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AN INVESTIGATION OF THE NOVEL ANTI-INFLAMMATORY AGENTS ONO-3144 AND MK-447

STUDIES ON THEIR POTENTIAL ANTIOXIDANT ACTIVITY

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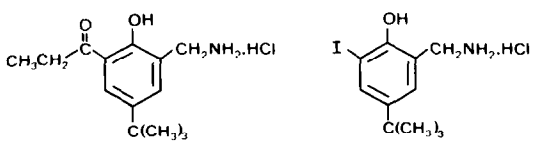
Abstract—Oxygen-derived free radicals have been implicated as contributors to inflammatory disorders and it has been suggested that certain anti-inflammatory drugs act by scavenging free radicals. In this paper we have studied the free radical scavenging activity of two such experimental anti-inflammatory drugs MK-447 and ONO-3144. Using the technique of pulse radiolysis we have been able to obtain rate constants for the reactions of these compounds with specific free radicals including $OH^{\cdot}$ and $O_2^{\cdot-}$. We have also investigated the antioxidant capacity of these compounds using rat liver microsomal lipid peroxidation systems. It is suggested that this approach yielding quantitative data concerning defined free radical species will lead to a better understanding of the role of radical scavenging in anti-inflammatory activity.

The development of acute inflammation following tissue injury involves several cell types as well as an array of chemical mediators including histamine, kinins, the complement system and not least the prostaglandins and oxygen metabolites [1]. Prostaglandins contribute to various aspects of the inflammatory response: tissue damage induces damaged cells to generate prostaglandins which evoke vasodilation, increased vascular permeability and chemotaxis. Further tissue injury following polymorphonuclear lymphocyte (PMN) infiltration in turn leads to further prostaglandin release [2–4]. Free radicals are now thought to play a major role in the progression of acute inflammation as well as many other biochemical processes [5, 6]. The production of oxygen-derived free radicals by phagocytic cells has proved of particular interest [6, 7]. PMNs are known to produce both the superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) on activation of the respiratory burst system which provides a mechanism for the bacteriocidal action of neutrophils. This proposed role for the free radical metabolites of oxygen is particularly evident in the case of chronic granulomatous disease (CGD) where the phagocytic cells from affected individuals fail to produce such metabolites [8–10]. Leucocytes from patients with CGD have the ability to phagocytose bacteria but the cells do not exhibit a respiratory burst. The basis of this disorder is thought to be an abnormality in the NADPH dependent $O_2^{\cdot-}$ generating enzyme system which catalyses the following reaction

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot-} + NADP^+ + H^+ \quad (1)$$

As a consequence the affected individual is susceptible to a range of opportunistic bacterial and fungal infections.

Thus, both the prostaglandins and free radicals are intimately involved in the development of acute inflammation. The role of the prostaglandins in the inflammatory process has been further elucidated since the discovery that many non-steroidal anti-inflammatory agents (NSAID) are potent inhibitors of the enzyme prostaglandin synthetase [12–14]. However, some reports have raised the point that many NSAIDSs may also be effective in preventing free radical mediated damage [15]. It follows that some anti-inflammatory agents may evoke their action through antioxidant free radical-scavenging-properties. The novel anti-inflammatory agent 2-aminomethyl-4-*t*-butyl-6-iodophenol (MK-447:Fig. 1) has been shown to inhibit the inflammatory process in certain model systems [16] although it is inactive as an inhibitor of PG synthetase [17]. It has been proposed that MK-447 may exert its action by removing activated oxygen metabolites arising during the conversion of PGG_2 to PGH_2 as well as that released from PMNs [18]. Similarly, the proprionyl derivative (ONO-3144:Fig. 1) has been found to be comparable in activity to indomethacin though, as with MK-447, no inhibition of prostaglandin synthesis was demonstrated. Again, free radical scavenging has been proposed as the basis of its anti-inflammatory properties [19, 20].



ONO-3144 MK-447

Fig. 1. Structure of (a) MK-447 and (b) ONO-3144.

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In this study the technique of pulse radiolysis has been used to generate defined free radical species and rat liver microsomal systems have been used to test the activity of these compounds in scavenging the radicals involved in lipid peroxidation. During investigations into the latter topic it was necessary to test the interaction of these compounds with the enzymes of the microsomal cytochrome P-450 system: a preliminary report of the inhibition of P-450-dependent drug metabolism by ONO-3144 has already appeared [21].

MATERIALS AND METHODS

Chemicals. MK-447 and ONO-3144 were generous gifts of Merck, Sharp and Dohme (Hoddesdon, Herts, U.K.) and ONO Pharmaceutical Co. (Osaka, Japan) respectively. Sodium azide, potassium thiocyanate and ascorbic acid were supplied from Sigma Chemical Co. (St Louis, MO). All other chemicals used were of the highest purity available and used as supplied. Nitrous oxide was supplied by the British Oxygen Corporation.

Animals. The rats used were males of the Wistar strain, average body weight 250 g, obtained from Charles River Co. (Margate, Kent, U.K.). They were fed on a standard laboratory diet (Expanded Breeder Diet No. 5; Special Diet Services, Witham, Essex, U.K.) and water *ad libitum* until 18 hr before killing at which time the food was removed. For the drug metabolism studies the rats were induced with sodium phenobarbitone (1 mg/ml) in the drinking water for 7 days.

Liver microsomal preparation. The rats were killed by cervical dislocation and liver microsomes prepared as described by Slater and Sawyer [22]. They were washed once by resuspension and recentrifugation in 0.1 M Tris : HCl, pH 7.4, and the pellets stored at −20° for no more than two weeks before use, when they were resuspended in 0.15 M KCl such that 1 ml of suspension was equivalent to 1 g wet weight of liver. This suspension was assayed for protein [23] and usually contained 15–20 mg protein/ml.

Lipid peroxidation estimations. Microsomal lipid peroxidation was measured as malondialdehyde (MDA) production by the thiobarbituric acid (TBA) test except where indicated. Carbon tetrachloride-induced lipid peroxidation was measured as described by Slater and Sawyer [24] as modified by Cheeseman *et al.* [25]. Cumene hydroperoxide-stimulated, ascorbate/iron-stimulated and NADPH/ADP/iron-stimulated MDA production was measured as described previously [26]. NADPH/ADP/iron-dependent lipid peroxidation was also measured as oxygen-uptake using an oxygen electrode as described by Slater [27]. Cysteine/iron-stimulated MDA production was measured by the method of Searle and Willson [28] but with an incubation time of 15 min.

