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Organic Free Radicals and Proteins in Biochemical Injury: Electron- or Hydrogen-Transfer Reactions? [and Discussion]

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Organic free radicals and proteins in biochemical injury: electron- or hydrogen-transfer reactions?

By R. L. Willson, Christina A. Dunster, L. G. Forni, Carolyn A. Gee and Katherine J. Kittridge

Biochemistry Department, Brunel University of West London, Uxbridge, Middlesex UB8 3PH, U.K.

The reactions of organic free radicals, acting as either reductants or oxidants, have been studied by pulse radiolysis in neutral aqueous solution at room temperature. Many hydroxyl-substituted aliphatic carbon-centred radicals and one-electron adducts have been shown to be good one-electron reductants, while several oxygen-, sulphur- and nitrogen- (but not carbon-) centred free radicals have been shown to be good one-electron oxidants. Several carbon-centred radicals can be reduced rapidly by hydrogen transfer, from undissociated thiol compounds which can thus act as catalysts facilitating the overall reduction of a carbon-centred radical by an electron-donating molecule.

Kinetic considerations influenced by the one-electron redox potentials of the radical–molecule couples involved, determine whether a particular reaction predominates.

In this paper examples of such reactions, involving a water-soluble derivative of vitamin E (Trolox C) and the coenzyme NADH, are described, together with studies showing (*a*) that even in complex multi-solute systems some organic peroxy radicals can inactiviate alcohol dehydrogenase under conditions where the superoxide radical does not, and (*b*) the superoxide radical can be damaging if urate is also present, and this damage can be reduced by the presence of superoxide dismutase.

Introduction

Although free radicals have long been accepted as important intermediates in a large number of chemical processes of industrial importance, there is still a considerable reluctance to believe that they have any role in biology, let alone medicine. Radiation chemistry, likewise, is still often regarded as a somewhat esoteric subject, relevant only to areas such as nuclear safety, radiation sterilization and radiation therapy. Indeed, it is still widely believed that when a system is exposed to radiation a host of more or less random reactions takes place and that the intermediates involved, even if they are free radicals, are somewhat different from those that might be formed by conventional chemistry. This is just not so. It cannot be overstressed that, in most instances (indeed in all the experiments of the type referred to here), radiation-induced free radicals are of thermal energy and follow the same laws of chemistry as other free radicals. We hope this presentation will demonstrate that radiation effects, free radicals and contemporary biology, even medicine, can provide an exciting multidisciplinary area of study for physical organic chemists, biochemists, biologists and clinicians alike.

Clearly, when a biological system is exposed to free radicals a host of molecules will be affected. Some of these molecules may be important, for example DNA, or perhaps a key protein whose normal rate of synthesis is comparatively slow. The repercussions may then be

serious. Other molecules effected may be less important. For example, destruction of a small amount of glucose is unlikely to effect a biological system *per se*, although there does remain the possibility that products derived from such innocuous molecules may themselves be toxic. We must not forget that in organic chemistry, when one free radical reacts with another molecule, another free radical is generally formed and the reactions of this radical must be taken into consideration (see, for example, Adams *et al.* 1970, 1972; Willson 1976, 1977*a*, *b*, 1978, 1979, 1982, 1983).

Let us consider, for example, the many questions that can arise when we consider how free radicals react with a protein. For example, if we irradiate an enzyme in solution what happens?

(*a*) Is its activity lost?
(*b*) If so, by how much?
(*c*) Which free radicals are involved?
(*d*) What chemical changes occur?
(*e*) Can only one type of radical initiate a certain type of damage or can other radicals initiate the same or different damage?
(*f*) Are certain amino-acid residues attacked preferentially or is the free-radical attack simply random?
(*g*) Can some residues be destroyed without loss of activity?
(*h*) Does the damage remain on one particular residue or can it be transferred to another?
(*i*) If so, how fast are these processes?
(*j*) Do they occur intramolecularly or intermolecularly?
(*k*) What type of compounds can prevent these reactions?
(*l*) If such reactions can be prevented, do the compounds act by loosely binding to the enzyme, so modifying the active site and making it less susceptible to damage?
(*m*) Or, do they block the active site, shielding it from free-radical attack?
(*n*) Do other protective compounds act indirectly by scavenging free radicals generally?
(*o*) Or, do they act by repairing damage that has already been caused?
(*p*) If so, what reaction mechanisms are involved: do the processes occur, for example, by electron- or hydrogen-transfer reactions?

Clearly the number of possible reactions that can take place in such complex systems is enormous. Furthermore, any other inorganic or organic compounds present, even in relatively small concentrations, may have a significant effect on the overall processes that take place. To minimize such complications, radiation chemists traditionally have worked in systems which are as clean as possible. Materials have often been highly purified. Water, not just distilled, but often triply distilled, has been used for making up solutions and in many cases oxygen has been rigorously excluded. The fact that the biological relevance of information obtained in such sterile *in vitro* systems has sometimes been viewed with scepticism is perhaps, therefore, not surprising.

It is in this light that we have been undertaking experiments not only in pure chemical systems but also in 'dirty' but defined model biological systems containing an assortment of foreign compounds ('Analar dirt') and natural substances such as α-tocopherol (vitamin E), a water-soluble derivative of vitamin E (Trolox C), ascorbate (vitCH), and NADH.

When a neutral aqueous solution is irradiated at room temperature, whether it be pulsed radiation from an electron linear accelerator or continuous radiation from a ^{60}Co source, most chemical changes that take place can be attributed to initial reactions of the solvated electron

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and hydroxyl radicals formed after the ionization of water. A small amount of hydrogen atoms (*ca.* 10%) is also formed, but this can often, but not always, be neglected

$$H_2O \rightarrow e^-_{aq} + OH^{\cdot} + H_3O^+. \quad (1)$$

The solvated electron, a strongly reducing free radical, and the hydroxyl radical, a strongly oxidizing species, will react rapidly either with themselves or with other substances present, the extent depending on the relative concentrations and the respective rate constants (k) involved:

$$e^-_{aq} + A \xrightarrow{k_1} A^{-\cdot}; \quad (2)$$

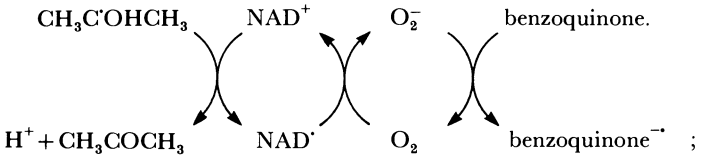
$$e^-_{aq} + B \xrightarrow{k_2} B^{-\cdot}; \quad (3)$$

$$e^-_{aq} + C \xrightarrow{k_3} C^{-\cdot}; \quad (4)$$

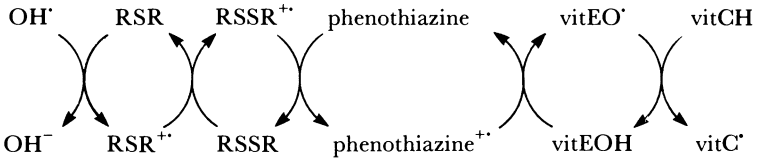
$$[A^{-\cdot}]:[B^{-\cdot}]:[C^{-\cdot}] = k_1[A]:k_2[B]:k_3[C].$$

