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PRO-OXIDANT AND ANTI-OXIDANT MECHANISM(S) OF BHT AND b-CAROTENE IN PHOTOCARCINOGENESIS

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1. ABSTRACT

An hypothesis for the role of free radicals in cancer was elaborated by D. Harman in 1962 who suggested that it might be possible to reduce the extent of damage caused by free radicals through three dietary changes: (*i*) caloric reduction, i.e., lowering the level of free radical reactions arising in the course of normal metabolism; (*ii*) minimize dietary components that tend to increase the level of free radical reactions (e.g., polyunsaturated fats); and (*iii*) supplement the diet with one or more free radical reaction inhibitors (anti-oxidants). With respect to (*ii*) and (*iii*), lipid peroxidation exemplifies the type of chain reaction initiated by free radicals, with unsaturated fatty acids being the primary center of free radical attack. Anti-oxidants act as free radical scavengers and are able to terminate these reactions. Indeed, the phenolic anti-oxidant butylated hydroxytoluene (BHT), and the carotenoid β-carotene, have both been shown to influence photocarcinogenesis, although the lack of correlation between physicochemical parameters and pathophysiological responses is apparent in both instances. The bimolecular rate constant for reaction of BHT with model peroxyl radicals is low while β-carotene is highly reactive. However, both are able to efficiently inhibit lipid peroxidation reactions in biological membranes. Indeed, the influence of photocarcinogenesis by both BHT and β-carotene is diminished as the level of dietary fat decreases, pointing to the involvement of lipid peroxidative reactions. Nevertheless, the mode of action of BHT in inhibiting photocarcinogenesis appears to be related to dose-diminution resulting from an increased spectral absorbance of the stratum corneum. On the other hand, β-carotene has no such effect and may actually exacerbate photocarcinogenesis under certain dietary conditions. This paradox points to the complex relationship between chemical mechanisms and biological mode of action of anti-oxidants in photocarcinogenesis. Recent clinical and experimental data also suggest that supplementation of the complex and intricately balanced natural antioxidant defense system with one or more anti-oxidants as a cancer prevention strategy will demand extreme caution.

2. INTRODUCTION

"Pro-oxidants" have been considered synonymous with "reactive species", toxic substances that can cause oxidative damage to major constituents of biological systems, e.g., lipids, proteins, and nucleic acids (1). An "anti-oxidant", in contradistinction, is any substance that, when present at low concentrations compared with that of the oxidizable substrate, significantly prevents or delays a pro-oxidant initiated oxidation of that substrate. The idea that reactive species, or free radicals, and the reactions mediated by them, play an important role in the deleterious effects of ultraviolet (UV) radiation upon skin has now generally been accepted as *feat accompli*. This has occurred only after an overwhelming body of evidence, albeit mostly circumstantial, had overcome a respected level of skepticism, and parallels the evolving knowledge accrued

with respect to the principal participants, i.e., pro-oxidants, free radicals, and anti-oxidants, in actinically-mediated pathophysiology. It should be emphasized, however, that despite advances in instrumentation (2), such as pulse spectrometry, free radical spin trapping, and chemiluminescence techniques, it remains unclear whether free radical reactions represent important causal steps in UV-carcinogenesis, or simply represent concomitant processes that exacerbate the pathology (3). Proctor and Reynolds (4) provided some direction in addressing this question when they outlined criteria of acceptance for free radical participation in a disease state. These are:

1. The disease state must be associated with abnormal production of free radicals or intermediates.

2. It must be demonstrated that specific free radical species, or their unique reaction products, occur at the lesion site.

3. It must be demonstrated, *in vitro*, that free radical species are involved in important mechanisms relevant to the specific disease entity.

4. Fulfill a "Common Symptom Test", i.e., production of similar lesions by otherwise dissimilar etiologic agents that produce common free radical species.

5. Demonstrate the ability to modulate pathogenesis through administration of antioxidants or free radical quenchers.

Although there is compelling evidence for important roles of free radical mediated reactions in the deleterious effects of UV upon skin, it should be emphasized that the preceding criteria imply participation of free radicals in a disease process but do not indicate a causal role (5). Certainly these criteria are less stringent than those imposed for causal involvement of a radical-induced biogenic compound (cholesterol-5α-6α-epoxide) in UV-carcinogenesis (6). Furthermore, there are at least two of these criteria that may provide illusory leads with regard to events that occur *in vivo*. For example, criterion 3, i.e., “demonstration, *in vitro*, that free radical species are involved in important mechanisms relevant to the specific disease entity”. While seeking potentially important mechanisms of action of pro- and anti-oxidants under controlled physicochemical conditions can provide important insight, the action of these agents cannot currently be examined *in vivo* through direct kinetic experiments. Other concerns such as tissue absorption of the agent, target tissue concentrations, rate constants for radical reactions, localization and mobility with respect to hydrophobic and hydrophilic domains, turnover rates of the respective agent in the tissue and rate of generation and cycling are but some of the complicating factors that will have impact upon an agents pro-or anti-oxidant efficacy *in vivo* (7).

