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REVIEW ARTICLE

Free-radical mechanisms in tissue injury

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Introduction

There are very many ways to injure and kill cells: amongst these is an important class of reactions dependent on the production of free radical intermediates to trigger an expanding network of multifarious disturbances (see Slater, 1972, 1979*a*). If we are to adopt rational approaches to the recognition and control of such disturbances, to develop effective protection against the types of injury involved and, where necessary, to apply specific therapies, it is essential to understand more of the basic biochemical mechanisms that are involved in such free radical-mediated injuries to cells. In this Review I will discuss a number of types of cell injury that are dependent on free radical intermediates; firstly, however, some background information on free radical reactions will be given.

What are free radicals and how are they formed in cells?

Free radicals can be defined as molecules or molecular fragments with an unpaired electron; this Review will exclude discussion of atoms and transitional metal ions that would otherwise qualify under the broad definition given. A good review on the roles of transitional metal ions in the formation and reactions of free radicals, especially in relation to the superoxide anion radical $O_2^{-\bullet}$, is by Halliwell & Gutteridge (1984) in this Journal. The present Review has been written to complement the article by Halliwell & Gutteridge (1984), to widen the range of discussion of cell injuries that are mediated by free radical intermediates, and to introduce a number of general concepts relevant to free radical processes and to mechanisms of protection against such types of injury.

The unpaired electron gives certain characteristic properties to the free radical, such as paramagnetism; the chemical reactivity of free radicals is usually high. The presence of the unpaired electron in the free radical is conventionally represented by a superscript bold dot: $R^{\bullet}$. Free radicals can be positively charged, negatively charged or electrically neutral as shown:

$$\text{Promethazine (Pr)} + OH^{\bullet} \rightarrow Pr^{+\bullet} + OH^{-} \quad (1)$$

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

$$CCl_4 + e^{-} \rightarrow CCl_4^{-\bullet} \rightarrow CCl_3^{\bullet} + Cl^{-} \quad (3)$$

In (1) the phenothiazine drug promethazine is oxidized by the hydroxyl radical to the promethazine radical cation; in (2), oxygen is reduced to the superoxide radical anion; in (3), CCl_4 undergoes dissociative electron capture to form the trichloromethyl radical.

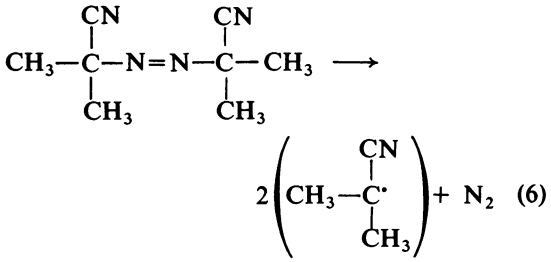
Free radicals can be formed by processes of homolytic bond fission (4) or by electron-transfer reactions (5):

$$A:B \rightarrow A^{\bullet} + B^{\bullet} \quad (4)$$

$$A^{-}:+B \rightarrow A^{\bullet} + B^{-\bullet} \quad (5)$$

In general, such processes proceed either (i) through the absorption of radiation: ionizing, u.v., visible or thermal, or (ii) by redox reactions such as non-enzymic electron transfer reactions, metal-catalysed reactions or enzyme-catalysed processes. The impact of ionizing radiation on biological material can produce a complex variety of free radical products; ionizing radiation produces mainly $H^{\bullet}$, $OH^{\bullet}$ and $e_{aq.}^{-}$ when directed onto aqueous solutions, and these primary free radicals and the hydrated electron can interact readily with neighbouring biomolecules. If non-ionizing radiation has sufficient intrinsic energy to produce homolysis of covalent bonds (the C–C bond requires approx. $350\,kJ\cdot mol^{-1}$ for dissociation, equivalent to u.v. radiation of wavelength approx. 300nm) then free radicals can be formed. Even radiation of relatively low intrinsic energy can produce free radicals if an appropriate photosensitizer is present. In such cases the photosensitizer absorbs light of appropriate wavelengths and is thereby converted first to an excited singlet and this may pass to an excited triplet state; then in the presence of oxygen, itself a triplet in the ground state, energy transfer occurs and singlet oxygen is produced (for review see Foote, 1976). Singlet oxygen is not a free radical, but can interact with

substances such as polyunsaturated fatty acids to initiate lipid peroxidation (see Krinsky, 1979), a free radical chain process. This type of photosensitization reaction is important in the skin damage that occurs in some abnormalities of porphyrin metabolism (Magnus, 1976; see also the section on 'Injuries due to incident radiation' below). Thermal energy can also produce free radicals. In general high temperatures are required to break covalent bonds, but some bonds are relatively unstable and break homolytically at temperatures of 30–50°C; very often these compounds are used as initiators of free radical reactions: an example is azobisisobutyronitrile:



(6)

Organic materials exposed to high temperatures, as in garden bonfires or the burning tips of cigarettes, produce complex mixtures of free radicals; the free radical content of cigarette smoke is very high (Pryor *et al.*, 1983).

As already mentioned, free radicals can be formed by redox reactions that include non-enzymic electron transfers such as:

$$\text{Reduced quinone} + O_2 \rightarrow \text{semiquinone}^{\bullet} + O_2^{-\bullet} + H^+ \quad (7)$$

The importance of the enzyme DT-diaphorase (EC 1.6.99.2) (Ernster, 1967) in protecting cells against this pathway of $O_2^{-\bullet}$-mediated injury has been emphasized by Ernster *et al.* (1982) and Lind *et al.* (1982). Many other redox reactions that produce free radical intermediates require either a transitional metal for catalysis (see Kochi, 1973; Sheldon & Kochi, 1981; Wong *et al.*, 1981; Aust & Svingen, 1982) or an enzyme, usually a metalloprotein or flavoprotein (see Yamazaki, 1977; Walsh, 1978; Hemmerich & Wessiak, 1979). Some normal metabolic processes involve radical intermediates (for example, ribonucleotide reductase; Reichard & Ehrenberg, 1983); such 'normal' radicals are often transient intermediates of regulated enzymic reactions, and may not occur as free (i.e. diffusable) radicals but are 'frozen' at the reaction site and then pass under controlled conditions to the appropriate product.