Drug metabolism estimations. NADPH: cytochrome P-450 reductase was measured as cytochrome *c* reductase activity as described previously [22] except at 25°. The binding of test compounds to cytochrome P-450 was determined by optical difference spectroscopy [29]. Aminopyrine demethylase and aniline hydroxylase were measured as previously [30, 31].

Pulse radiolysis experiments. The pulse radiolysis experiments were undertaken using the Brunel 4 MeV linear accelerator and associated equipment for kinetic spectroscopy and computer analysis [32, 33]. In the present study a 0.2 μsec electron pulse producing a radiation dose of 2–10 Gy (Jkg^{-1}) in an irradiation cell of 1.5 cm optical path length was used. All solutions were prepared in doubly distilled or Millipore filtered water. Solutions were saturated, where necessary, with N_2O using the syringe bubbling technique [34].

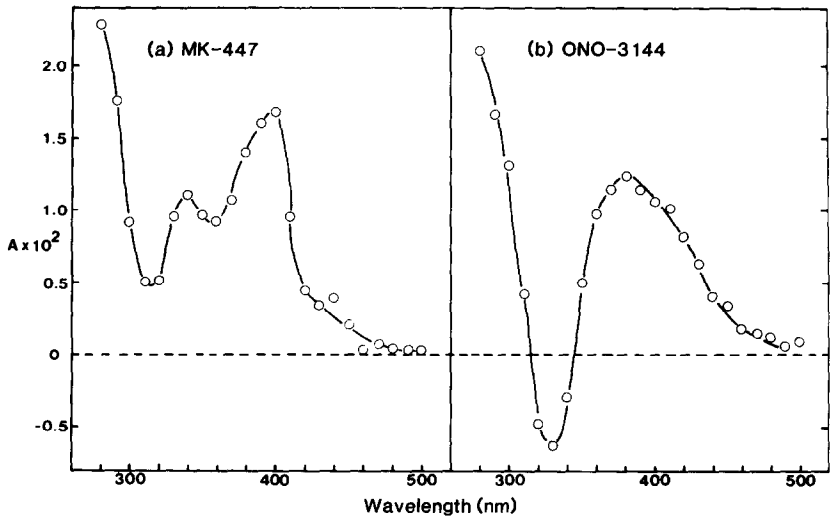

Fig. 2. Spectrum of the transient produced on pulse radiolysis of a nitrous oxide saturated solution containing 0.1 mM MK-447 at pH 7.8 (a) or 0.1 mM ONO-3144 at pH 7.4 (b). Dose = 10 Gy; path-length = 1.5 cm.

RESULTS

(A) Pulse radiolysis studies

(i) *Reaction of hydroxyl radicals.* The radiolysis of water yields hydroxyl radicals ($OH^{\cdot}$) and solvated electrons $e^-(aq)$ both with a yield of *ca.* 0.28 mol J^{-1}, together with hydrogen atoms ($H^{\cdot}$), with a yield of *ca.* 0.06 mol J^{-1}. In the presence of saturating nitrous oxide solvated electrons react rapidly to effectively double the yield of $OH^{\cdot}$ [32]:

$$e^-(aq) + N_2O \rightarrow OH^- + N_2 + OH^{\cdot} \quad (2)$$

with $k_{(2)} = 5.8 \times 10^9$ mol^{-1}dm^{3}sec^{-1}. On pulse radiolysis of a nitrous oxide saturated solution containing 0.1 mM MK-447 (MK-OH) at pH 7.8 a strong transient absorption was observed (Fig. 2a).

Radiolysis of similar solutions containing 0.1 mM ONO-3144 (ONO-OH) at pH 7.4 resulted in the transient shown in Fig. 2(b). In this case there is a decrease in absorption between 310 and 340 nm, attributable to a decrease in the ground state absorption of the parent molecule, the spectrum shown being the difference between the absorption of the ground state parent molecule and that of the generated free radical. In both cases the formation of the transient was exponential and dependent on either the MK-OH or ONO-OH concentration. Kinetic analysis of the reactions:

$$OH^{\cdot} + \text{MK-OH} \rightarrow \text{Products} \quad (3)$$

$$OH^{\cdot} + \text{ONO-OH} \rightarrow \text{Products} \quad (4)$$

was performed by using competition kinetic methods using either thiocyanate or ABTS (2,2′azino-di-(3-ethylbenzthiazoline-6-sulphonate) as the competitor. In the case of MK-OH, SCN^- was used. On pulse radiolysis of a nitrous oxide saturated solution containing 0.1 mM SCN^- at pH 6.7 the characteristic absorption [32] of the thiocyanate radical anion, $(SCN)_2^{\cdot-}$, was found. In the additional presence of MK-OH the observed absorption at 480 nm (λ_{max} for $(SCN)_2^{\cdot-}$) decreased on increasing MK-OH concentration. Plots of A°/A' against [MK-OH]/[SCN^-] (where A° is the absorption immediately after the pulse where no MK-OH is present and A' is the absorption on addition of MK-OH) were linear, in agreement with the following competing reactions

$$OH^{\cdot} + 2SCN^- \rightarrow OH^- + (SCN)_2^{\cdot-} \quad (5)$$

$$OH^{\cdot} + \text{MK-OH} \rightarrow \text{Products} \quad (3)$$

The relative rate constant being calculated from

$$\frac{A^0}{A'} = 1 + \frac{k_{(3)} \cdot [\text{MK-OH}]}{k_{(5)} \cdot [SCN^-]}$$

This yields the sort of plot shown in Fig. 3 and the derived rate constants listed in Table 1.

Table 1. Second order rate constants for the reaction of MK-447 and ONO-3144 with a range of free radical species generated radiolytically

Radical	pH	k(mol^{-1}dm^{3}sec^{-1}) MK-OH	ONO-OH
$OH^{\cdot}$	7.8	1.9×10^{10}	—
$OH^{\cdot}$	7.4	—	3.9×10^9
$(SCN)_2^{\cdot-}$	6.7	1.1×10^9	1.2×10^8
	12.0	1.1×10^9	1.2×10^9
$N_3^{\cdot}$	7.0	1×10^9	$<10^5$
	12.0	1×10^9	ca.1×10^8
$CCl_3O_2^{\cdot}$	7.0	6×10^8	$<10^5$
$NO_2^{\cdot}$	6.5	4.6×10^7	$<10^5$
	12.8	1.6×10^8	$<10^5$
$O_2^{\cdot-}$	6.6	$<10^5$	$<10^5$
	3.0	$<10^5$	$<10^5$

