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β-Carotene: An Unusual Type of Lipid Antioxidant

G. W. Burton and K. U. Ingold

Epidemiological studies indicate that the incidence of cancer may be slightly lower among individuals with an above-average intake of β-carotene and other carotenoids (*1*). Although the association between dietary β-carotene and decreased cancer incidence may not reflect a causal relationship, β-carotene may exert a genuine protective effect against the onset of cancer (*1*). Several mechanisms for its possible protective action have been suggested (*1*). One of the most interesting proposals is that β-carotene deactivates reactive chemical species, such as singlet oxygen and free radicals, that would otherwise initiate harmful reactions such as lipid peroxidation (*2*) and, through this process, eventually induce cancer (*3–15*). There is ample evidence that β-carotene is a very effective quencher of singlet oxygen (*16, 17*), but the scope of its radical-trapping abilities has not yet been defined.

Summary. The mechanism of lipid peroxidation and the manner in which antioxidants function is reviewed. β-Carotene is a purported anticancer agent, which is believed by some to have antioxidant action of a radical-trapping type. However, definitive experimental support for such action has been lacking. New experiments in vitro show that β-carotene belongs to a previously unknown class of biological antioxidants. Specifically, it exhibits good radical-trapping antioxidant behavior only at partial pressures of oxygen significantly less than 150 torr, the pressure of oxygen in normal air. Such low oxygen partial pressures are found in most tissues under physiological conditions. At higher oxygen pressures, β-carotene loses its antioxidant activity and shows an autocatalytic, prooxidant effect, particularly at relatively high concentrations. Similar oxygen-pressure-dependent behavior may be shown by other compounds containing many conjugated double bonds.

Experimental evidence from our laboratory shows that β-carotene can indeed function as an effective radical-trapping antioxidant. However, because β-carotene represents a previously unknown class of biological antioxidants it is appropriate first to review the mechanism of lipid peroxidation (autoxidation) and the ways in which conventional antioxidants work.

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Mechanism of Autoxidation

Lipid peroxidation in vivo provides a steady supply of free radicals since it is a chain reaction in which the chain is carried by free radicals. This process is generally represented as

Initiation: production of R· from a molecular precursor (1)

Propagation:

$$R\cdot + O_2 \rightarrow ROO\cdot \quad (2)$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot \quad (3)$$

Termination:

$$ROO\cdot + ROO\cdot \rightarrow \text{nonradical products} \quad (4)$$

In this reaction scheme, RH represents the lipid (generally a polyunsaturated fatty acid moiety) and R· the carbon-centered radical derived from it. The overall rate of autoxidation can be represented by

$$-d[O_2]/dt = k_3[RH]R_i^{1/2}/(2\ k_4)^{1/2} \quad (5)$$

where R_i is the rate of initiation and the k's are the rate constants for the indicated reactions. It should be noted that the overall rate of autoxidation does not depend on the oxygen pressure. This somewhat unexpected result has been shown to apply to many different classes of oxidizable substrates at ambient temperatures and at oxygen pressures equal to or greater than those of normal air (*18–20*). Reaction 4 is very much slower than most reactions between two free radicals.

Mechanism of Antioxidant Action

The molecular precursor for the initiation process is generally the hydroperoxide product, ROOH. Lipid peroxidation is therefore a branching chain reaction with potentially devastating effects on a living organism. To control and reduce lipid peroxidation, nature makes use of

compounds known collectively as antioxidants. Such compounds are divided into two broad classes referred to as preventive antioxidants and chain-breaking antioxidants (*21*). Preventive antioxidants reduce the rate of chain initiation, whereas chain-breaking antioxidants interfere with chain propagation by trapping the chain-carrying radicals ROO· or R·, or both. The mechanisms by which these two classes of antioxidants act are described below (*21*).

Peroxide-decomposing (preventive) antioxidants. Antioxidants of this class either stoichiometrically reduce hydroperoxides to the corresponding alcohol

$$ROOH \xrightarrow{2H} ROH + H_2O \quad (6)$$

or catalytically decompose it to nonradical products

$$R_3COOH \xrightarrow{(H^+)} R_2C{=}O + ROH \quad (7)$$

In living organisms a variety of enzymes (such as catalase, glutathione, and peroxidase) act as preventive antioxidants by destroying hydroperoxides without generating free radicals.

