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Vitamin E analogue Trolox C

E.s.r. and pulse-radiolysis studies of free-radical reactions

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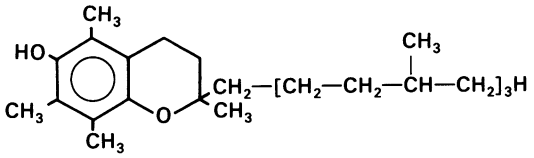
The reactions between Trolox C, a water-soluble vitamin E analogue, and several oxidizing free radicals including the hydroxyl radical and various peroxy radicals were examined by using the pulse-radiolysis technique. The results demonstrate that Trolox C may undergo rapid one-electron-transfer reactions as well as hydrogen-transfer processes; the resulting phenoxyl radical is shown to be relatively stable, in common with the phenoxyl radical derived from vitamin E. The reactions between the Trolox C phenoxyl radical and a variety of biologically relevant reducing compounds were examined by using both pulse radiolysis and e.s.r. The results demonstrate that the Trolox C phenoxyl radical is readily repaired by ascorbate ($k = 8.3 \times 10^6$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$) and certain thiols ($k < 10^5$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$) but not by urate, NADH or propyl gallate. Evidence from e.s.r. studies indicates that thiol-containing compounds may also enter into similar repair reactions with the α-tocopherol phenoxyl radical. Kinetic evidence is presented that suggests that Trolox C may 'repair' proteins that have been oxidized by free radicals.

INTRODUCTION

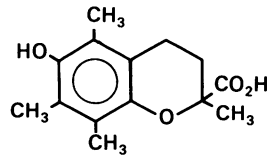
The prevention of the autoxidation of polyunsaturated fatty acids by chain-breaking phenolic antioxidants continues to attract considerable interest. The autoxidation of membrane lipids following free-radical insult has been implicated in some pathological conditions, and several inherent protective mechanisms have been characterized (Pryor, 1976–1983; Fridovich, 1976; Porter & Whelan, 1983; Slater, 1984). Vitamin E, which is present in cells mainly as α-tocopherol (Witting, 1980), has been shown to be a potent inhibitor of free-radical-mediated membrane damage and acts by scavenging chain-carrying peroxyl radicals, resulting in the formation of the α-tocopherol phenoxyl radical (Burton & Ingold, 1983). Clearly, any regeneration of the vitamin through repair of this radical will result in greater antioxidant efficiency.

Such a synergistic process between vitamin E and vitamin C (Tappel, 1968) has been directly observed (Packer *et al.*, 1979). Several reports have confirmed this synergism (Leung *et al.*, 1981; Niki *et al.*, 1982, 1984; Barclay *et al.*, 1983). Other reports suggest that thiol compounds may also repair the α-tocopherol phenoxyl radical (Niki *et al.*, 1982, 1984; Tsuchiya *et al.*, 1983; Rousseau *et al.*, 1984). Indeed, some synergism between GSH and α-tocopherol has been observed in microsomal systems (Reddy *et al.*, 1982). To date, most kinetic studies with α-tocopherol have been undertaken in organic and liposomal systems, studies in aqueous systems having been hampered by the vitamin's poor solubility (Burton & Ingold, 1981, 1983; Winterle *et al.*, 1984).

The compound 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Trolox C (Fig. 1), a water-soluble analogue of α-tocopherol, has been found to compare well with several commercial antioxidants, and some kinetic studies on this compound have been reported (Scott *et al.*, 1974; Bisby *et al.*, 1982, 1984). In the present paper we describe studies on the oxidation of Trolox C by several free radicals of biological interest. The reactions of the resulting phenoxyl radical with a variety of biological reducing agents have also been investigated in order to assess which compounds may act synergistically with α-tocopherol with respect to biological protection.



Vitamin E



Trolox C

Fig. 1. Structural formulae of vitamin E and Trolox C

Abbreviations used: TxOH, Trolox C; TxO·, Trolox C phenoxyl radical.

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MATERIALS AND METHODS

Propanone, 2-methylpropan-1-ol, propan-2-ol, CCl_4, $CHCl_3$ and CH_2Cl_2 (AnalaR) were supplied by BDH Chemicals. Trolox C (> 97%), KBr, KSCN, *o*-cresol, *m*-cresol, *p*-cresol, ^2H_2O (99%), $NaNO_2$, phenol, salicylic acid and 5,5-dimethyl-1-pyrroline *N*-oxide were from the Aldrich Chemical Co. Sodium formate was supplied by Fluka and 2,2′-azinodi-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) from Boehringer Mannheim. All other chemicals used were of the highest purity available and were supplied by Sigma Chemical Co. The gases employed, N_2O and N_2, were from British Oxygen Co.

The pulse-radiolysis experiments were undertaken on the Brunel 4 MeV linear accelerator (200 ns electron pulse) and associated equipment as described previously (Willson, 1978, 1982). Doses of up to 10 $J \cdot kg^{-1}$, determined by thiocyanate dosimetry, were employed (Adams *et al.*, 1965). All solutions were prepared just before use in Millipore-filtered distilled water and were exposed to the minimum of light. Where necessary solutions were saturated with N_2 or N_2O by using the syringe bubbling technique (Willson, 1970).

E.s.r. experiments were performed on a Bruker ER 200D spectrometer equipped with 100 kHz modulation and a Bruker ER 035M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the spectra by using the gaussmeter marker signals as calibration. U.v. irradiation was carried out with the diffused unfocused output of a Heraeus 200 W mercury/xenon arc as described previously (Davies & Slater, 1986).

RESULTS AND DISCUSSION

Hydroxyl radical and Trolox C

On pulse radiolysis of aqueous N_2O-saturated solutions of Trolox C (0.1 mM) at pH 7 a strong transient absorption spectrum ($\lambda_{max.}$ 430 nm) was observed (Fig. 2). Immediately after the pulse (1 μs) there was a sharp increase in absorbance at 330 and 435 nm. This was followed by a slower process that led to further formation of the same species (Fig. 2). This spectrum, which is similar to that previously assigned to the α-tocopherol phenoxyl radical (Packer *et al.*, 1979) and that reported for the Trolox C radical (Bisby *et al.*, 1984), is assigned to this latter species. When the experiment was repeated at pH 11 only a rapid increase in absorption was observed, with no evidence for a second slower reaction. Increasing the pH also increased the yield of the absorption at 435 nm ($G\epsilon$ = 10500 at pH 7, $G\epsilon$ = 23300 at pH 11). The rate of increase at 435 nm was biphasic and kinetic analysis proved difficult. The initial rapid component of the absorption increase was first-order in Trolox C at pH 7. On increasing the Trolox C concentration the initial fast process at 435 nm increased in rate whereas the slower process did not. If it is assumed that the absorption is due to the Trolox C phenoxyl radical (TxO$^\bullet$), then the slower appearance at pH 7 can be attributed to an intramolecular process (k approx. 10^4 s^{-1}) following OH$^\bullet$ attack, which is not evident at pH 11. This is consistent with studies with phenol, where the initial radical observed was identified as the OH$^\bullet$ adduct, which subsequently eliminates water in an acid/base-catalysed reaction with k approx. 10^3 s^{-1} (Land & Ebert, 1967). The OH$^\bullet$ adducts to phenols absorb at approx. 320 nm, and if such a species was formed with Trolox C its presence may be masked by the TxO$^\bullet$ absorption at 330 nm. There are several sites on Trolox C that could be susceptible to OH$^\bullet$ attack, including hydrogen abstraction at the phenolic group (giving TxO$^\bullet$ directly), addition to the phenolic ring and abstraction at the chroman ring. This last process would not result in phenoxyl radical formation, whereas an OH$^\bullet$ adduct formed by addition to the phenolic ring might yield the phenoxyl radical via the elimination of water (yielding the Trolox C radical cation, which could then undergo rapid deprotonation to generate TxO$^\bullet$). Such a process would be expected to be acid/base-catalysed (cf. similar reactions with phenol; Land & Ebert, 1967), in agreement with the fact that the slower process found at pH 7 becomes too rapid to observe at pH 11. These results are consistent with the predominant reactions:

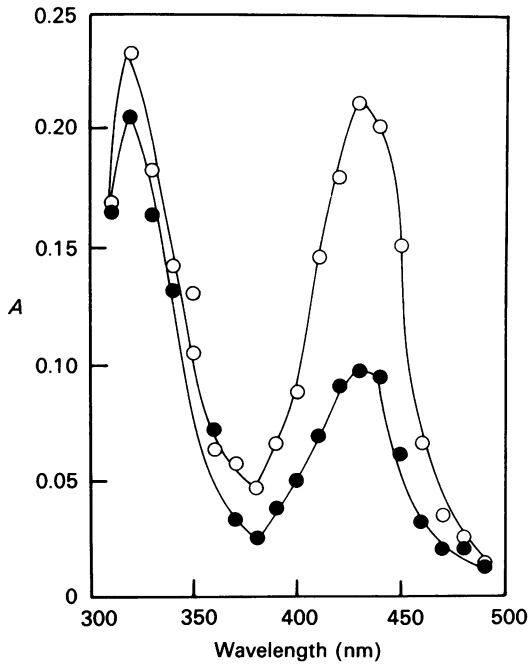


Fig. 2. Transient absorption spectra observed 1 μs (●) and 125 μs (○) after pulse radiolysis of an N_2O-saturated solution containing 0.1 mM-Trolox C at pH 7

$$H_2O \xrightarrow{\text{Radiation}} e^-_{(aq.)} + OH^\bullet + H^\bullet \quad (1)$$

$$e^-_{(aq.)} + N_2O \longrightarrow N_2 + OH^- + OH^\bullet \quad (2)$$

$$TxOH + OH^\bullet \longrightarrow TxO^\bullet + H_2O \quad (3)$$

$$TxOH + OH^\bullet \longrightarrow TxOH(OH)^\bullet \quad (4)$$

$$TxO^- + OH^\bullet \longrightarrow TxO(OH)^{\bullet -} \quad (5)$$

$$TxOH(OH)^\bullet \longrightarrow TxO^\bullet + H_2O \quad (6)$$

$$TxO(OH)^{\bullet -} \longrightarrow TxO^\bullet + OH^- \quad (7)$$

with the overal rate constant for TxO$^\bullet$ formation 2.2×10^9 $dm^3 \cdot mol^{-1} \cdot s^{-1}$ at pH 7 and $k_4 + k_5 = 3.8 \times 10^9$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$ at pH 11. In order to confirm this interpretation, the reaction between OH$^\bullet$ and Trolox C at pH 7 and pH 11 was studied by using competition kinetic methods with 2,2′-azinodi-(3-ethyl-

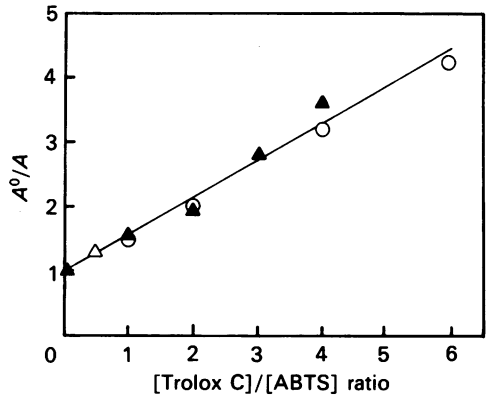


Fig. 3. Competition plot obtained for the reaction of OH· with 2,2′-azinodi-(3-ethylbenzothiazoline-6-sulphonate) and Trolox C at pH 7 (○) and pH 11 (▲)

The results were obtained from pulse radiolysis of N_2O-saturated solutions containing 0.1 mM-2,2′-azinodi-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and Trolox C. The dose was 4 Gy.

benzothiazoline-6-sulphonate) as the reference (with k 1.2×10^{10} $dm^3 \cdot mol^{-1} \cdot s^{-1}$; Wolfenden & Willson, 1982). Fig. 3 shows the competition plots obtained. Clearly there is no difference in rate constant at pH 7 or pH 11, in agreement with the competition method measuring the overall rate of reaction of OH· with Trolox C (i.e. attack at all sites, determined as k_8 6.9×10^9 $dm^3 \cdot mol^{-1} \cdot s^{-1}$). The direct method measures the rate of formation of TxO· arising from both direct reaction of OH· with the phenol group and the slower addition/elimination process, resulting in a lower value of the rate constant:

$$OH^{\cdot} + TxOH/TxO^- \rightarrow \text{Products} \quad (8)$$

This value is lower than a previously reported value of 2.2×10^{10} $dm^3 \cdot mol^{-1} \cdot s^{-1}$ determined by competition kinetic methods with the radical $(SCN)_2^{\cdot -}$ as a reference (Bisby *et al.*, 1982). As shown below, $(SCN)_2^{\cdot -}$ reacts rapidly with Trolox C, which could lead to an overestimation of the rate constant.

Reactions of the inorganic radicals with Trolox C

On pulse radiolysis of an N_2O-saturated solution containing 0.1 M-NaN_3 and 10 mM-Trolox C at pH 7 a similar transient to that observed with OH· was found but with an enhanced yield ($G\epsilon = 38800$) attributable to the overall reaction:

$$N_3^{\cdot} + TxOH \rightarrow TxO^{\cdot} + H^+ + N_3^- \quad (9)$$

with $k_9 = 5.0 \times 10^8$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$. Assuming that the yield of azide radicals is 0.6 $\mu mol \cdot J^{-1}$, then this corresponds to a molar decadic extinction coefficient of 7100 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ for TxO·.