The product radicals from these reactions may, however, enter into further reactions with the other solutes present, the relative probabilities again depending on the respective concentrations and rate constants. Thus, although damage may occur initially with a compound present in high concentration, it may nevertheless be transferred subsequently to a compound present only in minor amounts. Such electron-transfer (or 'hole'-transfer) processes, taking place either intermolecularly or intramolecularly, can be readily envisaged when one considers the following cycling processes which have been characterized by pulse radiolysis (Forni & Willson 1984; Willson, 1970, 1971; Asmus *et al.* 1979; Mahood *et al.* 1980):

(i) radical as a reducing agent:



(ii) radical as an oxidizing agent:



Free-radical inactivation of lysozyme

Let us consider the irradiation of the enzyme lysozyme, in doubly distilled water containing a little phosphate buffer to adjust the solution to neutral pH. If we measure the extent of inactivation after a certain dose of radiation, we find that it is reduced by approximately half (table 1). These effects can be attributed to the reaction of the water free radicals OH· and

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Table 1. Percentage remaining activity of a lysozyme solution[a] after irradiation in solution[b] in the presence of an increasing assortment of other solutes

	additional solutes	percentage remaining activity
	air	40
plus	thymine (100 μM) leucine (100 μM) acetate (100 μM) glucose (100 μM) lactate (100 μM) citrate (100 μM) isocitrate (100 μM) NAD^+ (100 μM)	60
plus	Br^- (5 mM)	45
plus	C_2H_5OH (0.5 M)	90
plus	$CHCl_3$ (5 mM)	25
plus	acetone–isopropanol (1 M)	90
plus	CCl_4 (10 mM)	40
plus	NADH (1 mM)	90
or	Trolox C (1 mM)	85
or	tryptophan (1 mM)	85
or	glutathione (1 mM)	55

[a] Concentration 50 μg ml^{-1}.
[b] With 150 Gy (*ca.* 100 μM free radicals).

e^-_{aq} with the enzyme and oxygen respectively, but with the superoxide radical formed having no effect on the enzyme activity (Adams *et al.* 1970).

$$OH^\cdot + \text{lysozyme} \rightarrow \text{inactivation}, \quad (5)$$

$$e^-_{aq} + O_2 \rightarrow O^\cdot_2, \quad (6)$$

$$O^\cdot_2 + \text{lysozyme} \rightarrow \text{no inactivation}. \quad (7)$$

We may then repeat the experiment, but include in the irradiated solution an assortment of biological molecules. For example, if in addition to the DNA base thymine, we include an amino acid such as leucine, a fatty acid such as acetate, a monosaccharide such as glucose, some intermediates of the citric acid cycle such as citrate and isocitrate, as well as lactate and the coenzyme NAD^+, all at 100 μM concentration, the remaining activity is now increased (see figure 1). This can be attributed to the scavenging of the hydroxyl free radicals by the additional substances that have been included in the irradiated solution:

$$OH^\cdot + S \rightarrow \text{`products'}, \quad (8)$$

$$\text{`products'} + \text{lysozyme} \rightarrow \text{no inactivation}. \quad (9)$$

If bromide (5 mM) is added before irradiation, the extent of damage is increased. The extent is decreased again, however, if ethanol (100 mM) in high concentration is also included. The effects can be attributed to the action of bromide ion scavenging the hydroxyl radical, and the resulting bromine radical-anion reacting with the enzyme and inactivating it (Adams *et al.* 1969, 1972; Posner *et al.* 1976):

$$OH^\cdot + Br^- \rightarrow Br^\cdot, \quad (10)$$

$$Br^\cdot + Br^- \rightarrow Br^{-\cdot}_2, \quad (11)$$

$$Br^{-\cdot}_2 + \text{lysozyme} \rightarrow \text{inactivation}. \quad (12)$$

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In the further presence of ethanol, the hydroxyl radicals are now selectively scavenged by the alcohol. Because the ethanol radical or radicals derived from it, such as the peroxy radical or superoxide radical, do not inactivate lysozyme, considerable protection is observed.

$$OH^{\cdot} + CH_3CH_2OH \rightarrow CH_3C^{\cdot}HOH + H_2O, \quad (13)$$

$$CH_3C^{\cdot}HOH + O_2 \rightarrow CH_3CH(O_2^{\cdot})OH, \quad (14)$$

$$CH_3CH(O_2^{\cdot})OH + OH^- \rightarrow CH_3CH(O_2^{\cdot})O^- + H_2O, \quad (15)$$

$$CH_3CH(O_2^{\cdot})O^- \rightarrow CH_3CHO + O_2^- + H_2O. \quad (16)$$

If chloroform (5 mM) is now included before irradiation, the solvated electrons are preferentially scavenged by the halocarbon to form the chloroform peroxy radical, $CHCl_2O_2^{\cdot}$. This peroxy radical can react with the enzyme and inactivate it (Willson 1982).

$$e_{aq}^- + CHCl_3 \rightarrow CHCl_2 + Cl^-, \quad (17)$$

$$CHCl_2 + O_2 \rightarrow CHCl_2O_2^{\cdot}, \quad (18)$$

$$CHCl_2O_2^{\cdot} + \text{lysozyme} \rightarrow \text{inactivation}. \quad (19)$$

However, if isopropanol and acetone in high concentration (1 M) are also included, the water free radicals are now selectively scavenged by these compounds. The resulting isopropanol radical, or the corresponding peroxy radical or superoxide radical derived from it, do not react with the enzyme and therefore protection is again observed.