Conventional chain-breaking antioxidants. Antioxidants of this class are generally phenols or aromatic amines. They owe their antioxidant activity to their ability to trap peroxyl radicals. For a phenol, such as one of the four tocopherols (α, β, γ, and δ) that together constitute vitamin E (*22*), the initial step involves a very rapid transfer of the phenolic hydrogen

$$ROO\cdot + ArOH \rightarrow ROOH + ArO\cdot \quad (8)$$

The phenoxyl radical, ArO·, is resonance stabilized and is relatively unreactive toward RH and O_2, and therefore it does not continue the chain. It is eventually either destroyed by reaction with a second peroxyl radical

$$ROO\cdot + ArO\cdot \rightarrow \text{nonradical products} \quad (9)$$

or, in certain systems, it may be "repaired," that is, reduced to the starting phenol by reaction with a water-soluble reducing agent such as vitamin C (*23, 24*)

$$ArO\cdot + AH^- \rightarrow ArOH + A^{\cdot -} \quad (10)$$

The rate of a phenol (or aromatic amine) inhibited oxidation in model systems is independent of the oxygen pressure and can be represented by

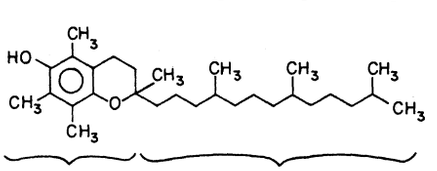
$$-d[O_2]/dt = k_3[RH]R_i/2k_8[ArOH] \quad (11)$$

The activity of such an antioxidant is directly related to the magnitude of k_8. This quantity can be most readily determined by measuring the inhibited rate of autoxidation of some substrate RH, for which k_3 has been measured, at a constant and known rate of chain initiation and at a known antioxidant concentration (*22*). We have used this technique to measure k_8 values for a variety of chain-breaking antioxidants (*22*). Rates of autoxidation in the presence of a suitable, oxidizable substrate were determined at 30°C under an O_2 pressure of 760 torr in an automatic apparatus in which the pressure change in a sealed system was continuously recorded with a pressure transducer. The unique feature of this method is that the autoxidation chains are initiated at a uniform, reproducible rate by the thermal decomposition of azobisisobutyronitrile (AIBN).

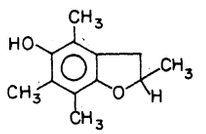
$$(CH_3)_2C(CN)N{=}NC(CN)(CH_3)_2 \longrightarrow N_2 + 2(CH_3)_2\dot{C}CN \xrightarrow{O_2} \xrightarrow{RH} ROO\cdot \quad (12)$$

The absence of this feature in other methods, together with other poorly controlled experimental parameters, has led to considerable and widespread confusion in both the understanding of mechanisms of antioxidant action and in the determination of the relative activities of different antioxidants. For example, earlier studies of the relative in vitro antioxidant activity of the vitamin E tocopherols led to a widespread belief that γ-tocopherol was superior to α-tocopherol [see (*22*)]. By contrast, our technique, which focuses solely on the rate of reaction between an antioxidant and a peroxyl radical, has provided values of k_8 for the tocopherols that decrease in the order α > β > γ > δ (*22*). This is the same as the order of their biological potencies; that is, α-tocopherol is biologically the most active.

The principal chain-breaking antioxidants in living organisms are superoxide dismutase, which acts in the aqueous phase to trap the superoxide radical anion, $O_2^{\cdot -}$ (*25*), and vitamin E which acts in the lipid phase to trap ROO· radicals. We showed earlier (*26*) that vitamin E (principally α-tocopherol) is the major and probably the only peroxyl radical–trapping, lipid-soluble, chain-breaking antioxidant present in human blood. This result is consistent with the fact that, of all the antioxidants (both natural and synthetic) that we originally tested, the largest value for k_8 was obtained with α-tocopherol (*22*). The heterocyclic ring of the chroman "head group" in α-tocopherol plays a very important part in optimizing antioxidant activity (*22, 27*). The realization of this fact has subsequently led to the discovery of a hydroxydihydrobenzofuran, with an even larger value for k_8 (*27*). It will be of interest to determine whether a hydroxydihydrobenzofuran having a properly located phytyl tail (which appears to be required to provide membrane solubility) will also possess superior vitamin E activity.