Similar results were obtained with 0.1 M-KBr instead of NaN_3 at pH 10. In this case the absorption due to $Br_2^{\cdot -}$ was observed immediately after the pulse, but this was rapidly replaced by that assigned to TxO·, the appearance of which was exponential and first-order in Trolox C concentration with $k = 4.3 \times 10^8$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$ (cf. a value of 3.8×10^8 $dm^3 \cdot mol^{-1} \cdot s^{-1}$ at pH 7 reported previously; Bisby *et al.*, 1984). Similarly, when the thiocyanate radical anion $(SCN)_2^{\cdot -}$ (generated from 0.1 M-KSCN at pH 7) was used as the oxidant, the rates of appearance of TxO· at 330 nm and the decay of $(SCN)_2^{\cdot -}$ at 480 nm were found to be exponential, first-order in Trolox C and independent of pH within the range 7–11, in agreement with $k_{10} + k_{11} = 2.2 \times 10^8$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$:

$$(SCN)_2^{\cdot -} + TxOH \rightarrow 2SCN^- + TxO^{\cdot} + H^+ \quad (10)$$

$$(SCN)_2^{\cdot -} + TxO^- \rightarrow 2SCN^- + TxO^{\cdot} \quad (11)$$

Thiyl radicals, generated by pulse radiolysis of free thiols or the corresponding disulphides (Adams *et al.*, 1968), can enter into one-electron-transfer reactions (Wolfenden & Willson, 1982; Forni *et al.*, 1983*a*; Stratford *et al.*, 1984; Ahmad & Armstrong, 1984; Forni & Willson, 1986). On pulse radiolysis of an N_2-saturated solution containing 1 M-2-methylpropan-2-ol, 10 mM-cystamine and 0.1 mM-Trolox C at pH 12.1 the characteristic absorption due to TxO· was observed, in agreement with:

$$e^-_{(aq.)} + CySSCy \rightarrow CySSCy^{\cdot -} \quad (12)$$

$$CySSCy^{\cdot -} \rightleftharpoons CyS^- + Cys^{\cdot} \quad (13)$$

$$CyS^{\cdot} + TxO^- \rightarrow CyS^- + TxO^{\cdot} \quad (14)$$

with $k_{14} = 8 \times 10^8$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$ at pH 12.1. At pH 6.2 the TxO· spectrum was again apparent, but the rate of appearance of TxO· was slower and the yield approx. 25% lower, with $k_{15} = 1 \times 10^8$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$ at pH 7:

$$CyS^{\cdot} + TxOH \rightarrow CyS^- + H^+ + TxO^{\cdot} \quad (15)$$

Nitrogen dioxide, $NO_2^{\cdot}$, which can be formed by reaction of OH· with $NaNO_2$ in accordance with reaction (16) with $k_{16} = 1.3 \times 10^9$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$, has been shown to undergo one-electron-transfer reactions in aqueous solution and hence might be expected to react with Trolox C (Forni *et al.*, 1986):

$$OH^{\cdot} + NO_2^- \rightarrow OH^- + NO_2^{\cdot} \quad (16)$$

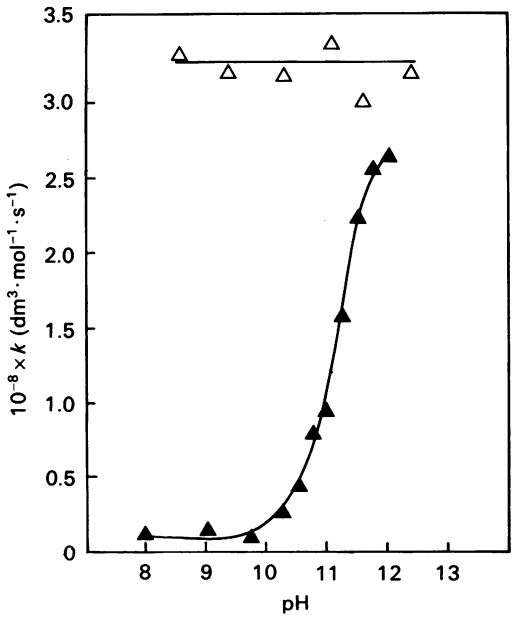


Fig. 4. Effect of pH on the first-order rate constant for the increase in absorption at 435 nM following pulse radiolysis of N_2O-saturated solutions containing 2 M-$NaNO_2$ and 0.2 mM-Trolox C (▲) or 0.1 M-KSCN and 0.1 mM-Trolox C (△)

On pulse radiolysis of N_2O-saturated solutions containing 0.1 mM-Trolox C and 0.1 M-$NaNO_2$ at pH 6.6 no absorption due to TxO$^\bullet$ could be detected, indicating that reaction (17) is relatively slow (i.e. $k < 10^5$ dm$^3\cdot$mol$^{-1}\cdot$s^{-1}):

$$NO_2^\bullet + TxOH \rightarrow NO_2^- + TxO^\bullet + H^+ \quad (17)$$

However, at pH 11.5 the absorption spectrum of TxO$^\bullet$ appeared exponentially with the rate of appearance being first-order in Trolox C concentration in agreement with $k_{18} = 5\times10^8$ dm$^3\cdot$mol$^{-1}\cdot$s^{-1}:

$$NO_2^\bullet + TxO^- \rightarrow NO_2^- + TxO^\bullet \quad (18)$$

The effect of pH on the overall rate constant (Fig. 4) is in agreement with a pK_a of 11.7 for the TxOH/TxO$^-$ couple; this compares favourably with a previously reported value of 11.9 (Steenken & Neta, 1982).

These results show that TxO$^\bullet$ is formed rapidly on reaction of $N_3^\bullet$, $Br_2^{\bullet-}$ and Cys$^\bullet$ at neutral pH, and with these radicals plus $NO_2^\bullet$ when the Trolox C phenol group is ionized. At neutral pH the reactions might be thought to involve initial one-electron transfer followed by rapid deprotonation, as is thought to occur on oxidation of tyrosine by $SO_4^{\bullet-}$ or $N_3^\bullet$ (Bansal & Fessenden, 1970; Alfassi & Schuler, 1985), e.g.:

$$Br_2^{\bullet-} + TxOH \rightarrow 2Br^- + TxOH^{\bullet+} \quad (19)$$

$$TxOH^{\bullet+} \rightleftharpoons TxO^\bullet + H^+ \quad (20)$$

In the present study no spectral evidence for a radical cation was obtained. Generally, phenoxyl radical cations have pK_a values of approx. -5 (Land *et al.*, 1961). Therefore the Trolox C radical cation TxOH$^{\bullet+}$ would be expected to deprotonate extremely rapidly. The formation of such a species (with a lifetime of several minutes) has been demonstrated with a homologue of vitamin E by using cyclic voltammetry and e.s.r. in acidic acetonitrile, where the rate of deprotonation is less rapid (Svanholm *et al.*, 1974).