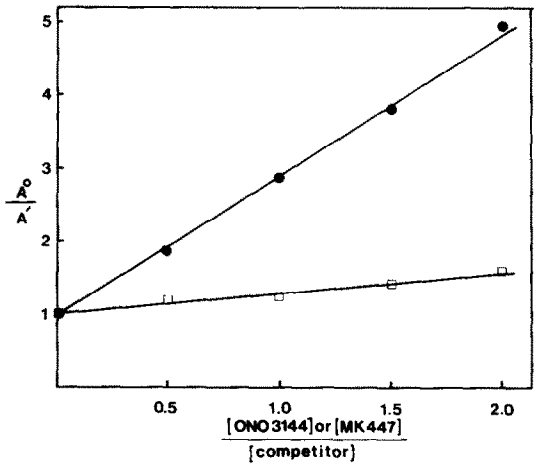

Fig. 3. Kinetic competition plots (see text) for the effect of increasing the MK-447 concentration on the magnitude of the $(SCN)_2^{\cdot-}$ absorption at 480 nm (●) or ONO-3144 concentration on the magnitude of the $ABTS^+$ absorption at 415 nm (□). Note that for ease of comparison between MK-447 and ONO-3144 the plot shown has a maximum [ONO-3144]:[ABTS] ratio of 2.0; higher ratios were actually used to obtain the accurate rate constant listed in Table 1. Dose = 10 Gy.

(ii) *Reactions of the inorganic radicals.* The azide radical ($N_3^{\cdot}$) is known to react rapidly and selectively with phenolic compounds to generate the corresponding phenoxy radicals [35]. On pulse radiolysis of a nitrous oxide saturated solution containing 0.1 M sodium azide and 0.1 mM MK-447 at pH 7 a strong signal similar to that observed previously (during the $OH^{\cdot}$ generating system) was observed. However, in this case the yield was higher than that observed previously with $\varepsilon = 1420$ dm^{3}mol^{-1}cm^{-1} at 400 nm on $N_3^{\cdot}$ oxidation and $\varepsilon = 1120$ dm^{3}mol^{-1} cm^{-1} following $OH^{\cdot}$ oxidation (assuming a radical yield of 0.55 mol J^{-1}). When the bromide radical anion ($Br_2^{\cdot-}$) was used as the oxidant, similar results were obtained (data not shown).

On substituting ONO-3144 for MK-447 in the $N_3^{\cdot}$ generating system no reaction was observed at pH 7. However, on raising the pH to 12.5 the same spectrum as found with $OH^{\cdot}$ oxidation was observed. Again the yield was increased with $\varepsilon =$ 4075 dm^{3}mol^{-1}cm^{-1} at 290 nm as opposed to $\varepsilon =$ 1850 dm^{3}mol^{-1}cm^{-1} on $OH^{\cdot}$ oxidation.

The thiocyanate radical anion behaves in a similar manner to $Br_2^{\cdot-}$ and $N_3^{\cdot}$. Again, pulse radiolysis of a nitrous oxide saturated solution containing 0.1 M

KSCN and 0.1 mM MK-447 at pH 7.4 the same transient as observed previously was observed. This transient is attributed to the MK-447 phenoxyl radical, MK-O$^{\cdot}$. The appearance of the MK-O$^{\cdot}$ radical was exponential and first order in MK-447 concentration with $k = 1.1 \times 10^9$ mol^{-1} dm^{3}sec^{-1}, according to:

$$(SCN)_2^{\cdot-} + \text{MK-OH} \rightarrow 2SCN^- + \text{MK-O}^{\cdot} + H^+ \quad (7)$$

When similar solutions containing 0.1 mM ONO-3144 were irradiated at pH 7.4 the transient observed with OH$^{\cdot}$ and $N_3^{\cdot}$ oxidation was seen and again the formation of the transient was exponential and first order in ONO-3144 concentration with $k = 1.2 \times 10^8$ mol^{-1}dm^{3}sec^{-1}. However, on raising the pH to 12.0 the rate of appearance of the transient species was accelerated with $k = 1.2 \times 10^9$ mol^{-1}dm^{3}sec^{-1}. The effect of changing the pH on nitrous oxide saturated solutions of 0.1 M KSCN and 0.1 mM ONO-3144 is shown in Fig. 4. There is a sharp increase in first order rate constant above pH 9 although throughout the pH range used there is no significant change in the primary species generated. This effect is attributed to the ionisation of the hydroxyl group on the ONO-3144 molecule. The data shown in Fig. 4 indicate that the process:

$$\text{ONO-OH} \rightleftharpoons \text{ONO-O}^- + H^+ \quad (8)$$

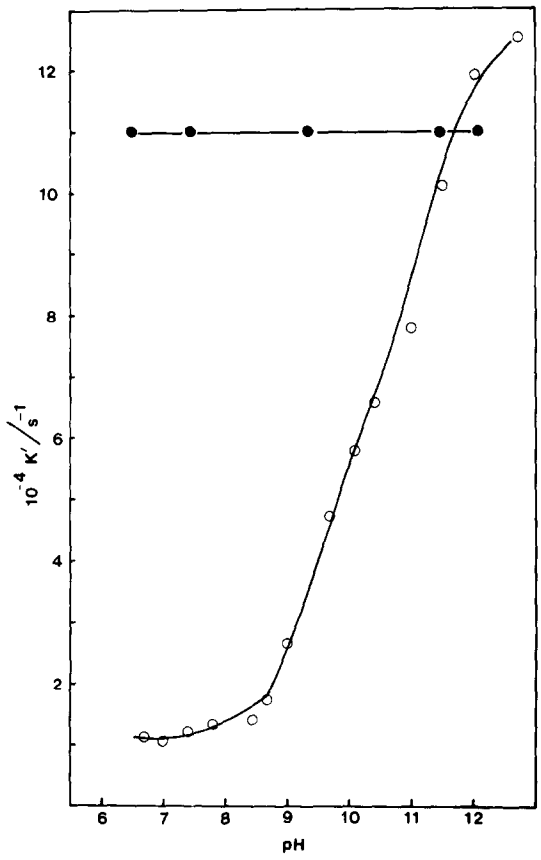

Fig. 4. Effect of pH on the observed first order rate constant for ONO-3144 (○) or MK-447 (●) oxidation by the thiocyanate radical anion. Dose = 9 Gy.

has a pK of *ca.* 10.5. Also shown in Fig. 4 is the effect of changing pH on MK-447 oxidation by $(SCN)_2^{\cdot-}$.