Criterion 5, i.e., “demonstration of the ability to modulate pathogenesis through administration of anti-oxidants or free radical quenchers” has particular relevance to carcinogenesis as it is congruent with Harmon’s (8, 9) approach to minimizing the extent of free-radical damage through dietary changes such as:

1. Caloric reduction, i.e., lowering the level of free radical reactions arising in the course of normal metabolism. The rationale of this tenet rests with the observation that endogenous factors that influence mitochondrial radical production are those that regulate respiration and includes the availability of NAD-linked substrates, succinate, and oxygen. If oxygen is present at concentrations that limit its reduction to water by cytochrome oxidase, if the respiratory chain is highly reduced, or if an accumulation of reduced cofactors occurs, superoxide anion formation will be favored (10, 11). Superoxide anion and hydrogen peroxide production usually accounts for about 1-2% of the total oxygen consumption under normal reduced conditions (12, 13). However, one can readily see that under conditions where the metabolic fires are stoked with additional calories, more oxygen consumed in respiratory reactions may be lost as free radicals, perhaps as much as 10%.

2. Minimize dietary components that tend to increase the level of free-radical reactions (e.g., polyunsaturated lipids. Lipid peroxidation exemplifies the type of chain reaction initiated by free radicals with unsaturated fatty acids being a primary center of free radical attack.)

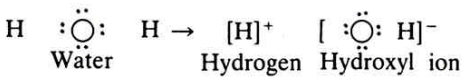
3. Supplement the diet with one or more free radical reaction inhibitors (anti-oxidants).

With respect to the first and second tenets, a number of studies in the 1940s clearly pointed to caloric restriction and dietary fat reduction as important avenues to prevent or moderate the course of cancer for several organ sites, including skin (14-17). Recent studies have `shown that a decrease in per cent of calories consumed as fat reduces the occurrence of pre-cancerous keratosis and nonmelanoma skin cancer (18-20). In addition to a general reduction in dietary fat, the composition of fat intake is an important factor that influences photo-induced responses (21, 22). Whereas a considerable body of evidence has accrued that demonstrates the influence of polyunsaturated fatty acids on carcinogenesis, it is now clear that a distinction must be made between those ω-6 fatty acids that may enhance carcinogenesis in some animal models (23), and ω-3 fatty acids that are protective (24). As the degree of unsaturation of ω-3 fatty acids is even greater than that of ω-6 fatty acids, the anti-carcinogenic properties of ω-3 fatty acids may be related to the reduced formation of prostaglandin E_2 (PGE_2), an immunomodulator (25). Indeed, it has been shown in randomized trials that dietary supplementation with ω-3 fatty acids significantly increases the threshold to UVB-induced erythema and reduces UVB-generated skin PGE_2 while *increasing* oxidative stress as exemplified by lipid peroxidation (26, 27). Thus, the rationale of tenet 2, i.e., "minimizing dietary components that tend to increase the level of free-radical reactions" is not as straightforward as it must have appeared in the 1960s. Nor, in view of recent studies with β-carotene, is tenet 3, i.e., "supplementing the diet with one or more free radical reaction inhibitors", any longer a reasonable approach to minimizing presumed free-radical damage

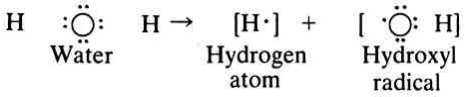
associated with carcinogenesis. As Hensley and colleagues (28) astutely noted "*the main problem faced by clinicians and basic scientists is that antioxidant function is much more complex than simple free radical scavenging…*" Indeed, dietary supplementation with a single antioxidant to an intricate and delicately balanced natural anti-oxidant defense system may have a detrimental, rather than the desired beneficial response. After examining some of the basic mechanisms of free-radical and anti-oxidant reactions, two anti-oxidants, butylated hydrotoluene (BHT) and β-carotene will serve as examples of how physicochemical mechanisms observed *in vitro* may be illusory with respect to pathophysiologic responses such as UV-carcinogenesis, and why recommendations of supplementing the diet with one or more anti-oxidants may be imprudent.

3. HOW FREE RADICALS ARE FORMED

The basis for skepticism of free radical involvement in disease was formidable, not only from a pathophysiology standpoint, but from a chemical consideration, as well. Before the 1900s, it was axiomatic that the principles of valence and molecular weight, the foundation of modern chemistry, forbade the type of molecular fission that would result in free radical formation (29). Normally a compound undergoing ionization does so whereby one fragment retains two of the shared bonding electrons and thus acquires a formal negative charge. The other fragment retains neither electron and thus acquires a formal positive charge. The ionization of water represents this usual type of *heterolytic* fission [Eq. 1]:



However, if adequate energy is provided, UV irradiation being a potential energy source, it is possible for a bond to split in such a manner that the two fragments retain one bonding electron each. This is known as *homolytic* fission and when this occurs a free radical has been formed [30]. In the following example, a dangerous hydroxyl radical (•OH) results [Eq. 2]:



A free radical, then, is any molecule that has an odd number of electrons in an outer orbital. This definition is expanded to include chemical species characterized by pairs of electrons of similar directional spin isolated singly in separate orbitals. Because of these properties, free radicals are extremely reactive. In the 1930s chemists demonstrated that even though free radicals might have an independent life span of only microseconds, or less, because of their extreme reactivity the reactions that resulted accounted for some very important and spectacular chemistry.