Chemical properties of free radicals

In brief, the typical reactions of free radicals are (*a*) electron donation (from a reducing radical) and electron acceptance (for an oxidizing radical; (*b*) hydrogen abstraction; (*c*) addition reactions; (*d*) self-annihilation reactions; and (*e*) disproportionations. Examples are:

(*a*) $$CO_2^{-\bullet} + O_2 \rightarrow CO_2 + O_2^{-\bullet} \quad (8)$$

(*a*) $$OH^{\bullet} + RS^- \rightarrow OH^- + RS^{\bullet} \quad (9)$$

(*b*) $$CCl_3^{\bullet} + RH \rightarrow CHCl_3 + R^{\bullet} \quad (10)$$

(*c*) $$CCl_3^{\bullet} + CH_2{=}CH_2 \rightarrow CH_2(CCl_3){-}\dot{C}H_2 \quad (11)$$

(*d*) $$CCl_3^{\bullet} + CCl_3^{\bullet} \rightarrow C_2Cl_6 \quad (12)$$

(*e*) $$CH_3CH_2^{\bullet} + CH_3CH_2^{\bullet} \rightarrow CH_2{=}CH_2 + CH_3{-}CH_3 \quad (13)$$

General accounts on free radical reactions are by Pryor (1966) and Nonhebel *et al.* (1979).

Detection of free radicals

A direct procedure for the study of free radical intermediates in biological systems is e.s.r. spectroscopy; good introductory reviews are by Borg (1976) and Knowles *et al.* (1976). The technique is not very sensitive, however, (in biological samples the concentration required is usually in the range 10^{-5}–10^{-7}M; see Knowles *et al.*, 1976) and if the rate of production of free radical intermediate is low, and the lifetime short due to its high chemical reactivity, then the concentration of the free radical may be far too small for direct detection by e.s.r. In such situations it may be possible to detect and characterize the transient intermediate by spin trapping (see Janzen, 1971, 1980). In this development of e.s.r. spectroscopy the free radical under study interacts with a non-radical trap forming thereby a relatively stable free radical adduct. In most cases the adducts formed have been nitroxy radicals that have characteristic e.s.r. spectra; Fig. 1 illustrates a reaction that is referred to later, the trapping of the trichloromethyl radical by phenyl-*t*-butylnitrone. Spin trapping permits the progressive accumulation of the adduct, and may be experimentally successful even when the production rate of the primary free radical species is very low.

The application of direct e.s.r. spectroscopy, of e.s.r. spin trapping technique and the use of less-direct methods (see below) have demonstrated that free radical intermediates are produced *in vivo* under normal as well as abnormal conditions.

Other methods that can be used on occasion for detection and identification of free radical intermediates include absorption spectroscopy, provided that the molar absorption coefficient is sufficiently high, and product analysis including covalent binding; with the latter technique it is only possible to infer that a particular free radical

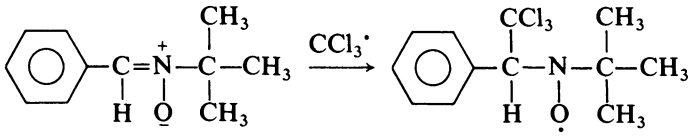


Fig. 1. *Reaction of the trichloromethyl free radical with the spin trap phenylbutylnitrone*
For details of the application and interpretation of this reaction see McCay *et al.* (1980) and Albano *et al.* (1982).

species has been produced by analysis of the reaction products.

Techniques that allow the study of free-radical kinetics include e.s.r. spectroscopy, flash photolysis and pulse radiolysis (see Bensasson *et al.*, 1983).

Damaging reactions produced by free radicals

Extensive studies with model systems, and with biological materials *in vitro*, have clearly shown that reactive free radicals are able to produce chemical modifications of, and damage to, proteins, lipids, carbohydrates and nucleotides. Therefore, if such reactive free radicals are produced *in vivo*, or in cells *in vitro*, in amounts sufficient to overcome the normally efficient protective mechanisms, we can expect metabolic and cellular disturbances to occur in various major ways as summarized in Fig. 2.

If the reactive free radical is formed close to DNA (see the section on 'Diffusion and biological effects of free radicals' below), then it may produce a change in the structure resulting in a mutation or cytotoxicity. Oxidizing free radicals can alter the redox state of the $NAD^+/NADH$ and $NADP^+/NADPH$ couples and may produce $NAD(P)^\bullet$ that can dimerize (Land & Swallow, 1968). Free radicals can also add to nucleotides to produce significant changes in their biological properties. Protein and non-protein thiol groups are readily oxidized by many free radicals and the thiyl radicals produced may dimerize:

$$YSH + X^\bullet \rightarrow YS^\bullet + XH \quad (14)$$

$$R_1S^\bullet + R_2S^\bullet \rightarrow R_1SSR_2 \quad (15)$$

Such types of free-radical-mediated disturbances of thiol groups may lead to profound changes in enzyme activity.

Another major pathway of metabolic disturbance frequently occurring when free radicals are produced is that dependent on covalent binding. In this case the reactive free radical intermediate interacts with cell components such as protein, lipid and nucleic acid, to form a stable covalently-bound adduct that may grossly distort structure and function.

Reactive free radicals may also damage cells through a pathway dependent essentially on membrane damage: (*a*) by covalent binding of the free radical to membrane enzymes and/or receptors, thereby modifying the activities of membrane components; (*b*) by covalent binding to membrane components, thereby changing structure and producing effects on membrane function and/or antigenic character; (*c*) by disturbance of transport processes through covalent binding, thiol-group oxidation, or change in polyunsaturated fatty acid: protein ratios; and (*d*) by initiation of lipid peroxidation of polyunsaturated fatty acids with direct effects on membrane structure, and associated influences of the products of peroxidation on membrane fluidity, cross-linking, structure and function.

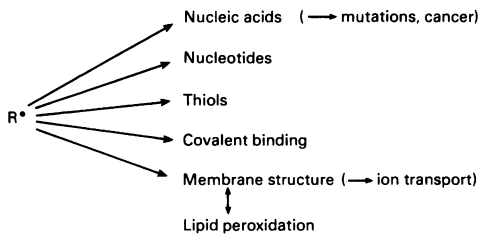


Fig. 2. *Free radicals and cellular injury*
Major routes are shown in which a reactive free radical (R•) can interact with neighbouring components in cells to disturb their metabolic function(s) are indicated by the arrows.

If reactive free radicals formed inside cells can produce such a wide diversity of severely damaging reactions, it might well be asked 'how do we survive at all?' The answer is that we have normally very efficient and widely distributed protective mechanisms of various kinds (see below), and it is only when these are overcome that damage results.

