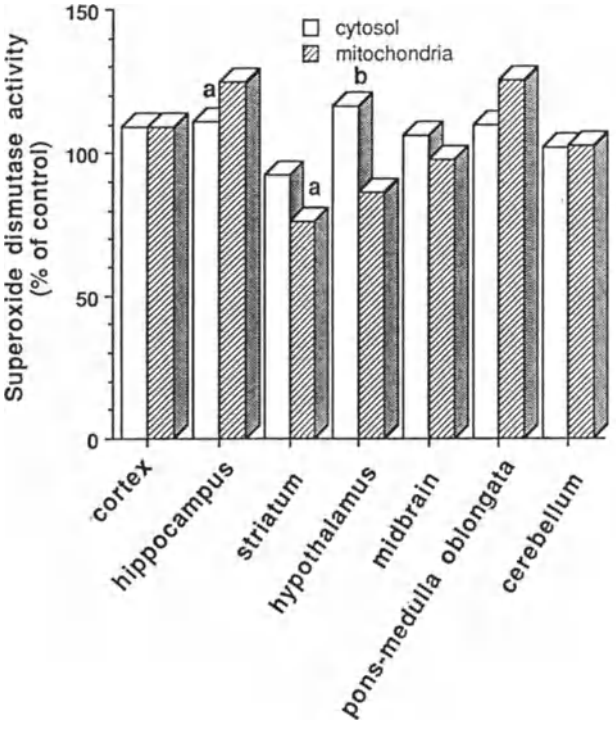

Fig. 11 Effect of TJ 960 on superoxide dismutase activity in cytosol and mitochondrial fractions of aged rat brain. Each value was obtained from 12 ~ 15 animals. Significant differences of $^{a}p < 0.05$ and $^{b}p < O.Ol$ were found compared with control using student's t-test.

al., 1980; Osterburg et al., 1981; Simpkins et al., 1977) and in Alzheimer's disease (Gottfries et al.,1976; Yates et al.,1981; Gottfries et al.,1983; Yates et al., 1983). The dopamine-β-hydroxylase (DBH) activities in the cerebrospinal fluid of aged people and patients with Alzheimer's disease are decreased (Fujita et al.,1982; Cross et al., 1981; Perry et al., 1981). In addition, cell loss in the locus coerulus, reduced nucleolar volume in locus coeruleus neurones, and loss of norepinephrine are reported in the norepinephrinergic system in Alzheimer's disease (Hardy et al.,1985). These reports paved the analysis of catecholamine levels in aged rat brain using high performance liquid chromatography. The administration of TJ-960 for 3 weeks increased the norepinephrine level in the hypothalamus and had no effect on the level in any other brain regions. (Fig.12). Dopamine level in seven parts of aged rat brain was not affected by the treatment of TJ-960 (Hiramatsu et al., 1986). However, 5-hydroxytryptamine level in the hypothalamus was decreased and the level in the cerebellum was increased after the chronic administration of TJ-960 (Hiramatsu et al., 1988).

There have been few reports on the brain levels of amino acids in aged rats . Our studies have shown that overall brain amino acid levels are lower in aged than in adult rats. Cerebral cortical γ-aminobutyric acid, glutamine, and lysine levels are significantly higher in aged than in adult rats, while in the cerebellum, glutamate levels are higher and alanine levels lower.

Administration of TJ-960 to rats resulted in significant increase in cortical levels of taurine, serine and alanine in aged rats (Fig.13) and significant increases in cortical taurine, glutamate, glutamine, glycine and alanine in adult rats.

In the cerebellum, TJ-960 produced increases in glycine and γ-aminobutyric acid levels in adult rats but no significant changes were seen in aged rats. (Hiramatsu et al., 1988) . Thus, TJ-960 increased only cerebral cortical taurine, serine and alanine in aged rats (Fig. 13) . It also increased taurine levels in adult rats. Taurine levels are not altered with age. These findings suggest that the antiepileptic action of TJ-960 may be partly the result of the anticonvulsant action of taurine.

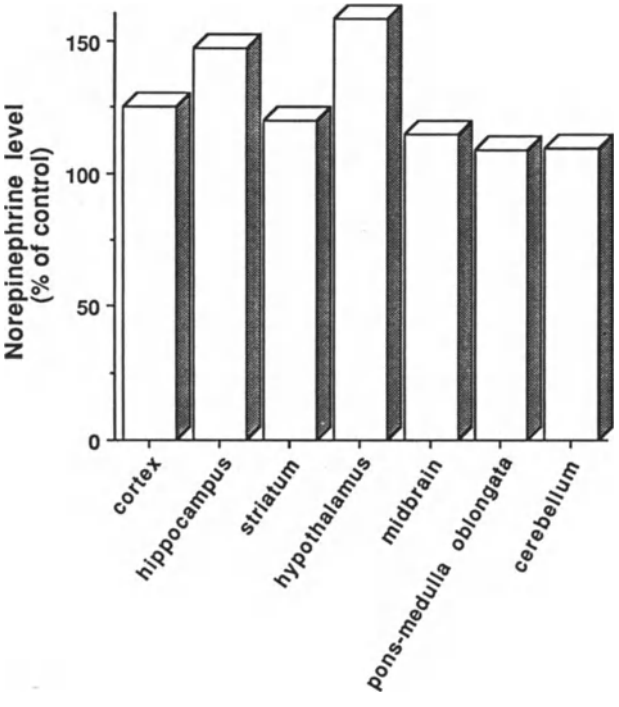

Fig. 12 Effect of TJ-960 on norepinephrine level in aged rat brain. Each value was obtained from 6～9 animals. Significant difference of $p < 0.05$ was found compared with control using student's t-test.

5. CAT Activity in the Aged Rat Brain

Previous reports have shown decreased CAT activity in the striatum and hippocampus of aged rats (Allen et al., 1983; Haba et al., 1988; Sims et al., 1982.) CAT activity was measured according to a modification of Fonnum's method (1974) as described by Haba et al., (1988 In comparison with adult rat brain, CAT activity in the hippocampus, pons-medulla oblongata and striatum was lower and the activity in the cerebellum was higher. The enzyme activity in the cortex of aged rats was also lower than that in adult rats but it was not significant (Fig.14).

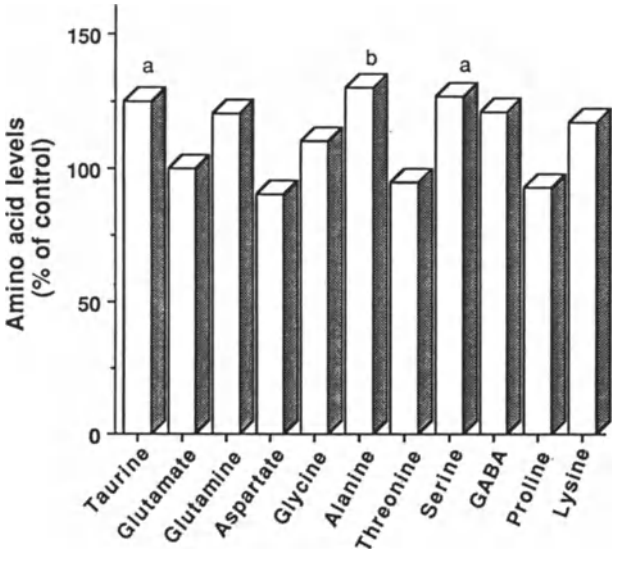

Fig.13 Effect of TJ-960 on cerebral cortical amino acid levels of aged rat brain. Each value was obtained from 7～9 animals. Significant differences of $^{a}p<0.05$ and $^{b}p<O.Ol$ were found compared with control using student's t-test.

However, the administration of TJ-960 increased the CAT activity in the hippocampus and striatum but it did not affect the activity in the seven brain parts of adult rats (Hiramatsu et al., 1989) (Fig.14). While the mechanism underlying this activity still remains to be clarified, the observed increase in CAT activity may suggest a protective effect of TJ-960 against aging in the brain.

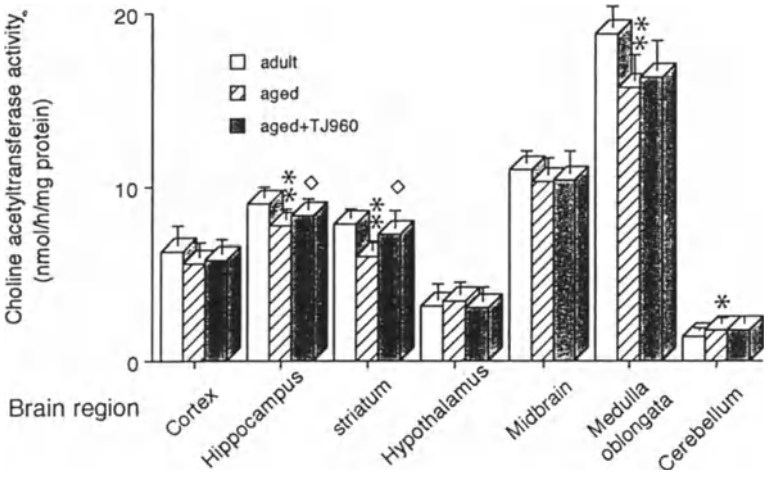

Fig. 14 Effect of TJ-960 on choline acetyltransferase activity in the aged rat brain. Each value was obtained from 7～12 animals. Significant differences of $^{*}p<0.01$, $^{**}p<0.001$ and $\diamond p<0.05$ were found compared with adult and aged rats, respectively, using student's t-test.

Conclusion

TJ-960 was first developed as anticonvulsants using experimental animal model for epilepsy (Hiramatsu et al., 1981; Hiramatsu et al., 1985; Sugaya et al., 1988). The

authors described here that TJ-960 has a free radical scavenging action in *vitro* and *in vivo*. Paeoniflorin, which is one element of Paeoniae radix, a component of TJ-960, does not have any scavenging action on free radicals. Traditionally, it has been believed that Chinese herbal medicine exhibits the effect only as a cocktail of complex plant extracts. It therefore remains an open question as to which component of TJ-960 may exert the effect seen as the antiaging effect, a free radical scavenging action and an increased CAT activity. Anyhow, it may be allowed to say that TJ960 is an excellent prophylactive agent against aging.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
A.S.H. Ong & L. Packer (eds)

LIPOPROTEIN OXIDATION

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Introduction

The earliest recognized gross lesion in atherogenesis is the fatty streak characterized by an accumulation of foam cells derived from circulating monocytes and, to a lesser degree, from smooth muscle cells (Ross, 1986; Steinberg et al., 1989).

The fatty streak usually develops under a structurally intact endothelium and begins to form when circulating monocytes adhere to the arterial endothelium. After adhesion, the monocytes penetrate into the subendothelial space and adopt the phenotypic properties of the resident arterial macrophages (Steinberg et al., 1989).

These macrophages play a significant role in atherogenesis because they are capable of taking up and degrading considerable quantities of lipoproteins. They then appear as foam cells, this aspect being the consequence of an accumulation of cholesteryl esters in the cytoplasm of cells (Steinberg et al., 1989). Cholesteryl esters from foam cells derived

mainly from low density lipoproteins (LDL) a well known atherogenic risk-factor (Ross,1986 ; Steinberg et al., 1989).

The study of patients with familial hypercholesterolemia (FH) has shown the essential function played by the LDL receptor in the LDL metabolism (Brown et al., 1981; Goldstein et al., 1989). Apolipoprotein (apo) B100, the protein moiety of LDL is the ligand for the LDL receptor. However, homozygous FH patients with no LDL receptor activity clearly have an accelerated atherosclerosis with cholesterol deposits in the macrophages of the artery wall (Goldstein et al., 1989). Similarly, rabbits deficient in LDL receptor (Watanabe heritable hyperlipidemic rabbits; WHHL) demonstrate early atherosclerosis with lesions rich in macrophage-derived foam cells (Tanzawa et al., 1980). Therefore, the accumulation of cholesterol in macrophages could not occur by the LDL-receptor pathway, but by another pathway known as the "scavenger-pathway" (Gorden et al., 1981). The capacity for removal is important, because if the catabolism of LDL is very slow in receptor-negative patients or animals, the LDL production and removal are very high, about three fold that of normolipidemia subjects. Secondly, the incubation of native LDL with monocytes-macrophages in culture did not lead to the formation of foam cells. This observation could be explained by a low number of LDL receptors on the surface of macrophages. Moreover, these LDL receptors, like LDL as the LDL receptors present in other tissues, can be down regulated in the presence of LDL and no accumulation of cholesterol appears during such an experiment.

So, apparent contradictory observations of the absence of native LDL uptake by the monocytes-macrophages on one hand, and the accumulation of cholesterol in monocytes-macrophages occurring essentially from LDL on another hand, have led to the hypothesis that LDL could undergo one or more modifications and that this modified LDL could then be taken up by these cells.

Modifications of Lipoproteins and their Role in the Pathogenesis of Atherosclerosis

Modifications of Lipoproteins

Goldstein et al.,and Mahley et al., have demonstrated that cultured mouse peritoneal macrophages accumulate massive amounts of cholesterol when exposed to chemically modified LDL such as acetylated or acetoacetylated modified LDL (Goldstein et al., 1979; Mahley et al., 1979). These studies have been extended to other modifications of LDL such as malondialdehydation (Fogelman et al., 1980) or by incubation with some types of cells. The capacity for cells to modify LDL varies with the type of cells and the species. So, modification of LDL has been reported for endothelial cells from rabbits (Henriksen et al., 1981), human umbilical veins and adult arteries and veins (Van Hinsbergh et al., 1986), bovine and human smooth muscle cells (Heinecke et al., 1987 ; Morel et al., 1984) and human monocytes/macrophages (Hiramatsu et al., 1987 ; Cathcart et al., 1985), but it did not occur with bovine aorta endothelial cells or human fibroblasts (Morel et al., 1984 ; Henriksen et al., 1983). Some of these in vitro modifications (malondialdehydation, modification by cells) could occur in vivo. The modification of LDL induced by incubation with cells has been extensively studied since Henriksen et al., demonstrated the structural changes of LDL under these conditions (Henriksen et al., 1981) . The structural and biological properties of cell-modified LDL can be mimicked by incubating LDL in a serum-free medium in the presence of copper or iron (Heinecke et al., 1987 ; Steinbrecher et al., 1984 ; Steinbrecher et al., 1987). These modifications occur through an oxidative phenomenon, on the polyunsaturated fatty acids (PUFAs) of LDL.

Cells may produce oxidatively modified LDL by at least two distinct mechanisms. The first, which involves the generation of superoxide radical and the direct participation of these radicals in the oxidation of LDL lipids, is discussed in this paper. The second mechanism, which suggests a role of cellular lipoxygenases and particularly 15-lipoxygenase in the oxidation of LDL is suggested by several lines of evidence.

The oxidation of LDL induces a large number of structural modifications with the disappearance of some components and the synthesis of others. Studies of these modifications as a function of time have shown that the earliest change was the fast decrease in Vitamin E content (Esterbauer et al., 1989 (**); Esterbauer et al., 1987). LDL preparations contained variable quantity of Vitamin E, from 3.15 to 9.90 moles/mole LDL with a mean at 6.4. This Vitamin E was completely consumed during LDL oxidation in 1 to 6 hours, the time involved being a function of the Vitamin E content in LDL and of the experimental conditions. Other antioxidants in lesser quantities in LDL (γ-tocopherol, β-carotene, lycopene, retinyl stearate) also disappeared during the LDL oxidation. The stage of antioxidant consumption could be defined as a lag phase, because no oxidation of PUFAs occured. The second phase was the propagation phase. When LDL was completely depleted of its antioxidants, the PUFAs were rapidly oxidized. Thus, PUFAs 18:2 and 20:4 decreased dramatically during an incubation of LDL with 10 μM copper 10 μM. Indeed, the content of these two fatty acids in LDL dropped to 10 % of the value present in native LDL during a 24 hour incubation (Esterbauer et al., 1989 (*)). The importance of PUFAs in the susceptibility of LDL to peroxidation was suggested by the resistance of LDL to oxidation when these particles were enriched by monounsaturated fatty acid such as oleic acid (Parthasarathy et al., 1990). Furthermore, the modification of LDL by endothelial cells, appeared to be accompanied by extensive hydrolysis of the phosphatidylcholine in LDL to lysophosphatidylcholine (Steinbrecher et al., 1984). About 40 % of the phosphatidylcholine disappeared during the 24 hour incubation.

The lysophosphatidylcholine remained associated with LDL; so the phospholipid/protein ratio remains constant in cell-modified LDL (Henriksen et al., 1982). Studies using radiolabelled phosphatidylcholine showed that the second position fatty acid was cleaved during modification by incubation with endothelial cells or with Cu^{2+}. The specificity of hydrolysis suggested the presence of a phospholipase A_2 activity.