Obviously, as a result of high bond dissociation energies of biomolecules, homolysis will occur only upon input of substantial levels of energy, e.g., UV irradiation, or by processes that lower the energy of activation for these reactions to occur under cellular conditions. As an example, unsaturated lipids undergo auto-oxidation to yield hydroperoxides. Because of the high bond dissociation energy of the **O-O** bond of the hydroperoxide, the uncatalyzed, unimolecular decomposition to produce lipid radicals would not be expected to occur in the cell. On the contrary, lipid hydroperoxides are known to undergo rapid decomposition, due, in part, to other free radicals that may be present. This free radical-initiated decomposition proceeds *via* a *hydrogen abstraction* reaction similar to that occurring in the initial step in polyunsaturated fatty acid (PUFA) peroxidation [Eq. 3]:

$$R\cdot + PUFA \rightarrow PUFA\cdot + RH$$

Moreover, in some cases free radical formation may proceed with the assistance of other molecules that can hydrogen bond and thereby reduce the activation energy of homolysis. This is called *Molecule Assisted Homolysis* (31). One can see that in the presence of a strongly hydrogen bonding agent, (**C=C**), a hydroperoxide (**ROOH**) is decomposed to yield free radicals [Eq. 4]:

$$C{=}C + ROOH \rightarrow \dot{C}{-}C{-}H + ROO\cdot$$

Aside from homolysis, the other major process that results in net formation of free radicals in biological systems is electron transfer from transition metal ions to organic species. The redox reaction between transition metals and peroxide compounds, as exemplified by the Haber/Weiss cycle, may play an important role in free radical formation *in vivo*. Indeed, the non-enzymatic reduction of hydrogen peroxide (H_2O_2) to •OH radical proceeds slowly. However, in the presence of a heavy metal, the reaction progresses rapidly by the Haber/Weiss cycle [Eq. 5, 6]. The latter equation is known as the Fenton reaction in which the metal ion is first reduced by superoxide anion ($\mathbf{O_2\cdot^-}$) and then oxidized by H_2O_2 to yield a •OH radical:

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$

4. BIOLOGICALLY RELEVANT FREE RADICALS

Although there exists a number of free radical species to which cells may be exposed, endogenous sources of free radicals, for the most part, tend to be derivatives of molecular oxygen, polyunsaturated fatty acids, sulphydryl compounds and compounds such as quinones that can easily transfer single electrons (32).

4.1. Reactive Oxygen Species

Superoxide anion is the primary radical formed by the univalent reduction of molecular oxygen (33) [Eq. 7]:

$$^3O_2 \xrightarrow[+H^+]{+e-} O_2^-$$

This radical may result from a variety of other biochemical processes as well, e.g., when chelated Fe^{3+} is reduced and undergoes auto-oxidation (34).

Normally, endogenous factors that influence radical production in the cell are those that regulate cellular respiration and includes the availability of NAD-linked substrates, succinate, and oxygen. If oxygen is present at concentrations that limit its reduction to water by cytochrome oxidase, if the respiratory chain is highly reduced, or if an accumulation of reduced cofactors occurs, superoxide anion formation will be favored [Eq. 8] (35):

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

Although the endogenous metabolism of superoxide anion, itself, may occur spontaneously or through a variety of biochemical steps, subsequent reductions from the superoxide radical are 'spin-forbidden' and the non-enzymatic reactions proceed very slowly unless catalyzed by a heavy metal ion as exemplified in equations 5 and 6. Enzymatically, superoxide dismutase (SOD) first dismutes the superoxide anion to H_2O_2 (33), [Eq. 9]:

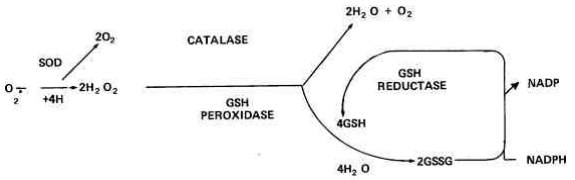
$$O_2^- \xrightarrow{SOD} H_2O_2 + O_2$$

Hydrogen peroxide may then be enzymatically reduced to water (H_2O) by catalase (CAT) or glutathione (GSH) peroxidase (GP) [Eq. 10, 11]:

$$H_2O_2 \xrightarrow{catalase} O_2 + H_2O,$$

$$H_2O_2 + GSH \xrightarrow[peroxidase]{GSH} H_2O + GSSG.$$

Hydrogen peroxide has been detected after UVB irradiation in human skin under *in vivo* conditions by application of non-invasive Fourier Transform Raman spectroscopy (36). In equation 11, the oxidized glutathione (GSSG) may be re-reduced to GSH in the presence of NADPH. The enzymatic reactions of the di- and trivalent reduction of oxygen in the cytoplasm are represented in the simplified schema below [Eq. 12]:



Singlet oxygen ($^1O_2$), although not a free radical, is an excited state species created by absorption of energy that shifts an electron to an orbit of higher energy level. It is highly reactive and may produce biological effects similar to other reactive oxygen species. In principle, $^1O_2$ could be formed from reactions of the Haber/Weiss cycle or *via* spontaneous dismutation of superoxide anion [Eq. 13, 14]:

$$O_2^- + H_2O_2 \rightarrow {}^1O_2 + \cdot OH + OH^-$$

$$2O_2^- + 2H^+ \rightarrow {}^1O_2 + H_2O_2$$

Photosensitized reactions leading to the formation of $^1O_2$ have been extensively studied (37). Energy transfer, the most common process, involves interaction of a triplet sensitizer (^3S) with triplet-state (ground-state) oxygen ($^3O_2$). The ground state sensitizer (S) may be excited ($^1S^*$) to the triplet (^3S) by photons ($h\nu$) [Eq. 15, 16]:

$$S + h\nu \rightarrow {}^1S^* \rightarrow {}^3S$$

$$^3S + O_2 \rightarrow S + {}^1O_2$$

4.2. Lipid Peroxidation

Biological membranes are rich in polyunsaturated fatty acids (PUFA) and are vulnerable targets for free radical attack. Of particular importance are those free radical-mediated reactions that result in lipid peroxidation (38, 39). This type of chain-propagated reaction usually proceeds by free radical ($R^\bullet$) attack upon PUFA to yield lipid radicals [Eq. 3]: The newly formed PUFA radical ($PUFA^\bullet$) then reacts with molecular oxygen to form fatty acid peroxy radicals [Eq. 17]:

$$PUFA\cdot + O_2 \rightarrow PUFA\text{-}O\text{-}O\cdot$$

The fatty acid peroxy radical, *via* hydrogen abstraction from adjacent PUFA, yields unsaturated hydroperoxides [Eq. 18]:

$$PUFA\text{-}O\text{-}O\cdot + PUFA \rightarrow PUFA\cdot + PUFA\text{-}O\text{-}OH$$

Participation of the newly formed PUFA radical in a propagating chain reaction can lead to extensive disruption of the finely ordered architecture and function of the cellular membranes. Whereas lipid peroxidation may occur at late stages in some disease processes, it is certainly an early event associated with cutaneous UV-insult (23, 40, 41). In addition, the formation of a number of vasoactive molecules and immunopotentiators, important participants in the inflammatory process, are dependent upon PUFA peroxidation (42, 43). These might express themselves in UV-carcinogenesis through immunoregulatory actions occurring at later stages of carcinogenesis. The role, and importance, of each reactive species in UV-carcinogenesis remains speculative, but one can readily see how radical-initiated processes can lead to secondary and tertiary free radicals, with a consequent cascade of damaging effects. Fortunately, cells possess a number of enzymatic and non-enzymatic defense mechanisms to control these reactions.

5. ANTI-OXIDANT DEFENSES

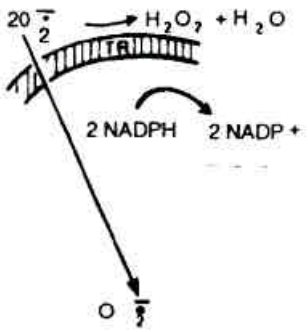
Free radical defense mechanisms may be broadly classified into enzymatic and quenching reactions. Some of the enzymatic reactions involved in the reduction of molecular oxygen have already been mentioned, as exemplified in equations 9-12. The metalloprotein, superoxide dismutase (SOD), dismutes superoxide to

Table 1. Agents that may act as biological anti-oxidants

• Phenols (Polyphenols)
• Carotenoids
• Ascorbate
• Tocopherol
• Reduced glutathione
• Sulfhydryl compounds
• Mannitol
• Uric acid
• Metal chelators
• Selenium

hydrogen peroxide. Other enzymes considered as radical scavengers are catalase and peroxidases. Glutathione peroxidase, as does catalase, reduces hydrogen peroxide to water. The oxidized glutathione (GSSH) is re-reduced to GSH by glutathione reductase in the presence of NADPH [Eq. 12].

In addition to the cytoplasmic anti-oxidant defenses, membrane-associated thioredoxin reductase (TR) acts as a free radical trap at the external surface of the epidermis (44). Thioredoxin reductases are homodimeric proteins, with each monomer including a FAD prosthetic group and a NADPH binding site (45). Furthermore, TR has a regulatory selenocysteine residue. This active site of the cytosolic TR rapidly reduces H_2O_2. Generally, TR reduces oxidized thioredoxin in the presence of NADPH. Reduced thioredoxin serves as an electron donor for thioredoxin peroxidase (TPx). Superoxide anion, in the presence of TR and NADPH, would be reduced to H_2O_2 and H_2O *via* TPx, with the subsequent regeneration of $NADP^+$ (as depicted in schema 19):



Earlier concerns were raised regarding methods employed in assessing TR activity (46). Responses to these concerns have been addressed (47) and it appears that TR plays a much more important role in epidermal free radical defense than previously recognized (48).

There are many low molecular weight agents that may serve as free radical scavengers. Some are listed in Table 1.

The type of process in which anti-oxidants act as free radical scavengers is exemplified by the reaction of ascorbic acid (AAH_2) with the stable free radical diphenylpicryhydrazyl (DPPH•) in equations 20, 21:

$$DPPH\cdot + AAH_2 \rightarrow DPPH_2 + AAH\cdot$$
$$DPPH\cdot + AAH\cdot \rightarrow DPPH_2 + AA$$

Ascorbic acid is first oxidized to yield an intermediate, monodehydroascorbic acid radical (AAH•). This radical then reacts with a second radical of DPPH from which dehydroascorbic acid (AA) is formed. One molecule of ascorbic acid effectively scavenges two DPPH radicals.

Anti-oxidant-mediated termination of chain propagation may proceed *via* reactions similar to those depicted with lipid peroxidation [Eq. 3, 17, 18]. It is generally believed that the lipid peroxy radical is the most likely site at which an anti-oxidant intervenes [Eq. 22].

$$PUFA\text{-}O\text{-}O\cdot + AH_2 + PUFA \rightarrow PUFA\text{-}O\text{-}OH + AH\cdot + PUFA$$

The PUFA of this reaction would, in the absence of an anti-oxidant (AH_2), have reacted with the fatty acid peroxyl radical (PUFA-O-O•) to yield another lipid radical (PUFA•). In the presence of AH_2, the PUFA is spared and the anti-oxidant reacts with the peroxy radical instead, to yield an anti-oxidant radical (AH•).