Biological specificity of free radical reactions

It can be concluded from the discussion so far that free radicals can cause metabolic disturbances and cell injury in a variety of ways: this appears to be firmly and unequivocally established. It might be thought, on first considerations, that the chemical reactivity of a free radical species will be directly related to its biologically damaging effects for a given rate of production of the free radical; this, however, does not appear generally valid as it neglects the important aspect of specificity in the

interactions of a free radical with biologically important materials. A highly reactive free radical, $OH^{\bullet}$ for example, will react with everything in its immediate environment; only some of these interactions may result in specific and biologically significant disturbances such as changes in enzyme activity and the proportion of interactions of the free radical with essential targets will be diluted by virtue of the indiscriminately high reactivity of the free radical. Another free radical species, at the same initial concentration, however, may react less indiscriminately with non-essential groups (in the biological sense) but as efficiently as the more reactive free radical with essential targets; in such a situation the free radical with lower chemical reactivity will have more profound biological consequences with respect to the particular biological property under study. An example of this type of behaviour can be found illustrated in the data reported by Hiller *et al.* (1983). The survival of bacteriophage T_2 was studied after exposure to $OH^{\bullet}$ and the chemically less reactive $CCl_3O_2^{\bullet}$ free radical; the less reactive species ($CCl_3O_2^{\bullet}$) was biologically more efficient in destroying phage.

The above example concerns two free radical species that are chemically very reactive, and where the difference in biological effects probably depends largely on a 'dilution' of $OH^{\bullet}$ with respect to essential target interactions. Such highly reactive free radicals cannot diffuse very far from their locus of formation (see the following section) so that questions of penetration and of chemical specificity are not critical. With free radicals of intermediate chemical reactivity, however, the penetration of the free radical to an essential biological target may become of significance and this is clearly dependent on molecular features additional to chemical reactivity: steric factors including size, lipophilicity, electrical charge, etc.

Diffusion and biological effects of free radicals

Highly reactive free radicals produced in biological systems are essentially confined to their immediate vicinity as a direct consequence of their extreme chemical reactivity; in consequence, their average radius of diffusion will be very small. It has been calculated that a free radical such as $CCl_3^{\bullet}$ or $OH^{\bullet}$ will have a half-life in biological systems of only a few microseconds (Slater, 1976, 1979*a*; Borg & Schaich, 1984); in consequence the diffusion of such a species from its locus of production will be, on average, less than 100nm (Slater, 1976).

If the reactive free radical is produced by metabolic activation in the endoplasmic reticulum then it is most unlikely that significant direct effects can be exerted by that free radical species on components present in other intracellular compartments such as DNA in the nucleus. Free radicals of much lower reactivity may be successful in diffusing such relatively long distances and still have enough chemical reactivity to produce a biologically significant event. Free radicals of very low reactivity, while able to diffuse further, may have such low chemical reactivity that they are unable to produce significant cellular disturbances. These considerations suggest that free radical intermediates of chemical carcinogens, produced by metabolic activation in the endoplasmic reticulum, will have moderate but not extreme chemical reactivity if the free radical is the active carcinogenic species (Slater, 1976).

Lipid peroxidation

The peroxidation of polyunsaturated fatty acids, especially $C_{20:4}$ and $C_{22:6}$, is a feature of many types of cell injury in which free radical intermediates are produced in excess of local defence mechanisms. Lipid peroxidation is a free radical-mediated process; the polyunsaturated fatty acid substrates do not have to be in the free carboxylate form but can also undergo peroxidation while esterified as in phospholipids. Lipid peroxidation is quite different, thereby, from the reaction catalysed by cyclo-oxygenase that requires free fatty acid in order to initiate the prostaglandin cascade via a peroxidation event (see Porter, 1980; Flower, 1981; Pace-Asciak & Granström, 1983).

In lipid peroxidation a primary reactive free radical $R^{\bullet}$ interacts with a polyunsaturated fatty acid (PUFA) to initiate a complex series of reactions (16–19) that result in a variety of degradation products:

Reactions (16) and (17) can be followed by

$$R^{\bullet} + PUFA \rightarrow RH + PUFA^{\bullet} \quad (16)$$

$$PUFA^{\bullet} + O_2 \rightarrow PUFAO_2^{\bullet} \quad (17)$$

$$PUFAO_2^{\bullet} + PUFAH \text{ (or XH)} \rightarrow PUFAO_2H + PUFA^{\bullet} \text{ (or X}^{\bullet}) \quad (18)$$

$$PUFAO_2^{\bullet},\ PUFAO_2H,\ \text{metal ions} \rightarrow \begin{matrix}\text{alkanes}\\ \text{alkanals}\\ \text{alkenals}\\ \text{4-hydroxyalkenals}\end{matrix} \quad (19)$$

measuring the loss of fatty acid substrate or the uptake of O_2 respectively; the formation of the PUFA$^{\bullet}$ free radical is accompanied by bond rearrangement that results in a diene-bond character; reaction (18) can be measured by determining the formation of lipid hydroperoxide; reaction (19), which is a group of complex degradative reactions involving metal catalysis, results not only in products such as ethane, pentane and malonaldehyde, but also fluorescent materials and chemiluminescence (for an overview of methods used in such estimations see Slater, 1984).

A simplified description of the peroxidation of a polyunsaturated fatty acid such as arachidonic acid by a reactive free radical R$^{\bullet}$ has been given above and has illustrated the variety of components and products that have been used as measures of lipid peroxidation (e.g. alkanes, malondialdehyde, lipid hydroperoxides, diene conjugates). Since some of these components and products may be metabolized rather rapidly (for instance, lipid hydroperoxides to hydroxy fatty acids) it is important to check experiments that are found negative in respect of the production of such substances by following the disappearance of polyunsaturated fatty acids, and by one or more other experimental procedures of measurement. The relatively unspecific diene conjugation method has been recently improved by a modification based on the second differential derivative of the absorption spectrum (Corongiu & Milia, 1983). New methods for the estimation of lipid hydroperoxide are also available using h.p.l.c. (see Packer, 1984). Although the thiobarbituric acid reaction for malonaldehyde is well known to give positive reactions with many substances that may occur in biological extracts, or in incubation mixtures, it has been shown by direct h.p.l.c. to be a good measure of malonaldehyde in rat liver microsomes peroxidized by ADP/Fe^{2+} (Esterbauer & Slater, 1981). The advantages and disadvantages of the methods used to estimate lipid peroxidation have been discussed by Slater (1984); detailed chapters on individual methods can be found in the appropriate volume of *Methods in Enzymology* (Packer, 1984).

