J. Pharm. Pharmacol. 1989, 41: 505–511
Received December 14, 1988

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Mechanistic Toxicology: A Radical Perspective*

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Toxicology affects our everyday lives in many diverse ways. Water and air quality, dietary toxins and the hazards of toxic waste are just three examples of important and emotive topics on which the public are rightly demanding information and quantitative assessments of human health risk. Taken together with increasing occupational and regulatory concerns, it is not surprising that the toxicological sciences are experiencing rapid expansion. One of the most critical questions when examining a particular toxic response relates to the mechanism of toxicity. Without knowledge of mechanism it is very difficult to make rational decisions regarding antidotal therapy or the setting of regulatory limits for exposure.

In many cases the metabolism of a particular drug or toxin plays a pivotal role in the expression of its toxicity. Over the last decade a major emphasis has been placed on the role of free radical metabolites of xenobiotics in toxicity. This review will briefly examine the utility of studying mechanism in toxicology and discuss the role of free radical metabolites of drugs and environmental toxins in the toxic process.

Toxicology deals with the study of adverse effects of poisons and a toxic response is defined as "a damaging, noxious or deleterious effect on a living system" (Timbrell 1982). Mechanistic toxicology is concerned with the events at the molecular level which result in the expression of a toxic effect. These events can be many and varied but in general, mechanisms of toxicity may include altered physiology or, at the cellular level, covalent modification of cellular constituents or disturbance of cellular homeostatic mechanisms (Table 1). The study of mechanism in toxicology has impact on every area of the discipline. Relevant examples are listed in Table 2 and discussed below.

Prediction of drug toxicity

The solvents hexane and 2-hexanone induced a polyneuropathy in exposed workers (Yamamura 1969). The disease was associated with sensory loss and motor weakness and characterized pathologically by nerve fibre degeneration and a "dying-back" syndrome. Structure-toxicity studies by Spencer et al (1978), demonstrated 2,5-hexanedione as the major neurotoxic metabolite which was formed from both hexane and 2-hexanone. Structurally related compounds which did not generate this metabolite did not induce neuropathy. Thus, if new drugs or chemicals are likely to produce 2,5-hexanedione as a metabolite, it can be predicted that neuropathy may be a toxic side effect.

Rational design of antidotal therapies

Paracetamol (acetaminophen) induces a centrilobular hepatic necrosis due to its bioactivation to reactive metabolites which include *N*-acetyl-*p*-benzoquinone imine (Mitchell et al 1973). The latter metabolite is an electrophilic compound and will react with cellular nucleophiles such as those contained in nucleic acids and proteins. The thiol-containing tripeptide, glutathione, is able to detoxify reactive electrophilic species but, after large doses of paracetamol, glutathione becomes depleted and covalent binding of paracetamol derivatives to critical cellular nucleophiles occurs. This has been suggested to be the mechanism of paracetamol induced cellular damage (Mitchell et al 1973). The thiol, *N*-acetylcysteine, was found to protect against paracetamol-induced hepatotoxicity and has been used as antidotal therapy for overdosage. *N*-Acetylcysteine can react directly with electrophiles or, after deacetylation to cysteine, can be utilized by the hepatocyte to synthesize glutathione (Thor et al 1979). This results in greater protection of the cell against reactive electrophilic metabolites of acetaminophen.

Estimation of human health risk. Regulatory limits

A current example of how mechanism may contribute to assessment of human health risk is benzene—a myelotoxin and leukaemogen. As discussed by Goldstein (1983), benzene may induce pancytopenia/aplastic anaemia and there is presumably a threshold dose for these effects. Regulatory treatments of carcinogens currently incorporate a no-threshold approach which associates some finite risk of carcinogenesis with every molecule of the carcinogen. Thus an important mechanistic question in terms of regulation is whether overt pancytopenia is a necessary prerequisite to benzene-induced leukaemogenesis. If, indeed, this were the case, it would imply that some threshold may exist for benzene-induced leukaemia. Such a finding would have an immense impact in both regulatory and economic terms.

Monitoring of exposed populations

If a particular mechanism of toxicity is known and reactions of the compound or its metabolites with macromolecules have been characterized, then the formation of these adducts can be used to monitor the extent of damage. For example, if a compound reacts with DNA or protein and this product can be detected in accessible tissues such as blood cells after exposure to the compound, then this approach may be used as an in-situ dosimeter of biological damage. Various "biological indicators of exposure" are now being developed with an emphasis on DNA and protein adducts (Ehrenberg et al 1974; Farmer et al 1980; Phillips et al 1988). These methods are particularly useful when dealing with compounds which are known to alkylate nucleophiles such as many carcinogens. Other indicators of exposure that may be useful include neurobehavioural endpoints (NIOSH 1987) and metabolite analysis (Drummond et al 1988). Obviously

* Conference Science Award 1987 lecture presented at the British Pharmaceutical Conference, University of Aberdeen, September 1988.