(iii) *Reactions of the superoxide and hydroperoxy radicals.* The superoxide radical ($O_2^{\cdot-}$) can be generated radiolytically by pulse radiolysis of nitrous oxide/oxygen (80:20) saturated solutions containing excess formate. Under these conditions the following reactions occur

$$OH^{\cdot} + HCOO^- \rightarrow H_2O + COO^{\cdot-} \quad (9)$$

$$COO^{\cdot-} + O_2 \rightarrow CO_2 + O_2^{\cdot-} \quad (10)$$

$$O_2^{\cdot-} + H^+ \rightleftharpoons HO_2^{\cdot} \quad (pK = 4.9) \quad (11)$$

with $k_{(9)}$ and $k_{(10)}$ equal to 0.4×10^9 and 2.0×10^9 mol^{-1}dm^{3}sec^{-1} respectively [32]. Pulse radiolysis of such a solution (1 M formate) containing 0.5 mM MK-447 at pH 6.6 gave a transient absorption immediately after the pulse with $\lambda_{max} = 260$ nm, characteristic of the superoxide radical anion. However, no formation of any other transient was observed; and no effect of MK-447 concentration on the decay of $O_2^{\cdot-}$ was demonstrated. On changing the pH to 3, where $HO_2^{\cdot}$ is the major species formed, again no reaction was observed. Similarly, when ONO-3144 was used rather than MK-447 no reaction with either $O_2^{\cdot-}$ or $HO_2^{\cdot}$ over the timescale used was seen. This implies that if $O_2^{\cdot-}$ or $HO_2^{\cdot}$ react with either of the anti-inflammatory agents then the rate is relatively slow, i.e. $<10^5$ mol^{-1}dm^{3}sec^{-1}.

(iv) *Reactions of the trichloromethylperoxy radical.* Some phenolic antioxidants, such as vitamin E and Trolox C have been shown to react rapidly with the trichloromethylperoxy radical ($CCl_3O_2^{\cdot}$) [36, 37]. $CCl_3O_2^{\cdot}$ may be readily generated in air saturated solutions containing 20% *t*-butanol and 5 mM CCl_4 through the following reactions

$$OH^{\cdot} + CH_3(CH_3)_2COH \rightarrow H_2O + {}^{\cdot}CH_2(CH_3)_2COH \quad (12)$$

$$e^-(aq) + CCl_4 \rightarrow CCl_4^{\cdot-} \quad (13)$$

$$CCl_4^{\cdot-} \rightarrow CCl_3^{\cdot} + Cl^- \quad (14)$$

$$CCl_3^{\cdot} + O_2 \rightarrow CCl_3O_2^{\cdot} \quad (15)$$

On addition of 0.1 mM MK-447 at pH 7 a transient identical to that observed previously was found. Again, the appearance of the transient was exponential and dependent on the MK-447 concentration according to:

$$CCl_3O_2^{\cdot} + \text{MK-OH} \rightarrow CCl_3OOH + \text{MK-O}^{\cdot} \quad (16)$$

with $k_{(16)} = 6 \times 10^8$ mol^{-1}dm^{3}sec^{-1}. Where ONO-3144 was substituted for MK-447, at pH 7 no product attributable to the phenoxyl radical was found. However, in keeping with the earlier results, raising the pH to 13 resulted in the observation of ONO-O$^{\cdot}$.

(v) *Reaction of nitrogen dioxide.* The nitrogen oxide, $NO_2^{\cdot}$, has been shown to undergo both one-electron and hydrogen atom transfer reactions [38], and may be generated on pulse radiolysis of nitrous

oxide saturated solutions containing sodium nitrite according to:

$$OH^{\cdot} + NO_2^- \rightarrow OH^- + NO_2^{\cdot} \quad (17)$$

with $k_{(17)} = 1.3 \times 10^9\ mol^{-1}dm^3sec^{-1}$. Radiolysis of 0.1 M $NaNO_2$ and 0.1 mM MK-447, pH 6.5, nitrous oxide saturated, resulted in the formation of MK-O$^{\cdot}$ with $k = 4.6 \times 10^7\ mol^{-1}dm^{-3}sec^{-1}$. On raising the pH to 12 the rate also increased to $1.6 \times 10^8\ mol^{-1}dm^3sec^{-1}$.

(vi) *Reactions of the MK-447 phenoxyl radical.* The results outlined above indicate that under conditions of neutral pH MK-447 is more readily oxidised than is ONO-3144. Given that recent results have shown that some phenoxyl radicals may themselves oxidise certain biological reducing agents [39], it was of interest to investigate the phenoxyl radical generated from MK-447 to assess whether it could enter into reactions with known antioxidants such as vitamin C and Trolox C (a water-souble analogue of vitamin E). On pulse radiolysis of a nitrous oxide saturated solution containing 0.1 M KBr, 10 mM MK-447 and 0.1 mM Trolox C at pH 3.5, the MK-O$^{\cdot}$ was formed immediately after the pulse according to:

$$Br_2^- + MK\text{-}OH \rightarrow 2Br^- + H^+ + MK\text{-}O^{\cdot} \quad (18)$$

The pH was maintained below 4 as high concentrations of MK-447 (i.e. > 5 mM) were poorly soluble above pH 4. The MK-O$^{\cdot}$ spectrum was replaced by that of the Trolox phenoxy radical (TxO$^{\cdot}$) but spectral resolution of the two species proved difficult. Nevertheless, on increasing the Trolox C concentration it was evident that the rate of appearance of TxO$^{\cdot}$ was increased. Therefore, it appears that MK-O$^{\cdot}$ can oxidise Trolox C but the spectral overlap means that no reliable kinetic data could be obtained. Ascorbic acid (vitamin C) has been shown to be oxidised by both the vitamin E and the Trolox C phenoxyl radical [36] and the ascorbyl radical ($A^{\cdot-}$), has an absorption maximum at 360 nm which allows spectral resolution from that of MK-447. On pulse radiolysis of a nitrous oxide saturated solution containing 0.1 M KBr, 5 mM MK-447 and 0.1 mM ascorbate at pH 3.2 the characteristic absorption attributed to MK-O$^{\cdot}$ was found immediately after the pulse. This was replaced by a species with λ_{max} 360 nm corresponding to the ascorbyl radical. On increasing the ascorbate concentration the rate of appearance of $A^{\cdot-}$ increased, in accordance with the reaction:

$$MK\text{-}O^{\cdot} + AH^- \rightarrow MK\text{-}O^- + A^{\cdot-} + H^+ \quad (19)$$

Kinetic analysis of the reaction gave $k = 8.4 \times 10^8\ mol^{-1}dm^3sec^{-1}$.