Lipid peroxidation in the sense used till now in this review is a general phrase covering the processes in which a polyunsaturated fatty acid radical reacts with oxygen to yield a fatty acid peroxy radical that then undergoes a complex variety of reactions. The process may be nonenzymic or enzymically catalysed: where the process is nonenzymic, or involves an ill-defined enzymic route, or an uncontrolled and deleterious enzymic diversion, it will be referred to as a lipid peroxidation reaction; where the process involves a characterized enzyme that gives a defined set of products it is usual to refer to the activity as a lipoxygenase type of activity. The products of lipoxygenases have been much studied in recent years, especially in relation to the prostaglandin field; the first products with arachidonic acid as substrate are hydroperoxyeicosatetraenoic acids that are usually accompanied by the corresponding hydroxy derivatives, hydroxyeicosatetraenoic acids; hydroperoxyeicosatetraenoic acids can also be converted to leukotrienes with pronounced pharmacological activity (Samuelsson & Paoletti, 1982; Hammarstrom, 1983). Reviews on lipoxygenases that provide background details and references are by Vliegenthart & Veldink (1982) and by Taylor & Morris (1983).

The range of products resulting from lipid peroxidation has already been briefly referred to; recent studies have demonstrated the variety of compounds that occur in some of the classes of product already noted. For example, Capdevila *et al.* (1982) have separated a number of hydroxy derivatives of arachidonic acid produced by a peroxidative reaction involving cytochrome *P*-450 in rat liver microsomes. Esterbauer *et al.* (1982) have isolated and identified a considerable number of aldehydes, including unsaturated and 4-hydroxy unsaturated aldehydes, from rat liver microsomes peroxidised by the addition of ADP/Fe^{2+}; results obtained with different types of peroxidation are reviewed by Esterbauer (1982). The products of lipid peroxidation can produce a wide range of biological effects that will be illustrated here by reference to the 4-hydroxyalkenals; background information on other products such as aldehydes and lipid hydroperoxides can be found in reviews by Schauenstein *et al.* (1977) and by Yagi (1982).

Initial studies on the biological reactivity of 4-hydroxyalkenals concentrated on 4-hydroxy-pent-2-en-1-al; this was shown to react rapidly with thiol groups and to inhibit a number of thiol dependent enzymes (see Schauenstein *et al.*, 1977). Hydroxypentenal was also found to decrease DNA synthesis in a variety of experimental systems and to have an antitumour effect on certain murine tumours (see Schauenstein *et al.*, 1969; Conroy *et al.*, 1975). Following the separation and identification of a wide range of other 4-hydroxyalkenals in peroxidizing microsomal lipid extracts (Esterbauer *et al.*, 1982) attention has concentrated on longer chain analogues such as 4-hydroxynonenal, which are biologically much more reactive (Benedetti *et al.*, 1980). Hydroxynonenal causes substantial inhibition of membrane enzymes such as glucose-6-phosphatase (Ferrali *et al.*, 1980) and adenylate cyclase (Dianzani, 1982) at very low (μM) concentrations; it affects chemotaxis when added to nM concentrations (Curzio *et al.*, 1982). Lipid peroxidation of polyunsaturated fatty acids in mem-

branes such as endoplasmic reticulum can thus be seen to be capable of producing a variety of products that have powerful biological activities.

The above discussion leads to an important concept: the production of a highly reactive free radical in a biomembrane leads to primary reactions and damage in the immediate surroundings of where the free radical is produced; it is trapped in its microenvironment by virtue of its high chemical reactivity. However, if one of its primary local interactions is to initiate lipid peroxidation then secondary products such as lipid hydroperoxy radicals and lipid hydroperoxides may diffuse in the plane of the membrane before reacting further, thereby spreading the biochemical lesion. These secondary products can undergo degradation reactions leading to the complex variety of products already referred to above. Some of these, like the lower M_r hydroperoxides, aldehydes and 4-hydroxyalkenals, can escape from the membrane and produce disturbances at a distance (Slater, 1976). A reaction that originally produces a spatially constrained reactive free radical may thereby produce a wave of secondary and later events that spread disturbances throughout the cell, even into the extracellular domain.

Lipid peroxidation can produce a range of enzymically damaging consequences, such as those dependent on thiol oxidation or thiol adduct formation as already mentioned. In addition, extensive lipid peroxidation can result in membrane disorganization by peroxidizing mainly the highly unsaturated polyunsaturated fatty acids and thereby changing the composition of the polyunsaturated fatty acid and phospholipid fractions, leading to changes in the ratio of polyunsaturated to other fatty acids. Such changes, and lipid peroxidation itself, are associated with decreases in membrane fluidity (Dobretsov *et al.*, 1977; Slater, 1979*a*; Eichenberger *et al.*, 1982; Bruch & Thayer, 1983), and by crosslinking reactions involving bifunctional aldehydes (Chio & Tappel, 1969*a*,*b*; Eichenberger *et al.*, 1982). The uncontrolled peroxidation of biomembranes can thus lead to profound effects on membrane structure and function, and may be sufficient to cause cell death. Background reviews on lipid peroxidation in relation to cell injury are by: Slater (1972, 1982*a*), Dianzani & Ugazio, (1978), Bus & Gibson (1979), Simic & Karel (1980) and Smith *et al.* (1983).

The above remarks have summarized the various ways in which lipid peroxidation can exert disparate damaging actions on cells and cell components. While it is relatively easy to follow the time course of lipid peroxidation under conditions *in vitro*, and to dissect out chemically those features that are primary causes of the ensuing cellular injury, it has proved much more difficult to accomplish the same results *in vivo* in other than a few instances. Lipid peroxidation can certainly be demonstrated to occur *in vivo* following a number of experimentally produced metabolic perturbations (and also in some clinically occurring diseases); for example, by the use of measurements on pentane exhalation, or by the determinations of lipid hydroperoxides, malondialdehyde, or diene conjugation absorption. However, it is not generally possible in such cases *in vivo* to be sure whether the lipid peroxidation that is detectable is one of the primary causes of injury, or is a sign that injury has already occurred and where the peroxidation is a *consequence* of major metabolic imbalance. While the unequivocal settlement of this problem is clearly of importance in understanding the primary mechanisms of cell injury, it is probably not unreasonable to say that if lipid peroxidation occurs at all to a significant extent, whether it is a primary factor or a secondary consequence, it will contribute to the overall complex network of metabolic disturbances that characterize the injurious process as a whole.