$$e_{aq}^- + CH_3COCH_3 \rightarrow CH_3COCH_3^{-\cdot}, \quad (20)$$

$$CH_3COCH_3^{-\cdot} + H_2O \rightarrow CH_3C^{\cdot}OHCH_3 + OH^-, \quad (21)$$

$$OH^{\cdot} + (CH_3)_2CHOH \rightarrow (CH_3)_2C^{\cdot}OH + H_2O, \quad (22)$$

$$(CH_3)_2C^{\cdot}OH + O_2 \rightarrow (CH_3)_2C(O_2^{\cdot})OH, \quad (23)$$

$$(CH_3)_2C(O_2^{\cdot})OH + OH^- \rightarrow (CH_3)_2C(O_2^{\cdot})O^- + H_2O, \quad (24)$$

$$(CH_3)_2C(O_2^{\cdot})O^- \rightarrow (CH_3)_2CO + O_2^-. \quad (25)$$

Finally, if carbon tetrachloride (10 mM) is added before irradiation, considerable inactivation again occurs. However, the extent is again reduced if any of the electron donors NADH, Trolox C (TxOH, a water soluble derivative of vitamin E), glutathione (GSH), tryptophan (trp), or the sulphur-containing amino acid, methionine, are also included. In the presence of carbon tetrachloride, the isopropanol radical is oxidized to acetone and the radical $CCl_3O_2^{\cdot}$ is formed (Koster & Asmus 1971; Willson & Slater 1975). This peroxy radical then reacts with lysozyme and inactivates it (Broadhurst *et al.* 1982; Willson 1983; Hiller *et al.* 1983):

$$CH_3C^{\cdot}OHCH_3 + CCl_4 \rightarrow CH_3COCH_3 + CCl_3^{\cdot} + Cl^- + H^+, \quad (26)$$

$$CCl_3^{\cdot} + O_2 \rightarrow CCl_3O_2^{\cdot}, \quad (27)$$

$$CCl_3O_2^{\cdot} + \text{lysozyme} \rightarrow \text{inactivation}. \quad (28)$$

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When NADH or any of the other electron-donating substances described are present at the time of radiation, $CCl_3O_2^{\cdot}$ is scavenged by these compounds to form a species which does not inactivate the enzyme appreciably, if at all.

$$CCl_3O_2^{\cdot} + NADH \rightarrow CCl_3O_2H + NAD^{\cdot}; \quad (29)$$

$$CCl_3O_2^{\cdot} + TxOH \rightarrow CCl_3O_2H + TxO^{\cdot}. \quad (30)$$

It is important to state that in none of these systems, nor in the systems containing alcohol dehydrogenase which will be described later, did the presence of the high concentrations of organic compounds used affect the activity of the enzymes significantly over the time course of the experiment, *ca.* 30 min.

Clearly, a host of chemical reactions are going on in these systems. Unfortunately, we cannot go into them in detail here. Let us concentrate, therefore, on the free radicals derived from isopropanol and carbon tetrachloride, and look at their reactions with NADH, Trolox C, GSH, tryptophan and tyrosine.

Reactions of organic peroxy radicals with Trolox C

Previous studies have shown that when aerated solutions containing vitamin E (α-tocopherol, vitEOH), isopropanol, acetone and carbon tetrachloride are pulse-irradiated, a transient absorbing species, ($\lambda_{max} = 435$ nm) can be observed (figure 1). The absorption

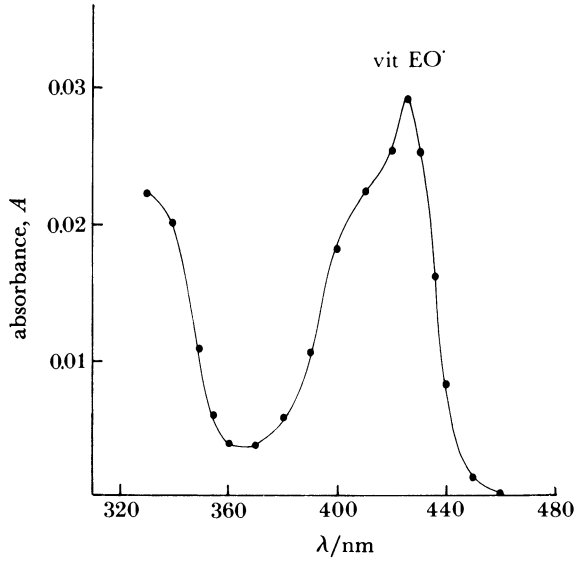
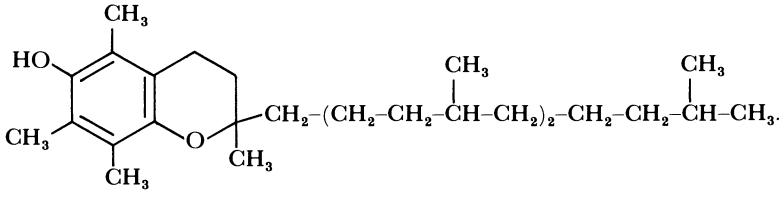


FIGURE 1. Absorption spectrum of the phenoxyl free radical from α-tocopherol observed by following the reaction of $CCl_3O_2^{\cdot}$; 25 ml solution contains 12 ml i-PrOH, 2.5 ml acetone, 0.04 M CCl_4, 8.8×10^{-4} M α-tocopherol. Structure of α-tocopherol:



is similar to the absorption recorded on flash photolysis of various phenols and has been attributed to the corresponding phenoxy free radical (Packer *et al.* 1979). No such absorptions are observed in the absence of CCl_4 or O_2 in agreement with the lack of any reaction of $CCl_3^{\cdot}$ or $CH_3C^{\cdot}OHCH_3$ with the vitamin (figure 2).

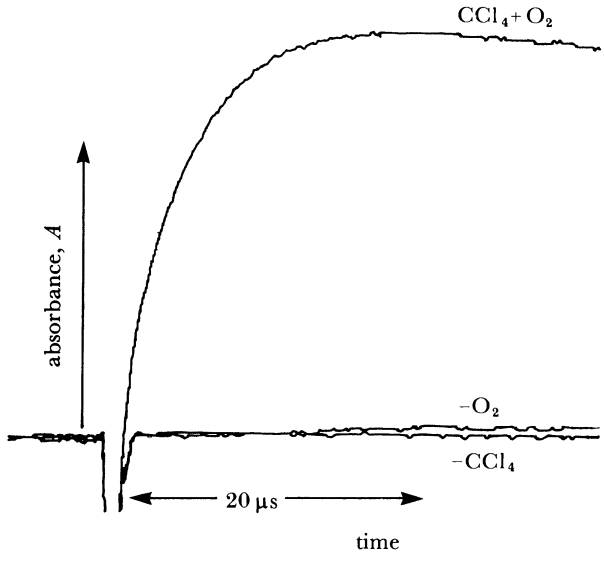


FIGURE. 2. Changes in absorption at 435 nm observed on pulse radiolysis of solutions containing isopropanol and acetone (1 M) and α-tocopherol (100 μm), in the absence and presence of air or CCl_4 (10^{-2} M).

