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# COMMENTARY

# ACTIVATION OF PHARMACOLOGIC AGENTS TO RADICAL INTERMEDIATES

## IMPLICATIONS FOR THE ROLE OF FREE RADICALS IN DRUG ACTION AND TOXICITY

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The biological effects of exogenous chemicals, including drugs, are initiated through physiochemical interactions between the chemical and specific tissue macromolecules such as enzymes, structural and receptor proteins, and nucleic acids. In most cases, such interactions provide the molecular basis for both the pharmacologic and toxic effects of a drug. One of the possible results of the interaction of a drug with enzyme systems is the metabolism of that compound to a chemically reactive intermediate, a concept extensively developed as a result of research in Gillette's laboratory at the National Institutes of Health [1–3]. The reaction of either this initial reactive metabolite or secondary reactive products with susceptible target molecules can then bring about changes in cellular function. The difficulty arises when one tries to determine whether the changes in cellular activity resulted from a sequence of events originating from a single biochemical lesion or from the concerted actions of a number of different initial biochemical lesions [1]. Nonetheless, in many instances, it has been possible to characterize the chemical identity of the reactive metabolite, to examine the interaction of the reactive metabolite with potential target molecules, and to characterize those cellular factors which facilitate and modulate such interactions.

It is becoming increasingly apparent that many reactive intermediates are free radicals [4]; that is, they have an odd- or spin-unpaired electron in their outer orbital [5]. Drug-derived radical intermediates may be carbon-, nitrogen- or oxygen-centered and include such compounds as acetaminophen [6], adriamycin [7], β-lapachone [8], carbon tetrachloride [9], halothane [10], morphine [11], nitrofurantoin [12], paraquat [13], and phenylhydrazine [14]. Because of the thermodynamic potential of the unpaired electron to form an electron pair, free radical intermediates are extremely reactive. As such, they can undergo a variety of reactions including adding across unsaturated bonds, abstracting hydrogen from other molecules, or combining with themselves to form dimers [5]. Another possibility to be considered is that the radical intermediate may be in an electronically excited state which could then transfer its electronic energy to acceptor molecules or utilize this energy to oxidize target molecules [15, 16]. In addition, free radical intermediates, such as the semiquinone anion, the azo anion, the nitroaromatic anion and the bipyridinium cation, can activate molecular oxygen by univalent reduction to the superoxide anion ($O_2^{\cdot}$), which in turn can rapidly dismutate to produce hydrogen peroxide ($H_2O_2$) [12, 17–19]. The metal catalyzed reaction of these two species of oxygen metabolites results in the formation of an extremely powerful oxidant, the hydroxyl radical (·OH) [20]. Hydroxyl radicals are also produced from water in aqueous solutions exposed to ionizing radiation and are considered integral to the mechanism of radiation-induced cellular damage [21, 22].

The generation of free radicals, including secondary molecular oxygen-derived radicals, presents a danger to cells because radicals are capable of interacting with, and subsequently damaging, the entire array of biomolecules which constitute cells. Moreover, radical-initiated processes are particularly deleterious because they are conservative and propagative; that is, radical interactions with cell constituents may produce secondary and tertiary free radicals derived from lipids, amino acids, glutathione, ascorbic acid and components of nucleic acids. The cumulative effects of such a cascade of radical-initiated reactions may be the immediate death of cells, possibly resulting in tissue necrosis and fibrosis, or may be more subtle and delayed as evidenced by the development of neoplasms (Fig. 1).

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### CELLULAR FACTORS WHICH MODULATE AND FACILITATE THE ACTIONS OF RADICALS

One of the basic premises that has emerged from the extensive investigations on the bioactivation of