Apparent absence of reaction of $O_2^{\bullet-}$ and $HO_2^\bullet$ radicals with Trolox C

On pulse radiolysis of N_2O/O_2-(4:1)-saturated solutions of Trolox C (0.1 mM) containing excess formate at pH 8 a transient absorption was observed immediately after the pulse with $\lambda_{max.} = 260$ nm, characteristic of the superoxide radical anion $O_2^{\bullet-}$ formed by reactions (21) and (22):

$$OH^\bullet + HCO_2^- \rightarrow H_2O + CO_2^{\bullet-} \quad (21)$$

$$CO_2^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + CO_2 \quad (22)$$

However, no formation of the TxO$^\bullet$ absorption was observed, and increasing the Trolox C concentration did not affect the rate of decay of the $O_2^{\bullet-}$ radical. At pH 3, where $O_2^{\bullet-}$ protonates to form the hydroperoxyl radical $HO_2^\bullet$ (pK_a 4.9), no absorbance due to TxO$^\bullet$ could be observed, indicating that the reactions:

$$O_2^{\bullet-} + TxOH \rightarrow O_2^{2-} + TxOH^{\bullet+} \quad (23)$$

$$HO_2^\bullet + TxOH \rightarrow H_2O_2 + TxO^\bullet \quad (24)$$

either have $k_{23} + k_{24} < 10^5$ dm$^3\cdot$m$^{-1}\cdot$s^{-1}, or a product other than the phenoxyl radical is formed that cannot be detected optically. E.s.r. evidence has been presented indicating that the superoxide radical can oxidize Trolox C, with the formation of the phenoxyl radical in accordance with eqn. (23) followed by eqn. (16) (Ozawa *et al.*, 1978). Other studies in aqueous systems where $O_2^{\bullet-}$ was generated enzymically also showed oxidation of α-tocopherol and Trolox C, though in these studies the involvement of $HO_2^\bullet$ cannot be ruled out (Nishikimi & Machlin, 1975; Nishikimi *et al.*, 1980). A value for $k_{23} = 1.7\times10^4$ dm$^3\cdot$mol$^{-1}\cdot$s^{-1} has been reported, and for the same reaction involving α-tocopherol a value of $k = 6$ dm$^3\cdot$mol$^{-1}\cdot$ s^{-1} (Nishikimi & Machlin, 1975; Fukuzawa & Gebicki, 1983). In the latter study the possible role of $HO_2^\bullet$ was also examined:

$$HO_2^\bullet + VitE\text{-}OH \rightarrow H_2O_2 + VitE\text{-}O^\bullet \quad (25)$$

and a value $k_{25} = 2\times10^5$ dm$^3\cdot$mol$^{-1}\cdot$s^{-1} was reported (Fukuzawa & Gebicki, 1983).

Reactions of halocarbonperoxy radicals with Trolox C

There is considerable evidence that free-radical intermediates are involved in the hepatotoxicity of several halogenated compounds (Slater, 1978), leading to lipid peroxidation *in vivo* as well as *in vitro*. Although the reaction between the trichloromethyl radical derived from CCl_4 and α-tocopherol has not been detected by pulse radiolysis, the corresponding peroxy radical has been shown to undergo rapid one-electron-transfer reactions with a variety of electron donors such as ascorbate and phenothiazines. The radical also reacts rapidly with polyunsaturated fatty acids and α-tocopherol (Packer *et al.*, 1978; Forni *et al.*, 1983*b*). On pulse radiolysis of air-saturated solutions containing 40% (v/v) 2-methylpropan-2-ol [to scavenge HO$^\bullet$ radicals; reaction (26)] and 10 mM-CCl_4 the trichloromethylperoxy radical $CCl_3OO^\bullet$ is generated in accordance with (Packer *et al.*, 1980, 1981):

$$OH^\bullet + CH_3(CH_3)_2COH \rightarrow H_2 + \dot{C}H_2(CH_3)_2COH \quad (26)$$

$$e^-_{(aq.)} + CCl_4 \rightarrow CCl_4^{\bullet-} \rightarrow CCl_3^\bullet + Cl^- \quad (27)$$

$$CCl_3^\bullet + O_2 \rightarrow CCl_3O_2^\bullet \quad (28)$$

On addition of 0.1 mM-Trolox C to the system the absorption spectrum of the Trolox C phenoxyl radical was observed in agreement with the overall reaction:

$$CCl_3O_2^\bullet + TxOH \rightarrow CCl_3O_2H + TxO^\bullet \quad (29)$$

The absorption appeared exponentially and was first-order in Trolox C concentration. On replacing CCl_4 by $CHCl_3$ or CH_2Cl_2 similar results were obtained. The rate constants derived decreased on decreasing halo-substitution, in agreement with previous studies (Packer *et al.*, 1980). The rate obtained of $k_{29} = 3.7\times10^8$ dm$^3\cdot$mol$^{-1}\cdot$s^{-1} compares with the value of 5.0×10^8 dm$^3\cdot$mol$^{-1}\cdot$s^{-1}obtained for the same reaction with α-tocopherol (Packer *et al.*, 1979), illustrating the similar properties of the two compounds. The reaction between Trolox C and $CCl_3OO^\bullet$, in common with the results for $NO_2^\bullet$ and RS$^\bullet$, may be described as either a hydrogen-atom-transfer (reaction 29) or a one-electron-transfer process:

$$CCl_3O_2^\bullet + TxOH \rightarrow CCl_3O_2^- + TxOH^{\bullet+} \quad (30)$$

In order to distinguish between these processes, studies were performed in ^2H_2O, where deuterated Trolox C is readily formed by hydrogen exchange. The results obtained did not demonstrate any kinetic isotope effect, i.e. k_H/k_D is unity, supporting the electron-transfer process (reaction 30).

Reactions involving phenoxyl radicals and Trolox C

Since previous studies have shown that various phenoxyl radicals can react rapidly with phenolates (Steenken & Neta, 1979, 1982; Steenken, 1979), the possibility that phenoxyl radicals may oxidize Trolox C was investigated. On pulse radiolysis of an N_2O-saturated solution containing 0.1 M-NaN_3, 10 mM-sodium salicylate (SalOH) and 50 μM-Trolox C at pH 7, the absorption spectra of the salicylate phenoxyl radical SalO$^\bullet$ was observed immediately after the pulse with subsequent formation after approx. 200 μs of TxO$^\bullet$:

$$SalO^\bullet + TxOH \rightarrow SalOH + TxO^\bullet \quad (31)$$

or

$$SalO^\bullet + TxOH \rightarrow SalO^- + H^+ + TxO^\bullet \quad (32)$$

The rate of appearance of TxO$^\bullet$ was first-order in Trolox C concentration and corresponded to $k_{31}+k_{32} = 3\times10^8$ dm^3·mol^{-1}·s^{-1}. Similar observations were obtained when experiments were undertaken with phenol, *m*-cresol or *p*-cresol and tyrosine in place of salicylate (see Table 1). No reaction was observed between the *o*-cresol phenoxyl radical and Trolox C, possibly because of steric hindrance. With tyrosine, the initial absorption of the tyrosine phenoxyl radical (Bansal & Fessenden, 1970) was replaced by that of TxO$^\bullet$, with a first-order rate constant of 3.2×10^8 dm^3·mol^{-1}·s^{-1}. This result suggests that tyrosine residues, which are susceptible to free-radical-mediated damage, can be readily repaired by antioxidants such as Trolox C, in agreement with studies that have shown that some proteins may be 'repaired' by phenolic antioxidants such as Trolox C (Hoey & Butler, 1985).