$$CH_3C^{\cdot}OHCH_3 + \text{vitEOH} \rightarrow CH_3CHOHCH_3 + \text{vitEO}^{\cdot} \text{ (relatively slow if at all)}; \quad (31)$$

$$CCl_3^{\cdot} + \text{vitEOH} \rightarrow CCl_3H + \text{vitEO}^{\cdot} \text{ (relatively slow if at all)}; \quad (32)$$

$$CCl_3O_2^{\cdot} + \text{vitEOH} \rightarrow CCl_3O_2H + \text{vitEO}^{\cdot} \text{ (fast)}. \quad (33)$$

Similar results have recently been obtained with Trolox C in agreement with:

$$CCl_3O_2^{\cdot} + \text{TxOH} \rightarrow CCl_3O_2H + \text{TxO}^{\cdot}. \quad (34)$$

Although (34) is written above as a hydrogen-transfer reaction it was of interest to know whether the reaction did occur in this manner, or was in fact an electron-transfer reaction, i.e.:

$$CCl_3O_2^{\cdot} + \text{TxOH} \rightarrow CCl_3O_2^{-} + \text{TxOH}^{+\cdot} \xrightarrow{\downarrow H_2O} \text{TxO}^{\cdot} + H_3O^{+}. \quad (35)$$

The peroxy radical $CCl_3O_2^{\cdot}$ and related peroxy radicals can also be formed on pulse radiolysis of aerated aqueous solutions of the halocarbon and excess t-butanol, according to:

$$OH^{\cdot} + (CH_3)_3COH \rightarrow {}^{\cdot}CH_2(CH_3)_2COH + H_2O, \quad (36)$$

$${}^{\cdot}CH_2(CH_3)_2COH + O_2 \rightarrow {}^{\cdot}O_2CH_2(CH_3)_2COH, \quad (37)$$

$$e_{aq}^{-} + CCl_4 \rightarrow CCl_3^{\cdot} + Cl^{-}. \quad (38)$$

On pulse radiolysis of solutions containing t-butanol and Trolox C no formation of the $TxO^{\cdot}$ could be observed. In the presence of CCl_4, however, an exponential formation of the

absorbtion was again apparent, in agreement with reaction (34) and reaction (39) being relatively slow.

$$^{\cdot}O_2CH_2(CH_3)_2COH + TxOH \rightarrow HO_2CH_2(CH_3)_2COH + TxO^{\cdot}. \quad (39)$$

On performing experiments with solutions of deuterated Trolox C, (TxOD), prepared from an alkaline solution of Trolox C ($pK_a = 11.9$) in D_2O, a similar absolute rate constant was obtained, in agreement with the occurrence of an electron-transfer (35) rather than a hydrogen-transfer reaction (34). Further support of an electron-transfer reaction comes from other studies, which have shown that $CCl_3O_2^{\cdot}$ reacts rapidly with a variety of one-electron donors (table 2) and that Trolox C reacts rapidly with a variety of other electrophilic radicals (Willson & Slater 1975; Packer *et al.* 1978, 1979, 1980, 1981; Bahnemann *et al.* 1981; Forni *et al.* 1983*b*).

TABLE 2. ABSOLUTE RATE CONSTANTS[a] OF FREE-RADICAL REACTIONS[b]

radical	TxOH	NADH	vitC$^-$	UO$^-$	PZ	cytII *c*	GSH	tyr	trp	MV^{2+}	O_2
$CH_3C^{\cdot}OHCH_3$	< 0.01	< 0.01	0.01	—	< 0.01	< 0.01	1.8	—	—	*ca.* 10	4.1
$OH^{\cdot}$	68	80	64	71	97	> 100	> 100	105	80		
$N_3^{\cdot}$	5	28	—	49	46	13	—	1	41		
$Br_2^{-\cdot}$	3.8	9.0	8.7	9	—	9.7	—	0.2	7.7		
$GS^{\cdot}$	—	2.5	6.0	0.14	0.33	2.5	0.14	—	—	—	6
$CCl_3O_2^{\cdot}$	3.7	5.6	2	7.0	4.5						
$CHCl_2O_2^{\cdot}$	1.1	3.3	2.6	—	0.67						
$NO_2^{\cdot}$	< 0.01[c]	—	0.3	—	0.3						
$TxO^{\cdot}$	—	—	0.4								
$tyrO^{\cdot}$	3.2	0.61									
$C_6H_5O^{\cdot}$	4.1										
m-$CH_3C_6H_4O^{\cdot}$	2.8										
p-$CH_3C_6H_4O^{\cdot}$	0.95										
o-$CH_3C_6H_4O^{\cdot}$	< 0.01										

[a] Units: $10^8\ M^{-1}\ s^{-1}$. [b] At *ca.* pH 7. [c] Takes value 5.0 at pH 11.

Reactions of peroxy radicals with NADH

The strong absorption of NADH at 340 nm provides a convenient handle for determining the rate of reaction of free radicals with the coenzyme (Swallow 1953, 1955; Land & Swallow 1971; Bielski & Chan 1973, 1974). On pulse radiolysis of aqueous solutions containing t-butanol or acetone, and isopropanol, carbon tetrachloride and NADH, an exponential bleaching at 340 nm was observed (figure 3), in agreement with the reactions (26) and (27) or (36), (37) and (38) followed by

$$CCl_3O_2^{\cdot} + NADH \rightarrow CCl_3O_2H + NAD^{\cdot}. \quad (40)$$

Again, although formerly written above as a hydrogen-transfer reaction, it was of interest to know whether the reaction occurs in this manner or was in fact an electron-transfer reaction:

$$CCl_3O_2^{\cdot} + NADH \rightarrow CCl_3O_2^{-} + NADH^{+\cdot} \quad (41)$$
$$\downarrow H_2O$$
$$NAD^{\cdot} + H_3O^{+}.$$

With solutions containing t-butanol and monodeuterated NADH, i.e. NADD (prepared by incubating C_2D_5OD and NAD^+ with YADH), no difference in absolute rate constants was obtained (figure 4). This is again in agreement with an electron-transfer reaction. Further