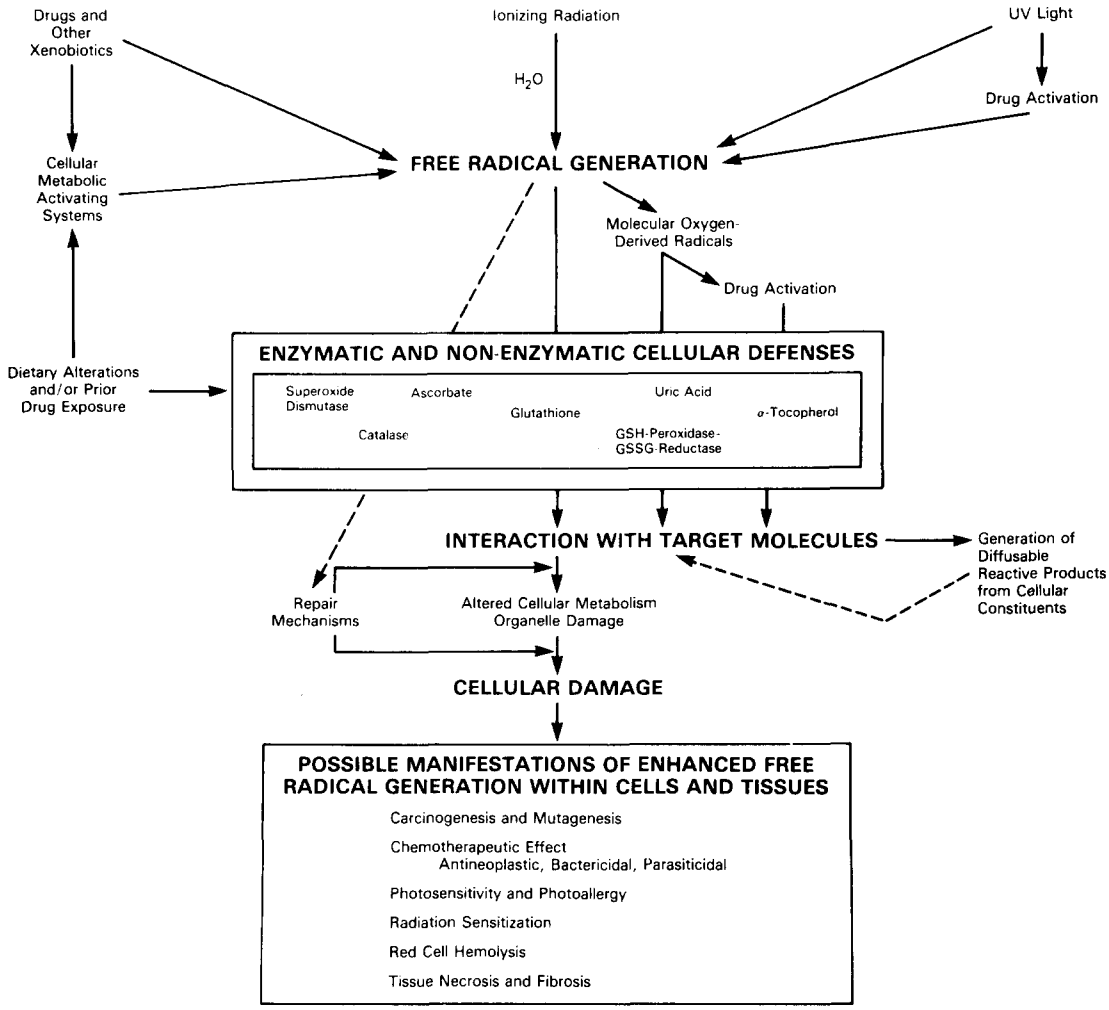



Fig. 1. General reaction scheme illustrating how enhanced free radical generation within cells may result in altered cellular function.

inert compounds to reactive intermediates is that, in spite of the fact that such intermediates are highly reactive chemically, cells are well protected against their actions [1–3]. In fact, significant cytotoxicity is usually observed only after cellular defenses are significantly depleted, particularly tissue glutathione. This same principle is applicable to radical-initiated reactions. Because of the variety of mechanisms by which compounds can be activated to radical intermediates it is fortunate that organisms have evolved a plethora of protective mechanisms which control radicals and their actions. These concepts will be expanded in the following sections.

