Br. J. Cancer (1987), **55**, Suppl. VIII, 46–52

Free radical mediated cell toxicity by redox cycling chemicals

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Summary Free radical formation has been implicated in the toxicity of a wide range of xenobiotics. In recent years, particular interest has been paid to compounds which can undergo a one electron reduction to form a radical species which can then react with oxygen forming superoxide ($O_2^{\cdot -}$) and regenerating the parent molecule. This process, which is called redox cycling, leads to a disproportionate consumption of O_2 and cellular reducing equivalents and the formation of active oxygen species, ultimately causing oxidative stress. It has been proposed that cell death results from a loss in control of Ca^{2+} homeostatis caused by thiol oxidation at critical enzyme sites. Physical properties of redox cycling compounds such as their one electron reduction potentials are important in determining their rate of reduction by cellular reductases and the reactivity of the radicals so formed with oxygen and other molecules.

Although redox cycling of many compounds can be clearly demonstrated *in vitro*, the unequivocal demonstration of this process *in vivo* and its involvement in *in vivo* toxicities remains a challenging area for future research.

Many chemicals require metabolic activation to reactive intermediates in order to exert their toxicity (Snyder *et al.*, 1982). Over the last few years there has been an increasing recognition that many of these reactive intermediates may be free radicals (Mason, 1982) i.e. they have an unpaired electron. The free radicals formed may be carbon-, nitrogen- or oxygen-centred and have been implicated in the action of a wide range of structurally and pharmacologically diverse compounds with an equally broad range of toxicities including carcinogenesis, mutagenesis, cell necrosis and lipid peroxidation (Mason, 1982; Mason & Chignell, 1982; Trush *et al.*, 1982; Halliwell & Gutteridge, 1985). The free radicals may be formed both enzymically and non-enzymically. These free radicals, because of their high reactivity, may undergo a number of reactions including: (i) electron transfer to molecular oxygen generating superoxide anion radical and other active oxygen species; (ii) hydrogen atom abstraction possibly leading to autoxidation of polyunsaturated fatty acids; and (iii) covalent binding to tissue macromolecules by radical addition to carbon–carbon double bonds or by radical combination (Halliwell & Gutteridge, 1985; Smith *et al.*, 1984).

This presentation deals mainly with (a) the first of the above reactions i.e. the transfer of an electron from the free radical to molecular oxygen forming superoxide anion radical ($O_2^{\cdot -}$) and reforming the parent molecule (R) (equation I) and (b) the ensuing toxicity to the organism.

$$R^{\cdot -} + O_2 \rightleftharpoons O_2^{\cdot -} + R \qquad (I)$$

The radical species may then be reformed from the parent molecule and react with oxygen again so a futile redox cycle is established. With many chemicals, such as quinones, paraquat and nitrofurantoin, the radical appears to be formed following one electron reduction by a cellular reductase utilizing reducing equivalents from NAD(P)H (Trush *et al.*, 1982; Kappus & Sies, 1981). Thus a redox cycle is established in which disproportionate amounts of both NAD(P)H and oxygen are utilized relative to the amount of chemical present and $O_2^{\cdot -}$ and other active oxygen species may be generated (Figure 1). This redox cycling of the chemical leads to an oxidative stress and subsequently cell death by a mechanism(s) which is still unclear but may involve alterations in intracellular calcium homeostasis. Such redox cycling has been implicated in the toxicity of a number of chemicals (Sies, 1985) including the pulmonary toxicities of paraquat and nitrofurantoin, the cardiotoxicity of adriamycin and the cytotoxicity of menadione and other structurally related naphthoquinones (Table I).