As has been previously indicated (49), anti-oxidant/free radical reactions are not "fail-safe" processes with respect to damage control *in vivo*. Some anti-oxidants, under certain conditions, act as pro-oxidants. Ascorbic and uric acids, listed in Table 1, may actually promote auto-oxidations by reducing oxygen activators such as transition metals or quinones, both of which can lead to free radical homolysis resulting in radical production. For example, ascorbic acid, in the presence of oxygen and a metal complex, can undergo a redox-induced homolysis that results in the production of perhydroxyl radical or superoxide anion (50). In addition to potential pro-oxidant action, termination of chain propagation, as depicted in equation 22, is contingent upon non-participation of the anti-oxidant radical (AH•) in further peroxidative reactions. Ideally, the anti-oxidant radical would be destroyed by harmless side reactions or restored to an active anti-oxidant state. Nevertheless, the anti-oxidant defense system is intricate, complex and interrelated as exemplified by the regulatory effect of SOD on reactive nitrogen species (RNS). Nitric oxide (•NO) is a reactive free radical gas generated enzymatically from the L-arginine pathway by nitric oxide synthase (NOS). Once produced, •NO can react with superoxide anion to yield peroxynitrate ($^-$OONO), with subsequent release of •OH radicals and destructive oxidations and nitrations (51, 52), [Eq. 23]:

$$^\bullet NO + O_2^{\bullet -} \rightarrow {}^-OONO \rightarrow [Intermediates] \rightarrow Oxidations/Nitrations$$

Thus, the level of nitric oxide that forms peroxynitrate is determined by the concentration of superoxide anion that, in turn, is controlled by SOD. Aside from the known detrimental effects of both reactive oxygen and nitrogen species, it is becoming apparent that these agents play important roles in cell redox-sensitive signal

transduction in pro-inflammatory and oxidative stress responses (28, 53).

In summary, the major cellular defense mechanisms are:

1. Enzymatic removal of superoxide anion and hydrogen peroxide by the detoxifying enzymes SOD, CAT, GP, GR, TR, and NOS.

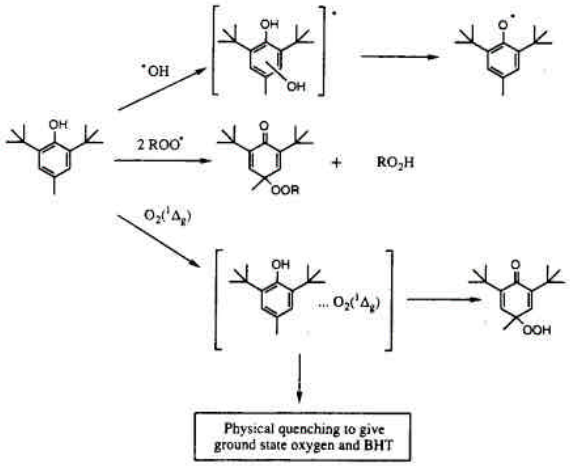
2. Decreasing the availability of metal catalysts that participate in Fenton reactions that yield •OH radicals.

3. Balance of lipid and water-soluble anti-oxidants such as tocopherol, ascorbic acid, and GSH.

The outcome of dietary addition of an anti-oxidant to such a complex system, with respect to UV-induced skin cancer, cannot, at present, be predicted. The hazards of such dietary intervention become obvious when the actions of two efficient anti-oxidants, BHT and β-carotene, are compared.

6. BHT AND UV-CARCINOGENESIS

BHT is a synthetic phenolic compound that has been widely used in the U.S. as a food anti-oxidant since 1954 when it was granted approval by the Food and Drug Administration (54). BHT is known to provide protection against the toxicity and carcinogenicity of a number of chemical and physical agents, including UV-irradiation. It has been assumed that the mechanism by which BHT exerts its anti-oxidant activity involves the quenching of reactive oxygen species and lipid soluble radicals, reactions that have been extensively studied over the past 25 years. The nature of the quenching reactions is known to vary, dependent upon the respective reactive oxygen species and the local environment. Some BHT reactions with reactive species are illustrated in the schema (55) [Eq. 24]:



As shown, the mechanism of reaction of BHT with • OH does not proceed by electron transfer directly, but rather by • OH addition followed by acid and base-catalyzed elimination to yield the phenoxyl radical.

The inhibition of radical chain reactions by BHT proceeds *via* reaction with alkyl peroxyl radicals rather than alkyl radicals. One molecule of BHT is able to react with two peroxyl radicals to yield products that are stable to further reaction. The mechanism is a two-step process involving a reversible reaction of BHT with a peroxyl radical to yield a peroxyl radical-BHT complex that undergoes an irreversible reaction with a second peroxyl radical by hydrogen transfer (56). In organic solvents, the rate-limiting step in this reaction is the formation of the complex.

The reaction of BHT with singlet oxygen, $O_2(^1\triangle_g)$, involves the formation of an encounter complex. Although the reaction is reversible, the collapse of the complex may give product formation or the parent ground state molecules. The fraction of quenching processes that lead to product formation is controlled by the balance between the rate constants for product formation and deactivation and, as shown by Foote *et al* (57), is solvent dependent.