*Cellular defense mechanisms*

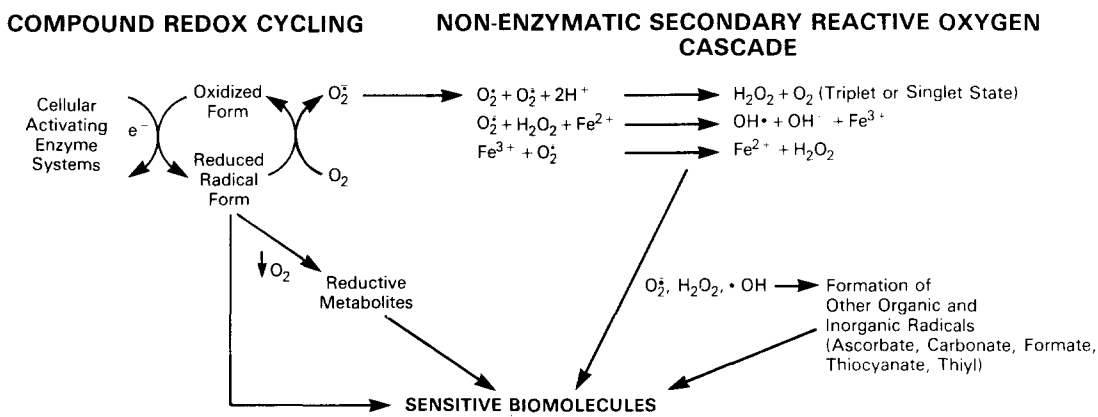
During intermediary metabolism, the flavoprotein enzymes embedded in the endoplasmic reticulum and the redox enzymes of the inner mitochondrial membrane electron transport system normally generate and concomitantly "leak" superoxide and hydrogen peroxide. However, the biological effects of these and other secondary radical species are sharply controlled by a wide spectrum of biochemical and enzymatic defenses including superoxide dismutase, catalase, the GSH peroxidase–GSSG reductase couple, $\alpha$-tocopherol, $\beta$-carotene, ascorbic acid, reduced glutathione [20, 23, 24], and possibly uric acid as recently hypothesized by Ames *et al.* [25]. Moreover, it appears that these defense mechanisms may function cooperatively as exemplified by the interplay between $\alpha$-tocopherol and ascorbic acid [26, 27] and the concerted actions of superoxide dismutase and catalase [20]. In addition, these cellular defenses are advantageously localized in either the hydrophobic or hydrophilic cell compartments as illustrated by $\alpha$-tocopherol and glutathione respectively. Therefore, so long as homeostasis is maintained between the rate of radical generation and the rate of radical dissipation, the cellular generation of radicals may not be harmful.

In contrast, this balance can be disturbed if cellular defenses are decreased or if there is a significant increase in the flux of radical generation, which then overwhelms or possibly even inactivates the defense mechanisms of the cells. Bus *et al.* [28] noted, for example, that following the administration of the

herbicide paraquat to mice, hepatic glutathione levels were significantly and selectively decreased while, in contrast, levels of lipid-soluble antioxidants, presumably α-tocopherol, were more severely depressed in the lung relative to the liver. In addition, prior depletion of glutathione by diethyl maleate shifted the organ specific toxicity of paraquat from the lung towards the liver [29]. Similarly, the pulmonary toxicity of paraquat was increased when it was given to animals maintained on a diet deficient in either selenium or α-tocopherol [30, 31] but was decreased following the induction of pulmonary superoxide dismutase by exposure to 85% oxygen or endotoxin [28, 32]. Moreover, Boyd *et al.* [33] have shown recently that the pulmonary toxicity of the antibiotic nitrofurantoin was enhanced significantly when administered to rats fed a diet deficient in α-tocopherol but high in polyunsaturated fats. Thus, the hypothesis that reactive oxygen metabolites initiate toxicogenic reactions *in vivo* is strengthened by these experiments in which manipulations of cellular defenses have resulted in either increased or decreased toxicity to the organism following administration of xenobiotics capable of augmenting the formation of $O_2^{\cdot}$.

*Activation of compounds to radical intermediates*

Compounds can be activated to radical intermediates by both enzymatic and non-enzymatic mechanisms. For example, by virtue of their chemical structures, xenobiotics such as dialuric acid [34], 6-hydroxydopamine [35, 36] and 7,8-dihydroxychlorpromazine [37] can readily autoxidize to radical intermediates, probably semiquinone radicals. Coincident with this autoxidation process is the generation of reactive oxygen metabolites. Metal ions, such as copper or iron, facilitate this autoxidation process, whereas reducing agents, such as ascorbic acid, mediate the regeneration of the original polyhydroxylated compound, in essence establishing a non-enzymatic redox cycle. Similarly, ultraviolet light can photoactivate sulfanilamide, an antibacterial drug, and 4-aminobenzoic acid, a component of sunscreen preparations, to radical intermediates