Metabolic activation and cell injury

Many examples are known where a substance is converted enzymically to a more toxic material; this process is often referred to as metabolic activation, and in some cases the product has been demonstrated to be a free radical which may then initiate damaging reactions as outlined above.

Examples of substances that have been shown to be metabolically activated to reactive free radical intermediates are halogenoalkanes such as CCl_4, $CHCl_3$, $CBrCl_3$, and halothane; nitro compounds, aromatic amines and nitrosamines; quinones; and polycyclic hydrocarbons. It is important to stress that with some of the examples just mentioned the free radical intermediate may be only one of several reactive intermediates produced from the parent material, and may not be the most important in relation to the ensuing injury. For instance, although free radical intermediates have been reported following metabolic activation of polycyclic hydrocarbons, there are extensive data available on the pronounced biological activity of non-radical intermediates such as expoxides and epoxydihydrodiols (see Sims *et al.*, 1974; Sims, 1980; Conney *et al.*, 1980; Jerina *et al.*, 1980).

Many substances are metabolically activated to a free radical intermediate through interactions with the NADPH–cytochrome *P*-450 electron transport chain, which is located in the endoplasmic reticulum in many types of cell and involves the flavoprotein NADPH–*P*-450 reductase and cytochrome *P*-450 together in a phospholipid environ-

ment. Although *P*-450 is mainly located in liver, small amounts of *P*-450 can be detected in many other tissues (see Orrenius & Ernster, 1974; Benedetto *et al.*, 1981*a*). The NADPH reductase flavoprotein, measured by the reduction of cytochrome *c* or ferricyanide, is also widely distributed and in significant amounts (Benedetto *et al.*, 1981*a*).

Metabolic activation by the NADPH–*P*-450 chain can involve electron donation directly from the flavoprotein, as with some nitrocompounds or quinones, or by interaction with the *P*-450 catalytic site. An important feature of metabolic activations that involve an initial electron transfer to the toxic agent (T) is the possibility of establishing redox cycles, which can result in the generation of relatively large concentrations of superoxide anion radicals (see Doroshow & Hochstein, 1982; Trush *et al.*, 1982):

$$\text{NADPH} \xrightarrow{e} \text{Flavoprotein} \xrightarrow{T} T^{-\cdot} \xrightarrow{O_2} T + O_2^{-\cdot} \qquad (20)$$

Another aspect of metabolic activations is that often the free radical species produced (or secondary products resulting from lipid peroxidation, for example) can decrease the enzymic ability of *P*-450 to a greater extent than occurs with the NADPH reductase flavoprotein. An illustration of this 'suicidal' metabolism resulting in *P*-450 damage is the metabolic activation of CCl_4 (Ugazio *et al.*, 1973).

Hepatotoxicity of CCl_4

The toxic effects of CCl_4 on the liver have been studied extensively by a large number of investigators, over many years (see Recknagel, 1967; Slater, 1972); a number of major concepts have emerged from such studies that have application to our understanding of the biochemical mechanisms of cell injury in general.

The main reasons why so much attention has been given to the pathology, toxicology and biochemistry of CCl_4-induced liver injury are: CCl_4 is readily available in pure form; CCl_4 consistently produces liver injury in many species including mouse, rat, rabbit and man; the type and severity of the liver injury produced can vary from triacylglycerol accumulation, through necrosis to cirrhosis and cancer depending on the dose and method of application; the acute lesion (fatty degeneration and necrosis) can be modified by various pretreatments that affect the activity of the NADPH–cytochrome *P*-450 chain, and by a variety of protective agents that include many free radical scavengers; CCl_4 is of considerable industrial and environmental importance, and is a natural product (Lovelock *et al.*, 1973; McConnell *et al.*, 1975; Bir Singh *et al.*, 1976).

A single dose of CCl_4 administered to a rat produces centrilobular necrosis and fatty degeneration of the liver (Cameron & Karunaratne, 1936). In the early 1960's experiments were reported that demonstrated a metabolism of CCl_4 to $CHCl_3$ and, to a lesser extent, CO_2 (Butler, 1961; Paul & Rubenstein, 1963); in addition, covalent binding of $^{14}CCl_4$ to liver protein and lipid was demonstrated (Reynolds, 1963). A general hypothesis was proposed in 1966 (Slater, 1966*a*) that CCl_4 has to undergo metabolic activation to a free radical intermediate, $CCl_3^{\cdot}$, in order to exert its full necrogenic potential; a similar view was put forward independently by Recknagel (see Ghoshal & Recknagel, 1965; Recknagel & Ghoshal, 1966).

An important study by McLean & McLean (1966) showed that the hepatotoxic action of CCl_4 was increased by prior induction of the drug-metabolizing enzyme system, while Gordis (1969) showed that the covalent binding was consistent with the addition of a CCl_3 group. Among the metabolic products detected during CCl_4 intoxication are hexachloroethane (Fowler, 1969) and phosgene (Shah *et al.*, 1979). Another key event that was recorded in the mid-1960's is the stimulatory action of CCl_4 on lipid peroxidation in the liver (see below).

Although there was much indirect evidence by 1970 for the metabolism of CCl_4 to a free radical intermediate, evidence such as the conversion to $CHCl_3$ and C_2Cl_6, the covalent binding results, and the stimulation of lipid peroxidation, the unequivocal demonstration of the production of $CCl_3^{\cdot}$ took much longer to achieve. Direct examination of liver or liver microsomes after exposure to CCl_4 using e.s.r. spectroscopy (see Ingall *et al.*, 1978, for references) was unsuccessful in establishing the formation of $CCl_3^{\cdot}$. Moreover, first attempts to trap the proposed intermediate free radical $CCl_3^{\cdot}$ using the spin trap *t*-butyl-*N*-oxide were also unsuccessful (Ingall *et al.*, 1978), although an intermediate radical was produced and trapped when CCl_4 was added to liver microsomes using that procedure; it was suggested that the radical trapped was a lipid-derived free radical. More recently, however, using the spin trap phenylbutylnitrone and $^{13}CCl_4$ unequivocal evidence has been obtained for the metabolic activa-

tion of CCl_4 to $CCl_3^{\bullet}$ by liver microsomes, by isolated hepatocytes and in the rat *in vivo* (Poyer *et al.*, 1980; Albano *et al.*, 1982).