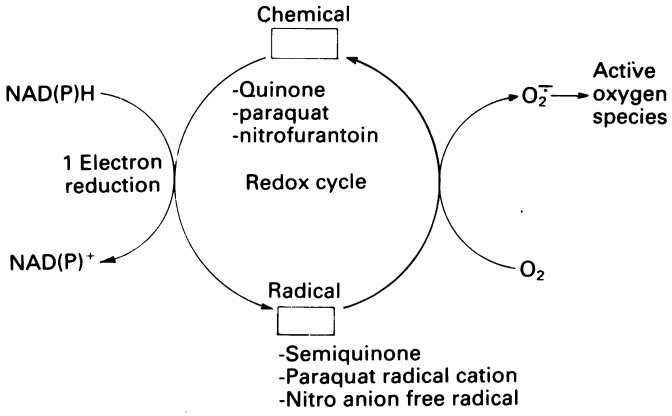

Figure 1 Redox cycling of chemicals. A free radical is formed when the chemical accepts an electron. This one electron reduction is often catalyzed by a flavoprotein such as NADPH-cytochrome P-450 reductase. The free radical then reacts rapidly with O_2 generating $O_2^{\cdot -}$ and other active oxygen species.

The remainder of this presentation will discuss four of these areas in more detail: (i) Formation of radicals which redox cycle, (ii) Reactivity of radicals with oxygen or other molecules, (iii) Possible mechanism(s) of toxicity of such

Table I Free radicals of these compounds implicated in the toxicity to specific tissues

Compound	*Use*	*Target organ of toxicity*
Paraquat	Herbicide	Lung
Nitrofurantoin	Antibacterial	Lung
Cephaloridine	Antibiotic	Kidney
Carbon tetrachloride	Organic solvent	Liver, lung
Adriamycin	Anticancer agent	Heart
Bleomycin	Anticancer agent	Lung
MPTP	Contaminant in illicit drugs	Brain
Alloxan	Production of diabetes in experimental animals	Pancreas

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redox cycling, and (iv) Evidence for redox cycling *in vivo* and *in vitro*.

Formation of radicals

Due to their chemical structures some chemicals, such as dialuric acid and 6-hydroxydopamine may readily autoxidize to radical intermediates (Trush *et al.*, 1982). The autoxidation is catalyzed by metal ions such as copper and iron and the parent molecule is reformed by reducing agents such as ascorbic acid so forming a non-enzymic redox cycle. Radical intermediates may also be formed following u.v. irradiation of either the antibacterial, sulphanilamide, or 4-aminobenzoic acid, which is present in sunscreen preparations or chlorpromazine, a major tranquillizer (Mason, 1982; Chignell *et al.*, 1985). These radicals may account for the phototoxic and photoallergic responses of these compounds (Chignell *et al.*, 1985).