Phospholipase activity and peroxidation of fatty acids could favor each other. Indeed, phospholipase could release peroxidized fatty acids which would propagate the peroxidation of other fatty acids. Furthermore, the oxidation of fatty acyl chains in

phosphatidylcholine could make a better substrate for phospholipase activity. Thus, the irreversible inhibition of phospholipase A_2 by p-bromophenacyl bromide blocked the phosphatidylcholine hydrolysis but only partially inhibited the peroxidation (Parthasarathy et al., 1985), indicating that lipid peroxidation did not require the hydrolysis of phosphatidylcholine. The peroxidation of fatty acids of LDL (essentially linoleic and arachidonic acids) generated a number of new products, the main mechanism for their formation being the so-called beta-cleavage reaction of the lipid alkoxyl radicals. When PUFAs were more or less completely oxidized during the decomposition phase, the lipids were converted to a variety of other products including complex hydroperoxy and hydroxy acid derivatives such as 13- or 9- hydroperoxy-octadecadienoate derivatives produced by oxidation of linoleic acid (Lenz et al., 1990) and lower molecular weight derivatives such as malonaldehyde, hexanal, propanal, 4-hydroxynonenal, butanal, hexadienal, pentanal, 4-hydroxyhexenal and 4-hydroxyoctenal.

The kinetics of the formation of the various aldehydes were different. Although the levels of propanal, hexanal and 4-hydroxyoctenal increased during the first 3 hours of incubation of LDL in a oxygenated buffer, those of 4-hydroxynonenal, 4-hydroxyhexenal, 2,4-heptadienal, pentanal and butanal only increased after this time. Hydroxy acids derived from linoleate and arachidonate increased with the incubation time of LDL with Cu^{++} (Henriksen et al., 1983).

Some of these products such as malonaldehyde were essentially hydrophilic and were found in the aqueous phase of the incubation mixture, while others were lipophilic and remained mostly in the lipid core of oxidized LDL (Esterbauer et al., 1987).

The oxidation of LDL was accompanied by an increase in thiobarbituric acid reactive substances (Heinecke et al., 1987 ; Steinbrecher et al., 1984), products used to evaluate LDL oxidation. This procedure is not specific and detect less than 10 % of the hydroperoxides derived from linoleic and arachidonic acids (Lenz et al., 1990), the main PUFAs in LDL.

The protein moiety of LDL, the apolipoprotein (apo) B100, was degraded during oxidation. The band corresponding to the apo B100 with a molecular weight 550,000 daltons on SDS-polyacrylamide gel electrophoresis disappeared almost completely in oxidized LDL and numerous bands of lower molecular weight ranging from 14,000 to 200,000 were detected after incubation of LDL with endothelial cells (Steinbrecher et al., 1984) or Cu^{++} (Fong et al., 1987). The disappearance of apo B100 was rapid: indeed, no intact apo B100 could be detected after 4 hours. This breakdown of apo B100, whether cell-induced or Cu^{2+} catalyzed was not due to proteolytic enzymes (Fong et al., 1987) and could be the result of the oxidative cleavage of the polypeptide chain, because it only appeared in media supporting oxidation and was inhibited by antioxidants such as butylated hydroxytoluene (BHT) or EDTA. This modification was accompanied simultaneously by a decrease in LDL amino group reactivity as shown by the TNBS (trinitrobenzene-sulfonic acid) reactivity for the free amino groups (Steinbrecher et al., 1987) . Amino acids already shown to be susceptible to oxidation, lysine, proline and histidine decreased of more than 10 %.

Acetylation of LDL involved lysine ϵ-amino groups (Goldstein et al., 1979). The oxidation of LDL is similarly accompanied by derivatization of lysine ϵ-amino groups that neutralized the positive charge in LDL, explaining the increase of electrophoretic migration of oxidized LDL. The fragmentation of apo B100 and modification of amino acids was the result of the reaction of lipid peroxidation product on lysine. The decomposition products of PUFAs could be candidates for this reaction (Steinbrecher, 1987) . Indeed, the incubation of one of the most reactive lipid peroxidation products, 4-hydroxynonenal with LDL revealed that most of this aldehyde became bound to the protein. 4-hydroxynonenal reacts mainly with lysine but also with serine, tyrosine, cysteine and histidine (Jurgens et al., 1986) . However, the incubation of LDL with 4-hydroxynonenal leads to higher molecular weight forms than apo B100. These forms were not being detected in endothelial-cell modified LDL. The structural modification of apo B100 during oxidation was reflected by a modified immunoreactivity. Indeed, the immunoreactivity of three different epitopes of apo B100 decreased during oxidation of

LDL with Cu^{2+}, but the immunoreactivity of another epitope located in the C-terminal 20 amino acids of apo B100, increased during the first 6 hours of oxidation and thereafter diminished (Zawadzki et al., 1989) . Using monoclonal antibodies developed against malondialdehyde modified LDL, 4-hydroxynonenal-LDL, it was demonstrated that malondialdehyde-lysine and 4-hydroxynonenallysine were generated during LDL oxidation in presence of Cu^{2+} (Palinski et al., 1990).

It appeared that the extent of LDL modification was extremely variable. We saw above that LDL isolated from rabbits fed with an oleic-rich diet were remarkably resistant to oxidation compared to LDL isolated from animals fed with a diet rich in PUFAs. The composition of the diet was partly reflected in LDL because LDL from the two groups contained 49.5 % and 24 % oleate and 16.5 and 39 % linoleate respectively (Parthasarathy et al., 1990). The density profile of isolated LDL also influenced its susceptibility to oxidation, denser LDL being less protected against oxidation than less dense LDL (De Graaf et al., 1990). Some of LDL preparations could not be modified at all (Van Hinsbergh et al., 1986), perhaps due to the presence of antioxidants. A high concentration of antioxidants in lipoproteins could be dependent on the content of vitamin E or other antioxidants in the diet.

Laboratory evidence now suggests that during the oxidation process, a minimally oxidized LDL, which is no longer recognized by the LDL receptor is first produced. Further oxidation produces what was originally termed oxidatively modified LDL (Berliner et al., 1990).

Other modifications of LDL can occur and play a role in atherosclerosis or formation of antibody LDL complexes. For example aggregation of LDL enhances their uptake by macrophages (Steinberg et al., 1989).

Biological properties of oxidized LDL

Table I summarizes the properties of oxidized LDL that account for its increased atherogenicity over native LDL.

Table I : Biological properties of oxidized LDL

1.	Recognized by the scavenger receptor whereas native LDL is not,
2.	Stimulates release of monocyte chemotactic proteins and of various colony-stimulating factors (CSFs),
3.	Stimulates the secretion of cytokines from macrophages and of endothelia 1 (ET1) from macrophages and endothelial cells,
4.	Inhibits endothelium-derived relaxation factor (EDRF).
5.	Cytotoxic
6.	Immunogenic

The primary biological properties of endothelial cell modified LDL was their quantitatively important uptake and degradation by macrophages and simultaneously, a decrease of its affinity for the LDL receptor (Henriksen et al.,1981 ; Henriksen et al., 1983) . The uptake and degradation of oxidized LDL delivers a sufficient amount of cholesterol into macrophages to produce foam cells.

One of the most attractive models for the deposition of lipoprotein cholesterol in macrophages is the scavenger receptor model. Macrophages express on their surfaces a receptor which can bind and mediate the internalization of oxidatively modified LDL (Kodama et al., 1988 Kodama et al., 1990; Rohrer et al., 1990; Freeman et al., 1991) The lipoprotein ligands for the scavenger receptor include chemically modified forms of LDL and oxidized LDL. This receptor is biochemically distinct from the native LDL receptor and its expression is not regulated by cholesterol. Its primary structure was determined by cDNA cloning (Fig. 1 and 2) and showed the existence of heterotrimers for Type I and type II receptors. Cysteine-rich domains which are known to be the ligand binding domains on the native LDL receptor are not responsible for scavenger receptor binding of modified LDL. Rather, it appears to be the fibrous collagen domain that plays the key role in ligand binding.

Minimally modified LDL and oxidized LDL stimulate both the release of chemotactic factors and growth factors for macrophage formation (Cushing et al., 1987; Berliner et al., 1986) for endothelial cells.

There is now also evidence that oxidized and MM LDL have a stimulatory effect on cytokine activity in macrophages. Very recently, we have shown that oxidized LDL may also induce the secretion from endothelial cells and macrophages of endothelia 1 a very potent vasoconstrictor peptide which presents a mitogenic activity on smooth muscle cells and fibroblasts (Martin-Nizard et al., 1991 ; Weissberg et al., 1990).

Because oxidized LDL inhibits EDRF it could also play a role in arterial spasm and we have recently shown that it has also in vitro arrhythmogenic effects (Simon et al., 1990).

The cytotoxicity of oxidized LDL has been demonstrated in vitro. When cultures of human endothelial cells or smooth muscle cells were exposed to even moderately oxidized LDL, cell survival was dramatically decreased (Quig et al., 1990 ; Tall et al., 1986 ; Francone et al., 1989) and the damage produced in vivo could contribute to atherosclerosis. Pretreatment of cell cultures with Vitamin E (Morel et al., 1989) or with HDL (Henriksen et al., 1979 ; Robertson et al., 1979) protects the cells against the toxic effects of oxidized LDL.

Oxidized LDL are also immunogenic and induce the formation of autoantibodies present in human and rabbit serum. The immune complexes made by autoantibodies of such oxidized LDL could be rapidly taken up by macrophages by way of the Fc receptor, another pathway for macrophages to take up oxidized LDL.

Potential Significance of Oxidative Modification of Lipoproteins

Studies by Shimano et al., (1991) have shown that a LDL subfraction resembling oxidized LDL exists in human plasma. However these modified lipoproteins do not seem to play a significant role and disappear rapidly from the plasma compartment (Nagelkerke

et al., 1984). LDL oxidation seems to take place mainly in a microenvironnment within the artery wall. We now have in vivo evidence that oxidative modification of LDL may be relevant in its atherogenicity. Using different monoclonal antibodies, several workers have detected malondialdehyde LDL and 4-hydroxynonenal-LDL in atherosclerotic lesions of WHHL rabbits, an animal model for human familial hypercholesterolemia (Haberland et al., 1988 ; Rosenfeld et al., 1990). Furthermore, the localization of epitopes specific for oxidized LDL was different from that of intact apo B100. In the fatty streaks, oxidized LDL were strongly detected in macrophages, while apo B100 was present in the extracellular space. In fibrous lesions, oxidized LDL and apo B100 co-localized in the extracellular environment (Rosenfeld et al., 1990). The results of these studies could be interpreted as follows, LDL is oxidized by the macrophages present in the fatty streak lesion, the interaction of LDL with arterial proteoglycans increasing the susceptibility of LDL to oxidative modification (Hurt-Camejo et al., 1990), and rapid uptake by these cells. When cells die, cellular oxidized LDL are released and trapped in the extracellular matrix. The small number of cells in the fibrous advanced atherosclerotic lesion does not permit the oxidation of LDL. This interpretation is consistent with the findings that relatively intact LDL and lipoprotein particles resembling oxidized LDL can be gently extracted from the normal or atherosclerotic arterial wall (Palinski et al., 1989 ; Hoff et al., 1979; Morton et al., 1986). Finally human and rabbit serum contain autoantibodies against malondialdehyde-LDL, suggesting in these subjects and animals, the presence of such modified-LDL.

In recent years, a number of reports suggested the increased presence of lipid peroxidation products in both tissue and plasma of human diabetic patients and experimental diabetic animals (Sato et al., 1979 ; Nishigaki et al., 1981; Higuchi et al., 1982; Karpen et al., 1982).

The mechanism by which lipoprotein oxidation occurs in vivo is unknown. However, in vitro evidence suggests that inflammatory leukocytes, in the presence of high levels of glucose, produce higher levels of reactive oxygen species than those exposed to normal glucose (Kitahara et al., 1980), and that these reactions could result in elevated levels of oxidized lipoproteins that can injure tissue.

Lipoprotein oxidation and lipoprotein mediated cytotoxicity associated with diabetes are inhibited by antioxidants such as vitamin E (Morel et al., 1989).

The ability of antioxidant therapy to prevent atherogenesis is currently being explored. Preliminary data obtained from β-carotene treatment of 333 men with documented coronary artery disease suggest that antioxidants can slow the progression of the disease (Gaziano et al., 1990).

Conclusion

Free radical-mediated lipid peroxidation has been implicated in the development of atherosclerosis. Oxidation of lipoprotein particles within the subendothelial space is one such modification that initiates the formation of foam cells. Antioxidants such as vitamin E that protect lipoprotein particles from oxidative processes may be clinically useful in the prevention of atherosclerosis in diabetics and patients with coronary heart disease.

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Figure 1. Scavenger Receptor Type I

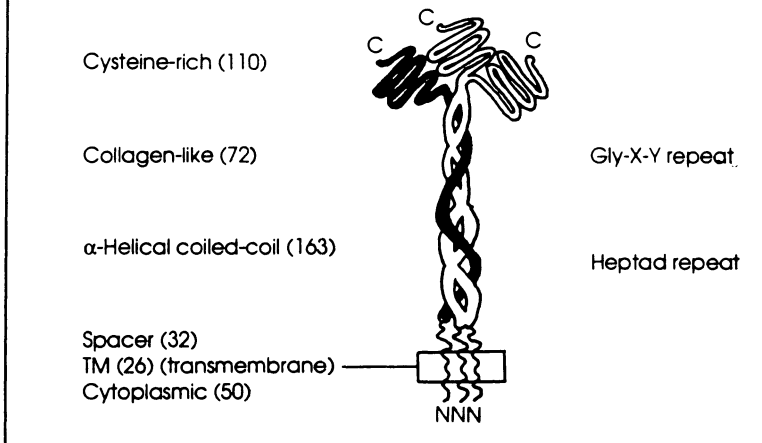
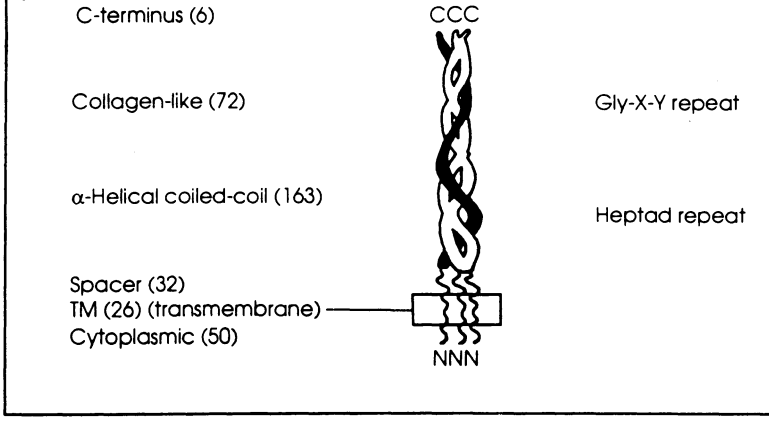


Figure 2. Scavenger Receptor Type II



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HEPATIC LIPID PEROXIDATION IN ETHANOL POTENTIATED AFLATOXIN B_1 HEPATOTOXICITY

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Summary

The possible role of hepatic lipid peroxidation in enhanced hepatotoxicity of aflatoxin B_1 (AFB_1) by ethanol was studied. Pretreatment with ethanol prior to AFB_1 administration caused a significant increase in the activities of plasma glutamic oxaloacetic transaminanse (PGOT), plasma glutamic pyruvic transaminase (PGPT) and liver triglycerides after AFB_1 administration. Hepatic lipid peroxide levels in subcellular fractions, particularly in microsomes, were significantly increased in rats treated with ethanol and AFB_1 following the hepatic Ca^{2+} and lysosomal enzymes activities and the decrease in mitochondrial respiratory enzymes activities in rats treated with ethanol and AFB_1 administration was a possible consequence of the increase in hepatic lipid peroxide. The pronounced effects of hepatic lipid peroxidation could play a role in ethanol potentiated AFB_1 hepatotoxicity in rats.

Introduction

Aflatoxin B_1 (AFB_1) is known as a highly toxic mycotoxin which is produced by many strains of Aspergillus flavus and Aspergillus parasiticus. It has been found to be one of the most potent naturally-occurring carcinogens and frequently contaminates

agricultural commodities such as peanuts, corn and feedstuffs. Aflatoxin problems become a potential hazard to human and animal health (Busby & Wogan, 1979). In addition, peanuts are commonly consumed by people in many parts of the world. In most places, peanuts are taken as a snack with alcoholic beverage (beer and whisky). It is possible that aflatoxins in peanuts will be consumed along with ethanol by human and the toxicity of aflatoxins might be modified. The ability of ethanol, administered acutely or chronically, to potentiate the hepatotoxicity of carbon tetrachloride, dimethylnitrosamine, paracetamol and other hepatotoxic agents (except aflatoxins) is well documented (Maling et al., 1975; Strubelt et al., 1978). Therefore, it is considered of interest to study the effect of ethanol on the hepatotoxicity of AFB_1.