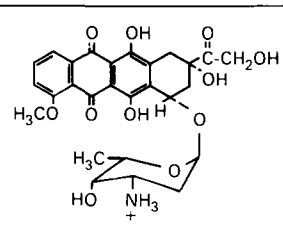
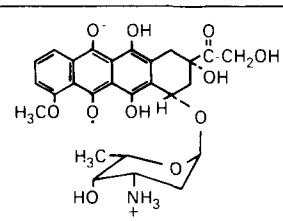
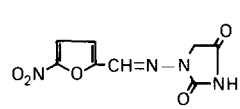
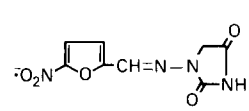
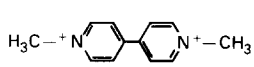
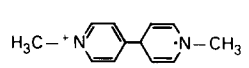

| COMPOUND | OXIDIZED FORM | REDUCED RADICAL FORM | TYPE OF RADICAL |
|---|---|---|---|
| Adriamycin |  |  | Semiquinone Anion Radical |
| Nitrofurantoin |  |  | Nitro Anion Radical |
| Paraquat |  |  | Bipyridinium Cation Radical |

Fig. 2. Processes involved in the redox cycling of drugs which results in the generation of the superoxide anion ($O_2^{\cdot}$) and accompanying non-enzymatic interactions between oxygen metabolites.

which are believed to account for the phototoxic and photoallergic responses elicited by these compounds [38]. In addition, the long-wave u.v. light (320–400 nm) activation of psoralens to radical intermediates that form photoadducts with pyrimidine bases in psoriatic epidermal cells has proven to be a beneficial mode of treatment for psoriasis [39]; however, the potential adverse effects of this reaction to the skin include the development of melanomas and squamous cell carcinomas.

While the above examples illustrate non-enzymatic mechanisms by which compounds can be activated to radical intermediates, clearly the predominant activation pathways for most compounds are enzyme-catalyzed. The ability of a compound to be enzymatically metabolized to a radical intermediate can generally be ascribed to its one-electron reduction potential. Compounds with high electron affinity have the greater propensity for accepting electrons while compounds with lower electron affinity are less easily, or possibly not at all, reduced by biological systems [40]. For example, chloroform ($CHCl_3$) does not initiate microsomal lipid peroxidation apparently because of its low ability to undergo one-electron reduction [41], whereas carbon tetrachloride ($CCl_4$) can be reductively metabolized to the trichloromethyl free radical ($\cdot CCl_3$) which is capable of abstracting hydrogen atoms from unsaturated fatty acids and, consequently, initiating lipid peroxidation. In addition, $\cdot CCl_3$ may either bind covalently to microsomal protein and lipid or react with oxygen, yielding a peroxy free-radical ($CCl_3O_2\cdot$) which can interact with tryptophan and tyrosine [42]. Interestingly, $\cdot CCl_3$ does not interact with these amino acids. The reductive metabolism of $CCl_4$ to $\cdot CCl_3$ is catalyzed by the microsomal mixed-function oxidase system consisting of the flavoproteins, NADPH–cytochrome P-450 reductase and NADH–cytochrome $b_5$ reductase, and the terminal oxidase cytochrome P-450 [43].

In the case of $CCl_4$, cytochrome P-450 is required for its metabolism to the trichloromethyl free-radical while, on the other hand, adriamycin, mitomycin C, nitrofurantoin, paraquat and other compounds can be metabolized to their respective radical intermediates solely by NADPH–cytochrome P-450 reductase as demonstrated by studies using the purified enzyme or antibodies raised against it [44–47]. The formation of a radical intermediate by these compounds can be demonstrated using electron spin resonance spectroscopy; however, the radical spectra can only be observed under anaerobic conditions since, in the presence of oxygen, the reduced radical intermediate rapidly reoxidizes to the parent substrate (Fig. 2). Depending on the degree of cell or tissue oxygenation, the reduced radical species may be further reductively metabolized, by accepting a second electron, to metabolites which may also interact with biomolecules. In such instances, formation of the radical drug intermediate appears to be a requisite step. In the presence of oxygen, however, the univalent transfer of the electron from the radical intermediate to molecular dioxygen results in the generation of the superoxide anion and concomitant secondary reactive oxygen metabolites and possibly even radical species of endogenous constituents (Fig. 2). Clearly, if this chemical redox cycling continues for a sustained period, then cellular protective mechanisms may be overwhelmed, allowing these various radical species to accumulate and damage biomolecules. This possibility would be further accentuated if the reactive oxygen species themselves damaged or oxidized defense mechanisms. Indeed, copper–zinc superoxide dismutase has been shown to be inactivated by $O_2^{\cdot}$ and/or $H_2O_2$ [48–50], glutathione peroxidase by $\cdot OH$ [51], and ascorbic acid and $\alpha$-tocopherol by $O_2^{\cdot}$ [52, 53]. Similarly, the interaction of adriamycin or mitomycin C with intact mitochondria, submitochondrial particles, or purified mitochondrial NADH:ubiquinone oxidoreductase results in the enhanced generation of superoxide [54–56].