α-Tocopherol (vitamin E)



β-Carotene

Evidence that β-carotene is not a conventional antioxidant. Where could β-carotene fit in the overall antioxidant picture? Evidence that it actually has antioxidant activity in addition to its ability to quench singlet oxygen is far from compelling and is certainly insufficient to define its mode of action (if any). Some early workers reported that β-carotene is a prooxidant (*28*), whereas others reported that it is an antioxidant (*29*). More recently, Yamane and Lamola (*30*) reported that β-carotene inhibits red blood cell hemolysis induced by cholesterol hydroperoxide; Kellogg and Fridovich (*31*) reported that β-carotene inhibits lipid peroxidations that have been initiated by xanthine oxidase (probably by its deactivating effect on singlet oxygen); Krinsky and Deneke (*32, 33*) reported that although β-carotene was an effective inhibitor of lipid peroxidation in an egg phosphatidylcholine liposome preparation under photosensitizing conditions, the results with other mechanisms for initiating lipid peroxidation were not consistent (for example, β-carotene prevented lipid peroxidation initiated by 0.1*M* $FeCl_2$ at 40°C but was ineffective in a much slower oxidation where the liposomes were merely incubated in air at the same temperature); finally, Kunert and Tappel (*34*) reported that lipid peroxidation of guinea pigs (in vivo) injected with CCl_4 (as measured by pentane and ethane production) can be reduced by a prior treatment with β-carotene.

We conducted a series of experiments showing that β-carotene can function as an antioxidant in systems in which singlet oxygen is not present. However, the following experimental results indicate that it is neither a peroxide-decomposing preventive antioxidant nor a conventional chain-breaking antioxidant.

The absence of preventive antioxidant properties was shown by the fact that β-

carotene did not noticeably accelerate the decomposition of organic hydroperoxides. For example, β-carotene ($8 \times 10^{-4}M$ and $8 \times 10^{-5}M$) did not undergo any observable reaction in the dark at room temperature over the course of 5 days with $2 \times 10^{-2}M$ *tert*-butyl hydroperoxide in chlorobenzene or in absolute ethanol.

Furthermore, β-carotene does not have the structural features commonly associated with chain-breaking antioxidants, although it reacts rapidly with $Cl_3COO^{\cdot}$ radicals (*35, 36*). However, a fast reaction with radicals, while a necessary condition for a good chain-breaking antioxidant, is not a sufficient condition. [To illustrate this point, trialkylboranes are extremely reactive toward $ROO^{\cdot}$ radicals, but they are prooxidants, often igniting spontaneously in air because they yield a radical that can continue the oxidation chain (*37, 38*)]. We find that under our normal experimental conditions—that is, 30°C; O_2 pressure, 760 torr; AIBN, $0.045M$ (which produces an R_i of approximately $4 \times 10^{-9}M$/sec)—β-carotene at a concentration of $5 \times 10^{-6}M$ is almost without effect on the rates of autoxidation of substrates such as Tetralin, styrene, and methyl linoleate. For comparison, α-tocopherol at the same concentration would have reduced the rate of autoxidation of these substrates to less than 1 percent of their uninhibited rates.

Effects of Oxygen Pressure

Other ways autoxidation can be retarded. Our discussion of autoxidation and antioxidants applies, so far, to model systems in which the uninhibited and inhibited rates of autoxidation are independent of the oxygen pressure. Such models are probably inappropriate for living organisms, since certain physiological malfunctions (such as retrolental fibroplasia and intraventricular hemorrhaging, both of which are presumed to involve lipid peroxidation because they can be controlled or moderated by vitamin E supplementation) are exacerbated at oxygen pressures above those present in normal air (*39–41*). Obviously the rate of lipid peroxidation in at least some tissues must be dependent on the oxygen pressure in the surrounding atmosphere. In principal, this could be due to the initiation of autoxidation chains by molecular oxygen

$$O_2 + RH \rightarrow R^{\cdot} + HOO^{\cdot} \qquad (13)$$

but in practice, this source of initiation is generally unimportant. The dependence of lipid peroxidation rates in vivo on oxygen pressure means, therefore, that not all the carbon-centered $R^{\cdot}$ radicals are irreversibly trapped by oxygen in reaction 2. Some of these $R^{\cdot}$ radicals must, instead, become involved in chain-terminating reactions (*18, 19*). Three possibilities are considered below.