Enhanced Hepatotoxicity of Aflatoxin B_1 by Ethanol

Hepatotoxicity of AFB1 as determined by plasma enzymes activities (GOT and GPT), liver triglycerides and histopathologic changes was enhanced in male and female Fischer rats pretreated with ethanol prior to AFBI administration (Glinsukon et al., 1978; Toskulkao et al., 1982; Toskulkao & Glinsukon, 1983). According to sex difference, male Fischer rats were more susceptible to ethanol induced AFB1 hepatotoxicity (Toskulkao & Glinsukon, 1983). Pretreatment with ethanol and treated with a single lower dose of AFB_1 was found to induce a significant increase in the activities of plasma GOT and GPT higher than those observed in female Fischer rats. The synergistic hepatotoxicity of ethanol and AFB1 was also supported by histopathologic examination. The hepatic lesions reveal the periportal zone necrosis with mild fatty infiltration were also observed in the rats treated with ethanol and AFB_1.

To clarify whether ethanol or its metabolite, acetaldehyde, is responsible for the potentiation of AFB_1 hepatotoxicity. The experiments were carried out by using pyrazole, an inhibitor of alcohol dehydrogenase to inhibit the biotransformation of ethanol to acetaldehyde. Pyrazole markedly increased by approximately 85% of the mortality rate of the rats treated with ethanol and AFB_1. However, pyrazole itself was not toxic to the

rats. In addition, rats treated with acetaldehyde and AFB_1, acetaldehyde caused neither significant change in the activities of plasma GOT and GPT nor liver triglycleride when compared to AFB1 treated rats (Toskulkao & Glinsukon, 1983). From these results, ethanol by itself enhances hepatotoxicity induced by AFB1 in rats.

According to the strain of rats, male Wistar rats were more susceptible to AFB_1 induced hepatotoxicity when compared with male Fischer rats (Toskulkao et al., 1986). Ethanol pretreatment prior to AFB_1 administration also caused a significant increase in the activities of plasma GOT and GPT, liver triglyceride and the severity of liver necrosis when compared to AFB_1. However, the activities of plasma GOT and GPT and liver triglyceride of rats treated simultaneously with ethanol and AFB_1 were not increased when compared to those rats treated with AFB_1, even with the higher doses of ethanol and AFB_1. Recently, Toskulkao et al.,(1991) reported the effect of ethanol pretreatment on acute toxicity and hepatic fat accumulation induced by AFB_1 in male Wistar rats. Ethanol pretreatment prior to AFB_1 administration caused a significant increase in the activities of plasma GOT and GPT, liver triglycerides and the severity of liver necrosis when compared to AFB_1 alone. The effect of ethanol on an increase in the accumulation of liver cholesterol and cholesterol esters induced by AFB_1 is additive. It was shown that liver necrosis and triglyceride, cholesterol and cholesterol esters accumulation occurred simultaneously in rats treated with AFB_1 and ethanol-AFB_1. These results suggest that fat accumulation is not a primary cause of liver necrosis induced by AFB_1 and ethanol-AFB_1. In addition, the ultrastructural changes in hepatocytes induced by AFB_1 and ethanol-AFB_1, were also studied by Sahaphong et al., (1991). The degree of nuclear edema, rough endoplasmic reticulum dilatation and mitochondrial swelling in hepatocytes from rats treated with ethanol and AFB_1 is much greater than that of the hepatocytes from rat treated with AFB_1 or ethanol alone.

Hepatic Lipid Peroxidation in Ethanol Potentiated Aflatoxin Bl Hepatotoxicity

The mechanism of acute toxicity of AFB_1 is not yet understood, but the initiation of carcinogenesis is thought to follow binding to DNA (Garner & Martin, 1979). It is not yet known whether or not lipid peroxide is involved in the AFB_1 hepatotoxicity. In fact, several GSH depleting compounds promoted lipid peroxidation and subsequent cellular lysis (Anundi et al., 1979; Younes & Siegers, 1980). Toskulkao & Glinsukon (1988) showed that AFBl caused a decrease in hepatic GSH content in a time-dependent fashion by which the maximal depletion occurred at 12 h. The fall in GSH content was followed by a temporary rebound to a level above those of the control in a similar report by other investigators (Corongin & Milia, 1982). Hepatic GSH content was further decreased in rats treated with ethanol and AFB_1 when compared to rats treated with AFB_1 alone at 12 h. This in turn subsequently induced a significant increase in hepatic lipid peroxide levels at 24 h in whole homogenate, microsome and cytosol with the exception of mitochondria (Figure). It is likely that an increase in hepatic lipid peroxide levels in various subcellular fractions are a consequence of GSH depletion may be due to an increase in the amount of AFB_1 reactive metabolite, AFB_1-8,9-oxide, in rats treated with ethanol and AFB_1 (Toskulkao & Glinsukon, 1986). Although a possible mechanism by which AFB_1 induced lipid peroxidation is still not elucidated, it could play a part in ethanol-induced potentiation of AFB_1 hepatotoxicity. Peroxidation of membrane lipids initiated the loss of membrane integrity and membrane bound enzymes activities which, in turn, brought about a disturbance of cellular homeostasis. Excessive Ca^{2+} influx has been suggested as a final common pathway leading to cellular death (Farber, 1981). A large increase in intracellular Ca^{2+} has been reported in liver cells injured by various substances (Farber et al., 1977; Moore, 1980), but no reports concerning AFB_1. Toskulkao and Glinsukon (1988) reported that AFB_1 could maximally increase hepatic Ca^{2+} in whole homogenate, mitochondria, microsome and cytosol at 48 h after administration. Hepatic Ca^{2+} accumulation was further increased in rats treated with ethanol and AFB_1. A massive

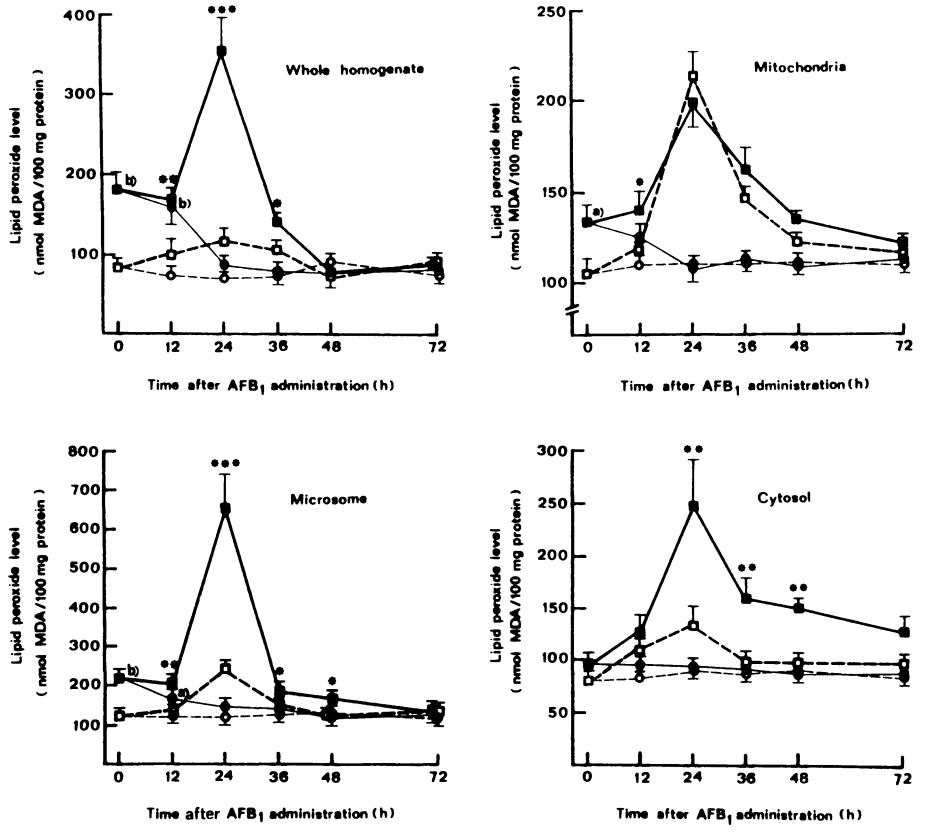

Figure.
Time-course of changes in lipid peroxide of hepatic subcellular fractions in rats pretreated with 4 oral doses of water or ethanol (4.0 g/kg BW) at 48, 45, 24 and 21 h prior to i.p. of AFB_1 (2.0 mg/kg BW). Rats were sacrificed at the time indicated. Values are means ± S.E. of 5 rats.
○, water-DMSO; ●, ethanol-DMSO; □, water-AFB_1; ■, ethanol-AFB_1
$p<0.05$, bp<0.0l, significantly different between water-DMSO and ethanol-DMSO.
*$P<0.05$, **$P<0.01$, ***$P<0.001$, significantly different between water-AFBl and ethanol-ABF1.
(Data, reprinted from C. Toskulkao & T. Glinsukon, 1988).

and progressive accumulation of Ca^{2+} was observed at 48 h after AFB_1 administration in rats treated with ethanol and AFB_1 as a consequence of an increase in the hepatic lipid peroxide level. Thus, the excessive accumulation of hepatic intracellular Ca^{2+} may be responsible for potentiation of hepatotoxicity in rats treated with ethanol and AFB^{2+}. However, this is rather a secondary effect of the dysfunction of mitochondria caused by lipid peroxidation and reduction of hepatic ATP content, which participates in Ca^{2+} extrusion and uptake mechanisms (Carloli & Tiozzo, 1968).

Hepatic injury or necrosis is related to the degree of inhibition of mitochondrial respiratory functions and the total ATP content (Obidoa & Obunwo, 1979). AFB_1 caused a maximum decrease in cytochrome C reductase activity at 36 h in rats. The hepatic ATP content also decrease in a similar pattern to those of mitochondrial enzymes activities. It is now certain that AFB_1 inhibits enzymes of the mitochondrial respiratory system and ATP production in liver cells which is caused by an increase of lipid peroxidation in mitochondrial membranes (Figure). In rats treated with ethanol and AFB_1, the activity of cytochrome oxidase did not change whereas NADH-cytochrome C reductase activity and ATP content were significantly increased when compared to rats treated with AFB_1 alone. Thus, the ATP content in rats treated with ethanol and AFB_1 decreased greater than in rats treated with AFB_1 alone due to the decrease in the activity of NADH-cytochrome C reductase. The major sites of inhibition of electron transport are localized at the second phosphorylation coupling sites. The degree of mitochondrial lipid peroxidation in rats treated with ethanol and AFB_1 was not different from rats treated with AFB_1 alone. The decrease in the activity of NADH-cytochrome C reductase in rats treated with ethanol and AFB_1 is due to the direct effect of AFB_1 on to this enzyme. The significant decrease in ATP content could play a part in ethanol potentiated AFB_1 hepatotoxicity. The lysis of subcellular organelles can often be attributed to lipid peroxidation in their membranes (Nagai et al., 1981; Ribarov & Benov, 1981). It has never been reported whether AFB_1 is capable of producing lipid peroxidation in lysosomes. Toskulkao and Glinsukon (1990) demonstrated that AFB_1 caused the most pronounced effect on the formation of lipid peroxide in rat liver lysosomes at 24 h after

AFB_1 administration. The activities of free lysosomal enzymes were markedly increased to maximum levels at 24-36 h after AFB_1 administration (Toskulkao & Glinsukon, 1990). In rats treated with ethanol and AFB_1, lysosomal lipid peroxide level and free lysosomal enzymes activities were significantly higher than rats treated with AFB_1 alone. The induction of lipid peroxidation by AFB_1 in accompany with a strong enhancement of lysosomal enzymes release is a time and concentration dependent. The increase in the activities of lysosomal enzymes is also responsible for ethanol potentiated AFB_1 hepatotoxicity.

Conclusion

Ethanol pretreatment enhanced hepatotoxicity of AFB_1 by raising reactive metabolites (Toskulkao & Glinsukon, 1986) after depletion of GSH content, a decrease in epoxide hydrolase activity and an increase in drug metabolizing enzymes activities (Toskulkao et al., 1986), to attack DNA and membranes of various organelles which lead to a reduction in hepatic ATP content and Ca^{2+} efflux.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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PALM OIL VITAMIN E EFFECTS IN HYPERCHOLESTEROLEMIA

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Summary

It has been shown that there is a relationship between atherogenesis and coronary artery disease (CAD) , elevated concentration of serum cholesterol, dietary fats, and cholesterol. The reduction of serum cholesterol concentration can be achieved by administering cholesterol lowering substances and by controlling dietary fat intake. Earlier we have demonstrated that Palmvitee capsules lower the serum cholesterol in human subjects. More recently we studied the effect of Palmvitee capsules and fiber preparation on the lipid profile of healthy Finnish male adolescents eating normal diets.

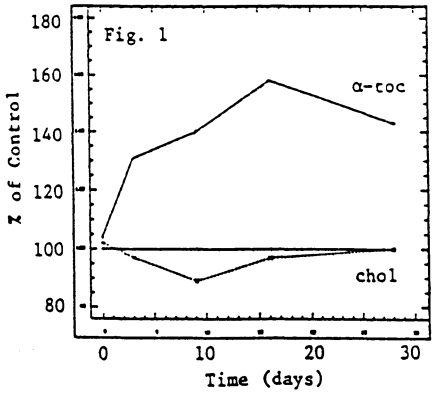
Hypercholesterolemia

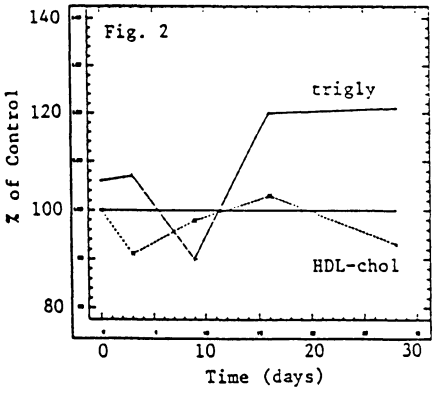
Hypercholesterolemia encompasses a heterogenous group of disorders in lipid metabolism characterized by elevated levels of plasma total cholesterol and low density lipoprotein (LDL)-derived cholesterol. It is one of the few independent risk factors definitively linked to increased morbidity and mortality due to myocardial infarction and considerable effort has been directed towards the development of effective cholesterol lowering agents. Cholesterol enters the body pool from two sources, either by absorption from the diet (exogenous source), which accounts for 300-500 mg per day in humans, or endogenous biosynthesis (700-900 mg per day) (Sliskovic & White, 1991).

Vitamin E in Atherogenesis

Natural vitamin E is a combination of tocopherols and tocotrienols. Vitamin E is present in crude palm oil (600-1000 ppm) and in refined palm oil (470-670) together with β-carotene and vitamin A. The major components of palm oil vitamin E are α-tocopherol, α-tocotrienol, τ-tocotrienol and δ-tocotrienol. Palm oil is commonly classified as saturated fat, containing palmitic acid (44%), oleic acid (39%) , linoleic acid (10%) and stearic acid (5%). The tocotrienol rich fraction of palm oil has recently become available in the form of gelatinous capsules, Palmvitee (Porim, Malaysia). One capsule contains 50 mg of the tocotrienol rich fraction including α-tocopherol (15-20%), α-tocotrienol (12-15%), τ-tocotrienol (35-40%) and δ-tocotrienol (25-30%).

We have examined the effect of Palmvitee capsules on serum cholesterol level in Finnish healthy adults (Atroshi et al., 1989, 1990, 1991). The double blind follow-up studies have lasted from four to six weeks. The supplementation dose has been from 1 to 4 capsules per day, corresponding to 18 to 72 mg of α-tocopherol and 42 to 168 mg of α-, τ-, and π-tocotrienols. Palmvitee supplementation showed, somehow, a reduction in total cholesterol and LDL-cholesterol and to elevate serum α-tocopherol level as seen in Figure 1 and 2. Tocotrienols were detected in traces.





Results from other studies on Palmvitee supplementation on human subjects in Malaysia (Tan & Khor, 1989; Tan et al., 1990), and in USA (Qureshi et al., 1989; Qureshi et al., 1991) have shown that Palmvitee supplementation lowers cholesterol level, apo B, thromboxane, and platelet factor concentrations in the blood.