Table 1. Mechanisms of toxicity may include:

(1) Altered physiology
(2) Covalent modification of cellular constituents
  (i) nucleic acids
  (ii) proteins
(3) Disturbance of cellular homeostatic mechanisms
  (i) ionic balances
  (ii) energy production
  (iii) redox balance

Table 2. Utility of studying mechanism in toxicology.

(1) Prediction of drug toxicity and synthesis of non-toxic analogues
(2) Rational design of antidotal therapies
(3) Estimation of human health risk and determination of appropriate regulatory limits
(4) Monitoring of exposed populations
(5) Elucidation of routes of selective toxicity. Prediction of activity of antitumour agents
(6) Information on cause/progression of disease states

the latter two approaches need not be restricted to alkylating agents. All of these monitoring methods may be particularly valuable in the occupational setting.

Selective toxicity. Prediction of activity of antitumour activity

There is a need to distinguish between a toxic and an antineoplastic effect. With chemotherapeutic agents attempts are being made to optimize *selective* toxicity but this still involves optimizing adverse effects on tumour cell function. An example of the predictive value of mechanism in this area can be taken from work on the antitumour agent hexamethylmelamine. The in-vivo antitumour activity of hexamethylmelamine analogues correlated well with their in-vivo metabolism to formaldehyde precursors which could be detected in plasma (Table 3; Ross et al 1984a). Thus, this provided a simple screening test based on the in-vivo metabolism of this series of antitumour agents. The development of predictive assays for antineoplastic activity is now a major area of research (McGuire et al 1988) and these assays, ideally, should be based on mechanistic studies of drug action.

Information on causes/progression of disease

Investigations of compounds which induce disease states may obviously yield information on that particular disease state. For example work using liver carcinogens has shown that cell proliferation can be an important aspect of hepatocarcinogenesis and has yielded information on the multistage nature of carcinogenesis (Farber 1984).

Mechanisms of toxicity and the role of free radical metabolites

Metabolism is often involved in the generation of reactive species in the body. Xenobiotics are metabolized to make them more water-soluble and during this process more reactive compounds may be generated. Amongst reactive metabolites which may be generated from drugs and environmental pollutants are "free radicals". A radical is a molecule with an unpaired electron and may be produced by

Table 3. Correlation of antitumour activity of HMM analogues with their in-vivo metabolism.

	Antitumour activity		Plasma levels of formaldehyde precursors	
Compound	M5076	ADJ/PCGA	Peak concn.	AUC
HMM[1]	+	+	111±3	14580
PMM[2]	+	+	243±27	21300
TMM[3]	+	+	101±15	7530
TriMM[4]	−	−	51±18	3060
CBDT[5]	−	−	16±26	96
HBDT[6]	−	−	33±9	3540

[1] Hexamethylmelamine. [2] Pentamethylenemelamine. [3] N_2,N_2,N_4,N_6-Tetramethylmelamine. [4] N_2,N_4,N_6-Trimethylmelamine. [5] 2-Chloro-4,6-bis(dimethylamino)-1,3,5-triazine. [6] 2-Hydrazino-4, 6-bis (dimethylamino)-1,3,5-triazine.

enzymatic one electron oxidation or reduction and during radiolysis or photolysis (Mason & Chignell 1982). A major group of enzymes responsible for one-electron oxidations are the peroxidases which are widely distributed in biological systems. Both mitochondrial and microsomal reductases are capable of catalysing one electron reductions of xenobiotics.

Free radicals have been known since the turn of the century and the first stable organic radical—the triphenylmethyl radical—was isolated by Gomberg (1900) in a work of fundamental importance. However, studies of free radical metabolites in biological systems is a relatively recent phenomenon. Analytical methodology to detect free radicals—electron spin resonance (ESR)—was developed in 1945 and the first biological samples analysed by ESR were reported in 1954 (see Borg 1976 for discussion). Although most radicals generated in biological systems tend to be reactive and/or unstable species, they can be stabilized, dependent on structure, and isolated in cystalline form. So, although most of the radicals discussed will be of toxicological interest and therefore, by definition, capable of inducing adverse effects in biological systems, not all are necessarily reactive. Many different chemicals can produce free radicals during metabolism and these include phenols, amines, hydrazines, sulphydryls, quinones, halogenated and aromatic hydrocarbons, alcohols and peroxides. Excellent reviews are available which discuss the wide variety of free radical metabolites of drugs and toxins (Mason 1979, 1982; Mason & Chignell 1982).