Following the early suggestions that CCl_4 was metabolically activated to $CCl_3^{\bullet}$ (Ghoshal & Recknagel, 1965; Slater, 1966*a*) it was generally assumed that subsequent damaging effects on the liver resulted from the reactivity of the primary free radical intermediate $CCl_3^{\bullet}$. No direct information concerning the reactivity of $CCl_3^{\bullet}$ under conditions similar to those that occur in liver endoplasmic reticulum was available, however. A major advance occurred in this respect when the kinetic behaviour of $CCl_3^{\bullet}$ was investigated in solution in the presence of various biologically important substances such as amino acids, nucleotides and fatty acids (Packer *et al.*, 1978, 1980, 1981). It was found that $CCl_3^{\bullet}$ was *relatively* unreactive with such substances, in that if reaction did occur it was too slow for observation with the pulse radiolysis technique being used; this put the rate constants of such reactions at less than $10^5 M^{-1} \cdot s^{-1}$. When the same reactions were studied in the presence of O_2, however, large increases in reaction rates were observed. The results are consistent with the formation of a highly reactive trichloromethyl peroxy radical, $CCl_3O_2^{\bullet}$, (Slater, 1982*b*,*c*; Symons *et al.*, 1982; Mönig *et al.*, 1983) which reacts rapidly with substances such as promethazine, ascorbate, β-carotene, vitamin E, and polyunsaturated fatty acids (Packer *et al.*, 1981; Slater, 1982*c*; Forni *et al.*, 1983).

It has already been mentioned that CCl_4 can stimulate lipid peroxidation in liver samples; this was reported in work with liver homogenates (Comporti *et al.*, 1965) and with post-mitochondrial fractions (Ghoshal & Recknagel, 1965). The stimulating ability of CCl_4 on lipid peroxidation was then demonstrated in liver microsomes provided that a source of NADPH was present (Slater, 1966*b*, 1967), suggesting the involvement of the NADPH–cytochrome *P*-450 system in the overall process. The general features of this effect of CCl_4 on lipid peroxidation, and the fact that other halogenomethanes (CCl_3Br, CCl_3F, CCl_3H) stimulated lipid peroxidation in proportion to the ease of homolytic bond cleavage (Slater & Sawyer, 1971*a*) are consistent with the metabolic activation of such compounds giving free radical products that initiate lipid peroxidation in the endoplasmic reticulum. Since $CCl_3O_2^{\bullet}$ reacts very much faster with polyunsaturated fatty acids than does $CCl_3^{\bullet}$ it appears likely that the major mechanism for the initiation of peroxidation involves $CCl_3O_2^{\bullet}$ rather than $CCl_3^{\bullet}$; the effects of CCl_4 on lipid peroxidation are strongly inhibited by promethazine (Slater & Sawyer, 1971*b*) which also reacts rapidly with $CCl_3O_2^{\bullet}$. Promethazine, however, has little effect on the covalent binding of $^{14}CCl_4$ to microsomal protein (Cheeseman, 1982) and reacts only slowly with $CCl_3^{\bullet}$. Since covalent binding by $CCl_3O_2^{\bullet}$, if it occurs, is unlikely to survive experimental work-up procedures, then it appears possible that covalent binding observed after exposure to CCl_4 results largely from $CCl_3^{\bullet}$, and that the stimulation of lipid peroxidation involves mainly $CCl_3O_2^{\bullet}$ (Fig. 3); other possibilities for covalent binding, such as reactions involving $CCl_3O^{\bullet}$, have also been considered (Slater, 1982*b*).

With regard to the contributions of covalent binding and lipid peroxidation to the overall liver damage produced by CCl_4 *in vivo*, it seems probable that the complex metabolic disturbances involve both phenomena to significant extents as well as other contributions such as changes in body temperature, blood flow and hormonal stimulations. From this viewpoint CCl_4-mediated liver injury exhibits a multicausal origin (Slater, 1982*b*) rather than a dependency on one or other predominant effects.

The remarks above on the metabolic activation of CCl_4 to $CCl_3^{\bullet}$, and the rapid interaction of $CCl_3^{\bullet}$ with O_2 to produce the highly reactive species $CCl_3O_2^{\bullet}$, provide information of relevance to a brief discussion of the mechanisms of attenuation of such free-radical-mediated disturbances. The criteria for successful scavenging of damaging free radicals, produced by metabolic activation in biomembranes, have been given by Slater (1981): in brief, a successful scavenger must not only be

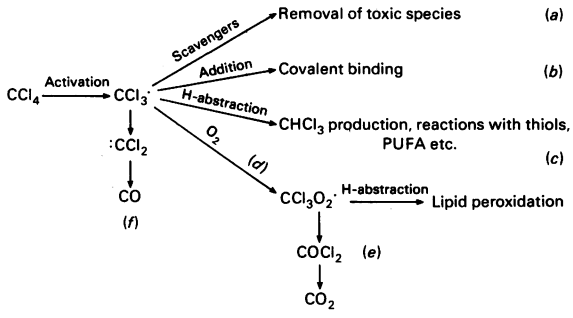


Fig. 3. *Activation and reactions of CCl_4*
The metabolic activation of CCl_4 to the $CCl_3^{\bullet}$ free radical is shown followed by a number of other reactions: (*a*) removal of $CCl_3^{\bullet}$ by reaction with scavengers; (*b*) covalent binding reactions; (*c*) hydrogen-abstraction reactions; (*d*) addition of O_2 to form the much more reactive $CCl_3O_2^{\bullet}$ species that can react with polyunsaturated fatty acids (PUFA) to initiate lipid peroxidation; (*e*) breakdown reactions of $CCl_3O_2^{\bullet}$ to yield CO_2; (*f*) reactions of $CCl_3^{\bullet}$ via a carbene intermediate. The illustration is modified from that in Slater (1982*b*).

able to react with the damaging free radical intermediate at a competitive rate with adjacent biomolecules, but also must be present in the right intracellular site at the right time, and in an effective concentration, in order to inhibit significantly the damaging free radical reactions that would otherwise take place. Moreover, since the interaction of a scavenger (SH) with a free radical intermediate results in the production of a scavenger-derived free radical:

$$R^{\bullet} + SH \rightarrow RH + S^{\bullet} \quad (21)$$

$$R^{\bullet} + S^{-} \rightarrow R^{-} + S^{\bullet} \quad (22)$$

then $S^{\bullet}$ by itself should have a low intrinsic toxicity. It is important to remember in addition that free radical scavengers that are very effective *in vitro*, such as promethazine, which inhibits CCl_4-stimulated lipid peroxidation when added in very small concentrations (Slater & Sawyer, 1971*b*), may have multitudinous effects influencing the overall toxicity when given *in vivo*. Promethazine has synergistic influence with CCl_4 on body temperature (thereby affecting metabolic activation); it has marked effects on respiration rate and expiration of volatile materials like CCl_4; and it has significant influence on the absorption and liver uptake of CCl_4 from the gastrointestinal tract (for references see Reddrop, 1981; Reddrop *et al.*, 1981*a*,*b*). In consequence, before concluding that a free radical scavenger is attenuating a type of cell injury by a free radical scavenging mechanism, it is necessary to study in depth the many other possible influences that such a scavenger may have under conditions *in vivo*.