There are several theoretical benefits of vitamin E supplementation in hypercholesterolemia and subsequent cardiovascular diseases. In addition to those involved in LDL cholesterol metabolism, the inhibitory effect of vitamin E on platelet aggregation, platelet adhesion (Steiner, 1983), eicosanoid metabolism, aryl sulfatase B inhibition and platelet-vascular interactions (Betteridge, 1987) have helped to retard the formation of arterial thrombosis. However, it should be remembered that some of the effects of different forms of vitamin E may be diverse. Absorption, transport and tissue uptake are specific for α-tocopherol. Alfa-tocopherol is the form of vitamin E that increases the most in the serum after oral intake. Finally, if vitamin E was conclusively shown to raise HDL-cholesterol in humans and in animals (Imaizumi et al., 1990), then this could further reduce the risk of atherosclerosis.

Antioxidants and Free Radical Stress in Atherogenesis

A number of antioxidant mechanisms prevent oxidative mechanisms by the reactive products of oxygen that are formed during normal metabolic events (Halliwell and Gutteridge, 1986). The primary intracellular defense mechanisms against this type of damage are superoxide dismutase, selenoenzyme glutathione peroxidase, catalase, and vitamin E localized in the lipid membrane. In the control of free radical formation and lipid peroxidation vitamin E and selenium may have similar functions. Despite its low molar concentration in membranes, vitamin E effectively serves as the major lipid-soluble, chain-breaking antioxidant (Oski, 1980). Vitamin E is known to be as the first line of defence against lipid peroxidation, protecting polyunsaturated fatty acids, the precursors for prostaglandins formation, in cell membranes through its free radical quenching activity

in biomembranes at an early stage of free radical attack (Van Gossum et al., 1988). The decomposition of hydrogen peroxide to water and oxygen can be catalysed by the enzymes catalase and glutathione peroxidase. The subsequent reduction of hydrogen peroxide, or lipid peroxides by glutathione peroxidase is accompanied by the oxidation of glutathione, resulting in the formation of glutathione disulfide. The myocardial concentrations of free radical scavengers such as glutathione, glutathione peroxidase and superoxide dismutase are reduced by hypoxia, which may increase the susceptibility of the hypoxic cells to injury by free radicals during reoxygenation of the tissues (Guarnieri et al., 1980).

Monounsaturated fatty acids may block the desaturation/elongation reactions, leading from linolenic to arachidonic acid. In vitro linoleic acid reacts with molecular oxygen 20 times more rapidly than oleic acid. This indicates the necessity of monounsaturates in lipid bilayers, where they may act even in a manner similar in principle to vitamin E. It has been shown that lipid peroxides accelerate the reactions catalyzed by cyclo-oxygenase and lipoxygenases in endothelial cells and platelets.

The formation of oxidatively modified LDL gives rise to high uptake of LDL not mediated by LDL receptors. In familial hypercholesterolemia the risk for oxidatively modified LDL is further increased. Goldstein et al.,(1984) have been showing that only oxidized and denatured LDL is incorporated and metabolized in macrophages. Capability of LDL-particles to resist free radical stress depends on the content of lipophilic antioxidants such as tocopherols and tocotrienols. Tocopherol content is shown to be very rapidly depleted. For example, in vitro incubation has shown a 97% drop within 6 hours. However, most of the circulating vitamin E is found in LDL-fraction (Traber & Kayden, 1984); thus one of the functions of LDL may be to provide vitamin E to the cells.

In addition to vitamin E many lipophilic antioxidants may prevent the oxidative modification of LDL. Some flavonoids (morin) have a sparing effect on α-tocopherol consumption and thereby protects LDL (DeWhalley et al., 1990 Jessup et al., 1990). In a cell free system where peroxidation of LDL was initiated by copper (II) even ascorbic acid seems to be more effective than α-tocopherol (Jialal et al., 1990). At the present,

the role of plasma antioxidants in the recycling of LDL vitamin E from chromanoxyl radicals remains an open question.

Free radicals may exert diverse biochemical effects on both intracellular and extracellular molecules, and there is extensive evidence of their involvement in the cardiovascular diseases. Free radicals may damage viable tissue via the peroxidation of lipids and oxidation of protein sulfhydryl groups, leading to perturbations of membrane permeability and enzyme function. Unesterified cholesterol may interact in the polar milieu behaving as both H donor and H acceptor. Furthermore, cholesterol has the ability to condense, i.e. restrict, the volume of phospholipid layers. This means that cholesterol decreases membrane fluidity i.e. increase the solidity of membrane (Esterbauer et al., 1987). Experimental results of cholesterol loading on platelets have indicated an increase in platelet membrane microviscosity which may affect the activity of phospholipase A responsible for the arachidonic acid release (Szczzeklik et al., 1981).

All human cells possess the capability for de novo synthesis of cholesterol. Human cells still accept and prefer exogenous and liver-derived cholesterol in the form of LDL which is particularly associated with sites of cellular damage and repair when cholesterol requirements are temporarily and rapidly increased (Stehbens, 1990). The control of cholesterol synthesis as well as that of ubiquinone and dolichol takes place through HMG-CoA-reductase. This enzyme is regulated by negative feedback mechanisms through end products, and enzymatically through HMG-CoA-reductase kinase. Normally uptake of LDL-cholesterol and nonsterol product derived from mevalonate keep HMG-CoA-reductase suppressed (Goldstein,et al., 1984). In addition to real end products as ubiquinone a variety of compounds possesses the ability to affect HMG-CoA-reductase, these include lovastatin and tocotrienol. Qureshi et al., (1991) have suggested that unlike lovastatin tocotrienois do not inhibit the enzyme activity but merely decrease the translation efficiency of HMG-CoA reductase mRNA.

LDL-cholesterol provided for the cells is internalized and metabolized in lysosomes. Some processes are promoted by vitamin E. Preincubation of vitamin E with LDL or

arterial wall increases cholesterol esterase activity. Conversely, cholesterol esterase activity is decreased in vitamin E deficiency. Stimulation of LDL metabolism may result also through vitamin E protection of peroxidative attack as reviewed by Saito & Morisaki (1989).

Conclusion

There are several theoretical promises for the benefits of vitamin E supplementation in hypercholesterolemia and subsequent cardiovascular diseases. In addition to those involved in LDL cholesterol metabolism, the inhibitory effect of vitamin E on platelet aggregation, platelet adhesion, eicosanoid metabolism, arylsulfatase B inhibition and platelet-vascular interactions might help retard the formation of arterial thrombosis.

We consider that habitual intakes of large quantities of polyunsaturated fat and recommendations to lower cholesterol are not always the right way to effect the risk for cardiovascular disease. In order to prevent atherogenesis a question will arise as to the mechanisms of LDL oxidation-internalization in the arterial wall and feedback regulation at the organismic level.

Palm oil tocotrienol has so far proven beneficial as a vitamin E nutrient in this sense. Daily intake as crude palm oil may be replaced by refined palm oil capsules, Palmvitee, which contains tocopherols and tocotrienols in a palm superolein mixture. Its measurable effects may depend on the diet and LDL-cholesterol status of the subject.

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FAT SOLUBLE ANTIOXIDANT VITAMINS IN CANCER PATIENTS

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Summary

Serum fat-soluble antioxidant vitamins A,E and carotenoids (Lutein, β-Cryptoxanthin, Lycopene, β-Carotenes) in 14 cancer patients were compared with 12 healthy subjects. All vitamins were measured by standard method using high performance liquid chromatography. Cholesterol as the indicator for fat intake was also measured by kit assay and the ratio of vitamin E : cholesterol was calculated. Total radical-trapping antioxidant activity (TRAP) and Carcinoembryonic antigen (CEA) were also measured. The results revealed that serum vitamin A in the cancer patients was lower than in control, 1.01 ± 0.58 vs 1.55 ± 0.68 μM ($p < 0.025$); vitamin E : cholesterol was 3.42 ± 1.41 vs 4.59 ± 1.29 ($p < 0.025$). Carotenoids, lutein was 0.36 ± 0.16 vs 0.71 ± 0.38 μM $p < 0.005$); β-cryptoxanthin was 0.16 ± 0.28 vs 0.14 ± 0.08 μM ($p = 0.8$); lycopene was 0.06 ± 0.09 vs 0.07 ± 0.05 μM ($p = 0.76$); α-carotene was 0.05 ± 0.04 vs 0.1 ± 0.1 μM ($p < 0.05$); and β-carotene was 0.16 ± 0.12 vs 0.32 ± 0.26 μM ($p < 0.05$). The total carotenes in patient group was lower than control, 1.57 ± 1.1 vs 2.32 ± 1.09 ($p<0..05$), due to lutein and β-carotene. There was no difference in TRAP activity among those 2 groups. CEA was positively correlated with TRAP activity ($r = 0.542$, $p < 0.05$). In conclusion, there is some evidence from this study to suggest that lower antioxidant vitamins A, E, carotenes in serum of the cancer patients, may be a risk factor for cancer aetiology among Thais.

Introduction

Many studies on the aetiology of cancer in relation to nutrition have been published. There is evidence that carcinogens are in some instances derived from foods, while dietary vitamins can inhibit the activities of certain mutagens (1,2,3). These observations have

always been clear in animal experiments, but it is difficult to find supporting evidence in humans.

The generation of oxygen radicals in vivo causes lipid peroxidation which may affect all aspects of cell organization (4,5) including the degeneration of the DNA system. This has been accepted as a mechanism of carcinogenesis. A variety of small molecules in the diet are known to act directly as antioxidants or contribute to antioxidant mechanisms. These include alpha-tocopherol, carotenoids, selenium, glutathione, ascorbic acid and uric acid.

The aim of this pilot study was to measure the total antioxidant capacity of serum obtained from patients with various types of cancer by measuring the total radical - trapping antioxidant activity, and the presence in it of specific antioxidants, such as vitamin E and β-carotene.

Vitamin A, which is known to control cell differentiation, was also measured. The study was conducted in patients and control subjects from Northern Thailand.

Materials and Methods

Specimen collection

Blood was collected from 14 cancer patients who had been admitted to the Chiang Mai University Hospital. Details on the individual patient are shown in Table I. Five ml of venous blood was drawn into glass bottles and left at room temperature for 30 minutes. Serum was obtained after centrifugation at 3,000 rpm for 10 minutes, followed by storage at -50°C before analysis. Control sera was obtained from 12 apparently healthy hospital staff (5 men and 7 women, with a median age of 40 years).

Fat-Soluble Vitamins Analysis

Vitamins E, A and carotenoids, were measured on Waters high performance liquid chromatography apparatus. Serum (0.25 ml) was mixed with 0.25 ml 10 mmol/L sodium dodecyl sulphate, 0.5 mL ethanol containing internal standard (tocopherol acetate) and

extracted into 1.0 mL heptane. After evaporation of heptane extract and reconstitution in the mobile phase (acetonitrile : methanol : chloroform, 47:47:6), 25 μL of the solution was injected onto a 60 mm, 3 μm Spherosorp ODS-2 cartridge chromatography column (LKB) and eluted at 0.5 mL/min. A Waters 490 spectrophotometric detector was set up at 325 nm up to measure vitamin A and then changed to 292 nm at 2.5 minutes to measure α-tocopherol and α-tocopherol acetate. On a second channel of the detector, total absorbance at 450 nm was monitored to measure the concentrations of carotene fractions and total carotenes were calculated from the total area of all substances measured at 450 nm using the β-carotene response factor.

Determination of the Carcinoembryonic Antigen (CEA)

The CEA-EIA test (Roche) which is a solid phase enzyme immunoassay based on the sandwich system was used.

TRAP Measurement

The TRAP assay based on that described by Thurnham et al., who modified it from that of Burton and colleagues (7, 9). 250 μL serum and vortexed for approximately 15 seconds with 10 μL linoleic acid. 50 μL of this mixture was placed in a reaction chamber containing 5 mmol/L potassium phosphate buffer pH 8.0 in normal saline at 37°C (Oxygen-Monitor Instrument Model 5331, Yellow Springs Instrument Co.).

Results and Discussion

There are three types of cancer in this group of study, gastrointestinal (stomach, colon and rectum), breast and lung. Table I shows the age range (19-80 years), sex (7 men, 7 women) and the severity of cancer as assessed by the measurement of carcinoembryonic antigen (CEA). CEA varied from 1.5 to greater than 20 ng/ml. The three highest CEA values 7.8, >20, >20 ng/ml were in cases of rectal cancer, even the number of cases is small, but it agrees with other reports (9, 11, 12).

Table II shows the concentrations of all the measurements of vitamin A status which tended to be lower in the cancer patients. The difference between the two groups would probably have been more significant if the number of patients had been greater. The wide range of results indicates that vitamin A status was quite variable in both groups. In the case of the cancer patients there were 14% (2/14) who were deficient in vitamin A (<0.35 μmol/L = 10 mg/dl), and there were several in this group whose carotenoid intake was negligible on the bases of the serum carotenoid measurements.

Low vitamin A and β-carotene concentrations have been implicated in the aetiology of carcinogenesis. This study suggests that serum concentration of these nutrients is low in Thai persons. In other work on adult Thai women reported from the same area however, it is suggested that the low concentrations of vitamin A nevertheless indicate normal vitamin A status since only 2% (1/49) of the cases showed any increase in serum vitamin A concentrations following the Relative Dose Response test (RDR). The RDR test is however a measure of liver vitamin A storage, the low circulating concentrations of vitamin A in Thai people may be sub-optimal for cancer prevention.

In the case of the serum carotenoid concentrations, the total amount present is similar o that reported in the West (15), but the amount of β-carotene is lower in both Thai control and patient groups. It is possible that low carotenoid concentrations may be more relevant in the aetiology of cancer risk (10,17), and the low concentrations of β-carotene reported in this study should be examined more closely in the Thai population for its possible importance as a risk factor in the aetiology of cancer.

Serum TRAP activity reported in Table II and shows that mean values in the cancer patient and control subjects were not significantly different, but was correlated with CEA values ($r = .542$, $p < 0.05$). But the relevance of this observation is questionable, since we would expect TRAP activity would be lower in those with more severe disease if the oxidation stress of free radical production was of importance. Unfortunately, it was not possible to obtain TRAP values in this group of cancer patient prior formation of cancer.

Table I

Carcinoembryonic antigen (CEA) concentrations in various types of cancer patients

Case No.	Site of Cancer	Age	Sex	CEA
		(year)		(ng/ml)
967	Colon	42	Male	3.3
972	Colon	59	Female	1.5
975	Rectum	65	Female	7.3
978	Rectum	56	Female	>20
991	Breast	53	Female	2.9
992	Breast	36	Male	2.1
993	*	75	Male	2.1
995	Lung	80	Male	10.8
997	Lung	19	Male	3.0
998	Lung	80	Male	4.0
1001	Stomach	*	Female	5.1
1005	Rectum	80	Male	>20
1007	Colon	48	Female	1.9
1009	Lung	62	Male	1.9

*Information can not be obtained

Table II
Serum vitamins E, A, carotenoid concentrations in cancer patients and healthy control subjects

	CANCER (n=14)	CONTROL (n=12)	T-test
Variables	Mean ± S	Mean ± SD	p-value
Vitamin A (μmol/L)	1.01 ± 0.58	1.55 ± 0.68	<0.025
Lutein (μmol/L)	0.36 ± 0.16	0.71 ± 0.38	<0.005
β-Cryptoxanthin (μmol/L)	0.16 ±0.28	0.15 ± 0.08	NS
Lycopene (μmol/L)	0.06 ± 0.09	0.07 ± 0.05	NS
Vitamin E (μmol/L)	15.42 ± 8.15	19.03 ± 7.48	NS
Cholesterol (mmol/L)	4.32 ± 1.41	4.02 ± 0.82	NS
Vitamin E: Cholesterol	3.42 ± 1.41	4.59 ± 1.30	<0.025
α-Carotene (μmolL)	0.05 ± 0.04	0.10 ± 0.11	<0.05
β-Carotene	0.17 ± 0.12	0.32 ± 1.09	<0.05
Total Carotenes (μmol/L)	1.57 ± 1.10	2.32 ± 1.09	<0.05
TRAP	503 ± 308	516 ± 335	NS

Vitamin E concentration was marginally lower in the cancer patients than of the control subjects but there was no difference in vitamin E status of the two groups unless the vitamin E : cholesterol ratios were considered, and the patient;s were significantly lower than the controls ($p < 0.025$).

It has been reported that TRAP activity in the serum is derived mainly from the concentration of urate in serum (65% - 70%) (10). Vitamin E contributes less than 15%; therefore concentrations are probably masked by high concentrations of urate, which were not measured in this study.

In conclusion, there is some evidence from these studies that free radical activity is increased in these cancer patients since TRAP values were similar to those in the control subjects. However, vitamin E and carotenoid concentrations were marginally lower in the cancer cases than the control subjects and may possibly be of importance in cancer aetiology.