Rate constants, depending upon the physicochemical parameters, of the order of 10^5-10^6 M^{-1} s^{-1} for BHT quenching of reactive oxygen species suggest that BHT, despite its potency as an anti-oxidant, reacts efficiently only with strongly oxidizing species such as • OH.

It is possible that a metabolite derived from or induced by BHT provides the strong anti-oxidant effects exhibited *in vivo*. The major metabolic pathway of BHT in the rodent appears to be oxidation to 3,5-di-*tert*-butyl-4-hydroxybenzoic acid with subsequent conjugation (58). However, when radiolabeled BHT was administered to animals, most of the lipophilic radiolabel recovered from skin co-chromatographed with authenic BHT. In fact, autoradiography of skin sections indicated that most of the radiolabel represented non-metabolized, non-conjugated BHT (59). The correlation between physicochemical parameters and biological function remains a paradox. Nevertheless, the activity of a number of anti-oxidants, including BHT, has been compared to that of β-carotene, both in solution and a model solid food system (60). Despite differences in solution chemistry, the anti-oxidant activities of BHT and β-carotene were found to be comparable.

In 1974, a mixture of dietary anti-oxidants, including BHT, was shown to provide hairless mice a very marked degree of protection to UV-induced actinic lesions and squamous cell carcinomas (61, 62). Dose-response studies indicated that the inhibitory response of the anti-oxidant mixture decreased with increasing UV doses (63). These data suggested that the natural anti-oxidant defense to UV-carcinogenesis could readily become overwhelmed and supported the tenet that anti-oxidant supplementation might be an effective means of reducing disease-related radical damage. When anti-oxidants of the mixture were administered individually at their respective concentrations, only BHT afforded protection to UV-carcinogenesis equivalent to the complete anti-oxidant mixture (64). Pauling *et al* (65) corroborated these findings.

Systemic administration of the anti-oxidant mixture or BHT also influences a number of other UV-mediated cutaneous responses. With increasing level of dietary lipid, with a presumed increase of lipid radical formation upon UV-irradiation, the anti-oxidant supplement markedly inhibited cutaneous levels of lipid peroxidation (23). This effect began to washout with *decreasing* dietary lipid level. It had been demonstrated earlier that formation of a putative carcinogen, cholesterol-5α, 6α-epoxide, a product of photo-oxidation, could be inhibited by the anti-oxidant supplement (66). Thus, BHT is an effective inhibitor of *in vivo* peroxidative processes that, if not causal, are concomitant or participating reactions associated with UV-carcinogenesis.

DeRios *et al* (67) demonstrated that after BHT treatment, twice the level of UV was required to induce erythema in mice when compared to non-supplemented controls. BHT also significantly decreased UV-induction of epidermal ornithine decarboxylase (ODC) activity (68). ODC catalyzes the rate-limiting step in synthesis of polyamines, changes in which reflect proliferative activity in cells. The degree of ODC stimulation has been related to the degree of malignant change although ODC induction appears to lack specificity as a marker for UV-induced tumor production (69). Nevertheless, UV mediated ODC induction has been useful in helping elucidate the mode of action of BHT's photoprotective properties. Forward scattering of UV-irradiation by the epidermis was found to be significantly less in BHT-fed animals than non-supplemented controls (70). It became apparent that a diminution of UV-irradiation penetrating the epidermis could account for BHT's inhibitory effect on all three observed biological responses, i.e., photocarcinogenesis, erythema, and ODC induction. Direct measurement of epidermal levels of resident BHT indicated that the agent, *per se*, would absorb no more than 1% of the incident UV radiation. Nor could increased absorption be explained by changes in the physical dimensions of the epidermal layer. The fact that most polyamines are localized within the basal layer of epidermis and thus the most likely site of ODC activity (71), and that the mouse epidermal layer is only about two cells thick, suggested that the protective effect of BHT might reside in the stratum corneum. Indeed, it was found that transmission between 280-320 nm was approximately 65% greater through stratum corneum obtained from control animals when compared with that of BHT-fed animals (72). Further evidence of the biological significance of this BHT effect upon stratum corneum absorption was obtained when stratum corneum was first removed by tape stripping, the animals irradiated to induce ODC, and ODC activity measured. BHT provided the usual inhibition of ODC activity in non-stripped animals, but ODC activity in BHT-treated, tape-stripped animals was restored to levels that did not significantly differ from controls. The protective effect exhibited by the stratum corneum could not be attributed to BHT-induced alteration of physical dimension of the tissue, as neither the number of stratum corneum layers, or thickness of the stratum corneum, differed from non-supplemented controls. These data supported the contention that systemically administered BHT resulted in diminished levels of UV reaching potential epidermal target sites and delimited a large component of the photoprotective effect to the stratum corneum.

As a consequence of these conclusions, BHT would be expected to exert its major anti-carcinogenesis effect during the initiation stage of photocarcinogenesis. Based upon this rationale, BHT's anti-carcinogenic action was examined in crossover feeding studies in which two of four groups of animals were fed diets containing either no BHT or diets supplemented with BHT. After a tumor-initiating dose of UV had been delivered, one group of each of the respective treatments was crossed to the opposite treatment. Tumor incidence data supported the contention that BHT exerted its major effect during the period in which the initiating incitant was administered and were compatible with a mode of action mediated *via* UV dose reduction (73).