However, most compounds are converted to radical intermediates by enzymes. This subject has been recently reviewed (Yamazaki *et al.*, 1985; Kappus, 1986) and may involve a number of different enzymes including peroxidase, ascorbate oxidase, prostaglandin endoperoxide synthetase, cytochrome P-450, microsomal flavoproteins, NADH dehydrogenase, xanthine oxidase and flavoproteins in phagocytosis. Particular attention has been paid to the reduction of quinone containing compounds to their corresponding semiquinones by various cellular enzymes. The quinone moiety may be present in the parent molecule or may be formed following metabolism. These quinones include active or potential antitumour agents such as adriamycin, daunomycin A, mitoxantrone, mitomycin C, aziridinylbenzoquinone (AZQ), etoposide (VP-16), β-lapachone, cytotoxic compounds such as 1,4-naphthoquinone, menadione and benzo(a)pyrene-3,6-quinone. Many of these quinones have been shown to be reduced and undergo redox cycling catalysed by NADPH-cytochrome P-450 reductase using either the isolated enzyme, microsomes or isolated nuclei (which also contain this enzyme). Many studies have demonstrated that many of these quinones may be reduced *in vitro* by such reductases and then be involved in redox cycling. Such studies have demonstrated the formation of free radicals *in vitro* by electron spin resonance (Bachur *et al.*, 1978), disproportionate oxygen consumption and NAD(P)H oxidation (Bachur *et al.*, 1978; Handa and Sato, 1975; Powis and Appel, 1980), formation of $O_2^{\cdot-}$ and other active oxygen species (Powis and Appel, 1980; Thor *et al.*, 1982; Thornalley *et al.*, 1984). *In vitro* many of these quinones may be reduced by microsomal NADPH-cytochrome P450 reductase, microsomal NADH-cytochrome b_5 reductase, mitochondrial NADH dehydrogenase, nuclear NADPH-cytochrome P-450 reductase or cytosolic xanthine oxidase (Yamazaki *et al.*, 1985; Kappus, 1986). Thus it is evident that under optimal *in vitro* conditions, many enzymes have the ability to catalyse the redox cycling of many different quinones. Powis and Appel (1980) have shown that the one electron reduction potential of quinones is an important determinant of their rates of metabolism *in vitro* by NADPH cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and NADH: ubiquinone oxidoreductase. Therefore redox potential of a compound is important not only in determining the rate of formation of the semiquinone but also its reactivity with molecular oxygen (see below). The relative rates of these two reactions may be an important determinant of toxicity. However, it should be emphasised that because redox cycling occurs *in vitro* does not mean that it will occur *in vivo* (see later for fuller discussion). The redox cycling of one such model quinone, 1,4-naphthoquinone (1,4-NQ), has been extensively investigated in our laboratory (Miller *et al.*, 1986). 1,4-NQ, which is cytotoxic to hepatocytes, is formed metabolically from 1-naphthol by either rat liver microsomes or a purified reconstituted cytochrome P-450 system (d'Arcy Doherty *et al.*, 1985). 1,4-NQ in the presence of microsomes and either NADH or NADPH forms $O_2^{\cdot-}$ which was detected by e.s.r. spin trapping (Thornalley *et al.*, 1984) and also caused a disproportionate consumption of both O_2 and NADPH (Miller *et al.*, 1986). In these experiments with rat liver microsomes, 2.5 nmoles of 1,4-NQ caused the oxidation of more than 100 nmoles of NADPH (Miller *et al.*, 1986). Both the O_2 consumption and NADPH oxidation were inhibited by $NADP^+$ an inhibitor of micrososmal NADPH-cytochrome P-450 reductase implicating this enzyme in the redox cycling of the compound (Miller *et al.*, 1986).

We should now like to consider formation of radicals from another class of chemicals i.e. the bipyridyls, in particular, paraquat. Paraquat (1,1'-dimethyl-4,4'-bipyridylium) is a contact herbicide, which after deliberate ingestion, has been responsible for many human fatalities (Smith & Heath, 1976). The most characteristic feature of these patients is pulmonary damage, the lung becoming progressively impaired leading to death from pulmonary fibrosis. The lung damage is believed to be caused by an initial selective uptake of paraquat into alveolar type I and II cells followed by redox cycling of paraquat (Bus & Gibson, 1984; Smith & Nemery, 1986. The exact mechanism of how this causes pulmonary toxicity is unclear but two main theories have been proposed involving NADPH depletion and lipid peroxidation (Bus & Gibson, 1984; Smith & Nemery, 1986). However irrespective of the detailed mechanism of toxicity most studies agree that the redox cycling of paraquat is an integral component of the mechanism of toxicity. Several enzymes including microsomal NADPH-cytochrome P-450 reductase, xanthine oxidase, and ferredoxin reductase, have been shown to reduce paraquat (Kappus, 1986). The paraquat radical cation can then react very readily with oxygen forming $O_2^{\cdot-}$ and reforming paraquat which can then be reduced again at the expense of cellular NADPH. A similar redox cycle as described above for quinones and bipyridyls may also occur with other molecules including nitro- and azo compounds as well as certain metal chelates as reviewed elsewhere (Kappus & Sies, 1981).