These results show that Trolox C may be oxidized by a variety of free radicals to the phenoxyl radical. In several cases the results obtained are similar to those previously obtained with α-tocopherol, suggesting that the two species have similar chemical reactivities, and that the function of the phytyl tail is to increase the lipophilicity of α-tocopherol rather than alter the chemical reactivity of the chroman ring. If the phenoxyl radical then reacts further to regenerate the parent Trolox C molecule, the antioxidant potential of Trolox C will be greatly enhanced. Further studies were therefore carried out on the reactions of this species with biological reducing agents to determine whether this process occurs.

Reaction of the Trolox C phenoxyl radical with ascorbate

The Trolox C phenoxyl radical was generated from 10 mM-Trolox C by using $Br_2^{\bullet-}$ at pH 7.2 (see above). Under these conditions TxO$^\bullet$ has a first half-life of at least 20 ms. Addition of 2 mM-ascorbate resulted in a rapid decay of the TxO$^\bullet$ absorption at 435 nm together with a concomitant increase in absorption at 360 nm (Fig. 5). This latter absorption is characteristic of the ascorbyl radical and is formed stoichiometrically (Bielski *et al.*, 1971; Redpath & Willson, 1973; Schuler, 1977). Both the increase in absorption at 360 nm and the decay at 435 nm were exponential and first-order with respect to ascorbate concentration. A plot of the observed first-order rate constant against ascorbate concentration is shown in Fig. 5 with $k = 8.3\times10^6 \pm 0.2\times10^6$ dm^3·mol^{-1}·s^{-1} for reaction (33):

$$TxO^\bullet + AH^- \rightarrow TxO^- + A^{\bullet-} + H^+ \quad (33)$$

Table 1. Absolute rate constants for reaction of selected radicals with Trolox C

All yields are calculated by using a molar decadic absorption coefficient for the Trolox C phenoxyl radical of 7100 dm^3·mol^{-1}·cm^{-1}.

Radical	pH	k (dm^3·mol^{-1}·s^{-1})	Yield (%)
OH$^\bullet$	7	2.2×10^9	25
	7	6.8×10^9	–
OH$^\bullet$	11	3.8×10^9	56
	11	6.8×10^9	–
$N_3^\bullet$	7	5.0×10^8	100
$Br_2^{\bullet-}$	10	4.3×10^8	60
$(SCN)_2^{\bullet-}$	6.5	2.2×10^8	96
$O_2^{\bullet-}$	8	$<10^5$	–
$HO_2^\bullet$	9	$<10^5$	–
$CCl_3O_2^\bullet$	7	3.7×10^8	75
$CHCl_2O_2^\bullet$	7	1.1×10^8	58
$CH_2ClO_2^\bullet$	7	1.6×10^7	21
$NO_2^\bullet$	6.6	$<10^5$	–
	11.5	5×10^8	75
Cysteamine$^\bullet$	6.2	1.0×10^8	75
	12.2	8.0×10^8	100
SalO$^\bullet$	7	3.0×10^8	75
PhO$^\bullet$	7	4.1×10^8	87
m-CreO$^\bullet$	7	2.8×10^8	70
p-CreO$^\bullet$	7	9.5×10^7	53
o-CreO$^\bullet$	7	$<10^5$	–
TyrO$^\bullet$	7	3.2×10^8	88

Confirmation of this repair process was obtained by using e.s.r. spectroscopy. It has been previously shown, by use of e.s.r. and the spin traps α-phenyl-*N*-butylnitrone and 2-methyl-2-nitrosopropane, that photolysis of halocarbons such as $CBrCl_3$ under anaerobic conditions leads to the production of $CCl_3^\bullet$ and bromine atoms through homolysis of the C–Br bond (Davies & Slater, 1986). Under aerobic conditions the $CCl_3^\bullet$ radicals are known to react rapidly with O_2 to give the corresponding peroxyl radical (Packer *et al.*, 1978, 1980, 1981). Photolysis of saturated aerobic solutions of $CBrCl_3$ in aq. 3 M-2-methylpropan-2-ol in the presence of Trolox C (10 mM) led to the detection of a complex spectrum assignable, by comparison with previous work on α-tocopherol in alcohol/water systems (Tsuchiya *et al.*, 1983; Niki *et al.*, 1984), to TxO$^\bullet$ with e.s.r. hyperfine coupling constants of a_{3H} 0.608, a_{3H} 0.456, a_{2H} 0.140 and a_{3H} approx. 0.09 mT (Figures 6 and 7). This spectrum was detectable with low intensities of u.v. light, though continuous generation of the TxO$^\bullet$ radical proved to be necessary owing to its relatively short half-life (of about 7.5 s; determined from the decay of an over-modulated signal with time). This value is in agreement with a previous report, where the half-life of similar phenoxyl radicals was found to be much shorter under aerobic conditions compared with the same species under vacuum (Niki *et al.*, 1984). The generation of TxO$^\bullet$ through reaction (29) rather than via $CCl_3^\bullet$ or Br$^\bullet$ was confirmed by carrying out similar experiments under strictly anaerobic conditions where no $CCl_3O_2^\bullet$ is produced; under these conditions no signal attributable to TxO$^\bullet$ was observed. Reaction (29) has $k_{29} = 3.7\times10^8$ dm^3·mol^{-1}·s^{-1} (see above); the

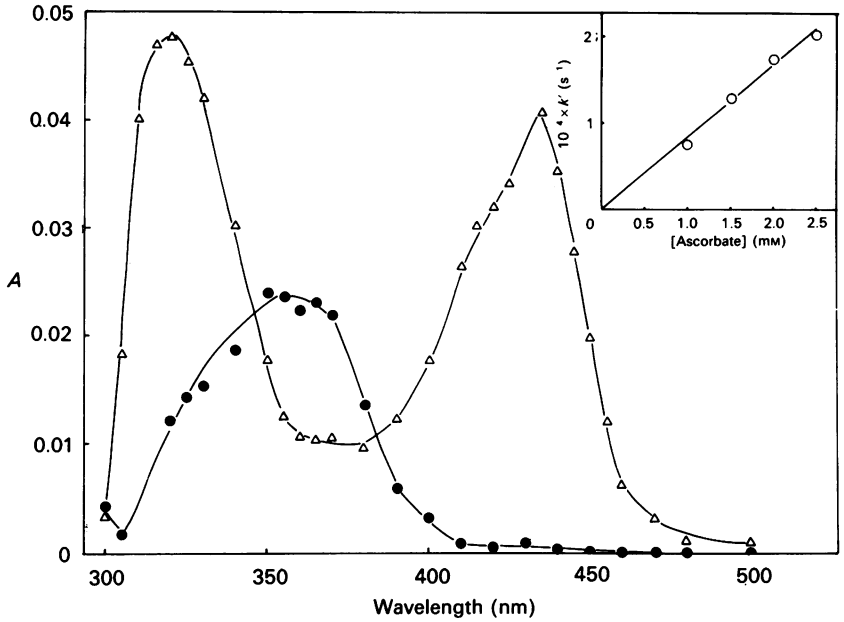


Fig. 5. Transient absorption spectra observed 5 μs (△) and 300 μs (●) after pulse radiolysis of an N_2O-saturated solution containing 0.1 M-KBr, 20 mM-Trolox C and 2 mM-ascorbate at pH 7.2