(B) *Lipid peroxidation studies*

MK-447 and ONO-3144 were tested for their antioxidant activity in a range of lipid peroxidation systems using rat liver microsomes. The antioxidant activity of MK-447 was greater than that of ONO-3144 in all of the lipid peroxidation systems tested, usually by a considerable degree (Table 2). In the system dependent on NADPH/ADP-iron, for example, the estimated ED_{50} for MK447 was approximately 50 μM whereas that for ONO-3144 was at

Table 2. Antioxidant activity of ONO-3144 and MK-447 in a range of rat liver microsomal lipid peroxidation systems

Drug	Conc. (μM)	Cumene hydroperoxide	Iron-cysteine	Iron-ascorbate	NADPH-CCl_4	NADPH-ADP-iron*	NADPH-ADP-iron†‡
ONO-3144	500	59 (1)	59 ± 3 (3)	N.D.	52 ± 1 (3)	21 ± 2 (3)	27 ± 9 (3)
	250	54 ± 5 (3)	42 ± 4 (3)	N.D.	46 ± 4 (3)	11 (2)	15 ± 3 (3)
	100	46 ± 3 (3)	24 ± 3 (3)	N.D.	34 ± 10 (3)	1 (2)	3 (2)
	50	34 ± 4 (3)	18 ± 3 (3)	N.D.	—		
	10	33 (2)	12 (2)	N.D.	—		
	Estimated ED_{50}	150 μM	350 μM	—	>500 μM	>500 μM	>500 μM
MK-447	100	64 ± 2 (3)	97 ± 1 (3)	99 ± 1 (3)	82 ± 6 (4)	93 ± 7 (4)	91 (2)
	50	37 ± 8 (3)	80 ± 6 (3)	79 (2)	67 ± 11 (4)	42 ± 9 (3)	63 (2)
	25	18 ± 3 (3)	58 ± 12 (3)	28 (2)	53 ± 13 (4)	16 ± 4 (4)	32 (2)
	10	7 ± 6 (3)	38 ± 8 (3)	16 (2)	45 ± 6 (3)	9 ± 3 (3)	10 (2)
	Estimated ED_{50}	75 μM	20 μM	30 μM	20 μM	60 μM	40 μM

Values are mean (±SD) percentage inhibition relative to controls incubated without antioxidant. Number of experiments, with samples in triplicate, in parentheses. The rate of lipid peroxidation in controls were as follows: cumene hydroperoxide, 0.33; iron-cysteine, 0.75; iron-ascorbate, 1.74; CCl_4, 0.22; NADPH/ADP-iron, 1.53 nmols/mg/min.

* MDA production.

† O_2 uptake.

N.D., not determined.

Table 3. Interaction of ONO-3144 and MK-447 with the liver microsomal cytochrome P-450 system

Agent		Percentage inhibition of: NADPH: cyt c reductase	Aminopyrine demethylase
ONO-3144	500 μM	−3 ± 2* (3)	95 ± 6 (3)
	100 μM	0 ± 3 (3)	—
	50 μM	4 ± 3 (3)	91 ± 3 (3)
	10 μM	—	68 ± 3 (3)
	5 μM	—	53 ± 2 (3)
	1 μM	—	21 ± 3 (3)
MK-447	100 μM	−1, +1	62 ± 7 (3)
	50 μM	0, +4	50 ± 8 (3)
	10 μM	—	29 ± 8 (3)
	5 μM	—	23 ± 9 (3)

* Net stimulation.

least 10-fold higher. In the CCl_4-stimulated system the ED_{50} for MK-447 was estimated at around 20 μM while for ONO-3144 it was approximately 25 times higher than this. Also in a non-enzymic system, that stimulated by iron-cysteine, MK-447 was some 17.5-fold more effective than ONO-3144 with an ED_{50} of around 20 μM. ONO-3144 was most effective against cumene hydroperoxide-dependent lipid oxidation (ED_{50} 150 μM) where, in contrast, MK-447 was at its least effective (ED_{50} 75 μM).

Since many of the lipid peroxidation systems involve enzymes of the microsomal drug metabolising system, the interaction of the test compounds with cytochrome P-450 and with NADPH: cytochrome P-450 reductase (measured as NADPH:cytochrome *c* reductase) was tested. Neither ONO-3144 nor MK-447 exhibited any inhibitory activity towards NADPH:cytochrome c reductase activity (Table 3). However, both compounds interacted strongly with cytochrome P-450 and inhibited mixed-function oxidase activity. Aminopyrine demethylase was inhibited by both compounds: 50% inhibition was achieved with around 5 μM ONO-3144 and 50 μM MK-447 (Table 3). Both ONO-3144 and MK-447 gave similar strong type II substrate-binding spectra with a peak around 430 nm and a broad trough around 393 nm (data not shown).

DISCUSSION

The pulse radiolysis data provide useful information as to the potential antioxidant activity of MK-447 and ONO-3144. On examination of Table 1 it is clear that at neutral pH the iodinated derivative MK-447 is far more easily oxidised by the radicals studied than ONO-3144. This implies that the redox couple E(ONO-O$^{\cdot}$/ONO-O$^-$) is greater than E(MK-O$^{\cdot}$/MK-O$^-$) and hence the radical scavenging ability of MK-447 should be greater than that of ONO-3144. As would be expected, both compounds react rapidly with the hydroxyl radical as determined by competition kinetic methods, though this method provides no evidence as to the mechanism of action. The species generated on OH$^{\cdot}$ oxidation could be the phenoxyl radical though alternatively OH$^{\cdot}$ oxidation could result in carbon-centred radical formation elsewhere on MK-447 or ONO-3144. In both cases the species resolved optically has been assigned to the phenoxyl radicals given that phenoxyl radicals tend to exhibit absorptions at *ca.* 400 nm and also that the yield of the observed species is enhanced when more selective oxidants were used. Furthermore, the oxidation of MK-447 by $NO_2^{\cdot}$ and ONO-3144 by $(SCN)_2^{\cdot-}$ demonstrate some pH dependence in common with the ionisation of the hydroxyl grouping facilitating electron transfer. The mechanism of OH$^{\cdot}$ oxidation probably involves OH$^{\cdot}$ addition in common with other phenols, e.g.

$$\text{OH}^{\cdot} + \text{MK-OH} \rightarrow \text{MK-OH(OH)}^{\cdot} \qquad (20)$$

The OH adduct may then undergo unimolecular acid/base-catalysed elimination of water to form the more resonance stabilised phenoxyl radical species as is the case with phenol [40]:

$$\text{MK-OH(OH)}^{\cdot} \rightarrow \text{MK-O}^{\cdot} + \text{H}_2\text{O} \qquad (21)$$

Previous studies on the oxidation of phenolic compounds by the inorganic radical anions have shown a marked pH dependence on the rate of oxidation as exhibited here by ONO-3144 [32, 42]. The mechanism at high pH is believed to proceed through a direct one electron transfer mechanism, i.e.