Reactions of radicals with oxygen and other molecules

Oxygen consumption and production of $O_2^{\cdot-}$ are frequently used as indices of redox cycling. Free radicals ($R^{\cdot}$), formed by one electron reduction of the parent molecules (R), may react with oxygen to form $O_2^{\cdot-}$ and regenerate the parent molecule (equation I – see above). Equation (I) is written as an equilibrium reaction as the extent and direction of this reaction depend not only on the concentration of the reactants but also on their one electron redox potentials (E_7^1) relative to the $O_2/O_2^{\cdot-}$ couple ($E_7^1 = -155$ mV, 1 M O_2 (Wood, 1974)). Compounds, which may undergo one electron reduction to form radical species, have a wide range of redox potentials. For example paraquat, adriamycin and benzoquinone have redox potentials of -450, -290 and $+99$ mV respectively (Willson, 1982). The log of the rate constants for the reactions of nitro radical anions, formed by the one electron reduction of nitroaromatic or nitroheterocyclic compounds, with oxygen is directly related to the difference between their one electron reduction potentials and that of the $O_2/O_2^{\cdot-}$ couple (-155 mV) (Wardman & Clarke, 1976). Compounds with more negative one electron redox potentials such as nitrobenzene ($E_7^1 = -486$ mV) react fastest with a rate constant of $7.7 \times 10^6\ M^{-1}\ sec^{-1}$ (Wardman & Clarke, 1976). In contrast, radicals formed by one electron reduction of more electron affinic compounds such as benzoquinone ($E_7^1 = +99$) do not readily react with oxygen. Pulse radiolysis studies have shown that the backward reaction i.e. the reduction of benzoquinone by $O_2^{\cdot-}$ proceeds with a rate constant of $9.6 \times 10^8\ M^{-1}\ sec^{-1}$ (Willson, 1971).

A practical implication of the reversibility of reaction (I) became evident when we were measuring the ability of naphthoquinones to stimulate the NAD(P)H dependent production of $O_2^{\cdot-}$ by rat liver microsomes in the presence of the spin trap DMPO (Thornalley *et al.*, 1984). A maximum stimulation was observed with 5 μM 1,4-NQ ($E_7^1 = -143$ mV). Increasing the naphthoquinone concentration above 5 μM produced a decrease in signal intensity which was due to the ability of increasing concentrations of 1,4-NQ to compete more effectively than DMPO (1 mM) for $O_2^{\cdot-}$.

In addition to the physical properties of the redox cycling compounds, other factors can effect the equilibrium reaction with oxygen. The presence of SOD which catalyses the dismutation of $O_2^{\cdot-}$ to H_2O_2 drives reaction (I) further to the right by removal of $O_2^{\cdot-}$ (Winterbourn *et al.*, 1978; Winterbourn, 1981*a*). In addition, the rates of disproportionation of the radicals (R$^{\cdot}$) relative to their rates of reaction with O_2 may be important, as well as their reactions with other cellular molecules such as ascorbate (Gutierrez *et al.*, 1985) and H_2O_2 (Winterbourn, 1981*b*) or the mitochrondrial electron transport chain (deGroot *et al.*, 1985).

Possible mechanism(s) of toxicity induced by redox cycling chemicals

The free radical intermediates formed can, as previously discussed, undergo several types of reaction including (i) direct reaction with cellular macromolecules possibly leading to toxicity or (ii) reaction with oxygen leading to formation of $O_2^{\cdot-}$ and other active oxygen species. The importance of reaction (ii) with redox cycling chemicals should lead to an oxidative stress caused by the generation of active oxygen species in susceptible cells or organs. The ability of any cell or organ to protect itself against these various active oxygen species may be crucial in determining their relative susceptibilities to such redox cycling induced oxidative stress. Thus the susceptibility of the heart to the cardiotoxicity of adriamycin has been attributed in part to its relatively low levels of catalase and superoxide dismutase (Doroshow *et al.*, 1980). The importance of these prooxidant and antioxidant activities will now be illustrated with two well documented examples i.e. the pulmonary toxicity of paraquat and the cytotoxicity of quinones such as menadione.