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BUTYLATED HYDROXYTOLUENE TOXICITY

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Summary

The phenolic antioxidant butylated hydroxytoluene (BHT) causes toxic effects in experimental animals including hemorrhage, lung,liver and kidney injury, and tumor promotion. ADI values of 0 - 0.125 (WHO) and 0 - 0.05 (EEC) mg/kg body weight have been derived from toxicological animal studies. Two aspects of the possible toxic mechanisms are discussed: 1) BHT interferes with certain components of calcium homeostasis and signal transduction. This may contribute to its hemorrhagic action. 2) During BHT metabolism, reactive oxidized BHT products and reactive oxygen species are formed. Among these, BHT hydroperoxide and/or BHT quinone methide are involved in the toxic as well as in the tumor-promoting activity of BHT.

Introduction

Butylated hydroxytoluene (BHT) has been introduced as an antioxidant for industrial use as well as for food preservation in the 1950's but has only recently attracted widespread attention. On the one hand, reports on the potential of antioxidants, including BHT, to prolong the life span of some animal species and to inhibit the toxic, mutagenic and carcinogenic action of other chemicals (reviewed in Kahl, 1984) have encouraged the public to consume antioxidants as a prophylactic means against aging and disease. BHT

capsules are available in health food stores in the United States and probably in other countries, and a few reports are available which describe neurotoxic symptoms possibly related to the ingestion of gram amounts of BHT taken for this purpose (Shlian & Goldstone 1986; Grogan 1986). On the other hand, BHT present in food has caused concern in health agencies because of the intrinsic toxicity of BHT expressed in animal experiments and, most notably, because of its tumor-promoting and carcinogenic properties. Consequently, the GRAS (generally recognized as safe) status given to BHT has been specified in a restrictive manner by lowering the old ADI (acceptable daily intake) value set by the WHO from 0.5 to 0.125 mg/kg body weight (Joint FAO/WHO Expert Committee on Food Additives, 1986). In the EEC, an even more restrictive ADI value of 0.05 mg/kg body weight is recommended (Commission of the European Communities, 1987) (Table I). Data on the consumption of BHT can tentatively be estimated from its production and sale and from the eating habits of the population. Such data (Kirkpatrick & Lauer 1986; Commission of the European Communities, 1987) as well as figures on the concentration of BHT in human body fat (Conacher et al., 1986) are collected in Table I.

Table I

Chemical structure and general aspects of BHT

Temporary ADI
WHO: 0 - 0.125 mg/kg
EEC: 0 - 0.05 mg/kg
Concentration in body fat 0.07-0.19 mg/kg
Estimated daily intake (BHT + BHA + Gallates)
< 0.4 mg/kg (Canada)
< 14 mg/person (probably 1 mg/person) (EEC)

The acute or subchronic administration of BHT to experimental animals results in intoxication symptoms which differ among species. Table II lists the main features of BHT toxicity with respect to the species in which they were reported.

Table II

Features of BHT toxicity
Bleeding disorders (rat, mouse, guinea pig, hamster, quail)
Lung injury (mouse)
Liver weight increase; liver injury (rat, rhesus monkey)
Disturbance of renal function; kidney injury (rat, rabbit)
Thyroid weight increase (rat, pig)
Retardation of development (rat)

Among these, the inhibition of blood clotting in the rat with a LOEL (lowest observed effect level) of 0.017% in the food (14.7 mg/kg body weight) (Takahashi & Hiraga, 1978a) and the increase in thyroid weight in the pregnant pig with a LOEL of 50 mg/kg body weight (Hansen et al., 1982) constitute the basis for the ADI values.

Three aspects of BHT intoxication have been subjected to indepth examination: hemorrhage, lung injury and liver injury. The coagulation disorder induced by BHT has been thoroughly studied by Suzuki and his coworkers and by Takahashi and Hiraga and their coworkers. Its severity differs among species, being most marked in the rat in which massive hemorrhage is the cause of death upon subacute BHT administration (Takahashi & Hiraga, 1978b; Suzuki et al.,1979) . Hemorrhage does also occur in mice and guinea pigs. In the hamster and in the quail, asymptomatic hypothrombinemia was observed (Takahashi et al.,1980) . The main mechanism is assumed to be inhibition of

vitamin K epoxide reductase resulting in decreased synthesis of the clotting factors II, VII, IX and X (Takahashi 1987). An effect on platelet function (Takahashi & Hiraga, 1984) and on vitamin K absorption (Suzuki et al., 1983) have also been described.

BHT-induced mouse lung injury has been characterized extensively enough to serve as a model syndrome for lung toxicants. A recent review provides an indepth description (Witschi et al., 1989). However, this syndrome appears to occur in the mouse only and thus has gained little impact on risk assessment of BHT as a food additive.

In the liver, proliferation accompanied by a characteristic pattern of enzyme induction is caused by BHT feeding (reviewed in Kahl, 1984). Phase II enzymes of drug metabolism are synthesized at an increased rate, among them glutathione transferase (Benson et al., 1978), glucuronyl transferase (Cha & Heine, 1982), epoxide hydrolase (Kahl & Wulff, 1979) and quinone reductase (Benson et al., 1980). Phase I drug metabolism is less affected. This leads to a predominance of detoxication reactions over activation reactions in the metabolism of a number of carcinogens and is assumed to be one of the factors involved in the anticarcinogenic activity of BHT. At high dosage (500 mg BHT/kg body weight and more) liver injury with enzyme leakage, inflammation, centrilobular necrosis, fibrosis and bile duct proliferation can occur (Nakagawa et al., 1984; Powell et al., 1986).

The molecular and cellular mechanisms by which BHT exerts its toxic effects have not completely been elucidated. In recent years, the perception has been growing that endogenous oxidants are involved in the regulation of multiple biological functions. Consequently, antioxidants may interfere with such functions in an opposite manner. This should result in protection from increased oxidant concentrations but may cause deterioration of physiological function at normal oxidant levels. Fig.1 demonstrates attacks of BHT in calcium homeostasis and signal transduction and some of their consequences. It should be noted that the events described in Fig.1 have not been observed in one cell type and at one BHT concentration. Two mechanisms by which BHT can decrease free cytosolic calcium are shown. In brain synaptosomes, BHT (100 uM) inhibits Ca^{2+} uptake via the voltage-operated calcium channel (Zoccarato et al., 1987). In platelets, calcium sequestration induced by agonists of the inositol triphosphate

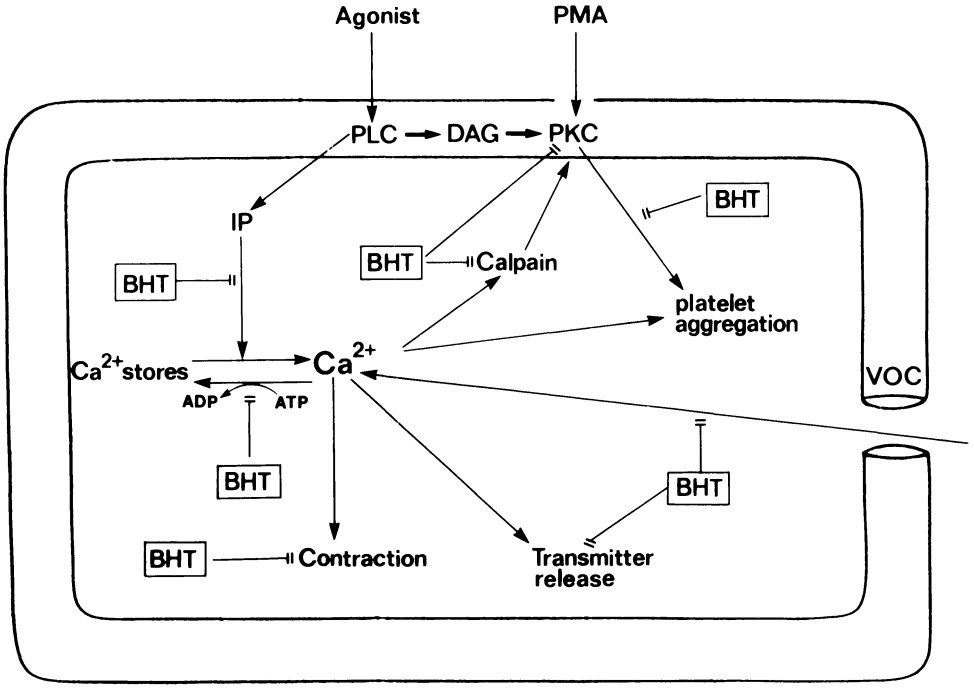

Fig.1 Attack of BHT in intracellular calcium homeostasis and signal transduction. DAG: diacylglycerol; IP3: inositol triphosphate; PKC: protein kinase C; PLC: phospholipase C; PMA: phorbol myristate acetate; VOC: voltage-operated calcium channel.

mechanism is inhibited by BHT (50 pM)(Alexandre et al., 1986). Notably, an effect acting in the opposite direction has also been established: BHT inhibits the sarcoplasmic Ca^{2+}-ATPase in skeletal muscle with an EC_{50} of 5 μM (Sokolove et al., 1986). Inhibition of Ca^{2+} translocases has not only been described for other phenolic antioxidants (Fewtrell & Gomperts, 1977) but is also a well known component of oxidant cell damage (Nicotera et al., 1985). This demonstrates that the concept of an antagonistic function of oxidants and antioxidants in cellular regulation processes may be oversimplified.

Among the consequences of decreased Ca^{2+} availability in the cytosol depicted in Fig.1, the inhibition of platelet aggregation occurring concomitantly with the inhibition

of agonist-dependent Ca^{2+} sequestration (Alexandre et al., 1986) may be directly related to the hemorrhagic disorder which occurs in various species upon administration of BHT: While inhibition of vitamin K epoxide reductase is assumed to be the main mechanism of bleeding (Takahashi 1987) a disturbance of platelet function has also been described (Takahashi and Hiraga, 1984) . Notably, inhibition of platelet aggregation by BHT is not restricted to the agonist-induced event but is also directed towards phorbolester-induced activation of protein kinase C (PKC) (Alexandre et al.,1986) . BHT inhibits PKC activity and the Ca^{2+}-dependent protease calpain in lung (Malkinson et al., 1985). It is conceivable that decreased Ca^{2+} availability for calpain which can irreversibly activate PKC contributes to PKC inhibition. However, direct inhibition of purified protein kinase C has been shown to be caused by other antioxidants (Ferriola et al., 1989).

In brain synaptosomes, the Ca^{2+}-dependent release of the neurotransmitters, glutamate and GABA, was suppressed concomitantly with the decrease of cytosolic Ca^{2+} concentration (Zoccarato et al., 1987). The suppression of contractility of cardiac muscle (5 μM) and the inhibition of methacholine-induced contraction of ileal smooth muscle (50 μM) (Gad et al., 1979) referred to in Fig.1 may be due to decreased Ca^{2+} availability in the cytosol although Ca^{2+} concentrations have not been measured in this study.

The inhibition by BHT of PKC activity opens a wide field of possible interactions with cellular signal transduction pathways. One effect for which inhibition of PKC may be crucial is the anticarcinogenic activity of BHT. PKC is the receptor for phorbol esters and related tumor promoters, and BHT may exert antipromotional actions by inhibiting PKC. However, BHT also acts as a tumor promoter itself. Both the antitumorigenic and the protumorigenic action can be expressed in the same tissues and against the same initiating carcinogens as demonstrated in Table III. Witschi and his coworkers have collected numerous data on time course, dose dependency and age factors of tumor promotion by BHT in a mouse lung model. They have shown that BHT will lead to increased tumor formation in the lung even when administered as much as 4 months after cessation of urethane treatment and that enhanced tumorigenesis can be obtained after no more than 6 weekly injections of 50 mf BHT/kg body weight, corresponding to a total dose of only 300 mg/kg (Witschi & Lock, 1979). BHT fulfills a number of criteria

Table III

Modulation of chemical carcinogenesis by BHT

Tissue	Chemical carcinogen	
	Antitumorigenic action of BHT	Protumorigenic action of BHT
Rat forestomach		N-Methyl-N'-nitro-N-nitrosoguanidine[1]
Rat stomach	N-Methyl,N'-nitro-N-nitrosoguanidine[2]	
Mouse intestine	1,2-Dimethylhydrazine[3]	1,2-Dimethylhydrazine[4]
Rat liver	2-Acetylaminofluorene[5]	2-Acetylaminofluorene[6]
Rat pancreas	Azaserine[7]	Azaserine[7]
Rat bladder		N-Methyl-N-nitrosourea[8]
Mouse lung	Isoniazid[9]	Urethane[10]
Rat mamma	7,12-Dimethylbenz(a)-anthracene[11]	
Mouse skin	7,12-Dimethylbenz(a)-anthracene[12]	

1 Shirai et al., 1984
2 Tatsuta et al., 1983
3 Clapp et al., 1979
4 Lindenschmidt et al., 1986
5 Ulland et al., 1973
6 Peraino et al., 1977
7 Thornton et al., 1989
8 Imaida et al., 1984
9 Maru & Bhide, 1982
10 Witschi and Lock, 1978
11 Cohen et al., 1984
12 Slaga & Bracken, 1977

elaborated in vitro for a genuine tumor promoter: It inhibits cell-cell cooperation in cell culture (Trosko et al., 1982; Williams et al., 1991), induces ornithine decarboxylase in rat liver (Kitchin & Brown, 1987), and increases transformation frequency in cell transformation tests (Djurhuus & Lillehaug, 1982; Potenberg et al., 1986). In addition, promutagenic actions operative in the initiation phase of carcinogenesis may also occur: In the Ames test, BHT potentiated the mutagenicity of aflatoxin B1 (Shelef & Chin, 1980), and the administration of BHT in vivo increased the mutagenicity of 2-acetylaminofluorene in the Ames test (Black & Gerguis, 1980) and of ethyl

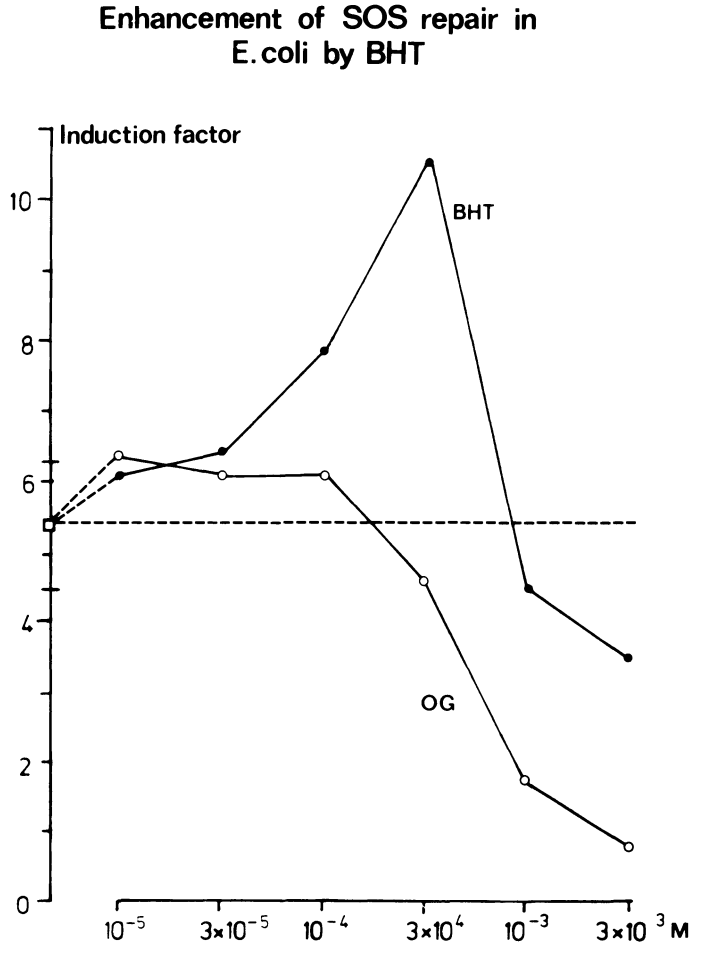

Fig.2 Enhancement of benzo(a)pyrene-induced SOS repair in E.coli PQ 37 by BHT as measured in the SOS chromotest. Data from Potenberg et al., (1988). The induction factor is plotted vs. the antioxidant concentration. Benzo(a)pyrene: 3 x 10^{-5} M; metabolic activation by S9 mix from rat liver. OG: octyl gallate.

methanesulfonate and diethylsulfate in Drosophila (Mazar Barnett & Munoz, 1983). We have previously shown that BHT in submillimolar concentrations increases benzo(a)pyrene-induced SOS function in E.coli, an indicator of mutagenicity (Potenberg et al., 1988). Fig.2 demonstrates this effect as compared to the inhibitory action of the gallic acid ester antioxidant, octyl gallate.