The dose was 10 Gy. Inset: plot of first-order rate constant for the decrease in absorption at 435 nm following pulse radiolysis of identical solutions containing different ascorbate concentrations.

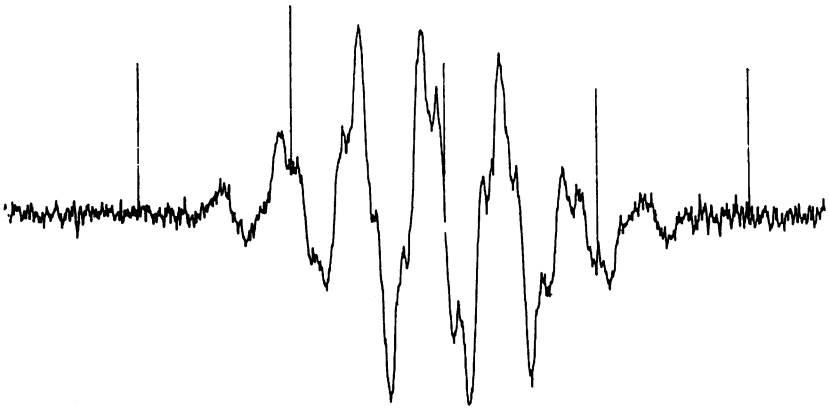


Fig. 6. E.s.r. spectrum assigned to the phenoxyl radical from Trolox C, observed on photolysis of saturated aqueous oxygenated solutions of $CBrCl_3$ in the presence of 1 mM-Trolox C under high-resolution conditions

E.s.r. spectrometer settings: gain 1.25×10^6, modulation amplitude 0.05 mT, time constant 0.5 s, scan time 500 s, field 347.5 mT, scan 6 mT, power 13 dB, frequency 9.78 GHz, room temperature. Marker lines are at 1 mT intervals.

corresponding rate of reaction with ascorbate is 1.6×10^8 $dm^3 \cdot mol^{-1} \cdot s^{-1}$ at pH 7 (Packer *et al.*, 1980), and therefore, provided that only low concentrations of ascorbate are added to the system, it is possible to ensure that the $CCl_3O_2^{\cdot}$ radicals react solely with Trolox C, allowing the effect of ascorbate on the concentration of TxO$^{\cdot}$ to be studied.

Addition of 0.1 mM-ascorbate to the photolysis system led to a complete loss of the TxO$^{\cdot}$ signal and the appearance of the well-documented ascorbyl trioxo radical doublet (Yamakazi & Piette, 1961), confirming the pulse-radiolysis observations. Under the conditions used, less than 0.4% of the $CCl_3O_2^{\cdot}$ radicals generated react directly with the ascorbate. These results indicate that TxO$^{\cdot}$ can be repaired rapidly by ascorbate. The rate constant obtained (8.3×10^6 $dm^3 \cdot mol^{-1} \cdot s^{-1}$) is greater than that previously determined for the repair of vitamin E by ascorbate (1.6×10^6 $dm^3 \cdot mol^{-1} \cdot s^{-1}$; Packer *et al.*, 1979). However, these previous studies were performed in a propan-2-ol/propanone/water (5:1:4, by vol.) solvent system, which may account to some degree for the lower rate. In addition, it has been suggested, on the basis of stereoelectronic factors, that the presence of the electron-withdrawing CO_2H group on the Trolox C molecule (as compared with the phytyl chain on α-tocopherol; see Fig. 1) hinders stabilization of the phenoxyl radical by the ether oxygen *p*-type lone pair of electrons (Burton *et al.*, 1983). Given that ascorbate, under normal cellular conditions, is present in higher concentrations than vitamin E (Baker *et al.*, 1970), this

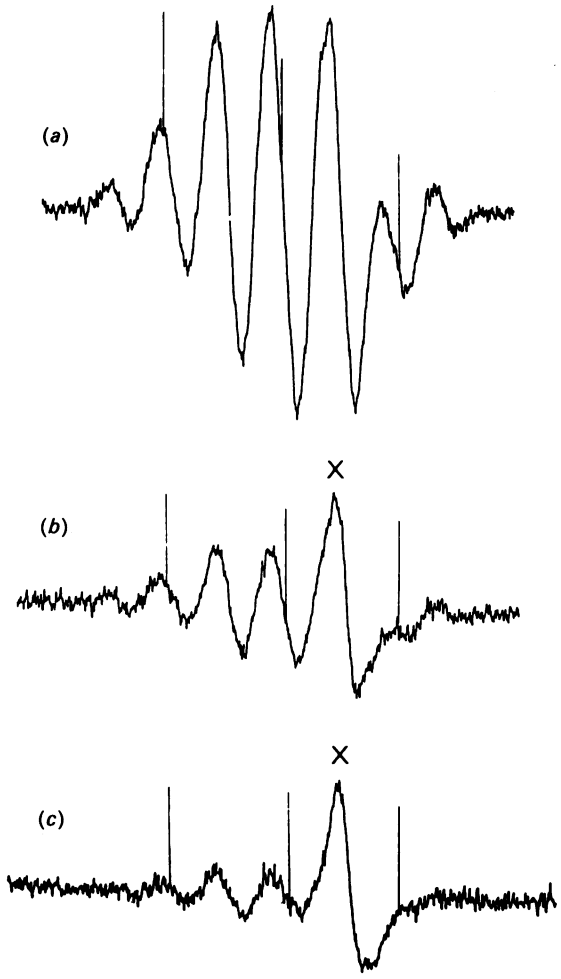


Fig. 7. E.s.r. spectrum assigned to the phenoxyl radical from Trolox C produced by photolysis of a saturated aqueous solution of $CBrCl_3$ containing 3 M-2-methylpropan-2-ol, 10 mM-Trolox C and O_2 under low-resolution conditions, and the effects of GSH on the steady-state concentration of this radical