Although the mode of action of BHT's photoprotective effects appears to be UV dose diminution, speculation still surrounds its mechanism(s) of action. It has been suggested that BHT present in the stratum corneum might, through its anti-oxidant properties, retard the natural oxidation or "maturation" of keratin and thus alter the physicochemical properties of this layer. It is well known that of the major effects of free radicals upon membranes and tissues, protein disulfide cross-links are prominent events. It seems possible that during normal keratin maturation (differentiation) processes, BHT might protect sulfhydryl moieties against oxidation, thereby resulting in fewer cross-links. The degree of cross-linking has long been known to alter X-ray diffraction patterns of keratin (74) and would be expected to influence optical properties, as well. Furthermore, the oxidation of two sulphydryl groups to a disulfide bond generates a molecule of H_20. Increased sensitivity of hydrated skin to sunlight is well documented and it has been demonstrated that degree of hydration of stratum corneum markedly affects its transmission characteristics (75). It is conceivable that by interfering with normal oxidation of keratin, BHT gives rise to the observed optical changes of stratum corneum that, in turn, provide photoprotection to acute responses to UV, as well as to carcinogenesis.

7. b-CAROTENE AND UV-CARCINOGENESIS

Of the 600 or so carotenoids that have been identified thus far, β-carotene has received special attention with respect to photoprotection. Chemically, it is a tetraterpenoid consisting of eight isoprenoid residues. β-carotene is a powerful singlet oxygen quencher and exhibits additional, strong anti-oxidant properties (76, 77). Based upon these properties, and epidemiological studies on the consumption of foodstuffs rich in β-carotene, it was suggested that individuals with an above average intake of the carotenoid might experience a lower cancer incidence (78). Indeed, experimental studies conducted in the 1970s and 1980s had shown that β-carotene provided significant photoprotection to UV-carcinogenesis (79-81). In addition, almost all of the large number of prospective and retrospective epidemiological studies of either the intake of

foods rich in β-carotene, or high levels of blood β-carotene, have reported a strong association with reduced risks of various kinds of cancer, including skin cancer (82, 83). However, the role of β-carotene as an anti-cancer dietary supplement has been questioned as a result of a clinical trial in which incidence of nonmelanoma skin cancer was unchanged in patients receiving a β-carotene supplement (50 mg/day) for five years (84). More alarming were the results from a trial in which smokers, provided a 20 mg daily supplement for five to eight years, suffered a significant increase in lung cancer incidence (85). Further, recent experimental studies have failed to demonstrate a photoprotective effect of β-carotene to UV-carcinogenesis (86), and significant *exacerbation* of UV-carcinogenic expression, with respect to tumor latent period and multiplicity, has been reported (87).

Although the rationale of β-carotene's presumed anti-cancer action was based upon the carotenoid's specific capacity to quench singlet oxygen, scavenge oxyradicals, and terminate other free radical reactions, it is apparent that the overall mechanism(s), with respect to its physiological influence, is complex. β-carotene can act as a pro-oxidant at high oxygen concentration (88) and it has been shown, that under oxidative stress conditions, the carotenoid exhibits either limited anti-oxidant properties or a pro-oxidant effect (89, 90).

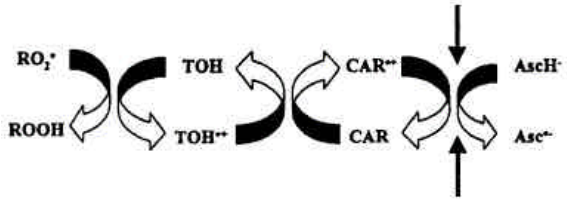
Truscott and associates (91) have shown that many strongly oxidizing species, especially peroxyl radicals, convert carotenoids to the one-electron oxidized form resulting in the radical cation [Eq. 25]:

$$RO_2^{\cdot} + CAR \rightarrow RO_2^{-} + CAR^{\cdot +}$$

The radical cation of β-carotene exhibits a reduction potential of about 1000 mV and represents a very strong oxidizing agent, itself. Furthermore, the decay rate of the β-carotene radical cation has, depending upon microenvironment, a rather long time scale, suggesting that if unrepaired, the radical cation could inflict considerable damage to biological membranes. Edge *et al* (92) have shown that β-carotene reacts with the α-tocopherol radical cation ($TOH^{\bullet +}$) to produce the carotenoid radical cation [Eq. 26] which, in turn, can be repaired by ascorbic acid [Eq. 27].

$$TOH^{\cdot -} + \beta\text{-}Car \rightarrow TOH + Car^{\cdot +}$$
$$CAR^{\bullet +} + AscH^{-} \rightarrow CAR + Asc^{\bullet} + H^{+}$$

Based upon the relative electron transfer rate constants for interactions between β-carotene, α-tocopherol (TOH), and vitamin C (ascorbic acid, $AscH^{-}$), a mechanism was proposed for the ultimate repair of the radical cation (93). In this scenario, developed from previous studies of the interaction of vitamins C and E (94), α-tocopherol would first intercept an oxy radical. In terminating the radical-propagating reaction, the tocopherol radical cation ($TOH^{\bullet +}$) would be formed which, in turn, would be repaired by β-carotene to form the carotenoid radical cation ($CAR^{\bullet +}$). This radical would be repaired by ascorbic acid. Because of its hydrophilic characteristics it is anticipated that the ascorbate radical would be formed in the hydration shell surrounding the membrane and either cleared or enzymatically repaired before it induced membrane damage (95). Equation 28 represents this schema:



Such a mechanism could explain how β-carotene not only quenches oxy radicals but could enhance the radical-protective properties of both vitamins E and C. It does not address the paradox of why β-carotene exacerbates UV-carcinogenesis and increases the incidence of lung cancer in smokers. In the latter case, smokers exhibit markedly reduced plasma vitamin C levels, a reflection of severe oxidant exposure (96). On the other hand mice used in the UV-carcinogenesis studies have no known vitamin C requirement for normal growth and development (97). The semi-defined diet used in studies in which exacerbation of UV-carcinogenesis occurred contained vitamin C, whereas those earlier studies in which photoprotection was observed employed closed-formula diets (commercial rodent chows) devoid of vitamin C. Either condition, i.e., lack of adequate vitamin C in smokers (upper arrow in Eq. 28) or excess vitamin C (lower arrow, Eq. 28) under oxidative stress (UV exposure), in which case vitamin C could act as a pro-oxidant, would lead to an increased level of the unrepaired β-carotene radical cation, and result in a "pro-oxidant" state. The unrepaired β-carotene radical cation could damage membranes by direct action or indirectly by stoichiometrically blocking effective antioxidant activity of α-tocopherol. Thus, the response to β-carotene supplementation might depend on the presence and interaction with other dietary factors, again reflecting the intricate balance of the natural anti-oxidant defense system. Indeed, in recent studies, β-carotene supplementation of a closed-formula diet (devoid of vitamin C) had no significant effect on tumor latent period or tumor multiplicity. However, there was a marked and statistically significant *exacerbation*, with respect to both tumor parameters, in animals receiving the semi-defined diet (vitamin C present) (98). Subsequent studies found that the presence or absence of dietary vitamin C in the semi-defined diet had no influence upon β-carotene-mediated exacerbation of UV-carcinogenesis (99). The latter findings weaken the argument for a mechanism as proposed in Eq. 28 to explain β-carotene's pro-carcinogenic action and, again, illustrate the difficulties of predicting *in vivo* responses from *in vitro* mechanistic studies. Nevertheless, the thesis of such a mechanism is conceptually sound. The *in vivo* data clearly demonstrate that diet can have a profound influence on β-carotene-modulated UV-carcinogenesis. The photoprotective, or non-injurious, effects of β-carotene might depend on the presence and interaction with other dietary factors that are either absent, or present in ineffectual concentrations, in the semi-defined diets. Those factors could be other carotenoids (91, 92),

their isomers, or some yet unidentified phytochemical(s) which, based upon redox potential, could repair the β-carotene radical cation. These possibilities are predicated upon the assumption that the β-carotene radical cation is formed *in vivo* in response to UV-irradiation, an assumption not yet confirmed.

8. CONCLUSIONS

Two inferences might be drawn from the foregoing discussion of the influence of BHT and β-carotene on UV-carcinogenesis. The first relates to the problematic attempts to predict pathophysiologic responses based upon *in vitro* mechanism(s) of action. Although measured rate constants would suggest that mechanisms other than simple competitive kinetics might have to be considered to explain BHT's activity, both BHT and β-carotene have been reported to exhibit comparable anti-oxidant capacity. The predicted anti-cancer properties of both were predicated upon their capacity to quench reactive oxygen species and terminate radical initiated reactions. While both agents exhibit anti-oxidant properties, the pathophysiologic response to each is in contrast. BHT exhibits effective anti-photocarcinogenic properties whereas β-carotene, under certain dietary conditions, exacerbates the process. Thus, while it is essential to understand *in vitro* mechanism(s) of free radical species or their antagonists, this represents only a part of the complex equation of how these agents will act *in vivo* and, as shown, may provide only illusory insights into disease prevention.

The second point reflects a broader application of the first. Agents, such as BHT, that inhibit UV-carcinogenesis may produce other untoward effects that make their use an as anti-photocarcinogenic agent of limited value. A number of these effects have been noted previously (100), but include induction of hepatomegaly; effects on synthesis of clotting factors; induction of pulmonary cell necrosis; alteration of kidney function; possible behavioral and developmental teratogen; and enhancement of certain chemically induced cancers. With respect to the latter response, it has been shown that dietary anti-oxidants, including BHT, enhance activation of N-2-fluorenylacetamide, a powerful hepatocarcinogen (101).

β-carotene, on the other hand, while also an effective anti-oxidant, may, under certain oxidative and dietary conditions, act in a pro-carcinogenic fashion. Although the carotenoid has been shown to produce a number of beneficial effects in a broad range of experimental systems, it has also has been shown to enhance proliferation and DNA synthesis of cells induced by a tumor promoter; to stimulate early induction of ODC (102); to enhance endothelial cell growth and TGF-alpha levels (103); and to induce cytochrome P450 enzymes with a subsequent *over-generation* of reactive oxygen species (104). In view of these effects, as well as it's influence on lung cancer incidence and experimental photocarcinogenesis, it may be prudent to weigh the potential risk-benefit when employing β-carotene therapeutically for other disorders, as well.

It appears that the addition of an antioxidant to the diet in order to limit potential free radical mediated damage no longer represents a simple nor failsafe approach to cancer prevention. The two agents discussed herein may represent the proverbial two-edged sword, acting under specific conditions as either anti-oxidant or pro-oxidant and in anti-cancer or pro-cancer roles, at times creating a threat to the very host they were intended to serve. Until more is known of the influence of anti-oxidants on the *in vivo* side of the equation, dietary supplementation with anti-oxidants as a means of disease prevention would appear to be a course not without risk and should be approached cautiously.

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