Table 1. Effect of the partial pressure of oxygen (PO_2) on the initial rate of oxidation of $5 \times 10^{-3}M$ β-carotene in chlorobenzene at 30°C. Thermal initiation was with AIBN at $4.5 \times 10^{-2}M$.

PO_2 (torr)	$10^8\ (-d[O_2]/dt)$ (M/sec)
760	28
150	14
15	8

Inhibition by cross-termination involving substrate-derived radicals. No antioxidant is present but the overall chain termination process contains a contribution from the cross-reaction

$$R^{\cdot} + ROO^{\cdot} \rightarrow \text{nonradical products} \qquad (14)$$

This reaction is very much faster than reaction 4, being essentially diffusion controlled. For most substrates at ambient temperatures and at oxygen pressures greater than 100 torr, the concentration of $R^{\cdot}$ is too low for reaction 14 to make an appreciable contribution to termination in comparison with reaction 4. However, reaction 14 can become important at lower oxygen pressures or higher temperatures, particularly for substrates yielding resonance-stabilized $R^{\cdot}$ radicals (*18*). This is not because reaction 2 is slow for such species (*42*). It is always a very fast reaction, being essentially diffusion controlled, even for resonance-stabilized carbon-centered radicals (*42*). However, reaction 2 is reversible and, other things being equal, reversibility is more important for resonance-stabilized $R^{\cdot}$ radicals (*20, 43–49*)

$$ROO^{\cdot} \rightarrow R^{\cdot} + O_2 \qquad (-2)$$

In living organisms, the partial pressure of oxygen in the capillaries of active muscle is only about 20 torr (*50*). In tissue that is more remote from the blood supply, the partial pressure of oxygen must be considerably lower. This will be particularly true of organelles such as the mitochondria that are within the cells and are actively metabolizing oxygen.

The peroxidation of lipids containing polyunsaturated fatty acid units generates radicals of the pentadienyl type

$$ROO^{\cdot} + R'CH{=}CHCH_2CH{=}CHR'' \rightarrow ROOH + R'\dot{C}H\bar{C}H\bar{C}H\bar{C}H\bar{C}HR'' \qquad (15)$$

These have been specifically demonstrated to undergo a reversible reaction with oxygen under ambient conditions (*46–48*), and the rates of autoxidation of polyunsaturated fatty acid esters decrease at oxygen partial pressures below approximately 20 torr at room temperature (*19, 51*)

$$R'\bar{C}H\bar{C}H\dot{C}H\bar{C}H\bar{C}HR'' + O_2 \rightleftharpoons R'CH(OO^{\cdot})CH{=}CH{-}CH{=}CHR'' \qquad (16)$$

It seems likely that such pentadienyl radicals will contribute significantly to chain termination in at least some tissue under normal physiological conditions.

Inhibition by trapping the carbon-centered radicals. An antioxidant is present that traps $R^{\cdot}$ radicals. There is, in fact, one class of commercial chain-breaking antioxidants that owe their activity to this reaction. These are certain persistent di-*tert*-alkylaminoxyl radicals (nitroxides) or their amine precursors. These free radicals do not react with oxygen and so do not undergo a radical chain oxidation. However, they are able to trap $R^{\cdot}$ radicals, but not $ROO^{\cdot}$ (*52*), at rates comparable to the rate of reaction 2 (*53*)

$$R^{\cdot} + R'_2NO^{\cdot} \rightarrow R'_2NOR \qquad (17)$$

The activity of this class of chain-breaking antioxidants increases as the oxygen pressure is decreased. They are not very effective under most conditions in solution because they must be used at relatively high concentrations to compete effectively with reaction 2.