Pulmonary toxicity of paraquat

The most characteristic feature of paraquat toxicity to humans is, pulmonary damage as previously discussed. The selective accumulation of paraquat in the lungs by an active transport process is of prime importance in the aetiology of the lung specific lesion (Smith *et al.*, 1979; Rose *et al.*, 1974). Paraquat in the lung undergoes a cyclical reduction and reoxidation with the concomitant production of superoxide anion as discussed earlier, ultimately leading to cell death. Although the mechanism of cell death is not clear, it is apparent that the selective accumulation of paraquat in the lung is a key factor in determining its organ specific toxicity. Following this accumulation, paraquat may then be reduced by various enzymes, as discussed earlier, forming the paraquat radical cation which may then redox cycle (Figure 1). Although there is no unequivocal evidence which enzyme(s) are responsible for the reduction *in vivo*, most data supports the involvement of NADPH-cytochrome P-450 reductase, which is present in lung in different cell types including alveolar type II cells. The generation of $O_2^{\cdot-}$ by redox cycling is then believed to lead to the generation of other active oxygen species. Superoxide may dismutate either spontaneously or in a reaction catalysed by superoxide dismutase (SOD)

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2. \qquad (II)$$

The H_2O_2 may then be detoxified by catalase or glutathione peroxidase or together with $O_2^{\cdot-}$ form the extremely reactive hydroxyl radical (OH$^{\cdot}$) in a metal catalysed Haber–Weiss reaction (Haber & Weiss, 1934) which is dealt with in more detail elsewhere in this volume. The occurrence of this reaction *in vivo* is still the subject of much controversy. Much indirect evidence supports the involvement of OH$^{\cdot}$ in paraquat induced lung toxicity. Thus OH$^{\cdot}$ are known to induce lipid peroxidation which subsequently leads to altered membrane functions and cell death. Based on the work of Bus and Gibson (1984) and others (Smith & Nemery, 1986) a mechanism of paraquat toxicity incorporating lipid peroxidation is shown in Figure 2. Whilst several groups have demonstrated an increase in lipid peroxidation of lung microsomes *in vitro*, there is again no unequivocal evidence that such reactions occur in the lung *in vivo* prior to cell death. An alternative mechanism of toxicity of paraquat has been proposed in which the primary event is the depletion of NADPH in the susceptible cells, where paraquat is accumulated. As seen in Figure 2, NADPH, which is formed in the pentose phosphate pathway, may be utilized directly in the redox cycling with NADPH-cytochrome P-450 reductase as well as in the reduction of GSSG to GSH catalyzed by glutathione reductase and thus indirectly in the reduction of lipid hydroperoxides to lipid alcohols. The rapid utilization of NADPH in the lungs following paraquat administration leads to a rapid increase in pentose phosphate pathway activity in order to regenerate NADPH and is accompanied by a decrease in NADPH-dependent fatty acid synthesis (Keeling & Smith, 1982). It is apparent that these two hypotheses are not mutually exclusive as NADPH depletion should render the cell more susceptible to the toxicity of H_2O_2 and/or lipid hydroperoxides (Smith & Nemery, 1986).