$$(\text{SCN})_2^{\cdot-} + \text{ONO-O}^- \rightarrow 2\text{SCN}^- + \text{ONO-O}^{\cdot} \qquad (22)$$

The fact that MK-447 does not exhibit such a pH dependence with $(SCN)_2^{\cdot-}$ reflects the lower oxidation potential of this compound. Thus any difference in redox potential between MK-OH and MK-O$^-$ does not significantly alter the equilibrium constant of the overall reaction. $NO_2^{\cdot}$ is a weaker oxidant than $(SCN)_2^{\cdot-}$, therefore the observed pH dependence in this case is not surprising. The mechanism involved at neutral pH may also be one of electron transfer:

$$(\text{SCN})_2^{\cdot-} + \text{MK-OH} \rightarrow 2\text{SCN}^- + \text{MK-OH}^{\cdot+} \qquad (23)$$

$$\text{MK-OH}^{\cdot+} \rightleftharpoons \text{MK-O}^{\cdot} + \text{H}^+ \qquad (24)$$

Phenolic radical cations tend to have p*K*a values of *ca.*-5 [42], therefore the MK-OH$^{\cdot+}$ if formed, should rapidly deprotonate according to reaction

[24]. A similar scheme has been proposed for the one electron oxidation of tyrosine by SO_4^{2-} [41]. Similarly, the results obtained on $CCl_3O_2^{\cdot}$ oxidation may be explained by electron transfer processes. Given that the redox couple (MK-O$^{\cdot}$/MK-O$^-$) is lower than that of (ONO-O$^{\cdot}$/ONO-O$^-$) then the oxidation of MK-OH will be favoured at neutral pH.

Perhaps the most intensively studied free radical in terms of biological significance is $O_2^{\cdot-}$ and its conjugate acid $HO_2^{\cdot}$. The potential toxicity of this species has invoked many studies on its reaction mechanisms, not least with potential antioxidants. Our results do not demonstrate any reaction between $O_2^{\cdot-}$ or $HO_2^{\cdot}$ and the compounds studied. $HO_2^{\cdot}$ has been shown to oxidise α-tocopherol with $k = 2 \times 10^5$ $mol^{-1}dm^3sec^{-1}$ [43] but no evidence for any reaction was shown here (Table 1).

Our pulse radiolysis data suggest that of the two compounds studied, MK-447 reacts more rapidly with oxidising free radicals. However, these results alone do not indicate whether MK-447 will function as an effective antioxidant. The inclusion of a phenolic hydroxyl group does not directly confer efficient antioxidant activity; the effectiveness depends on a number of factors [44]. Another example is that of *p*-cresol which could be viewed as structurally similar to both ONO-3144 and MK-447 in that it contains a phenolic −OH and acts efficiently as an antioxidant in hydrocarbon systems but not in aqueous systems [45]. Biological antioxidants such as vitamin E act by reacting with peroxyl radicals which are the chain carriers in lipid peroxidation. They trap radicals directly and as such shorten the chain length, hence they are chain breaking antioxidants. Clearly, the antioxidant ability of a compound will not only relate to the rate by which it may react with an oxidising species but also to the stability of the radical product formed. As indicated, the interactions between the α-tocopheroxyl radical with ascorbic acid are well established [36, 46, 47], and this may enhance the antioxidant activity of α-tocopherol. The results shown in Fig. 4 demonstrate that MK-O$^{\cdot}$ reacts rapidly with ascorbate. The rate obtained, 8.4×10^8 $mol^{-1}dm^3sec^{-1}$ is greater than that obtained for repair of the respective phenoxy radicals of Trolox C and tocopherol by ascorbate (39 and 1.6×10^6 $mol^{-1}dm^3sec^{-1}$ respectively). The slow rate obtained for the α-tocopheroxyl radical has been attributed in part to stereoelectronic factors which suggest that the radical is stabilised by the ethereal oxygen *p*-type lone pair of α-tocopherol [48]. Clearly, MK-O$^{\cdot}$ is more reactive; but these results suggest that the antioxidant efficacy of MK-447 may be enhanced by other compounds such as ascorbate. Thus, the pulse data suggest that the redox couple for ONO-O$^{\cdot}$/ONO-O$^-$ is high and as a consequence this makes MK-447 the more likely candidate to exhibit antioxidant properties. The lipid peroxidation systems examined go some way as to address this question. It is clear from the data shown in Table 2 that both compounds tested had antioxidant activity but MK-447 is, in fact, the better antioxidant of the two. In every lipid peroxidation system tested MK-447 was the more effective inhibitor and these data are clearly in line with the rate data obtained by pulse radiolysis. The ED_{50} values obtained for ONO-3144 in these microsomal lipid peroxidation systems are in concord with that obtained by Aishita *et al.* in H_2O_2-induced erythrocyte lipid peroxidation [19]. The mechanism of antioxidant action is not certain. The most likely mode of action is to break the chain reaction by intercepting lipid peroxyl radicals as in the case of the natural phenolic antioxidant α-tocopherol but other mechanisms cannot be ruled out. The scavenging of initiating radicals (such as $CCl_3O_2^{\cdot}$ in the CCl_4-dependent system) is unlikely given the greater concentration of target fatty acids. The NADPH/ADP-iron- and CCl_4-stimulated systems require the activity of NADPH : cytochrome P-450 reductase and this was not inhibited by either compound.

Cytochrome P-450 is essential for the systems stimulated by CCl_4 and cumene hydroperoxide and probably participates to some extent as a propagating catalyst in all the microsomal lipid peroxidation systems. For this reason it was necessary to test the interaction of MK-447 and ONO-3144 with cytochrome P-450. Both compounds interacted strongly with cytochrome P-450 producing type II binding spectra and inhibiting aminopyrene demethylase activity. In a previous preliminary report ONO-3144 was also shown to inhibit aniline hydroxylase and ethoxycoumarin deethylase [21]. Thus, the inhibitory activity of ONO-3144 and MK-447 may be in part due to binding to cytochrome P-450. The low inhibitory activity of ONO-3144 against CCl_4-induced lipid peroxidation is similar to that of other inhibitors of cytochrome P-450-dependent reactions [49] and may be due to displacement of the inhibitor by CCl_4. The P-450 binding activity of these compounds is interesting since ONO-3144 has been shown to inhibit thromboxane synthesis, despite enhancing arachidonic acid conversion overall [19, 20]. Thromboxane synthetase and prostacyclin synthetase have been shown to be "P-450-like" enzymes by Ullrich and colleagues [50]. Their respective active sites are sufficiently different for certain compounds to specifically inhibit purified thromboxane synthetase. It would be interesting to know if ONO-3144 also exhibited such activity with the purified enzyme.