In addition to its well-known localization in the endoplasmic reticulum, mixed-function oxidase activity has also been shown to be associated with the nuclear membrane [57]. Since chromatin material is juxtaposed with the nuclear membrane, activation of compounds to radical intermediates at this site would certainly pose a significant threat to DNA, either through the direct interaction of the reactive metabolite with DNA or indirectly through the generation of reactive oxygen metabolites, or possibly even products resulting from lipid peroxidation of the nuclear membrane [58]. In this regard, Kennedy and Sartorelli [59] have demonstrated that mitomycin C can be metabolically activated by isolated nuclei. Similarly, the interaction of adriamycin or bleomycin with purified NADPH–cytochrome P-450 reductase results in DNA damage [60, 61].

In addition to the enzymes associated with the endoplasmic reticulum, mitochondria and nuclear membrane, various cytoplasmic enzymes have the ability to activate xenobiotics to either radical intermediates or metabolites capable of activating oxygen. For example, xanthine oxidase has been shown to metabolize adriamycin [62], misonidazole [63], nitrofurantoin [64] and paraquat [65] to their one-electron reduction products; whether this enzyme can maintain the redox cycling of these compounds for a sustained period has not been investigated. Similarly, superoxide from xanthine oxidase can convert 2-hydroxyestrogens [66] and $\alpha$-methyldopa [67] to reactive intermediates, possibly quinones and/or semiquinones capable of covalently binding to protein. In addition, radicals, quinones and/or semiquinones, which are intermediates in the oxidation of diethylstilbestrol (DES) and 9-hydroxyellipticine by peroxidases [68–70], have been implicated in the toxicity of DES to the hamster kidney and mouse uterus and the cytotoxicity of 9-hydroxyellipticine to L1210 leukemia cells. Reactive quinone metabolites are also produced as the result of the interaction of compounds with tyrosinase [71, 72], an enzyme found in high activity in melanomas. Furthermore, cytoplasmic diaphorases can facilitate the redox cycling of paraquat [73] and benzo[*a*]pyrene quinones [74] resulting in reactive oxygen generation.

There are two types of primary processes involved in the enzymatic activation of benzo[*a*]pyrene (BP) by microsomal membranes, epoxidation and formation of 6-hydroxy-BP which rapidly autoxidizes