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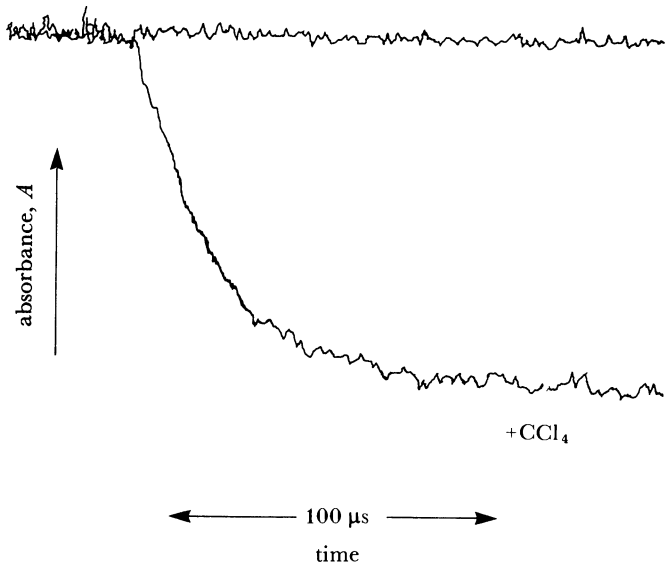


FIGURE. 3. Decrease in absorption at 340 nm observed on pulse radiolysis of an air-saturated solution containing t-butanol and NADH (100 μm), in the absence and presence of CCl_4 (10^{-2} M).

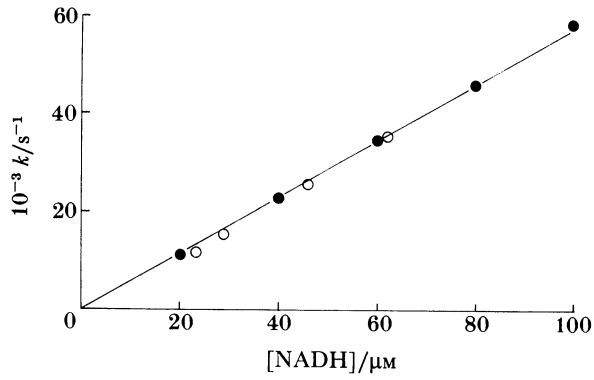


FIGURE 4. Plot of first-order rate constant against NADH (●) or NADD (○) concentration for the loss of absorption at 340 nm owing to reaction of CCl_3O_2 with the coenzyme (see text); $k = 5.6 \times 10^8$ M^{-1} s^{-1}.

support for such a mechanism also comes from other studies, showing that NADH reacts rapidly with a variety of electrophilic radicals (table 2). Furthermore, as with Trolox C, no reaction was apparent in the absence of O_2 or CCl_4, again indicating that the reaction of $CCl_3^{\cdot}$ or the carbon-centred t-butanol or isopropanol radicals with NADH, occur relatively slowly if at all.

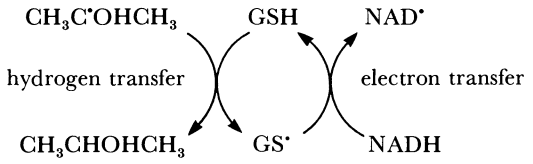
$$CH_3C^{\cdot}OHCH_3 + NADH \rightarrow CH_3CHOHCH_3 + NAD^{\cdot} \text{ (relatively slow if at all)}; \quad (42)$$

$$CCl_3^{\cdot} + NADH \rightarrow CCl_3H + NAD^{\cdot} \text{ (relatively slow if at all)}. \quad (43)$$

Reactions of thiyl radicals with NADH

The fact that thiyl radicals such as $GS^{\cdot}$ can also react rapidly with NADH again comes from pulse radiolysis studies of solutions containing isopropanol, acetone and NADH (Forni & Willson 1983). It was found that the exponential loss of the NADH absorption could also occur if GSH rather than CCl_4 was present in the solution. Thiols have been shown to repair organic

radicals by a comparatively rapid hydrogen-transfer reaction (Adams *et al.* 1967, 1968, 1969; Wolfenden & Willson 1983). The observations are then in agreement with the cycling sequence



If methyl viologen (paraquat), MV^{2+}, is also present in this system, a slow appearance of the characteristic absorption of the $MV^{+\bullet}$ radical cation is apparent. If NADH is not included in the irradiated solution the absorption is absent in agreement with the above reactions followed by the reaction (Anderson 1980; Farrington *et al.* 1980)

$$NAD^{\bullet} + MV^{2+} \rightarrow NAD^{+} + MV^{+\bullet}. \tag{44}$$

Again, further support that $GS^{\bullet}$ was reacting with NADH by an electron-transfer mechanism comes from other pulse-radiolysis studies, showing that thiyl radicals can react rapidly with a variety of electron donors (table 2) (Forni *et al.* 1983*a*; Forni & Willson 1983; Willson 1983). Furthermore, on pulse radiolysis of GSSG in the presence of t-butanol, bleaching of the NADH absorption was again observed and the same absolute rate constant was obtained whether NADH or NADD was used.

$$e^{-}_{aq} + RSSR \rightarrow RSSR^{-\bullet} \rightarrow RS^{\bullet} + RS^{-}, \tag{45}$$

$$RS^{\bullet} + NADH \rightarrow RS^{-} + NAD^{+\bullet} \xrightarrow{H_2O} NAD^{\bullet} + H_3O^{+}. \tag{46}$$

Reactions of tyrosine radicals with NADH *and intramolecular hydrogen transfer*

In the context of protein damage our finding that NADH can also be readily oxidized by phenoxyl free radicals, $\phi O^{\bullet}$, is particularly interesting. Previous studies on pulse radiolysis of solutions containing azide ion and tyrosine (Prutz 1979) have shown that the tyrosine phenoxyl radical can be formed rapidly:

$$OH^{\bullet} + N_3^{-} \rightarrow N_3^{\bullet} + OH^{-}, \tag{47}$$

$$N_3^{\bullet} + tyrOH \rightarrow N_3^{-} + tyrOH^{+\bullet} \xrightarrow{H2O\,(N_3^{-})} tyrO^{\bullet} + H_3O^{+}\,(N_3H). \tag{48}$$

In the additional presence of NADH the phenoxyl radical absorption decayed rapidly, with a parallel decrease in absorption at 340 nm. This is in agreement with

$$\begin{array}{ccc} tyrO^{\bullet} + NADH & \rightarrow & tyrO^{-} + NADH^{+\bullet} \\ {\scriptstyle H_2O}\downarrow & & \downarrow{\scriptstyle H_2O} \\ OH^{-} + tyrOH & & NAD^{\bullet} + H_3O^{+}. \end{array} \tag{49}$$

Furthermore, previous studies have shown that when $N_3^{\bullet}$ or $CCl_3O_2^{\bullet}$ reacts with the peptide tryptophanyl-tyrosine, the tryptophanyl radical (trp$^{\bullet}$) is soon formed, but a rapid