The significance of MK-447 and ONO-3144 lies in their ability to act as anti-inflammatory agents without inhibiting the synthesis of prostaglandins by prostaglandin synthetase. In this respect they differ from the typical, indomethacin-like, non-steroidal anti-inflammatory drugs. Kuehl and co-workers showed that MK-447 actually stimulated the synthesis of PGH_2 by enhancing cyclooxygenase activity [16, 18]. A number of free-radical scavengers, including phenol itself, were shown by this group to have this effect and their action was suggested to be the protection of the cyclooxygenase and peroxidase activity of prostaglandin synthetase by scavenging an activated oxygen species (postulated to be the OH$^{\cdot}$ radical) released during the enzymatic conversion of PGG_2 to PGH_2 [18, 50]. Similar results were reported for the MK-447 analogue, ONO-3144 [19, 20]. It was suggested that the activated oxygen species generated during PGH_2 production may be pro-inflammatory and that the anti-inflammatory activity of these compounds may be derived from their ability to scavenge this reactive intermediate

[18, 20]. It is unclear what the identity of this active oxygen species is, although $OH^{\cdot}$ has been suggested. Our own data make it clear that both MK-447 and ONO-3144 react very rapidly with $OH^{\cdot}$ but this is true of practically all organic compounds.

Neither the superoxide radical ($O_2^{\cdot-}$), nor its conjugate acid ($HO_2^{\cdot}$) reacted rapidly with the two test compounds. In fact, it seems unlikely that $O_2^{\cdot-}$ is sufficiently reactive itself to promote inflammation. The pulse radiolysis data and the lipid peroxidation data indicate that organic peroxyl radicals, such as lipoperoxyl radicals, are rapidly scavenged by both of these anti-inflammatory agents. This may represent a further mechanism for their anti-inflammatory effects as lipid peroxides may be pro-inflammatory and other antioxidant compounds have also been shown to have anti-inflammatory activity [52]. Overall, MK-447 exhibited better radical-scavenging and antioxidant activity than did ONO-3144. Likewise, MK-447 seems a better stimulator of PGH_2 production than is ONO-3144 judging by the respective data of Kuehl *et al.* [16] and Aishita *et al.* [19], although the compounds have not, to our knowledge, been tested simultaneously.

Free radicals are widely promoted as intermediates in the inflammatory response and anti-inflammatory activity is often attributed to radical scavenging, though the identity of the putative free radical species is often vague. In a recent publication the ability of a wide range of anti-inflammatory compounds to scavenge reactive oxygen species released by phagocytes was determined using a chemiluminescence assay [53]. This approach is valuable in that it gives a functional, pharmacological test of the ability of compounds to scavenge reactive oxygen species but the radical species involved are not well defined. In this paper we have shown that it is possible to obtain quantitative data on the reaction of defined free radicals with anti-inflammatory compounds postulated to be acting as free-radical scavengers. A combination of such approaches will lead to a better understanding of the role of free radicals in inflammation and of radical scavenging in anti-inflammatory activity.

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REFERENCES

1. Robbins S, Cotran R and Kumar V, *Pathological Basis of Disease*. W. B. Saunders, Philadelphia, 1984.
2. Samuelson B, Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220**: 568–575, 1983.
3. O'Flaherty JT, Lipid mediators of inflammation and allergy. *Lab Invest* **47**: 314–329, 1982.
4. Pinkard N, The 'new' chemical mediators of inflammation. In: *Current Topics in Inflammation & Infection* (Eds. Manjo G and Cotran RS), pp. 38–54. Williams & Wilkins, Baltimore, 1982.
5. Flohe L, Beckman R, Giertz H and Loschen G, Oxygen-centred free radicals as mediators of inflammation. In: *Oxidative Stress* (Ed. Sies H), pp. 403–435. Academic Press, London, 1985.
6. Fantone JC and Ward PA, Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am J Pathol* **107**: 397–418, 1982.
7. Babior BM, Oxygen-dependent microbial killing by phagocytes. N Eng J Med **298**: 659–668, 1978.
8. Quie PG, White JG, Holmes B and Good RA, In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease. *J Clin Invest* **46**: 668–674, 1967.
9. Mandell GL and Hook EW, Leukocyte bactericidal activity in chronic granulomatous disease: correlation of hydrogen peroxide production and susceptibility to intracellular killing. *J Bacteriol* **100**: 531–532, 1967.
10. Root RK, Comparison of other defects of granulocyte oxidative killing mechanisms with chronic granulomatous disease. In: *The Phagocytic Cell in Host Resistance* (Eds. Bellanti JA and Dayton DH), pp. 201–226. Raven Press, New York, 1975.
11. Gabig TG, Lefker BA, Ossanna PJ and Weiss SJ, Proton stoichiometry associated with human neutrophil respiratory burst reactions. *J Biol Chem* **259**: 13166–13171, 1984.
12. Vane JR, Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol* **231**: 232–235, 1971.
13. Smith JB and Willis AL, Aspirin selectively inhibits prostaglandin production in human platelets. *Nature New Biol* **231**: 235–237, 1971.
14. Ferreira SH, Moncada S and Vane JR, Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature New Biol* **231**: 237–239, 1971.
15. Hiller KO, Hodd PL and Willson RL, Anti-inflammatory drugs: protection of a bacterial virus as an in vitro biological measure of free radical activity. *Chem Biol Interact* **47**: 293–305, 1983.
16. Kuehl FA, Humes JL, Egan RW, Ham EA, Beveridge GC and Van Arman CG, Role of prostaglandin endoperoxide PGG_2 in inflammatory processes. *Nature (Lond)* **265**: 170–173, 1977.
17. Ham EA, Cirillo NJ, Zanetti M, Shen TY and Kuehl FA, Mode of action of non-steroidal anti-inflammatory agents. In: *Prostaglandins in Cellular Biology* eds. Ramwell PW and Pharris BB), pp. 345–352. Plenum Press, New York, 1972.
18. Kuehl FA, Humes JL, Ham EA, Egan RW and Dougherty HW, Inflammation: the role of peroxidase-drived products. In: *Advances in Prostaglandin & Thromboxane Research* (Eds. Samuelson B, Ramwell PW and Paoletti R), pp. 77–86. Raven Press, New York, 1980.
19. Aishita H, Morimura T, Obata T, Miura Y, Miyamoto T, Tsuboshima M and Mizushima Y, ONO-3144, a new anti-inflammatory drug and its possible mechanism of action. *Arch Int Pharmacodyn Ther* **261**: 316–327, 1983.
20. Crowshaw K, Role of inhibition of prostaglandin biosynthesis by anti-inflammatory drugs in the pathogenesis of renal damage. *Adv Inflammation Res* **6**: 149–158, 1984.
21. Cheeseman KH, ONO-3144 (2-aminomethyl-4-tert-butyl-6-propionylphenol): a potent inhibitor of cytochrome P-450-dependent drug metabolism in rat liver microsomes. *IRCS Med Sci* **12**: 306–307, 1984.
22. Slater TF and Sawyer B, The effects of carbon tetrachloride on rat liver microsomes during the first hour of poisoning *in vivo* and the modifying actions of promethazine. *Biochem J* **111**: 317–324, 1969.
23. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.