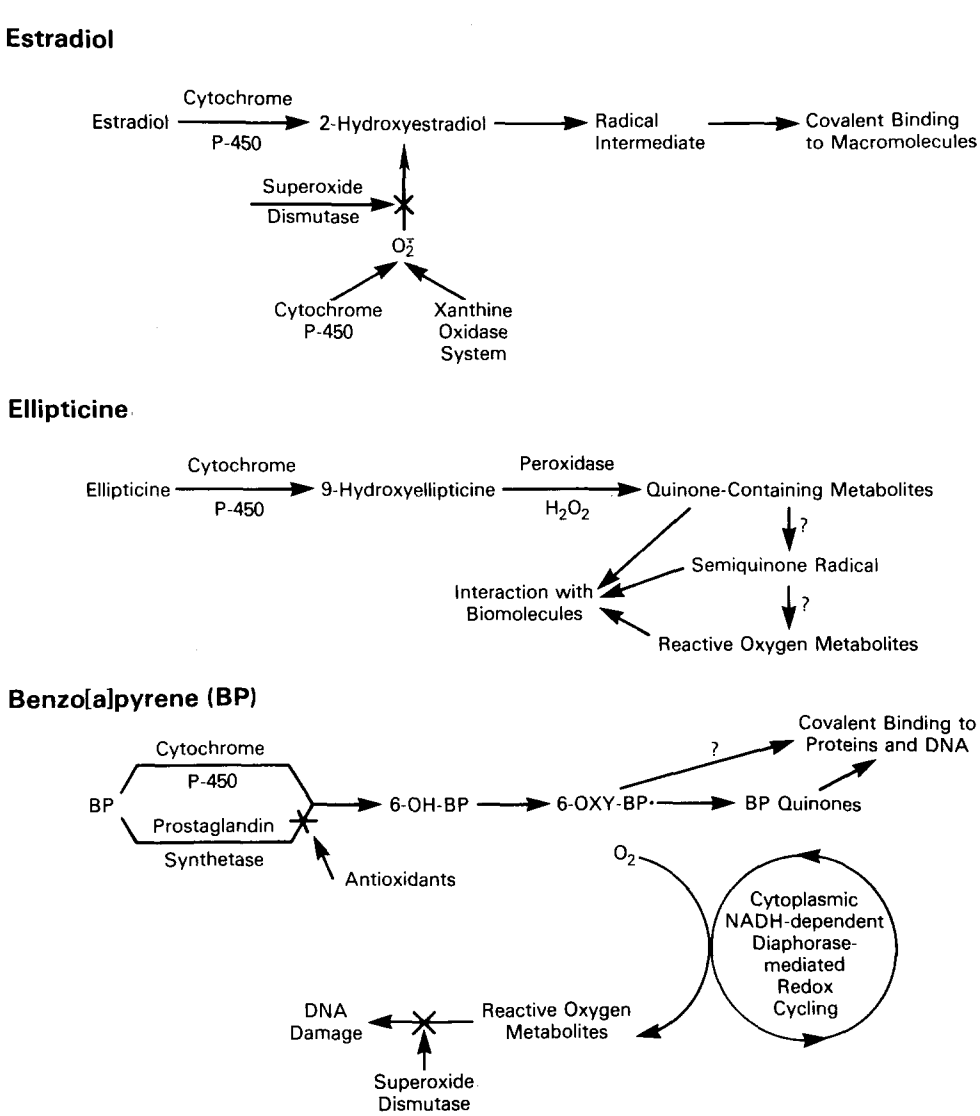



Fig. 3. Examples of compounds which may undergo enzymatic sequential metabolism to radical intermediates. The above examples illustrate that, within intact cells, the activation of a drug to a radical intermediate may result from the sequential metabolism by several enzymes.

to various quinone metabolites [75]. An intermediate in the autoxidation of 6-hydroxy-BP is the 6-oxy-BP radical which itself can interact with cellular macromolecules [76] in addition to molecular oxygen. Most of the investigations on the activation of BP have focused primarily on microsomal cytochrome P-450 and epoxide hydratase [77]; however, Marnett *et al.* [78] have shown recently that significant metabolism of BP to quinone metabolites results from the interaction with the arachidonic acid dependent prostaglandin-synthetase system. A number of other xenobiotics, including DES [79], acetaminophen [80] and the nitrofuran *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) [81], are also co-oxygenated through the peroxidative action of this enzyme system [82]; while it has not as yet been experimentally demonstrated, the generation of radical intermediates from these compounds by prostaglandin synthetase is a distinct possibility.

While the above discussion of the spectrum of enzymes which are capable of metabolizing drugs to radical intermediates is incomplete, it illustrates several key points relevant to the activation of xenobiotics in biological systems: once formed a radical intermediate may have several fates, possibly determined by the degree of tissue oxygenation; enhanced free radical generation may occur simultaneously at multiple sites within a cell; and the activation of xenobiotics to radicals may be the result of the sequential metabolism by several enzymes (Fig. 3). Similarly, not only may there be cooperativity between enzyme systems but chemical–chemical interactions may exist as well. For example, McManus and Davies [83] demonstrated that paraquat by virtue of its redox cycling capacity enhanced the superoxide-dependent cytochrome P-450 catalyzed covalent binding of dopa to microsomal protein. This observation demonstrates that reactive

oxygen generated during the redox cycling of one chemical may activate another compound to a reactive intermediate which is capable of damaging biomolecules. Such a drug–drug interaction may be the molecular basis for the enhancing effect of mitomycin C on bleomycin-mediated DNA damage and cytotoxicity [84, 85].

## RADICAL–TARGET INTERACTIONS WHICH COULD CONTRIBUTE TO ALTERATIONS IN CELLULAR FUNCTION

Although membrane lipids, free amino acids, nucleic acids, polysaccharides, enzymes, and receptor, structural and transport proteins have all been shown to be damaged by free radicals, the processes of lipid peroxidation and DNA damage are the best characterized. In the following two sections we will examine some concepts involved in the interaction of drug radical intermediates with these biochemical targets.