Inhibition by cross-termination involving radicals from a minor cooxidant. In many ways this possibility is similar to that involving substrate-derived radicals, but we are now considering a compound that is only a minor component of the total system. The compound is a cooxidant (or even a prooxidant) at high oxygen pressures but changes to become an antioxidant at reduced oxygen pressures. To our knowledge, only triphenylmethane and some related polyarylmethanes have been (more or less) shown to behave in this manner (*45, 54, 55*). Triphenylmethane (*54, 55*) and its hydroperoxide (*45*) have been shown to reduce the rates of autoxidation of other hydrocarbons, the effect being greater with higher temperatures (*55*) and lower oxygen pressures (*45*). The overall autoxidation scheme in the presence of triphenylmethane can be represented by reactions 1, 2, and 3, plus

$$ROO^{\cdot} + Ph_3CH \rightarrow ROOH + Ph_3C^{\cdot} \qquad (18)$$

$$Ph_3C^{\cdot} + O_2 \rightleftharpoons Ph_3COO^{\cdot} \qquad (19)$$

$$Ph_3COO^{\cdot} + RH \rightarrow Ph_3COOH + R^{\cdot} \qquad (20)$$

$$ROO^{\cdot} + Ph_3C^{\cdot} \rightarrow \text{nonradical products} \qquad (21)$$

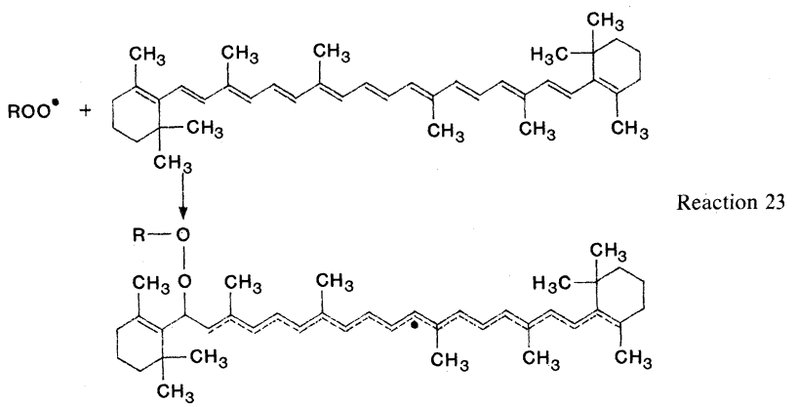
while, in the presence of triphenylmethyl hydroperoxide, the additional reaction

$$ROO^{\cdot} + Ph_3COOH \rightarrow ROOH + Ph_3COO^{\cdot} \quad (22)$$

must be included.

Experimental evidence that β-carotene can behave as an antioxidant at low oxygen pressures. As mentioned previously, we have shown that β-carotene is a rather ineffective antioxidant at 30°C and 760 torr oxygen pressure for various substrates, particularly when compared with vitamin E. Furthermore, we have found that β-carotene itself undergoes a fairly facile autoxidation in chlorobenzene under these conditions (see Table 1), and the reaction, even though initiated with AIBN, is autocatalytic (*56, 57*). However, the initial rates of β-carotene autoxidation decrease as the oxygen pressure is reduced (see Table 1) (*58*).

The effect of β-carotene on the rates of autoxidation of Tetralin and methyl linoleate at various oxygen pressures are summarized in Tables 2 and 3, respectively. Some data have also been included for triphenylmethane. Our results show that β-carotene, particularly when it is present at rather low concentrations, is a very effective antioxidant. Since β-carotene itself undergoes autoxidation, it could not be an antioxidant analogous to the aminoxyl radicals described above, which owe their oxygen-dependent antioxidant activity to an ability to trap R· but not ROO· radicals. The mechanism of its antioxidant action is, we believe, essentially the same as that for triphenylmethane, although β-carotene is certainly the better antioxidant. The inhibiting, resonance-stabilized, carbon-centered radical is probably formed by the addition of an ROO· (and, perhaps also of an R·) radical to the conjugated system of β-carotene, as shown, for example in reaction 23, rather than by an initial H-atom abstraction (reaction 18).



Reaction 23

To be effective, chain-breaking antioxidants must be very reactive toward the chain-carrying radicals since they are used at concentrations much lower than that of the oxidizable substrate, but they must still compete with the substrate for the radicals. β-Carotene is highly reactive towards peroxyl radicals and this reactivity, combined with an equilibrium (corresponding to reaction 2 or reaction 19) that favors the β-carotene–derived carbon-centered radicals, makes this compound a rather good reagent for reducing the concentration of the chain-carrying peroxyl radicals at low partial pressures of oxygen. The same will be true of other carotenoids, and also of the retinoids (*29, 59*), because antioxidant activity depends only on the production of a resonance-stabilized carbon-centered radical (*60*).