24. Slater TF and Sawyer B, The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions *in vitro*. *Biochem J* **123**: 805–814, 1971.
25. Cheeseman KH, Lai M and Slater TF, Enhancement of the metabolism of carbon tetrachloride metabolism by 2,2′-bipyridine and 1,10-phenanthroline in rat liver microsomes. *IRCS Med Sci* **9**: 600, 1984.
26. Cheeseman KH, Proudfoot K, Maddix SP, Collins MM, Milia A and Slater TF, Low rate of NADPH/ADP-iron dependent lipid peroxidation in hepatic microsomes of DBA/2 mice. *FEBS Lett* **184**: 343–346, 1985.
27. Slater TF, The inhibitory effects *in vitro* of phenothiazines and other drugs on lipid peroxidation systems in rat liver microsomes and their relationship to the liver necrosis produced by carbon tetrachloride. *Biochem J* **106**: 155–160, 1968.
28. Searle AJF and Willson RL, Stimulation of microsomal lipid peroxidation by iron and cysteine. Characterisation and the role of free radicals. *Biochem J* **212**: 549–554, 1983.
29. Jefcoate CR, Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical difference spectroscopy. *Methods Enzymol* **52**: 258–279, 1978.
30. Cheeseman, KH, The effect of the administration of cobaltic protoporphyrin IX on drug metabolism, carbon tetrachloride activation and lipid peroxidation. *Chem Biol Interact* **50**: 143–151, 1984.
31. Albano E, Cheeseman KH, Tomasi A, Carini R, Dianzani MU and Slater TF, Effect of spin traps in isolated rat hepatocytes and liver microsomes. *Biochem Pharmacol* **35**: 3955–3960, 1986.
32. Willson RL, Free radicals and tissue damage: mechanistic evidence from radiation studies. In: *Biochemical Mechanisms of Liver Injury* (Ed. Slater TF), pp. 123–224. Academic Press, London, 1978.
33. Willson RL, Iron and hydroxyl free radicals in enzyme inactivation and cancer. In: *Free Radicals, Lipid Peroxidation and Cancer* (Eds. McBrien DCH and Slater TF), pp. 275–303. Academic Press, London, 1982.
34. Willson RL, The reaction of oxygen with radiation-induced free radicals in DNA and related compounds. *Int J Radiat Biol* **17**: 349–358, 1970.
35. Prutz WA and Land EJ, Luminescence excitation and de-excitation involving one-electron transfer. *J Chem Soc Far Trans* **74**: 2077–2091, 1978.
36. Packer JE, Slater TF and Willson RL, Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* **278**: 737–738, 1979.
37. Davies, MJ, Forni LG and Willson RL, Free radical reactions of the vitamin E analogue Trolox C. Pulse radiolysis and e.s.r. studies. *Biochem J* (in press).
38. Forni LG, Mora-Arellano VO, Packer JE and Willson RL, Nitrogen dioxide and related free radicals: electron transfer reactions with organic compounds in solutions containing nitrite or nitrate. *J Chem Soc Perkin Trans* **II**: 1, 1986.
39. Forni LG and Willson RL, Thiyl and phenoxyl free radicals and NADH. Direct observation of one-electron oxidation. *Biochem J* **240**: 897–903, 1986.
40. Adams GE, Michael BD and Land EJ, Pulse radiolysis investigations of unimolecular elimination reactions in free radicals produced by $OH^{\cdot}$ addition to hydroxybenzenes. *Nature (Lond)* **211**: 293–294, 1966.
41. Bansal K and Fessenden RW, Pulse radiolysis studies of the oxidation of phenols by $SO_4^{\cdot -}$ and $Br_2^{\cdot -}$ in aqueous solutions *Radiat Res* **67**: 1–8, 1970.
42. Land EJ, Porter G and Strachen E, Primary photochemical processes in aromatic molecules Part 6. The absorption spectra and acidity constants of phenoxy radicals. *Trans Far Soc* **57**: 1885–1893, 1961.
43. Fukuzawa K and Gebicki JM, Oxidation of α-tocopherol in micelles and liposomes by the hydroxyl, perhydroxyl and superoxide free radicals, *Arch Biochem Biophys* **226**: 242–251, 1983.
44. Burton GW and Ingold KU, Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc Chem Res* **19**: 194–201, 1986.
45. Howard JA and Ingold KU, The kinetics of the inhibited autoxidation of tetralin II. *Can J Chem* **43**: 2724–2728, 1965.
46. Niki E, Saito T, Kawakain A and Kamiya Y, Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. *J Biol Chem* **259**: 4177–4182, 1984.
47. Doba T, Burton GW and Ingold KU, Antioxidant and co-antioxidant activity of vitamin C. *Biochim Biophys Acta* **835**: 298–303, 1985.
48. Burton GW and Ingold KU, Antioxidant activity of biological molecules I. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *J Am Chem Soc* **103**: 6472–6477, 1981.
49. Cheeseman KH, Effects of scavengers and inhibitors on lipid peroxidation in rat liver microsomes. In: *Free Radicals, Lipid Peroxidation and Cancer* (Eds. McBrien DCH and Slater TF), pp. 197–204. Academic Press, London, 1982.
50. Ullrich V and Graf H, Prostacyclin and thromboxane synthase as P-450 enzymes 352–355. *Trends Pharmacol Sci* 352–355, 1984.
51. Egan RW, Gale PH and Kuehl FA, Reduction of hydroperoxides in the prostaglandin biosynthetic pathway by a microsomal peroxidase. *J Biol Chem* **254**: 3295–3302, 1979.
52. Bragt PC, Bansberg JI and Bonty IL, Anti-inflammatory effects of free radical scavengers and antioxidants. Further support for proinflammatory roles of endogenous hydrogen peroxide and lipid peroxides. *Inflammation* **4**: 289–299, 1980.
53. Müller-Peddinghaus R and Wurl M, The amplified chemiluminescence test to characterize antirheumatic drugs as oxygen radical scavengers. *Biochem Pharmacol* **36**: 1125–1132, 1987.