(*a*) No added GSH; (*b*) 10 μM-GSH; (*c*) 1 mM-GSH. The asymmetrical signal marked × is due to an inherent paramagnetic impurity in the glass cell. E.s.r. spectrometer conditions were as given in Fig. 6 legend, except modulation amplitude 0.2 mT. Marker lines are at 1 mT intervals.

represents a mechanism that realizes the potential antioxidant properties of ascorbate, the ascorbate being subsequently regenerated via disproportionation of the ascorbyl radicals (reaction 34):

$$A^{\cdot -} + A^{\cdot -} \xrightarrow{H^+} A + AH^- \qquad (34)$$

Lack of reaction of the Trolox C phenoxyl radical with uric acid, NADH or propyl gallate

Uric acid has been proposed as an important biological antioxidant (Ames *et al.*, 1981), and therefore it was of interest to assess whether any synergism could be observed between TxO$^\cdot$ and urate. Trolox C phenoxyl radicals were generated by reaction of Trolox C with $N_3^\cdot$ (see above), and on addition of 0.5 mM-uric acid no absorption other than that due to TxO$^\cdot$ was observed nor was there any acceleration in the decay of the TxO$^\cdot$ signal. Therefore it appears that either urate does not repair the Trolox phenoxyl radical or that the rate is too low to be detected under pulse-radiolysis conditions (i.e. $k < 10^5$ dm$^3\cdot$mol$^{-1}\cdot$s^{-1}); this does not, however, necessarily mean that this reaction, if it occurs, is insignificant in biological situations. Similarly, when uric acid was replaced by either NADH or propyl gallate, no reaction was observed.

Reaction of the Trolox C phenoxyl radical and the α-tocopherol phenoxyl radical with thiols

Thiol-containing compounds have also been proposed as protective agents against free-radical-mediated injury. Indeed, they have long been known to protect cells against the lethal effects of ionizing radiation (Howard-Flanders, 1960). Therefore the possibility that the phenoxyl radical derived from Trolox C may be repaired by thiols was investigated. Under conditions where TxO$^\cdot$ was generated by $N_3^\cdot$ in the presence of thiols no reaction could be observed by pulse radiolysis at pH 7. On pulse radiolysis of similar solutions at pH 10 (i.e. above the pK_a of the thiol group) containing cysteamine (1 mM) no significant increase in the rate of decay of the TxO$^\cdot$ absorption was noted, implying that reactions (35) and (36) have rates less than 10^5 dm$^3\cdot$mol$^{-1}\cdot$s^{-1}:

$$TxO^\cdot + RSH \rightarrow TxOH + RS^\cdot \qquad (35)$$

$$TxO^\cdot + RS^- \rightarrow TxO^- + RS^\cdot \qquad (36)$$

On raising the pH to 13 (above the pK_a of Trolox C; see above) there was an acceleration in the decay of the TxO$^\cdot$ absorption in the presence of cysteamine with k approx. 2×10^5 dm$^3\cdot$mol$^{-1}\cdot$s^{-1}. This implies that the extent of ionization of TxOH governs the rate of reaction and that the electron-transfer process (reaction 36) is favoured only at very high pH. Clearly this is of little relevance to cellular systems, so further e.s.r. studies were therefore undertaken in an attempt to study any possible thiol repair process. Previous studies have shown that the rate of reaction of the trichloromethyl peroxyl radical with thiols shows a marked pH-dependence. The reaction with cysteine, for example, is rapid at pH 12 (i.e. when the SH group is unprotonated) with a rate constant of 5.3×10^7 dm$^3\cdot$mol$^{-1}\cdot$s^{-1} as measured by competition methods (J. E. Packer & R. L. Willson, unpublished work). At pH 7, however, no reaction is observed, and hence the rate constant is less than 10^5 dm$^3\cdot$mol$^{-1}\cdot$s^{-1}. By using the $CBrCl_3$ photolysis system and employing only low concentrations of thiol, the $CCl_3O_2^\cdot$ radicals generated will react solely with the Trolox C, thereby allowing the effects of thiols on TxO$^\cdot$ to be studied.

When 10 μM-GSH was included in the $CBrCl_3$ photolysis system (see the Materials and methods section) a marked decrease in the intensity of the e.s.r. signal due to TxO$^\cdot$ (which is directly proportional to the radical concentration) was observed, in agreement with the occurrence of reaction (35). Increasing the concentration of GSH to 1 mM led to a further decrease in the steady-state concentration of the phenoxyl radical signal (Fig. 7). Further evidence for the repair of TxO$^\cdot$ by thiols was obtained by observing the effect of added thiol on the decay of the phenoxyl radical when generation of the phenoxyl radical was halted by interruption of the light-source. Addition of GSH led to a decrease in the half-life

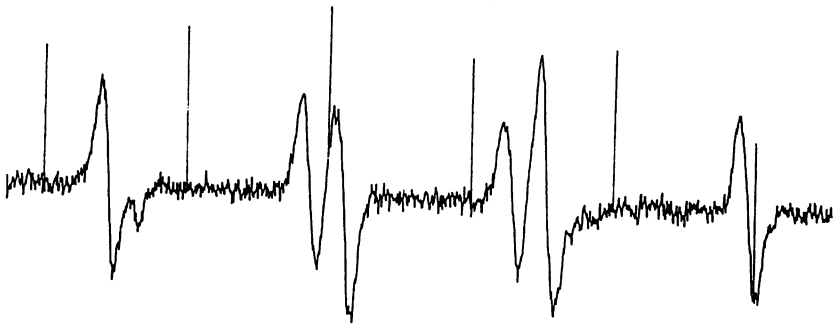


Fig. 8. E.s.r. spectrum observed on photolysis of a saturated solution of $CBrCl_3$ in aqueous 3 M-2-methylpropan-2-ol in the presence of Trolox C (1 mM), GSH (0.1 mM) and the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (25 mM), and assigned to the glutathione thiyl radical adduct to the spin trap

Omission of any of the components of this system led to the loss of the signal. E.s.r. spectrometer settings were as given in Fig. 6 legend.