BHT by itself has in most tests proven to be nonmutagenic (reviewed in Kahl, 1984). Its carcinogenic effect in the liver of very old rats (Olsen et al.,1986) and mice (Inai et al.,1988) may well be due to the expression of its tumor-promoting properties in latent tumor cells.

Evidence exists that the tumor-promoting action is not due to BHT itself but to more one or BHT metabolites. Fig.3 demonstrates the main pathways of metabolic activation of BHT. The phenoxy radical formed via ir electron oxidation is the basis of a cascade of prooxidative events. One main pathway leads to the BHT hydroperoxide (BHT-OOH) , probably via the peroxy radical (Taffe et al., 1989). The formation of BHT-OOH in liver microsomes by a process meeting the criteria for monoxygenation has been described as early as 1972 (Shaw & Chen, 1972). BHT-OOH is reduced to BHT quinol (BHT-OH) (Wand and Thompson, 1986). The quinoxy radical formed during homolytic scission of BHT-OOH can be rearranged to ring-contracted products or can be converted into the di-tert-butyl-benzoquinone (DBQ). The latter process involves the release of a methyl radical which has indeed been detected by ESR techniques (Wand & Thompson, 1986). BHT-OOH has been claimed to be an active metabolite of BHT. This is, on the one hand, suggested by the fact that the acute i.p. toxicity of BHT-OOH is 20 times that of BHT itself (Yamamoto et al.,1980). On the other hand, BHT-OOH is a potent tumor promoter in DMBA-initiated mouse skin (Taffe & Kensler, 1988) , where BHT itself does not possess promoting activity (Berry et al., 1978). BHT-OOH but none of the stable metabolites generated from BHT-OOH by hematin (BHT-OH, BHT quinone and BHT stilbenequinone) induced ornithine decarboxylase activity in mouse epidermis; BHT itself was also inactive (Taffe et al., 1989). Considerable tumor-promoting activity has also been demonstrated with BUOH-BHT, a metabolite oxidized in one of the butyl

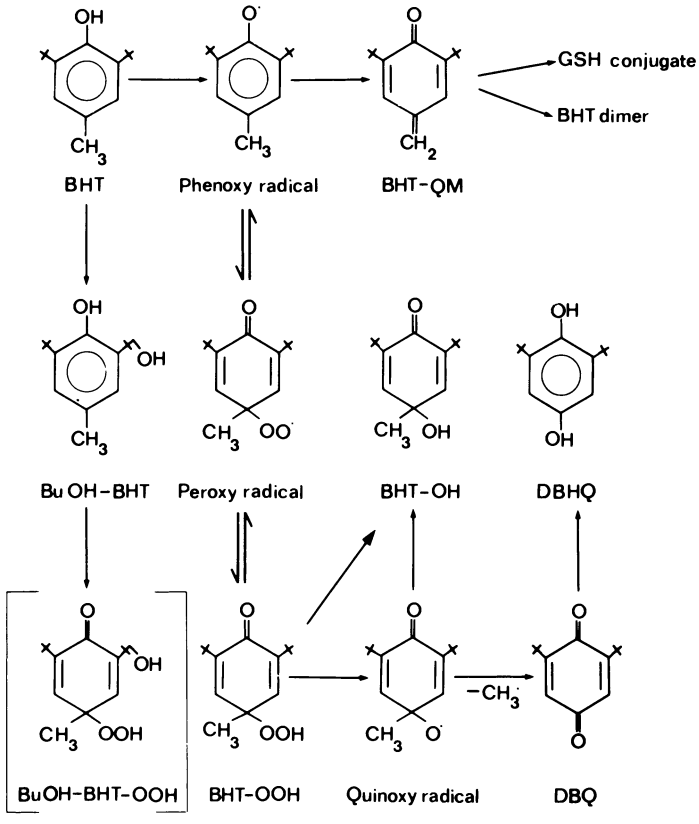

Fig.3 π-Electron oxidation pathways of BHT

moieties, in mouse lung with urethane as the initiator. In this model system, BUOH-BHT was 4 times more active than BHT. It has been discussed whether the ultimate promoting species might be the hydroperoxide of BuOH-BHT in analogy to the hydroperoxide of BHT itself. (Thompson et al., 1989) . BUOH-BHT is also 4 times more potent than BHT in eliciting pneumotoxicity in mice (Malkinson et al., 1989).

It has been suggested that the BHT quinone methide (QM) is formed from BHT-OOH and may be the ultimate tumor-promoting species derived from the latter (Guyton et al., 1991). During metabolism studies, the formation of QM along with BHT has been itself has, in spite of its high reactivity, been detected in a variety of studies. Its formation from BHT in vivo has long been reported (Takahashi and Hiraga, 1979) . Evidence for a role of cytochrome P-450 in its formation has been collected (Tajima et al.,1985) .

QM has become a favorite candidate for a reactive metabolite responsible not only for the tumor-promoting but also for the toxic effects of BHT. Covalent binding to protein of BHT-derived products (Nakagawa et al., 1983) as well as the formation of a glutathione conjugate and its derivatives (Tajima et al., 1985) have been ascribed to QM. BHT leads to glutathione depletion in lung (Mizutani et al., 1984) and liver (Nakagawa, 1987), and lung and liver damage should at least partially be due to QM. Mizutani et al., (1982) have shown that only BHT analogs capable of forming quinone methides will cause pulmonary toxicity in mice. Takahashi (1988a) reports to have administered QM to rats and demonstrates that it is far more potent in eliciting hemorrhage than BHT itself. This author also showed that QM very efficiently inhibits phylloquinone epoxide reductase in vitro (Takahashi, 1988b). The mechanism by which QM leads to the toxic effects is assumed to involve its covalent binding to critical target molecules.

In addition to the metabolic products listed in Fig.3, reactive oxygen species can also be formed from BHT. Fig.4 demonstrates BHT-induced excess production of hydrogen peroxide in rat lung microsomes (from Kahl et al.,1987) . Excess hydroxyl radical formation can also be induced by BHT in microsomal preparations as judged from the oxidation of dimethyl sulfoxide to formaldehyde (Fig. 5) It may be assumed that BHT, similar to BHA (Kahl et al.,1989) must be metabolized to yield a quinoid compound which is capable of producing reactive oxygen species. The quinone DBQ is assumed to be formed from BHT-OOH via the quinoxy radical (Wand & Thompson, 1986) but has also recently been suggested to be generated via the intermediate formation of 3, 5-di-tert-butyl-4-hydroxybenzoic acid (BHT acid) , a product of the main metabolic pathway of BHT, the oxidation in the p-methyl group (Yamamoto et al.,1991) . The corresponding hydroquinone is also formed indicating that redox cycling may take place to produce superoxide anion radical.

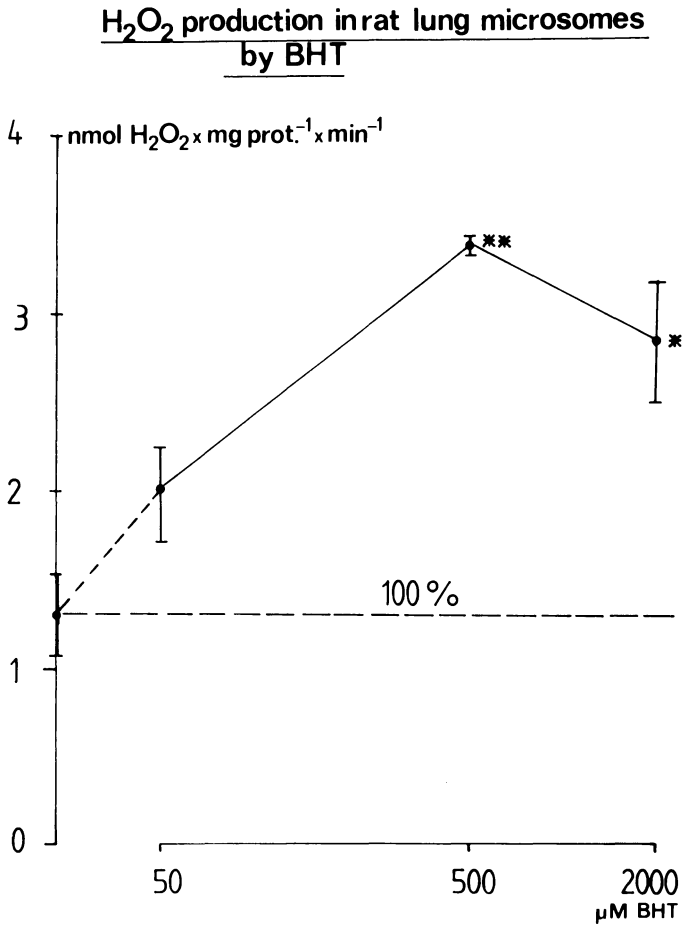

Fig.4 Excess production of hydrogen peroxide in rat lung microsomes induced by BHT. (From Kahl et al., 1987)

The contribution of superoxide and superoxide-derived oxidants to the biological effects of BHT has still to be elucidated. It is conceivable that one or several electrophilic BHT metabolites capable of producing reactive oxygen species are involved in the

generation of the antioxidant-specific pattern of hepatic drug metabolizing enzymes described above. Pickett and his coworkers have recently discovered a DNA sequence with considerable homology, in the rat NAD(P)H:quinone reductase gene (Favreau and Pickett, 1991) which responds with increased transcriptional activity to the metabolite of butylated hydroxyanisole, tert-butyl hydroquinone (TBHQ). They have named this sequence "antioxidant-responsive element (ARE)" and assume that a constitutively expressed transacting protein is modified by either chemical modification or a change in redox state to become capable of activating gene expression. Obviously, the antioxidant property by itself is not operating in activating ARE because the characteristic pattern of

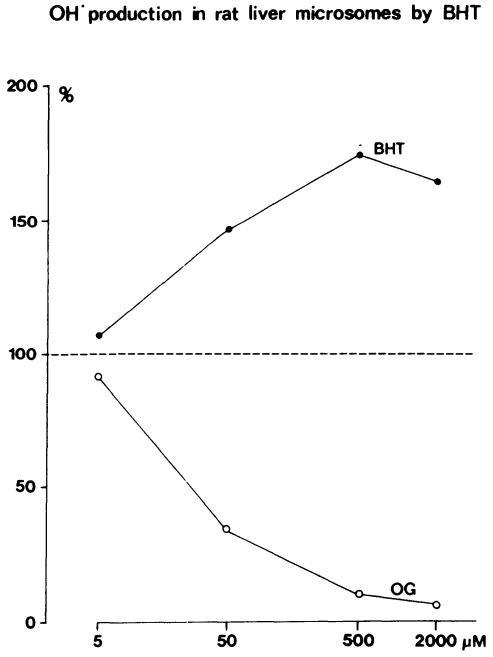

Fig.5 Excess production of hydroxyl radical in rat liver microsomes. Hydroxyl radical formation was estimated by the formation of formaldehyde from dimethyl sulfoxide. OG: octyl gallate.

enzyme induction including induction of glutathione transferase and NAD(P)H:quinone reductase is not obtained with all antioxidants. Besides TBHQ, BHA and BHT, only ethoxyquin appears to be effective in this respect (reviewed in Kahl 1984). The physiological phenolic antioxidant vitamin E, although quite similar to BHA and BHT with respect to its radical-scavenging properties, does not act as an enzyme inducer (reviewed in Kahl, 1984) . The gallic acid ester antioxidants do not induce glutathione transferase (Depner et al.,1982) . From a teleological point of view, the induction of glutathione transferase and of NAD(P)H:quinone reductase is an adaptation to oxidant stress while adaptation to antioxidants does not appear to make much sense. The regulatory sequence has been considered an electrophile-responsive element (Friling et al., 1990) , and the actual ARE activators among the antioxidant family are probably oxidant-producing electrophilic compounds which possess antioxidant activity themselves or are metabolically formed from antioxidants. In the case of BHA, TBHQ is the active compound. Since BHT elicits the same pattern of enzyme induction as BHA, a similar mechanism may be operative at the level of gene expression; however, it is at present unclear which metabolite might be involved. Among phenolic antioxidants, hepatic enzyme induction is associated with liver proliferation and tumor promotion. In tumor promotion, reactive oxygen is currently assumed to play an important role (Kensler and Taffe, 1986). Therefore, a trivial link may exist between the anticarcinogenic and the tumor-promoting properties of BHT, and it may be reasoned that the production of reactive oxygen species is associated with both. However, the genuine antioxidant properties of BHT are likely to contribute to the protective action since antioxidants inactive or only marginally active as enzyme inducers and sources of reactive oxygen species such as vitamin E and gallates are also anticarcinogens (Kahl, 1986).

In conclusion, BHT at doses considerably above those present in human foodstuff does not only act as an anticarcinogen but also as a blood, liver, lung, and kidney toxicant, as a promutagen and as a tumor promoter. Evidence exists that its biological actions are in part associated with inhibition of oxidant-dependent signal transduction pathways and require oxidation to active metabolites.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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EFFECTS OF TOCOTRIENOL-RICH VITAMIN E ON PATIENTS WITH PERIPHERAL VASCULAR DISEASE

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Summary

The important role played by free radicals in atherogenesis has stimulated interest in the effects of antioxidant vitamins on lipid peroxidation, platelet function and atherosclerosis. Palmvitee contains tocotrienol-rich Vitamin E (TVE) made up of 30% tocopherol and 70% tocotrienol, a strong antioxidant but not as well investigated as tocopherol. Patients with intermittent claudication were divided into 3 treatment groups (given either 300mg TVE, 300mg aspirin or placebo, daily for 12 weeks) in this randomised controlled clinical trial. Lipid peroxidation, measured as serum malondialdehyde, decreased by 8.2% in the TVE group compared to 2.32% and 4.32% in the placebo and aspirin groups respectively. A decrease in platelet aggregability was seen in the TVE and aspirin groups only. There were no significant differences in the lipid profiles between the treatment groups. At twelve weeks all the patients taking TVE had an improved treadmill walking distance, and the mean improvement of 46% was significantly greater than the 21.7% and 24.4% improvement seen in the other two groups. These results point towards a possible therapeutic role for tocotrienol and other antioxidant vitamins in atherosclerosis. More clinical trials using pure tocotrienols are needed to confirm its mode of action and efficacy.

Introduction

The important role played by free radicals and lipid peroxides in atherosclerosis is now established.[1-4] Lipid peroxidation induced by free radical reactions is believed to be one of the major causes of cell injury. Free radical modification to the apo-protein B

of low-density lipoprotein produces damaged LDL which are actively endocytosed by macrophages resulting in the formation of foam cells.[5,6] These lipid-laden cells are one of the many steps recognised in atherogenesis. The role of platelets too is important as platelet aggregation produces both thrombosis and increased lipid peroxides from the cyclooxygenase pathway.[7,8] We now have a better understanding of how the well-known risk factors like hypercholesterolaemia[4,9], smoking[10,11] and diabetes[12] are involved in atherogenesis. This has stimulated interest in the possible health benefits of using nutritional compounds like vitamin E (an effective lipid-soluble chain- breaking antioxidant)[13] and beta-carotene. Many studies on Vitamin E (α-tocopherol acetate) have shown an effective reduction in lipid peroxides and platelet aggregability.[3,12-15] Some studies have demonstrated beneficial effects on cholesterol levels.[16,17] However firm evidence about clinical benefit with Vitamin E supplements is still lacking.[13,18] The few clinical studies using Vitamin E on atherosclerotic disease were performed using only α-tocopherol[3,13,14,19-21] and have produced conflicting results.

Tocotrienol is interesting. A close relative of tocopherol, it has been shown in biochemical studies to be a stronger antioxidant.[22-24] It is also the predominant type of Vitamin E found in palm oil, a product of great economic importance and readily available in the tropical regions. There are however very few clinical studies using this product because adequate supplies of pure tocotrienols for clinical trials are difficult to obtain.

The aim of this study is to evaluate the clinical effects of Palmvitee, a tocotrienol-rich mixture of Vitamin E extracted from palm oil, on patients with intermittent claudication since clinical trials using α-tocopherols alone have previously not demonstrated any conclusive benefit. In particular we wished to assess the action of this tocotrienol-rich Vitamin E (TVE) on lipid peroxidation, platelet aggregation and cholesterol profile.

Patients and Methods

Patients with stable intermittent claudication were recruited from the vascular outpatient clinic. Excluded were those who needed urgent reconstructive arterial surgery or had other systemic illness. The pre-trial assessment included a full medical history, clinical examination and routine laboratory investigations (eg. renal profile, lipid profile, glucose, full blood count, ECG, chest X-ray). The patients' Ankle-Brachial Systolic Index (ABSI) and treadmill claudication distance were also measured. After the preliminary assessment all the patients were given standard advice about smoking, diet and exercise. All anti-platelet drugs and beta-blockers were stopped and a washout period of at least two weeks[19] was allowed before commencement of the trial. The patients were then randomised into 3 groups to receive either 5 capsules of Palmvitee containing 300mg of tocotrienol-rich Vitamin E (TVE), aspirin 300mg or placebo capsules daily for 12 weeks.