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intramolecular transfer process subsequently takes place in which the free-radical centre becomes located on the tyrosine (Prutz *et al.* 1980, 1981; Packer *et al.* 1981):

$$N_3^{\bullet} + \text{trpH-tyrOH} \rightarrow \text{trpH}^{+\bullet}\text{-tyrOH}, \quad (50)$$

$$\text{tryH}^{+\bullet}\text{-tyrOH} + N_3^- \rightarrow \text{trp}^{\bullet}\text{tyrOH} + N_3H, \quad (51)$$

$$\text{trp}^{\bullet}\text{-tyrOH} \rightarrow \text{trpH-tyrO}^{\bullet}. \quad (52)$$

When similar experiments were undertaken with NADH also present, the tryptophanyl radical, trp$^{\bullet}$, was soon observed, but this subsequently decayed with the formation of the tyrosine phenoxyl absorption at 410 nm. This in turn decayed and after 3 ms only a bleaching of the NADH absorption at 340 nm was apparent (figure 5). This can be represented:

$$\text{trpH-tyrO}^{\bullet} + \text{NADH} \rightarrow \text{trpH-tyrO}^- + \text{NADH}^{+\bullet} \quad (53)$$
$$\downarrow H_2O$$
$$\text{NAD}^{\bullet} + H_3O^+.$$

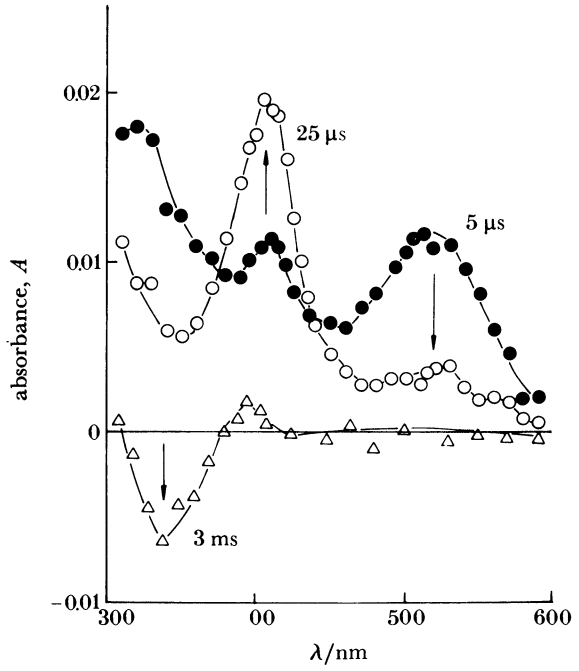

FIGURE 5. Absorption spectra observed on pulse radiolysis of N_2O-saturated aqueous solutions containing azide (50 mM), tryptophanyl-tyrosine (3 mM), and NADH (25 μM); ●, after 5 μs; ○, after 25 μs; △, after 3 ms.

Further support for an electron-transfer mechanism comes from other pulse-radiolysis studies showing that phenoxy radicals can react rapidly with a variety of electron donors, including ascorbate and various ionized phenols (Schuler 1977; Packer *et al.* 1979; Steenken & Neta 1979, 1982). Some rate constants are shown (table 2).

Reactions of peroxy radicals $RO_2^{\bullet}$ *with* NADH

Although reactions of $CCl_3O_2^{\bullet}$ and related halocarbon peroxy radicals with NADH have been characterized by pulse radiolysis, no reactions of the thymine hydroxyl radical-adduct peroxy

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radicals nor of aliphatic peroxy radicals ($RO_2^{\cdot}$) have been observed directly. However, stationary-state studies in which solutions of NADH saturated with a nitrous oxide–air mixture have been irradiated, have shown that such reactions do occur, albeit relatively slowly (table 3). In studies in which formate or ethanol were irradiated with the coenzyme, little loss of NADH absorption at 340 nm was apparent at pH = 8. With thymine at pH = 5–6 or at pH = 8 or

Table 3. Destruction of NADH by organic peroxy free radicals but not superoxide[a]

scavenger	pH 5–6	*ca.* pH 8
formate	90	100
ethanol	55	95
isopropanol	79	88
thymine (1 mM)	26	45

[a] Values given are percentage of coenzyme remaining after irradiation in $N_2O:O_2$ (4:1) saturated solutions in the presence of hydroxyl radical scavenger. Initial NADH concentration is 10^{-4} M; radiation dose is 100 Gy (*ca.* 60 μM free radicals).

with ethanol at pH = 5.9, however, some destruction was obtained, which is in agreement with O_2^- reacting only slowly, if at all, with the coenzyme and the thymine and ethanol peroxy radical reacting to a significant extent.

$$e_{aq}^- + N_2O \rightarrow N_2 + OH^{\cdot} + OH^-, \quad (54)$$

$$OH^{\cdot} + T \rightarrow T(OH)^{\cdot}, \quad (55)$$

$$T(OH^{\cdot}) + O_2 \rightarrow T(OH)O_2^{\cdot}, \quad (56)$$

$$T(OH)O_2^{\cdot} + NADH \rightarrow T(OH)O_2H + NAD^{\cdot}, \quad (57)$$

$$OH^{\cdot} + HCO_2^- \rightarrow CO_2^{-\cdot} + H_2O, \quad (58)$$

$$CO_2^{-\cdot} + O_2 \rightarrow CO_2 + O_2^-, \quad (59)$$

$$O_2^- + NADH \rightarrow HO_2^- + NAD^{\cdot} \text{ (relatively slow if at all)}. \quad (60)$$

The slow reaction of O_2^- with NADH is in agreement with previous studies where the rate constant $k_{60} = 10^4$ M^{-1} s^{-1} was determined when the enzyme was irradiated in the presence of lactate dehydrogenase, but was only $k_{60} = 28$ M^{-1} s^{-1} in the absence of the enzyme (Bielski & Chan 1973, 1974). It was suggested that this was a result of the enzyme affecting the conformation of NADH, making it more susceptible to oxidation. However, one wonders whether this large difference in rate constant may be partly because of the effect of the protein environment on the equilibrium concentration of the acid form of superoxide, $HO_2^{\cdot}$, in the vicinity of the coenzyme, with $k_{61} \gg k_{60}$:

$$HO_2^{\cdot} + NADH \rightarrow H_2O_2 + NAD^{\cdot}. \quad (61)$$

Such an effect of protein may have been observed with urate (see later).