of the phenoxyl radical from 7.5 s in the absence of thiol to 5.5 and 3.5 s in the presence of 10 μM- and 1 mM-GSH respectively. To confirm that reaction (35) was occurring, the effect of adding the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide, which has been previously shown to trap sulphur-centred radicals, was examined (Harman *et al.*, 1984; Davies *et al.*, 1987). With 25 mM-5,5-dimethyl-1-pyrroline *N*-oxide and 10 mM-GSH the signal due to $TxO^{\bullet}$ was almost completely eliminated, as expected, and an additional nitroxide signal, consisting of a doublet of triplets, was observed (Fig. 8). The parameters of this signal are similar to those previously observed for thiol radical adducts, suggesting that this signal is due to the trapping of the glutathione thiyl radical $GS^{\bullet}$ (Harman *et al.*, 1984; Davies *et al.*, 1987). Omission of either Trolox C or GSH from this system led to the loss of these signals, confirming that the generation of this radical adduct does not occur either via direct reaction of $TxO^{\bullet}$ with the trap, via direct reaction of $CCl_3O_2^{\bullet}$ with the thiol or via addition of $CCl_3O_2^{\bullet}$ to the trap. In experiments where Trolox C was omitted weak signals assignable to the hydroxyl radical adduct to the spin trap were observed; these probably arise through reaction of low concentrations of contaminating iron ions with the thiol as described previously (Langform & Nielson, 1957; Misra, 1974). Confirmation of the assignment of the signal observed in the complete system to a thiyl radical adduct was obtained by photolysis, in the presence of the same spin trap, of an aqueous solution of GSSG, a process known to produce homolytic cleavage of the disulphide linkage and hence $GS^{\bullet}$ radicals (Morine & Kuntz, 1981). Similar signals were observed, confirming the trapping of $GS^{\bullet}$ radicals, although the hyperfine coupling constants were slightly larger, presumably as a result of the change in solvent polarity, a factor known to affect the size of the coupling constants (Janzen, 1980). Analogous reactions were observed when the thiols cysteine, cysteamine and dithiothreitol were used rather than GSH. In each case there was a loss of the $TxO^{\bullet}$ signal and the appearance of the thiyl radical adduct to the spin trap with parameters similar to those observed previously (Davies *et al.*, 1987). When dithiothreitol was used the intensity of the spin adduct signal was low, presumably owing to competition between addition to the spin trap and the known cyclization of this thiyl radical (Chan & Bielski, 1973). The identity of the radical adduct observed with cysteine was confirmed by photo-

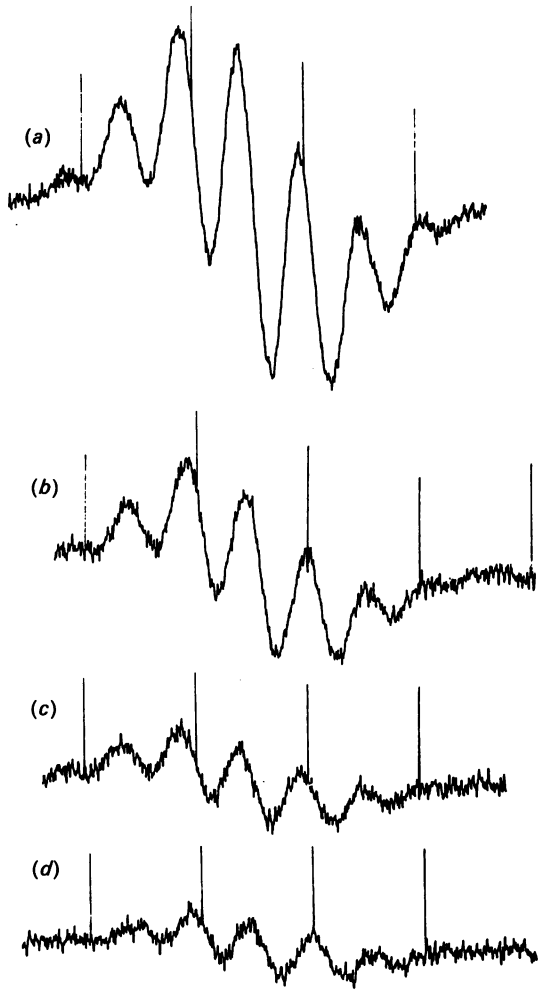
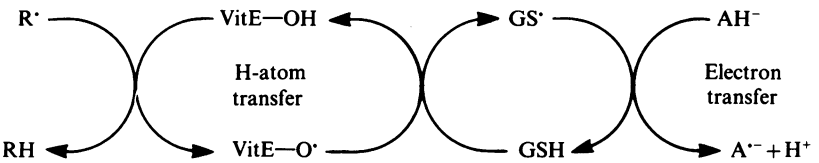


Fig. 9. E.s.r. spectrum observed on photolysis of $CBrCl_3$ (1 M) in the presence of α-tocopherol (0.4 M) in oxygenated toluene and the effects of dithiothreitol on the steady-state concentration of this radical

The spectrum is assigned to the α-tocopherol phenoxyl radical. (*a*) No added dithiothreitol; (*b*) 10 μM-dithiothreitol present; (*c*) 0.1 mM-dithiothreitol present; (*d*) 1 mM-dithiothreitol present. E.s.r. spectrometer conditions were as given in Fig. 6 legend, except gain 2.5×10^6, modulation amplitude 0.1 mT, time constant 2.0 s, scan time 2000 s.



Scheme 1. Coupled hydrogen-atom- and electron-transfer reactions that might participate in biological protection

lysis of the corresponding disulphide; identical signals were observed.

To confirm that this repair process (reaction 35) is not limited to highly polar solvent systems, similar photolytic experiments were performed in toluene. Photolysis of $CBrCl_3$ (1 M) in toluene under aerobic conditions in the presence of 0.4 M-α-tocopherol led to the observation of the α-tocopherol phenoxyl radical with parameters similar to those observed previously in benzene (Fig. 9) (Niki *et al.*, 1984). As with the aqueous systems, exclusion of O_2 led to a loss of this signal. Addition of dithiothreitol (0.1 mM) resulted in a marked decrease in the intensity of the radical signal, and the decrease was dependent on thiol concentration (Fig. 9). In contrast with the results obtained under aqueous conditions, addition of 5,5-dimethyl-1-pyrroline *N*-oxide did not result in trapping of the thiyl radical adduct, presumably owing to the competing cyclization reaction outlined above. No experiments were performed with other thiols because of solubility problems. The mechanism proposed for the radioprotective action of thiols is one of the hydrogen-atom-transfer reactions from a thiol to a carbon-centred radical lesion, thereby generating a thiyl free radical (Prevot-Bernas, 1953). In the case of GSH:

$$R^{\cdot} + GSH \rightarrow RH + GS^{\cdot} \quad (37)$$

GSH is a particularly abundant cellular thiol and therefore has been proposed as a cellular protective agent. Moreover, thiols have been shown to inhibit biphenol coupling of phenoxyl radicals (reaction 38) (Prutz *et al.*, 1983):

$$PheO^{\cdot} + RSH \rightarrow PheOH + RS^{\cdot} \quad (38)$$

The results presented here demonstrate that both the Trolox C phenoxyl radical and the α-tocopherol phenoxyl radical can be repaired by thiols, presumably through a similar hydrogen-atom-transfer reaction, generating a thiyl radical. Even though these results indicate that, when compared with ascorbate, the rate of reaction is low under cellular conditions, when one considers the concentrations of these two compounds the thiol repair reaction may be of some importance. Thiyl radicals, though previously viewed as relatively innocuous species, have been shown to react rapidly with a variety of suitable electron donors, including ascorbate (Forni *et al.*, 1983*a*; Ahmad & Armstrong, 1984; Stratford *et al.*, 1984). Therefore this raises the possibility of coupled hydrogen-atom- and electron-transfer reactions participating in biological protection (Scheme 1).

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