There are a variety of potential toxicological consequences of radical production in biological systems (Ross 1988): These include:

(i) Covalent binding to cellular macromolecules
(ii) DNA strand scission
(iii) Oxygen radical generation
(iv) Lipid peroxidation

(i) *Covalent binding to cellular macromolecules*

Covalent binding of radicals to cellular macromolecules in biological systems is often difficult to establish since radicals may undergo competing reactions to form other reactive products which also form adducts with cellular constituents. Thus, unequivocal assignment of a radical as a binding

species is not a simple process. Nevertheless, some radicals, such as cation radicals derived from aromatic hydrocarbons and the hydroxyl radical, have been reported to bind to DNA (Scholes et al 1969; Cavalieri & Rogan 1985).

(ii) *DNA strand scission*

Free radical generation from a wide variety of compounds, such as aromatic amines (Nordenskjold et al 1984), quinones (Morrison et al 1984) and molecular oxygen (Brown & Fridovitch 1981), has been implicated in DNA strand break induction. The mechanism of strand breakage is complex but has been reviewed (Schulte-Frolinde & Von Sonntag 1985).

(iii) *Oxygen radical generation*

Oxygen radical formation from xenobiotics in biological systems can occur in a number of different ways. Oxygen radicals are derived from molecular oxygen by successive one electron reductions (Del Maestro 1980; Bulkley 1983) producing superoxide anion radical, hydrogen peroxide (a non-radical) and hydroxyl radical (Fig. 1). Singlet oxygen, an electronically excited form of oxygen and powerful oxidizing agent, may also be produced in biochemical systems via photosensitization or enzymatic mechanisms (Sies 1986). One mechanism of formation of these reactive derivatives of oxygen involved a process known as redox cycling (Thor et al 1982). This process involves one electron reduction of a xenobiotic followed by interaction with molecular oxygen, resulting in superoxide production and regeneration of the parent xenobiotic. Once superoxide is formed, hydrogen peroxide can be generated by dismutation either enzymatically or non-enzymatically (Fig. 2). The enzyme responsible for the dismutation reaction—superoxide dismutase—was discovered by McCord & Fridovitch (1969) and this brought the study of the biological effects of oxygen radicals into mainstream biochemistry. Once hydrogen peroxide is generated, hydroxyl radicals can be produced as a result of metal-catalysed Fenton or Haber-Weiss reactions (Aust et al 1985). Hydroxyl radicals are extremely reactive species and will react in both addition and abstraction reactions with biological constituents. The other activated derivatives of oxygen may also act as oxidizing agents and thus they tend to shift the prooxidant/antioxidant balance in a biological system towards a more oxidized state. This can result in oxidation of critical enzymatic cofactors, proteins and nucleic acids. A variety of chemicals may exert their toxic effects via oxygen radical production (see Table 4) and these include antitumour quinones (doxorubicin), bipyridylium herbicides (paraquat) and nitro-containing antibiotics (nitrofurantoin).

(iv) *Lipid peroxidation*

Reactive radicals such as the hydroxyl radical may abstract a hydrogen atom from lipids which, in the presence of oxygen, leads to lipid destruction and formation of potentially toxic

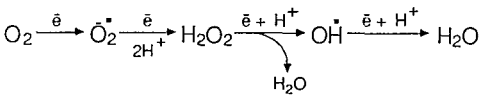

FIG. 1. Production of oxygen radicals by successive univalent reductions of molecular oxygen.

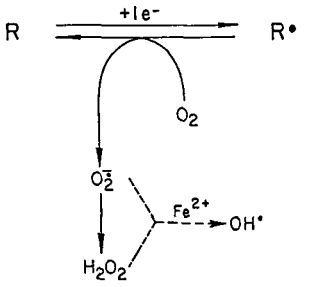

FIG. 2. General scheme for redox cycling of xenobiotics (R) with generation of oxygen radicals.

Table 4. Toxins whose effects may be mediated via oxygen radicals.

Alloxan	Paraquat, Diquat
Doxorubicin	Diethylstilboestrol
Asbestos	Gossypol
6-Hydroxydopamine	Mitomycin C
Naphthoquinones	AZQ
MPTP	Metronidazole
Nitrofurantoin	Bleomycin

degradation products such as malondialdehyde (Fig. 3, Comporti 1985).