There is no opportunity, within the confines of this brief Review, to refer in other than a fleeting manner to naturally-occurring protective mechanisms in relation to free-radical-mediated cell injuries. These mechanisms include endogenous antioxidants such as vitamin E, ubiquinone, ascorbic acid, glutathione, and enzymes including glutathione peroxidase, superoxide dismutase, catalase, and aldehyde dehydrogenases. For a review on mechanisms of protection in general see Slater (1978); for reviews on protection against free-radical-mediated damage see Slater (1972, 1979*b*), Sies & Wendel (1978) and Cohen & Greenwald (1983).

Other examples of cell injury dependent on free radical intermediates

A few examples have been selected for discussion below; more detailed comments and many more examples can be found in the articles listed at the end of this short Review.

Injuries due to incident radiation

Three illustrations will be given in this section: photosensitization, retrolental fibroplasia, and radiosensitization.

Many substances are known that will sensitize cells to the effects of visible radiation (see Blum, 1964; Magnus, 1976); in the whole animal, such effects are usually maximal on the skin. In certain disturbances of porphyrin metabolism there are accumulations of porphyrins in the body, including the skin, and exposure to light of appropriate wavelength (generally about 400 nm) may produce serious damage to the exposed areas of skin. A similar outcome results from disturbances of biliary secretion in herbivores where the normal pathway of excretion of the chlorophyll degradation product phylloerythrin is blocked. As a consequence, phylloerythrin, a porphyrin derivative, accumulates and produces photosensitization; a relevant example is the hepatotoxic effects of the fungal toxin sporidesmin (Slater *et al.*, 1964). The exposure of epidermal cells to visible light in the presence of low concentrations of porphyrins (P) results in rapid damage to lysosomal membranes (Slater & Riley, 1966); the damage has the features of a free-radical-mediated lipid peroxidation, probably through the intermediate formation of singlet oxygen that is known to be capable of initiating peroxidative reactions (Krinsky, 1979; Thomas & Pryor, 1980).

$$^1P \xrightarrow{h\nu} {}^1P^* \longrightarrow {}^3P^* \quad (23)$$

$$^3P^* + {}^3O_2 \longrightarrow {}^1P + {}^1O_2 \quad (24)$$

$$^1O_2 + PUFA \longrightarrow \text{lipid peroxidation} \quad (25)$$

Although this sequence of events is generally associated with unwelcome damage and side-effects, there is some attention being paid to the possibility of using the overall sequence in photodynamic therapy of tumour cells accessible to light; background references to these developments can be obtained from Kessel & Dougherty (1983).

Retrolental fibroplasia is a serious ocular disturbance that is associated with the exposure of neonates, especially pre-term infants, to elevated concentrations of oxygen. There is increasing evidence that the disturbance involves free radical processes, and is at least partially protected against by vitamin E pretreatment (Finer *et al.*, 1983; Hittner & Kretzer, 1983). It has also been speculated that the effects of high O_2 may be aggravated by the exposure of the neonates to visible light, thus stimulating peroxidative reactions during the periods of O_2 therapy for respiratory distress (Riley & Slater, 1969; Slater & Riley, 1970). Interesting reports concerning an increased lipid peroxidation during experimentally

produced retinal degeneration are by Magomedov *et al.* (1983), and by Anderson *et al.* (1984).

Some types of cancer are not very responsive to radiotherapy, and this may be a result of locally low concentrations of O_2 influencing the deleterious reactions that would otherwise follow the initial impact of high-energy radiation. Radiosensitizers have been used to try to improve radiotherapeutic treatment in such cases. One class of radiosensitizer is based on derivatives of nitroimidazoles (RNO_2) such as metronidazole or flagyl (Foster & Willson, 1973). When exposed to high-energy electron radiation the nitro compound is reduced to the anion radical:

$$RNO_2 + e^- \rightarrow RNO_2^{-\bullet} \quad (26)$$

which is toxic, and can produce cell damage when the O_2 concentration is very low. When O_2 is present, however, the following reaction occurs:

$$RNO_2^{-\bullet} + O_2 \rightarrow RNO_2 + O_2^{-\bullet} \quad (27)$$

which is then followed by removal of the superoxide anion radical by superoxide dismutase and catalase. A discussion of these types of reactions is by Willson (1978) and Biaglow *et al.* (1982).

Transitional-metal overload

Under normal conditions the absorption and effects of transitional metal ions that enter our bodies in our diet are carefully regulated (see Bothwell *et al.*, 1979). Under abnormal conditions, however, large amounts of such metal ions enter cells of various tissues and may then cause increased lipid peroxidation and cell injury or death. This is particularly true when either the body's normal defences against such redox events is diminished, or when the metal ion penetrates in a redox-active form to a particularly sensitive region of the cell; examples of each will be given.

Piglets born from vitamin-E-deficient sows are very sensitive to injections of an iron preparation, and some develop serious paralysis of the limbs (Patterson *et al.*, 1969). Under these conditions the administration of the iron preparation initiated a lipid peroxidative damage to muscle. Other references to the effects of iron overload, and iron toxicity in association with deficiency of vitamin E, are by Rachmilewitz *et al.* (1982) who studied thalassaemia, and by Jackson *et al.* (1983) who 'stressed' muscle homogenates by the addition of $FeCl_3$. Quintanilha & Packer (1983) have summarized their contributions to the role(s) of vitamin E in protecting muscle during severe exercise. A valuable review on the role of iron in enzymically catalysed lipid peroxidation is by Aust & Svingen (1982).

The clinically used antibiotic bleomycin has some toxic properties on normal lung. Bleomycin chelates iron (and other metals) and in this form can produce a redox cycling of electrons from a reduced substrate such as NADH to O_2. Since the bleomycin can intercalate into DNA it is possible to produce such damaging reduced O_2 species very close to genetic material (Lown *et al.*, 1982; Lown, 1983).