Reaction of peroxy radicals with alcohol dehydrogenase and protection by SOD

Recent studies have shown that, in addition to $CCl_3O_2^{\cdot}$ and related halocarbon peroxy radicals, peroxy radicals from a variety of aromatic compounds can inactivate yeast alcohol

dehydrogenase (YADH) under conditions where O_2^- is non-damaging (Kittridge & Willson 1984; Gee *et al.* 1985). Indeed, the extent of damage is often greater than that observed with a similar concentration of hydroxyl radicals, $OH^{\cdot}$. This is in agreement with a lower oxidizing ability conferring a greater degree of selectivity (Adams *et al.* 1969). When the enzyme was irradiated in the presence of both formate and thymine, the extent of inactivation observed depended on the relative formate and thymine concentrations, in agreement with the reactions (55) and (58) competing for hydroxyl radicals. Thus, in the presence of excess formate, little inactivation of the enzyme occurred.

Damage could also be prevented by the presence at the time of irradiation of a variety of electron donors (antioxidants) in relatively low concentration (100 μM). These included Trolox C, NADH, GSH and cysteamine, as well as cysteine, tryptophan and methionine. Other common amino acids did not protect the enzyme, perhaps indicating that the above residues were likely to be the vulnerable sites in YADH (Gee *et al.* 1985).

A further series of enzyme experiments in which YADH was irradiated in solutions of increasing complexity, hopefully serves to illustrate how, if a detailed biochemical description of a system is available, we may be able to predict the biological repercussions more accurately (table 4). Many of the reactions involved are similar to those in the

TABLE 4. PERCENTAGE REMAINING ACTIVITY OF A YEAST ALCOHOL DEHYDROGENASE SOLUTION[a] AFTER IRRADIATION[b] IN THE PRESENCE OF AN INCREASING ASSORTMENT OF OTHER MOLECULES KNOWN TO SCAVENGE FREE RADICALS

additional solutes	concentration	radical	10^{-9} rate constant/ $M^{-1}\,s^{-1}$	percentage remaining activity
oxygen (air)	250 μM	e^-_{aq}	12	41
plus formate	100 μM	$OH^{\cdot}$	4	72
plus thymine	500 μM	$OH^{\cdot}$	5	3
plus ethanol	5 mM	$OH^{\cdot}$	2	43
plus bromide	50 mM	$OH^{\cdot}$	1	0
plus isopropanol	1 M	$OH^{\cdot}$	2	55
plus chloroform	5 mM	e^-_{aq}	30	1
plus acetone	1 M	e^-_{aq}	6	53
plus CCl_4	10 mM	$CH_3C^{\cdot}OHCH_3$	0.2	0
plus methyl viologen	10 mM	$CH_3C^{\cdot}OHCH_3$	1	43
plus urate	1 mM	O_2^-/HO_2?	—	17
plus SOD	10 μg ml^{-1}	O_2^-	—	71

[a] Concentration 20 μg ml^{-1}.
[b] Dose 15 Gy (*ca.* 10 μM free radicals).

lysozyme system. When chloroform is added, the peroxy radical $CHClO_2^{\cdot}$ is formed according to (17) and (18). However, unlike reaction with carbon tetrachloride, the reaction of $CHCl_3$ with the isopropanol radical is relatively slow and it cannot compete effectively with oxygen for this radical: hence the observed sensitization on the addition of chloroform followed by protection on the addition of acetone.

$$CH_3C^{\cdot}OHCH_3 + CHCl_3 \rightarrow CHCl_2O_2^{\cdot} + CH^{\cdot}COCH_3 + Cl^- + H^+ \text{ (relatively slow if at all).} \quad (62)$$

The appropriate absolute rate constants are shown (table 4), together with the extent of enzyme inactivation observed.

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Uric acid, iron and the reactions of H_2O_2 *and* O_2^- *with enzymes*

In the irradiated system (table 4), it will have been noticed that the presence of urate (UO^-) increases the extent of inactivation, contrary to the suggestion that uric acid (UOH) is one of man's important antioxidants (Ames *et al.* 1981).

Even in simpler systems, the purine, like the pyrimidine thymine, does not protect alcohol dehydrogenase against inactivation. Indeed the extent of inactivation is again considerably increased (Kittridge & Willson 1984). What is particularly surprising is that extensive inactivation still occurs even when excess formate is present. This is in spite of the fact that when urate is irradiated in the presence of formate alone, little destruction of the purine occurs as measured spectrophotometrically at 292 nm. In the presence of excess formate, O_2^- is likely to be the only free radical able to react with urate or the enzyme and similar effects are also observed when O_2^- is generated in systems containing isopropanol, acetone (1 M) and methyl viologen (paraquat, 10 mM):

$$CH_3C^{\cdot}OHCH_3 + MV^{2+} \rightarrow CH_3COCH_3 + MV^{+\cdot} + H^+, \quad (63)$$

$$MV^{+\cdot} + O_2 \rightarrow MV^{2+} + O_2^-. \quad (64)$$

In both superoxide-generating systems the effect of urate is strongly pH-dependent, decreasing as the pH was increased from 6 to 8. No inactivation of YADH is apparent when the enzyme is added to a previously irradiated solution containing urate and excess formate.

Damage to the enzyme is therefore attributed to the radiation-induced formation of a short-lived toxin when O_2^- is generated in the presence of the protein and urate. The acid dissociation constant of urate is $pK_a = 5.5$ and that of the hydroperoxy radical, $HO_2^{\cdot}$, is $pK_a = 4.9$. Thus the damage may be due to $HO_2^{\cdot}$, a urate radical ($UO^{\cdot}$) or subsequent permanent product formed from urate in the presence of the enzyme:

$$UOH \rightarrow UO^- + H^+, \quad (65)$$

$$O_2^- + UOH \rightarrow UO^- + HO_2^{\cdot}, \quad (66)$$

$$HO_2^{\cdot} + UO^- \rightarrow HO_2^- + UO^{\cdot}, \quad (67)$$

$$UO^{\cdot} + YADH \rightarrow \text{inactivation}, \quad (68)$$

$$UO^{\cdot} \rightarrow \text{'toxin'}, \quad (69)$$

$$\text{'toxin'} + YADH \rightarrow \text{inactivation}, \quad (70)$$

$$\text{or} \quad HO_2^{\cdot} + YADH \rightarrow \text{inactivation}. \quad (71)$$

Interestingly, the above damaging action is again prevented by including Trolox C, ascorbate or NADH (100 μM) in the irradiated system. Superoxide dismutase also protects to a considerable extent. Whether these compounds are reacting with O_2^- in the presence of the enzyme or whether they are reacting directly with the urate toxin or with the damaged enzyme, remains to be elucidated.