Radical production in biological systems does not occur only from xenobiotic chemicals but also occurs endogenously. Oxygen radicals may be produced during respiration or during the oxidative burst of phagocytic cells (Fig. 4, Del Maestro 1980; Ames 1983). As a result of the reactivity of these radicals various disease states have been associated with their production e.g. cancer, inflammatory disease, ischaemia/reperfusion injury, neurodegenerative disease and respiratory distress syndrome (Demopoulos et al 1980; Ames 1983; McCord 1983; Halliwell & Gutteridge 1984). Ageing has also been linked to the effects of continuous endogenous oxygen radical production (Ames 1983).

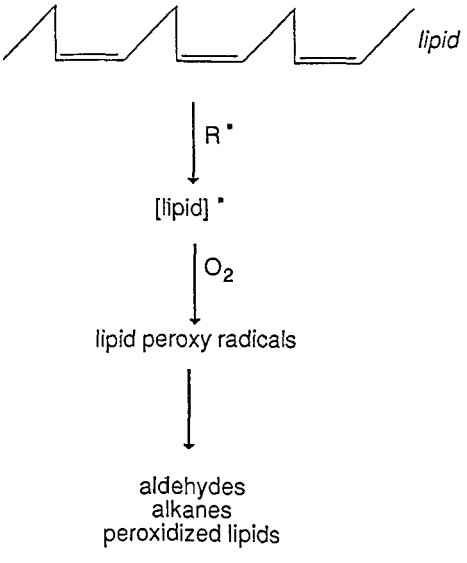

FIG. 3. General scheme for radical-induced lipid peroxidation.

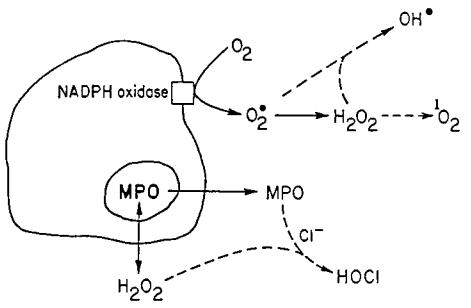

FIG. 4. Production of oxygen radicals during the oxidative burst of phagocytic cells.

Since the body produces free radicals during normal function it is appropriate that defense systems are also in place to protect biological constituents against free radical damage. One defense system involves the tripeptide glutathione which can reduce phenol- and amine-derived radicals back to their parent compounds (Ross et al 1984b, 1985). The further reactions of the glutathionyl radical thus formed in biological systems have also been extensively investigated (Quintilliani et al 1976; Ross et al 1985; Subrahmanyam & O'Brien 1985). The antioxidant defenses of cellular systems are diverse and present in different cellular compartments in order to combat localized oxidative insults. Glutathione for example is present mainly in cytosol whereas vitamin E, a lipophilic antioxidant, is localized in membrane sites. Antioxidant defense systems are listed in Tables 5 and 6. Enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, DNA repair enzymes) and non-enzymatic (vitamins C and E; β-carotene uric acid) defense systems combine to protect against endogenously-generated free radicals (Sies 1986). Glutathione serves as an essential cofactor for the enzyme glutathione peroxidase which removes hydroperoxides at the expense of the conversion of glutathione to its oxidized form—GSSG. Reduced glutathione is then regenerated via the enzyme GSSG reductase using reducing equivalents provided by NADPH. Thus providing the cell has adequate supplies of NADPH, hydroperoxides can be effectively detoxified via this coupled enzyme system (Reed 1986). Superoxide dismutase, together with glutathione peroxidase and/or catalase provides a defense system against superoxide and hydrogen peroxide production (Table 6). DT-diaphorase is also listed and although this flavoprotein does not directly interact with active oxygen species, it catalyses two-electron reduction of quinones to their hydroquinone derivatives which can be more easily conjugated and excreted. This reduces the extent of one-electron reduction of quinones to semiquinone radicals by enzymes such as cytochrome P450 reductase and subsequent oxygen radical production (Fig. 2). Thus DT-diaphorase is generally regarded as a protective enzyme against quinone-induced toxicity (Lind et al 1982; Thor et al 1982).