### *Redox cycling compounds and reactive oxygen-dependent lipid peroxidation*

Cytomembranes are largely composed of phospholipids (40–70% by dry weight), many of which contain unsaturated acyl fatty acids. Because of the unsaturated nature of the fatty acid chains of phospholipids and because allylic hydrogen atoms can easily be abstracted from methylene sites, cellular membranes are naturally predisposed to oxidation reactions mediated by radicals. This predisposition is amplified by the fact that molecular oxygen is more soluble (7- to 8-fold) in lipid hydrophobic regions than in hydrophilic areas. Thus, it is not unreasonable to expect that under normal oxygen tension any leakage of reactive oxygen from the electron transport systems associated with the endoplasmic reticulum or mitochondria could initiate peroxidation of membrane fatty acids. However, this "low-level leakage" of oxygen metabolites should be adequately controlled by cellular defense mechanisms such as $\alpha$-tocopherol. On the other hand, a significant increase in the flux of radical generation would result in the increased oxidation of this important radical scavenger. Significant peroxidation of membrane lipids would then have devastating effects on membrane fluidity and cell compartmentalization as manifested by an impairment of plasma membrane sodium–potassium ATPase activity [86], swelling of mitochondria [87], inhibition of microsomal mixed-function oxidase activity [88] and altered nuclear cytoplasmic communication [89]. Moreover, the toxic process of lipid peroxidation is amplified by the secondary formation of highly reactive and diffusable carbonyl products [90–93], which may, in fact, mediate some of these cellular changes. The demonstration that these products are stable enough to be isolated, chromatographed and, still, react with target molecules, in particular sulfhydryl-containing molecules [93], suggests that these products are capable of not only diffusing within the same cell but from cell to cell or from organ to organ.

Given that the ability of a compound to augment a reaction *in vitro*, such as lipid peroxidation, does not unequivocally establish that this same process would occur *in vivo*, certainly the failure to demonstrate a reaction *in vitro* subjects any hypothesis involving this reaction as a mechanism of *in vivo* toxicity to question. Since reactive oxygen and, in particular, the hydroxyl radical can initiate lipid peroxidation [94], it has been assumed that redox cycling compounds such as adriamycin, nitrofurantoin and paraquat are toxic because they stimulate reactive oxygen-mediated lipid peroxidation. Yet experimental evidence that these compounds do, in fact, produce tissue damage by virtue of their capacities to stimulate lipid peroxidation *in vivo* is presently lacking. Moreover, investigations designed to assess the ability of adriamycin or paraquat to stimulate *in vitro* microsomal lipid peroxidation have resulted in conflicting reports [44, 95–97].

In accord with the "balance concept" discussed previously, one would expect that before a radical intermediate such as $\cdot CCl_3$ or reactive oxygen metabolites could initiate membrane lipid peroxidation, $\alpha$-tocopherol levels would have to be decreased significantly; only then could radical intermediates and polyunsaturated fatty acids of membrane phospholipids interact and lipid peroxidation result. This is supported by the demonstration that microsomes isolated from $\alpha$-tocopherol-deficient animals are much more susceptible to radical-initiated lipid peroxidation than those from controls [98]. Thus, in regard to the ability of redox cycling compounds to augment microsomal lipid peroxidation, it is important to recognize that in order for reactive oxygen to create such an imbalance, it is necessary that radical generation be maintained for a sufficient period prior to observing any enhancement in lipid peroxidation. Theoretically, this lag in the time course of lipid peroxidation should correspond to the time it takes to decrease the microsomal $\alpha$-tocopherol level and, as such, should vary depending on the microsomal preparation since considerable species and organ differences are found in the $\alpha$-tocopherol levels of microsomes [99]. In this context, one of the consequences of the *in vitro* interaction of redox cycling compounds with microsomal NADPH–cytochrome P-450 reductase is a massive stimulation of NADPH oxidation [12, 19, 100]. Thus, should the NADPH be consumed prior to significantly depleting microsomal $\alpha$-tocopherol, then reactive oxygen generation would abate and endogenous lipid peroxidation would not be stimulated and perhaps even be inhibited. Failure to consider this possibility may result in erroneous conclusions as to the potential of such compounds to mediate microsomal lipid peroxidation *in vitro*. Therefore, *in vitro* studies designed to evaluate the ability of a redox cycling compound to stimulate lipid peroxidation in microsomes or other subcellular fractions must be conducted in the presence of relatively high concentrations of NADPH. The above points are emphasized in a recent series of papers from our laboratory which demonstrates that adriamycin and other anthracyline drugs, mitomycin C, nitrofurantoin and paraquat all stimulate reactive oxygen-dependent lipid peroxidation in microsomes isolated from various organs [101–105]. Moreover, the stimulation of lipid peroxidation by these compounds was significantly enhanced in microsomes isolated from

α-tocopherol-deficient animals. Considering that radical-generating compounds can enhance lipid peroxidation in intact cells [106] it appears that sufficient reducing equivalents are available to catalyze such a reaction *in vivo*.