Patient assessment was performed at the beginning, at 6 weeks and at 12 weeks. The patient was asked using a standard questionnaire about subjective improvement, smoking, dietary habits, drug compliance and treatment side effects. Supine systemic blood pressure was measured at the brachial artery using a mercury sphygmomanometer. The ABSI of both lower limbs were measured using a Doppler ultrasound 8 mHz probe on the posterior tibial artery. Claudication distances were measured on a treadmill using a standard walking speed of 2.58 Km/hr and a 10% gradient, the patients having been instructed to stop the moment the typical claudication pains were felt. Fasting venous blood samples were collected for lipid profile, platelet aggregation studies and malondialdehyde estimation.

Lipid profile: Total cholesterol was estimated using enzymatic determination.[16] In the HDL-cholesterol test the chylomicrons VLDL and LDL were precipitated by addition of phosphotungstic acid and magnesium chloride. After centrifugation the supernatant fluid contained the HDL fraction, which was assayed for HDL- cholesterol with the Human

Cholesterol Liquicolor test kit. Triglycerides were determined using the enzymatic colorimetric testing after enzymatic hydrolysis with lipases.

Platelet aggregability: 10ml of citrated venous blood collected from fasted patients were centrifuged at 150-200 g for 10 minutes at room temperature (25'C). The platelet-rich plasma was then warmed to 37'C on an aggregometer and stirred. Light transmission through the plasma was monitored continuously over a chart recorder. Standard concentrations of ADP (5.0um and 8.0um) and collagen (1.0ug) were used to test both the patient and control plasma, and in each case the aggregation curve was observed for up to 3 minutes. The maximum aggregation and rate of aggregation for each type of reagent used were recorded and calculated.[3,14]

Malondialdehyde (MDA): The concentration of MDA in serum is a measure of lipid peroxide production.[4] A modification of Yagi's fluorometric method was used.[5] In the thiobarbituric acid reaction a red-coloured complex is formed by heating the sample together with thiobarbituric acid: TCA: HCL (Uchiyama & Mihara, 1977). After cooling and centrifuging at 4000rpm the supernatant was removed for fluorometric measurement.

Palmvitee: These capsules were supplied by the Palm Oil Research Institute of Malaysia (PORIM). Each capsule contains 240mg superolein and 60mg tocotrienol-rich Vitamin E (consisting of 42mg tocotrienols and 18mg tocopherols).[16,17]

Results

Patient profile: To date the results of 41 patients in this on going trial are available for analysis.

See Table I.

Table I: Patient profile.

n = 41
Sex ratio = 38M : 3F
Mean age = 53 years (range 25 - 79 years)

	Placebo	Aspirin	TVE
Hypercholesterolaemic (HC)	7	2	6
Diabetic	4	1	3
HC + Diabetic	0	2	2
Neither	4	5	5
n	15	10	16

(TVE = Tocotrienol-rich Vitamin E)

Treadmill claudication distance: This is a useful objective measure of clinical performance and it correlated well with subjective estimation of improvement. Patients with intermittent claudication can usually walk a fairly constant and predictable distance before the onset of pain. This feature is useful, for it allows a reproducible comparison of performance. All patients were tested on the treadmill at a constant speed of 2.58 km/hr and a 10% incline.

All the patients (100%) taking TVE achieved a significant (greater than 10%) improvement in their walking distance at the end of twelve weeks compared to only 40% and 53% in the other 2 groups (See Table II). The mean within-patient variation was 1.87%. The TVE group of patients also achieved a higher mean percentage improvement (46%) in their walking distance at 12 weeks. (t test, $p < 0.05$) This compares with a mean improvement of 21.7% and 24.4% for the aspirin and placebo groups respectively.

Table II: Results of treadmill claudication distance.

	Placebo n=15	Aspirin n=10	TVE n=16
Number improved > 10% @ 12 wks	8 (53%)	4 (40%)	16 (100%)
Mean change in walking distance	10.8m	10.8m	32.6m
Mean % change	24.4%	21.7%	46.0%
Mean walking distance (0 wks)	86.0m	92.7m	72.1m
Mean walking distance (12 wks)	96.8m	103.5m	104.7m

Ankle-Brachial Systolic Index (ABSI): The ratio of the ankle to arm (brachial) systolic pressure is normally greater than one. When disease narrows the arteries of the lower limb this ratio (ABSI) becomes less than one. The systolic blood pressures were obtained by using a standard mercury sphygmomanometer and the pulse signals at the brachial and posterior tibial arteries detected using a Doppler ultrasound 8 MHz probe. The changes at 12 weeks were small in magnitude and not statistically significant except for the +11.6% change in the left limb of the TVE group. See Table III.

Table III: Ankle-Brachial Systolic Index changes after 12 weeks

		Placebo (15)	Aspirin (10)	TVE (16)
Mean % change:	Right	+4.1%	+1.9%	+2.7%
	Left	+4.5%	-4.5%	+11.6% *
Number improved:	Right	6 (40%)	4 (40%)	8 (50%)
	Left	8 (50%)	4 (40%)	12(75%)

* $p < 0.05$

Blood Pressure: After 12 weeks the systolic and diastolic pressures both showed a small drop of 2.0% and 1.8% in the Vitamin E group. In the placebo group the systolic pressure dropped by 4.0% but the diastolic pressure rose by 3.0%. The group taking aspirin had an increase in both the systolic and diastolic pressures of 11.4% and 4.1% respectively. None of these changes were statistically significant.

Malondialdehyde: The serum MDA levels were taken as a measure of lipid peroxidation. A trend is visible here. (Figure 1) There was a reduction in lipid peroxidation in all three groups at 6 weeks and a greater reduction after 12 weeks. The reduction in MDA levels seen at both 6 weeks and 12 weeks in the group taking TVE is significantly greater than in the other two groups (student t test $p < 0.05$). After 12 weeks the drop in MDA was 8.15% in the TVE group compared to 2.32% and 4.32% in the placebo and aspirin groups respectively. (Table IV)

Lipid Profile: The changes in the triglycerides and cholesterol profiles at 6 weeks and 12 weeks are shown in Figure 2. There appears to be a beneficial effect on the lipid profile (drop in total cholesterol, LDL and triglycerides but rise in HDL) of patients taking TVE, but the differences between the three groups were not statistically significant. (Table V)

Platelet Aggregability: Aggregation studies were performed on platelet-rich plasma and tested in response to both collagen and ADP. The aggregation curves obtained for each of these agents were then analysed to calculate the maximum and mean rate of aggregation. The TVE group showed a reduction in the maximum and mean rate of aggregation to both collagen and ADP after 12 weeks. (See **Tables VI and VII**). Aspirin produced a greater reduction in platelet aggregation when tested in response to collagen. No reduction was seen in the placebo group.

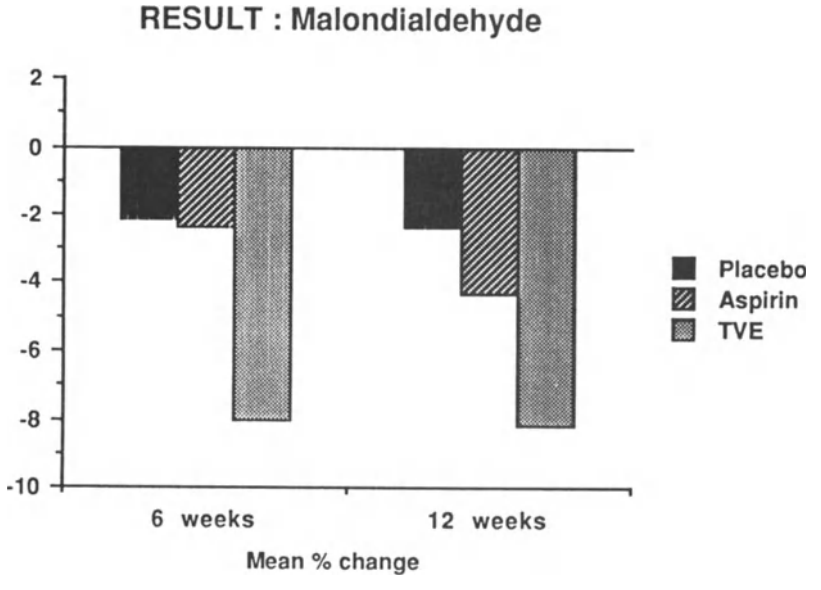

Figure 1: Comparing the percentage changes in MDA levels.

Table IV: Changes in serum Malondialdehyde (MDA) levels

		Placebo	Aspirin	TVE
Mean values @	0 weeks (μmol/l)	6.03	6.01	6.38
	6 weeks	5.90	5.87	5.87
	12 weeks	5.89	5.75	5.86
Mean % change @	6 weeks	-2.15%	-2.33%	-7.99%
	12 weeks	-2.32%	-4.32%	-8.15%

RESULTS: Lipid profile mean % changes after 12 weeks

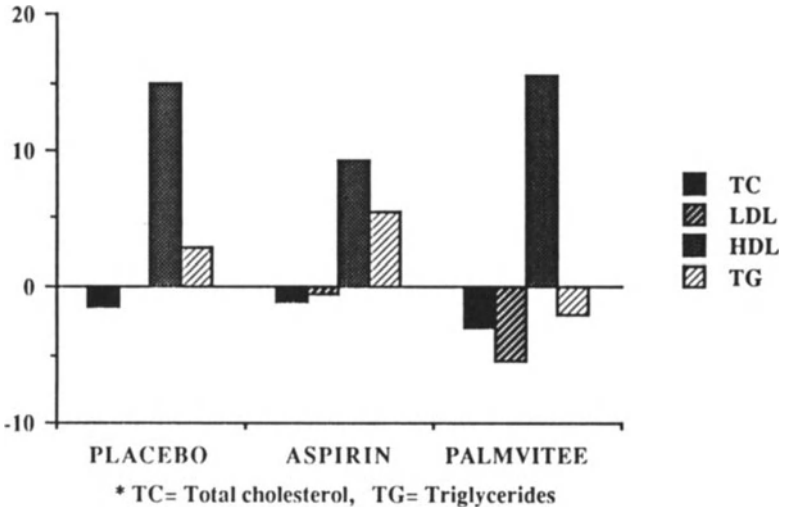

Figure 2: Mean % changes in lipid profile after 12 weeks o7 3

Discussion

This study, designed to assess both clinical benefit as well as the individual effects of tocotrienol-rich Vitamin E on patients with arterial disease, has produced some interesting results. Proving clinical benefit is always difficult but is the important bottom line. Data from previous Vitamin E studies suggests that tocopherol has anti-platelet and antioxidant effects[12-15] but evidence of clinical benefit has been conflicting.[18-20] Tocotrienol, a major constituent of the Vitamin E found in palm oil, appears from many studies to be an even stronger antioxidant than tocopherol[22-24] This suggests a potentially

Table V: Changes in Triglycerides, Total Cholesterol, LDL and HDL after 12 weeks for the three groups. (in mMol/l)

		Start	6 weeks	12 weeks
PLACEBO	Cholesterol	6.01	5.95	5.99
	LDL	3.78	3.75	3.86
	HDL	1.00	1.02	1.08
	Triglycerides	2.61	2.71	3.06
ASPIRIN	Cholesterol	5.90	5.71	5.93
	LDL	4.04	3.78	4.02
	HDL	1.07	1.09	1.11
	Triglycerides	1.63	1.82	1.78
TVE	Cholesterol	6.08	6.13	5.87
	LDL	3.60	3.58	3.45
	HDL	1.00	1.10	1.17
	Triglycerides	3.39	3.30	3.06

Table VI: Platelet aggregability changes @ 12 weeks (collagen)

		Placebo	Aspirin	TVE
Max aggregation (mean)	@ 0 wk	57.6%	49.7%	62.5%
	12 wk	66.4%	35.2%	58.3%
Mean % change		+6.7%	-29.4%*	-8.3%
Numbers improved		1 (6.7%)	7 (70%)	10 (67%)
Rate of aggregation (%/s)	@ 0 wk	0.66	0.80	0.68
	12 wk	0.77	0.56	0.61
Mean % change		+16.7%	-32.5%*	-6.0%
Numbers improved		1 (6.7%)	9 (90%)	10 (67%)

* $p < 0.05$

Table VII: Platelet aggregability changes after 12 weeks (ADP)

		Placebo	Aspirin	TVE
Max aggregation (mean)	@ 0 wk	60.7%	59.5%	62.8%
	12 wk	67.4%	63.0%	58.1%
Mean % change		+3.1%	+8.8%	-11.6%
Numbers improved		2 (13%)	3 (30%)	10 (67%)
Rate of aggregation	@ 0 wk (%/s)	1.02	1.06	1.03
	12 wk	1.01	1.10	0.89
Mean % change		+0.4%	+6.0%	-10.8%
Numbers improved		6 (40%)	5 (50%)	12 (80%)

greater clinical effect. Although it would be more interesting to study the effect of pure tocotrienol it was not possible to obtain sufficient amounts of this relatively new compound for a long clinical trial. We therefore used Palmvitee which contains 70% tocotrienol and 30% tocopherol, a natural product extracted from palm oil. In the light of the previous studies [22-24] any tocotrienol effect may reasonably be extrapolated from the data.

The patients in this double-blind controlled study were randomised with stratification for diabetes and hypercholesterolaemia. The 41 patients all satisfied the strict inclusion criteria which included a washout period of 2 weeks for related drug therapy, not smoking and treatment compliance. The sex ratio reflected the male preponderance in arterial disease. The main inclusion criteria and the parameter being assessed here is intermittent claudication due to arterial disease. Claudication distance is a convenient index of clinical improvement since a feature of claudication is that patients walk a consistent distance before the onset of pain. Any improvement in claudication distance however is due to a large number of factors and not just from improved blood flow to the limb. We therefore defined claudication distance as the distance patients can walk until

first experiencing the pain of claudication. This is a more sensitive index than the maximum ability to walk because the other confounding factors like tolerance of pain, motivation, and reaction to examiner and environment becomes less influential.[25] Although anti-platelet and other agents are commonly used in treating claudication, results are highly variable and patients who continue to deteriorate will eventually require arterial reconstructive surgery if the limb is to be saved. The lack of effective medical treatment for claudication underlines the need for more effective agents.[26] Tocotrienols, being naturally-occurring antioxidants, are logical substances to study.

It is notable that all the patients taking TVE achieved a greater than 10% improvement in their claudication distance after 12 weeks as compared to 53% of patients taking placebo and 40% who took aspirin. (The proportions of improvers in the placebo and aspirin groups are in agreement with published trials on this subject.) The 10% significance level for improvement was chosen in view of the potential error in measuring claudication distance on a treadmill as well as the expected normal variation in claudication distance when measured on any one day. However the reproducibility of the claudication distance measured in our patients during this study was surprisingly good. The mean within-patient variation was only 1.87% (2 treadmill claudication distances measured at an interval of 10 minutes on each of 7 patients). Interestingly, a greater **mean** percentage improvement too was observed in the group taking TVE: (46%) in their walking distance when compared to the placebo (24.4%) and aspirin (21.7%) groups ($p < 0.05$). Thus, these two components of the results are in agreement: that claudication distance improved significantly. What are the reasons for this improvement?

The changes in the Ankle-Brachial Systolic Index (ABSI) were small and the differences between the groups were not significant. Twelve weeks may be too short a time for there to be any detectable changes in the blood flow or arterial wall compliance as measured by the systolic pressure at the posterior tibial artery. The ABSI does not always reflect clinical improvement as many other factors are involved. However it remains a useful, convenient and reproducible bedside test. It is interesting to note that

there was a small drop of around 2% in both the systolic and diastolic blood pressure in the TVE group after 12 weeks. This small drop is not significant as the patient numbers are too small.

The effects on lipid profiles in all the groups were small and the differences were not statistically significant. It was only in the TVE group that a small beneficial effect appeared, with reduction in total cholesterol, LDL and triglycerides but an increase in HDL. The percentage reduction in total cholesterol and LDL was not only greater than in the other groups but the levels were lower after 12 weeks. A study using 300mg and 600mg of α-tocopherol daily for 2 weeks showed no significant effect[3] but two newer studies using 60mg and 200mg daily doses of TVE in Palmvitee for 4 weeks showed a hypocholesterolaemic effect.[16,17] A survey of patients with atherosclerosis showed no correlation between plasma lipid peroxide concentrations and plasma total cholesterol concentrations.[5] It is possible that this small positive change in the lipid profile is from the tocotrienol inhibition of enzymes in the cholesterol biosynthesis pathway (eg.HMGCoA)[16].