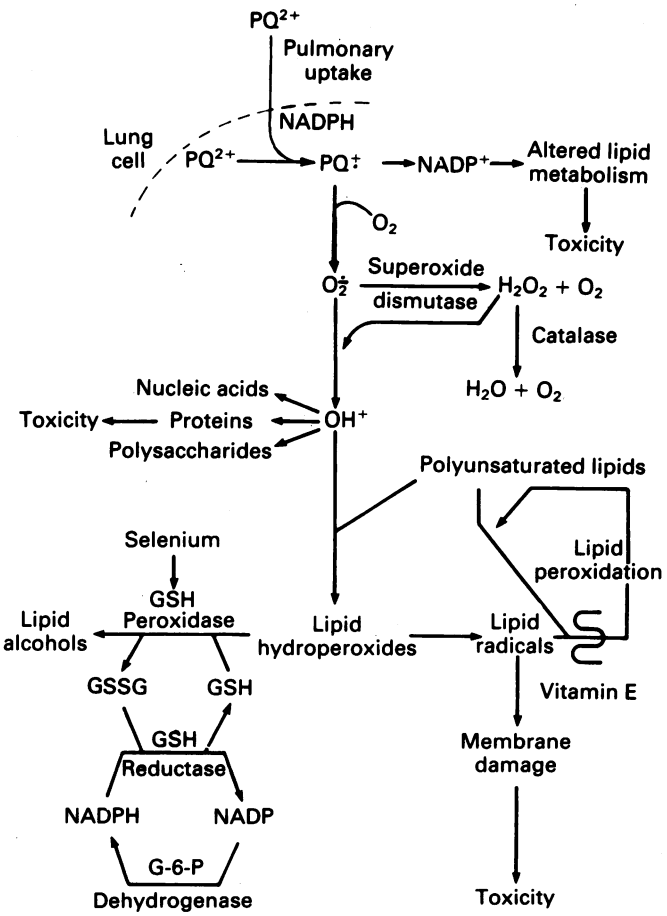

Figure 2 Proposed mechanism of toxicity of paraquat. Paraquat is accumulated selectively in the lung where it is believed to exert its toxicity by redox cycling followed by lipid peroxidation and/or NADPH depletion. Based on Bus and Gibson (1984).

Thus, based on current data, the most plausible mechanism of paraquat toxicity is believed to be a selective accumulation of paraquat into alveolar type I and II cells followed by its reduction and redox cycling, generating active oxygen species, depleting NADPH and increasing lipid peroxidation and ultimately leading to cell death.

Cytotoxicity of naphthoquinones to isolated hepatocytes

Quinones may exert their toxicity either by direct interaction with cellular macromolecules or as suggested by contemporary research by their ability to form free radicals, active oxygen species and redox cycle or by inhibition of mitochondrial electron transport. Cellular reductases may reduce quinones either by one or two electron reduction to the corresponding semiquinones or hydroquinones respectively. The semiquinones may be toxic *per se* or they may react with molecular oxygen to form superoxide anion radical and regenerate the parent quinone, which is then available to be rereduced and undergo a similar futile redox cycle as discussed earlier. Thus, for example, addition of 1,4-naphthoquinone (Miller *et al.*, 1986) or menadione (Thor *et al.*, 1982) to rat liver microsomes results in a disproportionate utilisation of both molecular oxygen and NADPH and generation of superoxide anion radical (Thor *et al.*, 1982; Thornalley *et al.*, 1984; Miller *et al.*, 1986). In contrast, the two electron reduction of the quinones to hydroquinones, catalysed by DT-diaphorase, is widely believed to be a detoxication reaction because hydroquinones are both generally less reactive and may also be readily excreted as their corresponding glucuronic acid or sulphate ester conjugate (Lind *et al.*, 1982). Some support for a role of DT-diaphorase in the detoxication of quinones is provided by the observation that dicoumarol, an inhibitor of DT-diaphorase, potentiates the toxicity of quinones to hepatocytes (Thor *et al.*, 1982; Miller *et al.*, 1986), presumably by blocking the two electron reduction to the relatively non-toxic hydroquinone and allowing more quinone to be available for one electron reduction to the semiquinone and redox cycling.

The cytotoxicity of naphthoquinones, in particular menadione (2-methyl-1,4-naphthoquinone) to isolated hepatocytes has been extensively studied (Thor *et al.*, 1982; DiMonte *et al.*, 1984*a*) particularly by Orrenius and co-workers. With menadione, cytotoxicity is preceded by a rapid depletion of reduced glutathione (GSH) together with the appearance of numerous small blebs on the plasma membrane, and an increase in oxidised glutathione (GSSG), possibly due to dismutation of $O_2^{\cdot -}$ to H_2O_2 and the subsequent oxidation of GSH by GSH peroxidase (Thor *et al.*, 1982; DiMonte *et al.*, 1984*a*). The cell surface blebbing was also observed in the presence of other toxic agents, such as bromobenzene and t-butylhydroperoxide, and of the calcium ionophore A 23187 in the absence of extracellular Ca^{2+} (Jewell *et al.*, 1982). The blebbing is most probably due to alterations in intracellular Ca^{2+}, which regulate the cytoskeletal structure as similar blebs were observed in the presence of cytochalasin B, which directly disrupts the cytoskeleton. A critical role for depletion of protein sulphydryls in the cytotoxicity of menadione has also been suggested (DiMonte *et al.*, 1984*a*, *b*).