The critical protective role of the tripeptide glutathione in chemical-induced toxicity in cellular systems can be illustrated by work using isolated hepatocyte model systems (Dimonte et al 1984a, b; Ross et al 1986; Gant et al 1988). The naphthoquinone, menadione, can be enzymatically reduced to a semiquinone radical which can be oxidized by molecular oxygen in a redox cycling reaction to generate superoxide and other aggressive oxygen species. Menadione is also electrophilic and can alkylate cellular nucleophiles (Fig. 5). The combined effects of alkylation and oxidation serve to deplete intracellular glutathione in isolated hepatocytes. This effect is not sufficient to kill the liver cell; cytotoxicity only becomes apparent after protein thiol groups have been depleted. The precise mechanisms of cytotoxicity which are responsible for cell death are still under investigation but are thought to involve disturbances in cellular calcium homeostasis. Thus a combination of three of the mechanisms discussed in Table 1—covalent binding, oxidation and disturbance of ionic homeostasis—may combine to induce toxicity.

Recent work on the myelotoxin and leukaemogen benzene has suggested that free radical mechanisms may also be involved in its toxicity. The metabolism of benzene is complex and although a consensus exists relating the metabolism of benzene to its toxicity (Sawahata et al 1985), the metabolites responsible and their sites of generation remain unclear. One mechanism which has received considerable attention concerns peroxidase-catalysed oxidation

Table 5. Non-enzymatic defense systems against oxygen radicals.

Tocopherols (Vitamin E)
Ascorbic acid (Vitamin C)
β-Carotene
Uric acid
Flavonoids
Proteins, e.g. caeruloplasmin
Bilirubin, Biliverdin

Table 6. Enzymatic defense systems against oxygen radicals.

Enzyme	Reaction
Superoxide dismutase	$2O_2^- \cdot + 2H^+ \rightarrow H_2O_2 + O_2$
GSH peroxidase	$2GSH + 2H_2O_2 \rightarrow GSSG + 2H_2O$
GSSG reductase	$GSSG + 2NADPH + H^+ \rightarrow 2GSH + 2NADP^+$
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
(DT-diaphorase)	Quinone → Hydroquinone

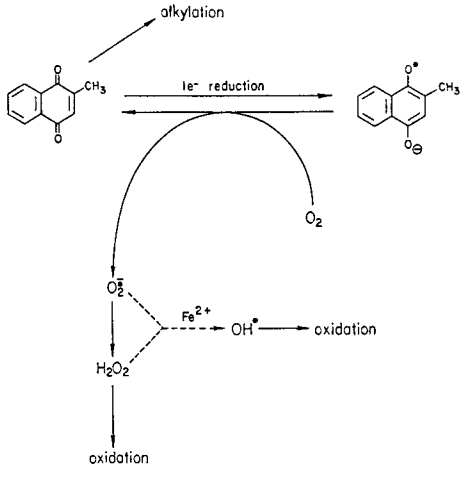

FIG. 5. Potential redox cycling and alkylation reactions of menadione.

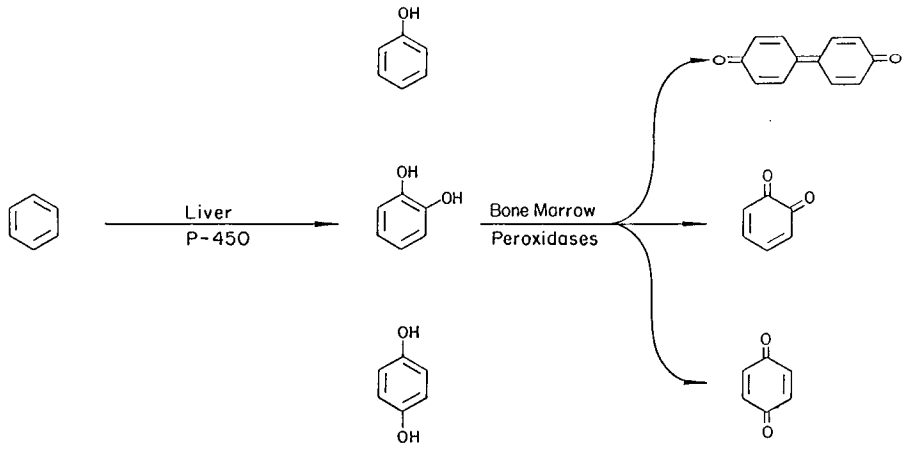

FIG. 6. Bioactivation of benzene via phenolic derivatives to quinones.