At elevated oxygen pressures, β-carotene and related compounds may act as prooxidants since the oxidation rates at oxygen pressures of 150 torr and higher with Tetralin or 760 torr or higher with methyl linoleate appear to have minimum values at a β-carotene concentration of approximately $5 \times 10^{-4} M$ (see Tables 2 and 3). This implies that if comparable concentrations of such compounds were present in the lipid portion of the tissue of a living organism, they might contribute to oxygen-induced cellular damage, particularly at elevated oxygen pressures.

Our results demonstrate that β-carotene has the potential to play an important role in protecting lipid tissue from peroxidation in vivo. The chain-breaking action of β-carotene complements that of vitamin E, since β-carotene is effective at low oxygen concentrations and vitamin E is effective at high oxygen concentrations (*61*). One might therefore predict that β-carotene and related compounds will tend to be concentrated (by those living organisms that can absorb such materials; for example, man) in those particular membranes and organelles that are exposed to the lowest partial pressures of oxygen. To the best of our knowledge, such detailed information is not available. However, as a corollary one might expect that vitamin E will tend to be concentrated in those lipid regions

Table 2. Effect of the partial pressure of oxygen (PO_2) on the initial rate of oxidation of 3.67*M* Tetralin in chlorobenzene at 30°C, showing the influence of β-carotene and triphenylmethane. Significant autocatalysis is indicated by entries giving rates at different times after the addition of the initiator. Thermal initiation was with AIBN at $4.5 \times 10^{-2} M$.

Substrate concentration (*M*)	10^8 ($-d[O_2]/dt$) (*M*/sec) at PO_2 values of		
	760 torr	150 torr	15 torr
β-Carotene			
0		52	50
5×10^{-5}	32	30	21
5×10^{-4}	19	11	10
5×10^{-3}	32 (4 minutes) 46 (14 minutes)	20	8
Triphenylmethane			
7×10^{-3}	50	44	38
5×10^{-2}	44	30	17

Table 3. Effect of the partial pressure of oxygen (PO_2) on the initial rate of oxidation of 1.51*M* methyl linoleate in chlorobenzene at 30°C, showing the influence of β-carotene. Significant autocatalysis is indicated by entries giving rates at different times after addition of the initiator. Thermal initiation was with AIBN at $4.5 \times 10^{-2} M$.

β-Carotene concentration (*M*)	10^8 ($-d[O_2]/dt$) (*M*/sec) at PO_2 values of		
	760 torr	150 torr	15 torr
0	144	132	100
5×10^{-5}	94	74	58
5×10^{-4}	51 (6 minutes) 64 (15 minutes)	33	20
5×10^{-3}	73 (4 minutes) 90 (14 minutes)	30	13

that are exposed to the highest partial pressures of oxygen; for example, cells lining the outer surface of the lung and red blood cell membranes. The results of Kornbrust and Mavis (*62*) suggest that this is the case.

In conclusion, our results provide a rationale for the possible anticancer activity of β-carotene, insofar as the onset and progress of cancer are affected by free radicals.

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60. It is interesting that the length of the β-carotene molecule corresponds approximately to the width of a typical membrane lipid bilayer. With an orientation perpendicular to the membrane plane, the conjugated system of double bonds spanning almost the entire length of the molecule could, in principle, trap radicals at any depth in the membrane.
61. Our proof that vitamin E is probably the only peroxyl radical–trapping, lipid-soluble, chain-breaking antioxidant in human blood relied on a titration for antioxidant with peroxyl radicals at an oxygen pressure of 760 torr (*26*, *27*). This titration would be relatively insensitive to antioxidants that are effective only at low partial pressures of oxygen.
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63. This work owes its origins to some exciting ideas presented to us by N. I. Krinsky and B. N. Ames regarding the possible reasons for β-carotene's effect on cancer incidence. A grant from the National Foundation for Cancer Research supported our investigation. We are also indebted to T. F. Slater for his comments, advice, and help once this work was under way. Finally, we thank T. Doba for his electron spin resonance study of the peroxyl radical plus β-carotene reaction at low temperature (*36*).