Inflammation

Considerable attention has been directed to a possible involvement of free radical intermediates in the inflammatory process (see Kuehl *et al.*, 1979; McCord & Wong, 1979); in particular, to the beneficial effects of superoxide dismutase that acts to decrease the concentration of $O_2^{-\bullet}$ generated by local metabolic disturbances and by stimulated white cells attracted to the site by chemotactic agents. Although $O_2^{-\bullet}$ is not extremely reactive chemically in aqueous solution (see Sawyer & Valentine, 1981), its protonated form, $O_2H^\bullet$, is more reactive (Bielski & Shiue, 1979; Bielski, 1981; Bielski *et al.*, 1983) and in the presence of transitional metal ions $O_2^{-\bullet}$ can generate $OH^\bullet$ which would contribute to the many toxic effects associated with superoxide production in biological systems (see Fridovich, 1981, 1983; Halliwell & Gutteridge, 1984).

Moreover, superoxide free radicals have considerable chemical reactivity when studied in aprotic media (Sawyer *et al.*, 1983). Among the consequences of the local production of $O_2^{-\bullet}$ and $OH^\bullet$ is a stimulation of lipid peroxidation, with all the associated damaging reactions that have been described earlier, and the production of chemotactic agents including aldehydes such as 4-hydroxynonenal. Background information on this interesting area of inflammation and free radical reactions can be obtained from reviews by McCord *et al.* (1980) and Torrielli & Dianzani (1984).

A similarly active field of study has been the role of free radical intermediates in cell killing by leukocytes (Badwey & Karnovsky, 1980; Babior, 1982). The phenomenon overall is complex and multiphasic, but an associated key process is the so-called 'metabolic burst' during which reduced oxygen species are produced. A clear summary of these events is by Baehner *et al.* (1982). Studies by Segal and collaborators have demonstrated a novel electron transport chain in the plasma membrane of phagocytosing cells, which is responsible for the metabolic burst and oxygen activation (Segal & Allison, 1979; Segal *et al.*, 1981); a deficiency in this system occurs in chronic granulomatous disease (Segal *et al.*, 1983).

Free radical intermediates have been linked recently to a number of clinically important disturbances including ischaemic attacks (Rao *et al.*, 1983) and to events consequent upon reperfu-

sion of organs. In my view the development of such studies is likely to have considerable impact on clinical procedures; background references are by Gardner *et al.* (1983) and Parks *et al.* (1983).

In all such studies it is necessary to view the free radical reactions and associated damaging processes in the broad context of cell injury, and not in a narrow sense in which free radical production, lipid peroxidation, etc. are considered in isolation. Endothelial damage, as occurs in the microvascular changes referred to above, will also have profound effects on the prostaglandin cascade, with attendant disturbances in the local prostacyclin:thromboxane ratio (see Vane *et al.*, 1982; Johnson *et al.*, 1983).

Chemical carcinogenesis and free radicals

The difficulties that arise in the direct application of e.s.r. to the study of free radical intermediates in fresh, frozen or lyophilized samples of tumour tissue have been critically reviewed by Swartz (1982). A striking example of a difference in e.s.r. signal between a cancer and its corresponding normal tissue has been given by Benedetto *et al.* (1981*b*) with human cervix; powdered frozen samples of normal cervix gave a very strong signal consistent with the presence of a peroxy radical, whereas the signal was much decreased in, or even absent from, corresponding samples of invasive cancer.

Many chemical carcinogens can be metabolically activated to free radical intermediates although, in many cases, the importance of such intermediates to the vital aspects of carcinogenesis is not fully established (Slater, 1972; Ts'o *et al.*, 1977; Demopoulos *et al.*, 1980). Polycyclic hydrocarbons can be metabolized to free radical intermediates (see Nagata *et al.*, 1982); if the intermediates can be formed close to DNA, by activation in the nuclear membrane (Baird *et al.*, 1980; Bachur *et al.*, 1982) for example, then consequences of relevance to the carcinogenic activity of the parent hydrocarbon may ensue. Aromatic amines are well known to undergo *N*-hydroxylation; if this is followed by an oxidation step then a nitroxy radical can be formed. A correlation has been found (Stier *et al.*, 1982) between the carcinogenic activities of a series of aromatic amines and the occurrence of e.s.r. signals indicative of nitroxy radical formation. An interesting facet of the production of nitroxy radicals by enzymic activation in the endoplasmic reticulum (Stier *et al.*, 1976, 1980) is the possibility that the nitroxy radical may be transported to other regions of the cell in the form of an adduct with polyunsaturated fatty acid (Stier *et al.*, 1982; Floyd, 1982*b*). Other examples of chemical carcinogens that are metabolically activated to free radical intermediates can be found in reviews by Mason *et al.* (1982) and in Floyd (1982*a*). An important aspect of carcinogen activation is that this may occur during and as a result of lipid peroxidation. Dix & Marnett (1983) have shown that polycyclic hydrocarbons are metabolized to epoxy derivatives when included in a peroxidizing microsomal system. Prostaglandin synthetase can also catalyse benzypyrene metabolism during a co-oxidation of arachidonate (Marnett *et al.*, 1977), and the endo-peroxide prostaglandin G_2 can promote the formation of benzpyrene quinone formation through a linked metabolic reaction (Marnett & Reed, 1979); for a discussion of peroxide-mediated metabolism of carcinogens see O'Brien (1982).

Considerable interest has been aroused by the suggestion that some promoters can initiate free radical reactions, and that this type of activity may be of significance to their mechanism of action in the carcinogenic process (see Copeland, 1983).

There have been numerous reports (see the review by Ts'o *et al.*, 1977) that the administration of antioxidants can have beneficial effects on the development of animal tumours, but unequivocal evidence that such activity results from the scavenging of free radical intermediates has been difficult to obtain. Important contributions to this point are by DeLong *et al.* (1983); Bollag (1983) and Boutwell (1983).

Concluding remarks

In this short Review I have drawn attention to a number of examples where free radical intermediates are involved in an important manner in metabolic disturbances that result in cell injury. The examples have been selected to illustrate the variety of studies in progress, and to stress a number of important concepts. There is an increasing number of reports in which free radical reactions have been studied in a clinical context. Recent reviews that provide a more extensive bibliography than can be given here are by: Autor (1982), Bulkley (1983), Cohen & Greenwald (1983), Dormandy (1983), Floyd (1982*a*), Halliwell & Gutteridge (1984), Lewis & Del Maestro (1980), McBrien & Slater (1982), Porter & Whelan (1983), Pryor (1976–1983), Rodgers & Powers (1981), and Yagi (1982).

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