At the present time, the possibility that a Fenton reaction involving hydrogen peroxide and trace iron contaminants also play some role, cannot be ruled out completely:

$$Fe^{II} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe^{III}. \quad (72)$$

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Hydrogen peroxide does inactivate such enzyme preparations in the absence of radiation. However, the concentrations of hydrogen peroxide and ferrous ion required for an appreciable effect between the end of the radiation and time of activity measurement (less than 30 min) are unlikely to be sufficiently high, unless a long chain reaction involving urate or the enzyme, or both, takes place. Furthermore, in the irradiated systems containing the enzyme and thymine, or the enzyme, formate and urate, the inclusion of ferrous ions (20 μM), like SOD (10 μg ml^{-1}), has a protective rather than a sensitizing effect.

Significance of in vitro studies and free-radical-induced protein damage

The vast wealth of free-radical reaction rate constants now available, and the steady accumulation of oxidation–reduction potentials of many free-radical–molecule couples are providing a very useful framework for the design of *in vitro* model experiments (Patel & Willson 1973; Wardman & Clark 1976; Steenken & Neta 1979, 1982; Butler *et al.* 1982; Anderson 1980; Farrington *et al.* 1980). Some examples of redox potentials are given (table 5). By

TABLE 5. REDOX POTENTIAL OF SOME FREE-RADICAL COUPLES[a]

couple	E/V
$OH^{\cdot}/OH^{-}$	2.00 (1.77)
$N_3^{\cdot}/N_3^{-}$	1.9
$Br_2^{-\cdot}/2Br^{-}$	1.7 (1.45)
$I^{\cdot}/I^{-}$	1.31
$I_2^{-\cdot}/2I^{-}$	1.0
$RS^{\cdot}, H^{+}/RSH$	*ca.* 1.0
$trp^{\cdot}, H^{+}/trpH$	0.98
$promethazine^{+\cdot}$/promethazine	0.896 (0.865)
$C_6H_5O^{\cdot}, H^{+}/C_6H_5OH$	> 0.8
$tyrO^{-\cdot}/H^{+}$, tyrOH	*ca.* 0.65
$p\text{-}CH_3OC_6H_5O^{\cdot}, H^{+}/p\text{-}CH_3OC_6H_5OH$	0.60
catechol $O^{\cdot}, H^{+}$/catechol OH	0.53
Trolox $O^{\cdot}, H^{+}$/Trolox OH	0.48
$ascorbate^{\cdot}$/$ascorbate^{-}$	0.30
$NAD^{\cdot}, H^{+}$/NADH	0.30
cyt(III) *c*/cyt(II) *c*	0.255
benzoquinone/$benzoquinone^{-\cdot}$	0.10
O_2/O_2^{-}	−0.16
methyl $viologen^{2+}$/methyl $viologen^{+\cdot}$	−0.46
$NAD^{+}/NAD^{\cdot}$	−0.93
$CH_3CHO, H^{+}/CH_3C^{\cdot}HOH$	−1.1
$CH_3COCH_3, H^{+}/CH_3C^{\cdot}OHCH_3$	−1.8
$CO_2/CO_2^{-\cdot}$	−2.0

[a] At pH 7.

ranking the couples in this manner it can be readily seen whether particular reactions are thermodynamically favourable: species on the left-hand side of a couple may oxidize those on the right-hand side of couples lower in the series, although kinetic factors must also be taken into consideration. Whether such studies will be relevant or have application *in vivo* remains to be seen. In the context of cell damage, it is clear that lysozyme or YADH are hardly likely to be key proteins. Nonetheless, the dehydrogenase enzyme, in particular, may serve as a model demonstrating what might occur when other more critical proteins are subject to free-radical attack. For example, some polymerase enzymes are known to be sensitive to oxidation and any serious depletion of these when they are required to repair damage to nucleic acid may be seriously detrimental. Various chemotactic factors and

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protease inhibitors are also known to be sensitive to oxidation, and again any damage to these at critical times within the cell cycle may have important repercussions. Clearly when proteins are exposed to free radicals, whether they are formed chemically, biochemically, or by exposure to radiation, the nature and extent of any damage will depend strongly on the local environment of the site of radical generation. Factors such as radical reactant concentrations, the polarity of the local medium and the presence of basic or acidic residues, not to mention simple steric constraints and the facility for intramolecular electron or hydrogen transfer, will all have a bearing on the biological outcome.

We hope that further stationary- and pulse-radiolysis studies will continue to provide information, enabling us to describe more fully the various criteria involved.

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Discussion

H. Sies (*Institut für Physiologische Chemie I, Universität Dusseldorf, F.R.G.*). The reaction of $GS^{\cdot}$ with NADH to form $NAD^{\cdot}$ is quite interesting *in vitro*. I wonder whether this reaction may occur to a significant extent in cells? The NADH concentration is buffered to 10^{-6} M by specialized binding proteins. On the other hand, there is a considerable capacity to reduce GSSG by GSSG reductase. Given that there are several reaction pathways to yield GSSG from $GS^{\cdot}$, I suppose that the latter pathway is more likely to occur in the cell than the reaction with NADH?

R. L. Willson. Surely we just don't know. The experiments I described were indeed undertaken *in vitro*, where we do know other reactions of $GS^{\cdot}$, for example with O_2 or GS^{-}, could occur if the reaction concentrations were favourable. But I am not at all sure whether we really know what the concentrations of the various reactants are at any given time in the cell, either overall

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or, more importantly, in particular intracellular micro-environments. All we can say at present is that such reactions can occur *in vitro* according to homogeneous kinetics. What occurs *in vivo* seems very much an open question.

G. Scott (*Department of Molecular Sciences, Aston University*). In several of Professor Willson's reaction schemes he had an alkyl radical reacting with a hydrogen donor. For a thiol I can accept this, but the reaction is known to be very slow for phenolic antioxidants. Would he indicate what was the partial oxygen pressure in the system because this determines the relative importance of hydrogen abstraction by alkylperoxyl as opposed to alkyl.

R. L. Willson. The reactions I have described were generally undertaken under deoxygenated conditions. As Professor Scott correctly infers, the reaction of carbon-centred radicals with hydrogen donors will occur in competition with the reaction of the radicals with oxygen. Such reactions will therefore only predominate at relatively high hydrogen-donor to oxygen concentrations. Nevertheless such reactions may occur within the cell: indeed this has long been accepted as one of the mechanisms by which oxygen sensitizes cells to radiation damage and by which thiol compounds often afford protection. With respect to phenolic antioxidants, we have not yet observed any reaction with carbon-centred radicals by pulse radiolysis. This is in agreement with Professor Scott's statement that such reactions are known to be very slow.

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