It is suggested that oxidation of critical -SH groups in key enzymes might then lead to changes in Ca^{2+} homeostatis and cell death. Because of the concentration gradient of Ca^{2+} across the plasma membrane, there is a continuous influx of Ca^{2+} into the cell which must be extruded if intracellular Ca^{2+} homeostatis is to be maintained. This is accomplished by a high affinity plasma membrane Ca^{2+} translocase, whose action is critically dependent on protein thiol groups (Bellomo *et al.*, 1983). Thus the oxidative stress caused by menadione is believed to result in oxidation of critical enzymes involved in intracellular Ca^{2+} homeostatis resulting in an increase in cytosolic free Ca^{2+} and cell death (Figure 3).

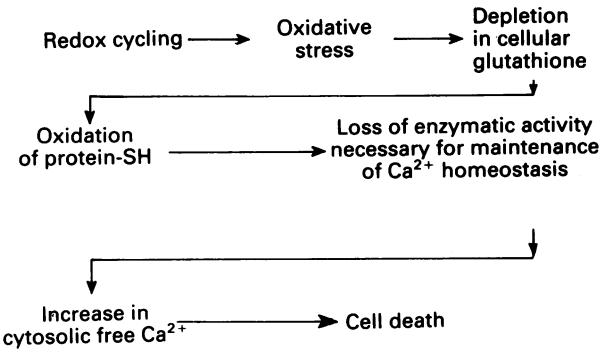

Figure 3 Mechanism of toxicity of menadione. Proposed involvement of glutathione and cytosolic free Ca^{2+} in the redox cycling and toxicity of naphthoquinones.

Evidence for redox cycling *in vivo* and *in vitro*

We have already discussed many indirect measures of redox cycling *in vitro*, such as ESR detection of free radicals, disproportionate oxygen consumption, NAD(P)H oxidation and lipid peroxidation. However, it should be emphasised that ability to form free radicals and redox cycle *in vitro* does not mean that this potential will be realised *in vivo*.

In order to demonstrate redox cycling *in vivo*, firstly it is necessary to show the formation of free radicals *in vivo*. In the last few years this has been successfully demonstrated using spin traps such as phenyl-tert-butyl nitrone (PBN). PBN has been used to trap free radicals *in vivo* formed from the hepatic metabolism of halothane (Poyer & McCay, 1981) and carbon tetrachloride and the pulmonary metabolism of 3-methylindole (Kubow *et al.*, 1984). Further work is required to determine the optimal use and limitations of such *in vivo* spin traps.

The radicals will react with oxygen forming active oxygen species, followed by oxidative stress and toxicity. Recently some studies have measured biliary and plasma GSSG as possible indicators of oxidative stress *in vivo* (Adams *et al.*, 1983; 1984). It should be possible to measure arterio-venous differences across susceptible organs in pO_2 and other markers of oxidative stress. These studies may further implicate redox cycling of chemicals as an important mechanism of toxicity *in vivo* but much further work is required in order to elucidate the critical events which ultimately lead to cell death.

Much of the work in the authors' laboratory has been supported by a grant from the Cancer Research Campaign. We thank Mrs M. Fagg for the typing of the manuscript and Mr D. King for preparation of the figures.

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Discussion

Adams: The electron transfer reaction as a mechanism of toxicity via production of O_2^- would predict that those quinones that have a less negative one electron reduction potential, those which, in other words, can oxidise O_2^- would be very non toxic. It is just the reverse. Benzoquinone which will oxidise O_2^-, is highly toxic to mammalian cells compared to, say, menadione. Also as you have just pointed out, nitroheterocyclics and nitrofurantoins are far more toxic in anoxia: that is why they are used as anaerobic antibodies or as bioreductive drugs for mammalian cells. Oxygen is highly protective. All this suggests that the damage does not arise from O_2^- as a precursor.

Cohen: A point that I didn't discuss in any detail and of course becomes a problem when you start comparing benzoquinone and menadione for instance in terms of redox cycling activity, is that we also have to consider their chemical reactivity.