of phenolic metabolites of benzene. Benzene is metabolized in the liver to phenol, catechol and hydroquinone which then accumulate in the bone marrow. The marrow is rich in peroxidase enzymes that can readily oxidize phenolics to free radicals which, via coupling or disproportionation reactions, can generate reactive quinones (Fig. 6). An important point to stress in this mechanism is the effect of mixtures, which is becoming a major problem in toxicological risk assessment. Although phenol, catechol and hydroquinone are all substrates for the same enzyme systems, the presence of phenol, for example, does not inhibit the bioactivation of hydroquinone. In fact, phenol can stimulate the conversion of both hydroquinone and catechol to reactive species via the following mechanism. Phenoxy radicals formed during enzymatic oxidation of phenol can abstract an H atom from either catechol or hydroquinone to generate a semiquinone radical which can then disproportionate to the appropriate quinone (Fig. 7, Yamazaki 1958; Kalyanaram et al 1985: Eastmond et al 1987; Bhat et al 1988; Subrahmanyam et al 1989). Thus interactions between phenolics should be considered as a mechanism of potential toxicological relevance.

Some of the major advances in elucidating toxicological mechanism in the next decade are likely to come from application of molecular biological and immunological techniques to toxicology. For example, some elegant immunotoxicological work concerning the target cells of benzene toxicity in bone marrow stroma has recently been performed by Wierda et al (King et al 1987, 1988; Gaido & Wierda 1987; Thomas et al 1989a). Benzene causes inhibition of maturation of B cells in bone marrow and this effect can be duplicated in-vitro by hydroquinone. Hydroquinone exerts a selective effect at the level of the macrophage by inhibiting interleukin (IL)-1 production. In the absence of IL-1 production, bone marrow fibroblastoid stromal cells are not stimulated to produce IL-4 which is a factor required to induce B cell maturation (Fig. 8). The production of IL-4 by stromal cell fibroblasts is not affected by hydroquinone. Together with Gaido & Wierda (1987) we have examined the bioactivation of hydroquinone in both of these cell types—the fibroblastoid stromal cell and the bone marrow macrophage—and have found that hydroquinone is selectively bioactivated by the macrophage (Thomas et al 1989b). Thus studies of metabolism and bioactivation may be able to explain the selective immunotoxicological effect by hydroquinone at the level of the macrophage.

In summary, it is clear that the study of mechanism can provide answers to toxicological problems and extensive work in recent decades has shown that free radicals often contribute to toxic mechanism. The final comment on the

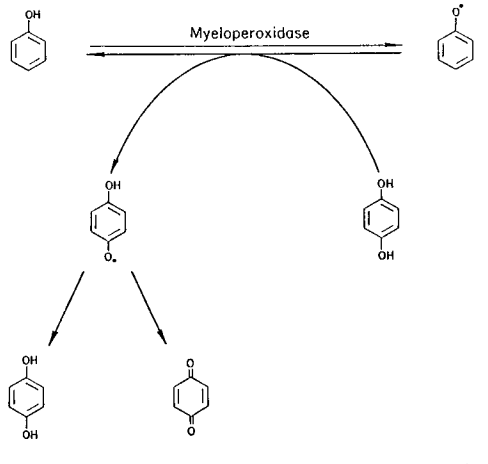

FIG. 7. Interaction of enzymatically-generated phenoxy radicals with hydroquinone.

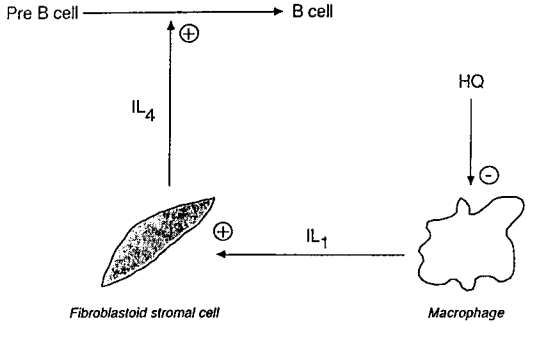

FIG. 8. Effect of hydroquinone on bone marrow stromal cells.

rapidly expanding area of free radical research should perhaps be left to Gomberg (1900), who in the concluding sentence of his seminal paper, dealing with preliminary characterization of an organic radical, wrote, "This work will be continued and I wish to reserve the field for myself."

Acknowledgements

The author thanks his many collaborators quoted in this review, the Royal Pharmaceutical Society of Great Britain for the invitation to present this Conference Science Award lecture, and Drs D. R. Petersen and D. Thompson for helpful discussions. The author is particularly grateful to Dr D. Wierda and colleagues for allowing discussion of unpublished data. Some of the author's work discussed in this review was supported by NIEHS grant ES04112.

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