There was a reduction of lipid peroxides in the TVE group. Estimation of lipid peroxide concentrations was with the well established Yagi's modification of measuring serum malondialdehyde by the thiobarbituric acid reaction.[5] Lipid peroxides are formed from the oxidation of polyunsaturated fatty acids as well as from the cyclooxygenase pathway during platelet aggregation. The lipid peroxides themselves can peroxidise other lipids (especially LDL) by a free radical chain reaction, damage endothelial cells and promote platelet aggregation by reducing prostacyclin synthesis and increasing thromboxane production.[4,5,13] The reduction of lipid peroxides in the TVE group was seen at both 6 weeks and 12 weeks supporting the view that Vitamin E can inhibit lipid peroxidation.[3-5] This protective effect against lipid peroxidation is important as it is now well established that free radical damage to plasma lipoproteins (of which LDL is the most susceptible) and membrane lipids in endothelial cells (giving rise to foam cells) are involved in atherosclerosis.[1,5] Additionally, TVE may also have an anti- thrombotic effect

(from a combination of reducing vascular endothelial damage by free radicals and an anti-platelet aggregating effect) which further helps in protection against atherosclerosis.

Thrombosis plays a large part in both gradual atheroma progression as well as in the sudden deterioration seen as a complication of stenotic atheromatous lesions. In this study TVE appeared to have an anti-platelet aggregating effect when tested in response to both collagen and ADP. When compared with aspirin, a cyclooxygenase pathway inhibitor,[27] TVE produced a greater anti-aggregatory effect with ADP but a lesser effect with collagen. α-tocopherol supplementation in doses of 400-1200IU/day for up to 6 weeks have shown little or no anti- aggregatory effect in some trials[3,14,19] but in other studies[12,13,15] decreased platelet aggregation in-vitro and in diabetic patients was seen. The reduction in platelet aggregation and MDA levels suggests a diminution of cyclooxygenase activity by Vitamin E.[12] Another effect of Vitamin E is the scavenging of free radicals and lipid peroxides that damage endothelium and inhibit prostacyclin synthetase thereby reducing platelet aggregation.[13] Platelet aggregation leads to atherogenesis by producing thrombosis and lipid peroxides.[2]

Conclusion

TVE significantly improved walking distance in patients with intermittent claudication and the improvement after 12 weeks was greater than in the groups taking placebo or aspirin. Together with this clinical improvement there was a significant reduction in lipid peroxides, an anti-platelet aggregatory effect and a small but beneficial change in the lipid profile. It is likely that the antioxidant effect of TVE in reducing free radical activity and lipid peroxidation is a key factor. In the long term the reduced endothelial damage, lipoprotein peroxidation, platelet aggregation and thrombosis would be expected to have the effect of increasing blood flow to the lower limbs. The symptomatic improvement may also result from other more immediate benefits like improved blood oxygen delivery, vasodilatation, increased muscle efficiency and reduced production of pain-causing metabolites during exercise. It is likely that a combination of

all of these factors is involved, and more clinical studies are needed to further elucidate the mechanisms of action. The activity profile of TVE is encouraging. By extrapolation, tocotrienol appears promising. We should compare its activity with that of tocopherol in future trials.

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Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications
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PROTECTION FROM AIR POLLUTION INJURY BY DIETARY VITAMIN E

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Summary

Ozone and nitrogen dioxide are the major photochemical oxidant constituents of polluted air with adverse health effects. Exposure to ambient or near ambient levels of these oxidants results in lung injury manifested in biochemical, morphological and physiological alterations. The magnitude of such alterations is proportional to the exposure dose. Dietary supplementation with vitamin E, a lipophilic antioxidant and a free radical quencher that is safe even at relatively high doses, was found to provide protection from oxidant-induced injury. A comparison between animals fed diets containing varied amounts of vitamin E, then exposed to ozone or nitrogen dioxide, indicates an inverse relationship between vitamin E level in the diet and the magnitude of oxidant-induced alterations in which the higher the vitamin level, the greater the attenuation of oxidation effects. However this association is limited, suggesting the possible presence of a critical level below which injury occurs, but above which no additional protection is afforded. Moreover, this hypothetical level may also be organ-specific i.e., vitamin E requirements may vary from organ to organ. The implication of these observations to human health is that moderate supplementaiton with vitamin E beyond the Recommended Daily Allowance (RDA), may be required to protect from oxidants in the air of highly polluted areas.

Introduction

The rapid growth in human population observed in the last few decades coupled with the ensuing industrialization and urbanization, has led to greater use of energy (predominantly from fossil fuels), and transportation (primarily the automobile). It was inevitable that such uncontrolled growth would become associated with undesirable side effects. One such effect threatening man's health and welfare is the alarming increase in

global environmental pollution of air, water and land. In this chapter, the pulmonary effects of exposure to environmental oxidants in polluted air will be discussed.

The most harmful components of polluted air are the photochemical oxidants, ozone (O_3) and oxides of nitrogen (NO_x) of which nitrogen dioxide (NO_2) is the most stable. Chemically, O_3 is about 10-15 times as reactive as NO_2 and thus more deleterious. Table I shows the concentration of O_3 in the air of several major cities around the world.

Table I Ozone Levels in Various Urban Areas of the World[A]

City	Concentration[B]	
	PPM	$\mu G/M^3$
Riverside, USA	0.372	744
Los Angeles, USA	0.274	548
Endhoven, Netherlands	0.210	420
Tokyo, Japan	0.190	380
Osaka, Japan	0.160	320
Washington, DC, USA	0.156	213
London, UK	0.147	294
Bonn, W. Germany	0.145	290

[A]World Health Organization, 1979.

[B]One-hour Maximum Concentration.

In this table, Los Angeles and Riverside, two cities in USA top the list. However, cities such as Houston, and Denver, USA; Mexico, Mexico; Athens, Greece; and Cairo, Egypt to name a few, have since joined the list of most polluted cities.

Inhalation of oxidants at levels above ambient (0.50 - 1.00 ppm) was shown to cause pulmonary and extra-pulmanry biochemical, morphological, and physiological

alterations in animals and humans (Stokinger and Coffin, 1968; Menzel, 1970; Plopper et al., 1973; Mudd and Freeman, 1977; Mustafa and Tierney, 1978; Evans, 1982; Crapo et al., 1984; Mustafa, 1990). Moreover, it has been shown in some experimental animals that chronic low-level exposure to environmental oxidants may be carcinogenic (Hassett et al., 1985; Mustafa et al., 1988; Witschi, 1988).

Pulmonary injury from oxidant inhalation may result from direct reactions of the oxidant (NO_2) acting as a free radical (Pryor et al., 1983) or indirectly, where the oxidant (O_3) initiates free radical-mediated reactions that can propagate after exposure (Pryor et al., 1983; 1991; Pryor, 1990). In either case it was proposed that oxidant exposure results in lipid peroxidation and protein oxidation (Menzel, 1970; 1976; Fletcher and Tappel, 1973; Mudd and Freeman, 1977; Mustafa et al, 1977; 1978; Mustafa, 1990; Sevanian et al., 1982; Pryor et al., 1983; 1991).

The lung are the major target for injury by environmental oxidants because of its size, as the organ with the largest surface area in the body, and its location, as the only internal organ in contact with the outside atmosphere. As a result, several antioxidant defense systems have evolved in the lung to protect not only from oxidants, but also from the reactive metabolites of atmospheric oxygen that the lung is continuously exposed to it. Oxygen constitutes a paradox, we need it to sustain life, but it is also a very powerful oxidant with reported toxicity (Mustafa, and Tierney, 1978; Heffner and Repine, 1989).

Vitamin E is a lipid soluble antioxidant and a free radical quencher in animals and man (Tappel, 1962; 1965; 1972; Burton et al., 1983) with little known toxicity even at high doses (Bendich and Machlin, 1988). Although the antioxidant properties of vitamin E were first proposed six decades ago (Mattil, 1931), it was not until Tappel demonstrated its ability to protect from oxidant-induced lipid peroxidation (Tappel, 1962, 1965, 1972), that vitamin E gained widespread interest as a protective biological antioxidant (Thomas et al., 1967; Golstein et al., 1970; Roehm et al., 1971; Fletcher and Tappel, 1973; Mustafa, 1975; Menzel, 1976; Mustafa and Tierney, 1978; Chow et al., 1981; Evans et al., 1981; Sagai et al., 1982; Elsayed and Mustafa, 1982; Elsayed, 1987; Elsayed et al., 1988; 1990). In addition to its protective role, vitamin E is suggested to contribute to membrane stability and fluidity based on its chemical and structural characteristics (Lucy, 1972; Patel and Edwards, 1988).

Methods

Diet

In conducting animal experiments involving vitamin E, one procedure that is commonly used and may be the cause of variability in results, is to purchase and feed the animals commercially available diets. Typically, one diet devoid of vitamin E, and another (or several) supplemented with the desired vitamin E concentration(s). In this regimen, lipids in the diet, particularly unsaturated fatty acids, would be left without the antixoidant protection afforded by vitamin E. To minimize this possibility, we implemented a procedure (Elsayed, 1987; Elsayed et al., 1990) in which the diet is purchased from a commercial supplier without both the lipids and vitmain E. The two are then mixed with the other dietary constituents in our laboratory just prior to feeding the animals. The complete diet is kept refrigerated until used and changed on a daily basis. Moreover, during oxidant exposures that lasted more than 24 hours, the diet was changed daily.

Animals

We purchased pregnant rats about 10 days from term and fed a vitamin E-deficient diet until birth. After birth, all animals received the same diet until the pups are weaned, then the dams are discarded. We fed the growing pups the same deficient diet for at least 8 weeks, then randomly divide them into dietary groups and fed the desired specified diets for 2 more weeks before using them experimentally. During the conditioning period, randomly selected animals are euthanized and some of their organs analyzed for vitamin E content.

Oxidant exposure

We have examined the protective effect of vitamin E from environmental oxidants in animals exposed to O_3 or NO_2. After conditioning the animals as described above, a number of animals were exposed to an oxidant in a stainless steel inhalation exposure chamber (Young and Burtke, Cincinnati, OH, USA). In another similar chamber a matched number of control animals were exposed to filtered room air. Oxidants were either purchased (NO_2) at a higher concentration, then diluted at the chamber inlet to the

desired concentration, or generated (O_3) by passing pure oxygen through an electric arc-type ozonizer. Both oxidants were continuously monitored in the exposure chamber and the monitors were calibrated periodically using established methods of calibration.

Results

Table II shows results from a study in which rats were fed either a vitamin E-deficient diet or a diet supplemented with vitamin E (50 IU/kg diet), then exposed to 3 ppm NO_2 or room air for 7 days (Elsayed and Mustafa, 1982). The table shows the activities of two antioxidant enzymes: glutathione peroxidase (GP) and glucose-6-phosphate dehydrogenase (G6PD). In lung tissues from vitamin E-deficient rats the activiites of both enzymes increased significantly after exposure compared to air-exposed controls (42% and 96%, $P<0.5$) for GP AND G6PD, respectively. In vitamin E-supplemented rats, the activities remained virtually unchanged (-17% and -8%, NS) respectively.

Table II Effects of exposure to 3 ppm NO_2 for 7 days on antioxidants enzyme activities in rat lungs.

Enzyme	Vitamin E (IU/kg diet)	Air-exposed group (n=6)	NO_2-exposed group (n=6)	Change (%)
GP	50	2.59 ± 0.61	2.16 ± 0.41	-17
(U/lung)[b]	0	2.23 ± 0.57	3.16 ± 0.38	+42[c]
G6PD	50	1.41 ± 0.27	1.30 ± 0.16	-8
(U/lung)[a]	0	1.17 ± 0.12	2.30 ± 0.42	+96[c]

[a]. G6PD Unit = μmol NADPH reduced $min^{-1}.lung^{-1}$.
[b]. GP Unit = μmol NADPH oxidized $min^{-1}.lung^{-1}$.
[c]. Significantly different from air-exposed control, $p<0.05$

Results from another study (Elsayed et al., 1988) in which the activities of the same enzymes in rats exposed to O_3, (0.8 ppm continuously for 4 days) are presented in Table III. In this study, the rats were fed 3 diets supplemented with different levels of vitamin E (10, 50, and 500 IU vitamin E/kg diet) before exposure to O_3. The results of the study showed that O_3 exposure resulted in increased activities of both enzymes compared to air-exposed controls. However, the magnitude of increases were progressively reduced as supplemental vitamin level increased.

Table III Effects of exposure to 0.8 ppm O_3 continuously for 4 days on antioxidant enzyme activities in rat lungs.

Enzyme	Vitamin E (IU/kg diet)	Air-exposed group (n=6)	O_3-exposed group (n=6)	Change (%)
GP	500	1.75 ± 0.36	6.73 ± 2.91	+285
(U/lung)[a]	50	1.91 ± 0.29	11.09 ± 5.82	+480
	10	1.64 ± 1.40	12.73 ± 4.50	+676
G6PD	500	1.41 ± 0.81	5.30 ± 1.13	+279
(U/lung)[b]	50	1.56 ± 0.90	7.22 ± 2.17	+363
	10	1.48 ± 0.52	8.70 ± 1.30	+488

[a]. GP Unit = μmol NADPH oxidized $min^{-1}.lung^{-1}$.
[b]. G6PD Unit = μmol NADPH reduced $min^{-1}.lung^{-1}$.

Red blood cell fragility, shown in Table (IV), was inveresely related to vitamin E level and was reduced significantly as vitmain E level increased. However, vitamin E supplementation had no effect on the fragility of the cells when compared to air-exposed controls.

Table IV Effects of exposure to 0.8 ppm O_3 continuously for 4 days or on the fragility of rat red blood cells.

Enzyme	Vitamin E (IU/kg diet)	Air-exposed group (n=6)	O_3-exposed group (n=6)	Change (%)
Fragility[a]	500	3.8 ± 2.7	3.4 ± 2.4	-10
	50	52.4 ± 14.5	41.7 ± 17.2	-20
	10	95.9 ± 3.1	94.8 ± 3.8	-1

[a] Fragility = % hemolysis of red blood cells

Discussion

The results presented in this chapter indicate that vitamin E deficiency is assciated with biochemical alterations that does not occur when vitamin E is adequately supplied. Although deficiency is a rare occurrence in man, it has been observed, particularly in newly born children with retrolental fibroplasia (Hittner et al., 1981). Supplemental vitamin E has been also used therapeutically in the treatment of Mediterranean-type glucose-6-phosphate dehydrogenase defiency (Corach, et al., 1980); neurological dysfunctions associated with chronic congenital cholestatic hepatobiliary diseases (Sokol et al., 1985); Spinocerebellar degeneration (Harding et al., 1985).

In areas characterized by high atmospheric pollution, the demand for antioxidant supplementation may be greater in order to sustain the cellular antioxidant defenses that are under oxidant attack. This proposal is supported by the findings of the second study prsented in Table III in which it was shown that increased vitamin E supplementation tended to decrease the magnitude of the biochemical alterations in the lung, i.e., the magnitude of lung injury.

Red blood cell fragility did not increase as a result of oxidant exposure. However, the degree of hemolysis decreased as vitamin E level in the diet increased which in itself

shows that red blood cells are protected by vitamin E, and that the protection increase with increased vitamin E supplementation.

Although it may be premature to extrapolate the protective effects observed in animal studies to humans, it is important to note, however, that other human activities would benefit from vitamin E supplementation. First, the progress in our understanding of the association between saturated lipids and cardiovascular diseases, has led many in the today's society to substitute their dietary lipid intake with mono- and polyunsaturated fatty acids, and with low and nonfat diets. Whereas low and nonfat diets can be very poor in vitamin E if not supplemented with the vitamin, diets containing unsaturated lipids increased the demand for vitamin E (Harris, 1963; Witting, 1980) beyond the Recommended Daily Allowance (RDA) in order to prevent lipid autoxidation. Second, increased popular awareness of the health benefits of exercise, drove many in the society to exercise regularly. A practice although beneficial to the cardiovascular system, increases free radical production and therefore, may benefit from increased vitamin E supplementation. However, the demand for supplemental vitamin E and the antioxidant protection gained by supplementation vary widely, because increased vitamin E intake does not confer proportionally increased protection from oxidant attacks (Elsayed et al., 1988; Pryor et al., 1990).

In conclusion, the results of these studies and many other, support the proposal that increased vitamin E supplementation may be beneficial to humans in today's society in general, but more paticularly to those living in areas characterized by highly polluted atmospheres in particular.

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Disclaimer

The opinions and assertions contained herein are the private views of the author and are not to be construed as official nor do they reflect the views of the Department of the Army or the Department of Defense (AR 360-5). The experimental studies described in this report adhered to the "Guide for the Care and Use of Laboratory Animals." DHEW Publication (NIH) 85-23.

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