It is important to note that the microsomal experiments described above were conducted under an oxygen atmosphere. The oxygen serves two functions: (1) molecular oxygen is an efficient electron acceptor from the enzymatically reduced radical species (Fig. 2) and (2) the possibility of further reductive metabolism of the compound is lessened or eliminated by ensuring a fully aerobic environment. Relative to this latter point, it has been our experience that, during the incubation of rat liver microsomes with adriamycin under air, the augmentation of lipid peroxidation is consistently less than under oxygen [101] and considerable quantities of aglycone metabolites are formed, a metabolic reaction which occurs only after an anaerobic environment is well established [107]. The fact that adriamycin aglycones are formed *in vivo* [108] suggests that the degree of tissue oxygenation, in addition to cellular defense mechanisms, may be an important factor in the ability of redox cycling compounds to initiate reactive oxygen-dependent lipid peroxidation *in vivo*. Thus, those tissues such as the heart and lung which are well oxygenated may be more susceptible to this process. In addition, while reductive metabolites formed anaerobically from nitrofurantoin bind covalently to pulmonary microsomal protein *in vitro*, significant *in vivo* binding of these metabolites in the lung does not occur [64]. Conversely, the pulmonary toxicity of nitrofurantoin is enhanced by oxygen exposure [33].

With the demonstration that adriamycin and paraquat can indeed significantly stimulate lipid peroxidation *in vitro*, the question arises as to whether the *in vivo* cytotoxicity of these compounds can be traced to reactive oxygen-dependent lipid peroxidation. Further, how much lipid peroxidation has to occur in an intact cell to initiate alterations in cellular function, especially in light of the fact that even a slight stimulation of lipid peroxidation may be significantly amplified by the aforementioned reactive carbonyl products [90–93]? Since glutathione functions as a defense against these peroxidized lipid-originating reactive carbonyl products [93], the potential for these compounds to interact with critical biomolecules would be greatly enhanced if cellular stores of glutathione were depleted. This could occur either through the direct interaction of glutathione with radicals or indirectly as a result of the excretion of glutathione disulfide (GSSG) from cells following the oxidation of glutathione by glutathione peroxidase. Interestingly, the excretion of GSSG in isolated perfused organs is enhanced by the infusion of hydroperoxides [109, 110]. Similarly, the infusion of paraquat into an isolated perfused liver brings about an increased release of GSSG, although this was interpreted to be an indirect effect of paraquat because at the time, there was some question as to the ability of paraquat to directly stimulate lipid peroxidation [111, 112]. Since reduction of GSSG by glutathione reductase is NADPH dependent, this sequence of events may be a link between the lipid peroxidation hypothesis [113], the toxic-product hypothesis [90–93] and the NADPH depletion hypothesis [114] as mechanisms for the toxicity of redox cycling compounds.

*The "bound-oxidant" concept and bleomycin-mediated DNA damage*

Redox cycling compounds are able to alter biomolecules and initiate changes in cellular function by significantly augmenting the flux of free $O_2^{\cdot}$, $H_2O_2$ and ·OH within cells. In contrast, the effects of other agents may be mediated through the formation of a "bound-oxidant"; that is, an activated molecule or complex having reactivity comparable to reactive oxygen species—probably the hydroxy radical—but not being released from its site of generation free into solution. Cederbaum *et al.* [115] have discussed this concept in relationship to the microsomal metabolism of alcohols, while Egan *et al.* [116] have proposed that the protaglandin synthetase-mediated co-oxygenation of certain substrates occurs via a "bound-oxidant". Another example of this concept and the one for which the details of the reaction at the molecular level have been most thoroughly characterized is the demonstration by Hodgson and Fridovich [48, 49] that an intermediate stage in the hydrogen peroxide-initiated inactivation of the copper–zinc form of superoxide dismutase (CuZn SOD) catalyzes the oxidation of a variety of substrates, including phospholipids. Moreover, they observed that when these substrates were present the inactivation of CuZn SOD was inhibited.

The mechanism suggested by these workers for the inactivation of CuZn SOD was that $H_2O_2$ first reduces the $Cu^{2+}$ of the SOD and then subsequently reacts with the $Cu^{+}$ to generate an enzyme-bound oxidant, possibly ENZ-$Cu^{2+}$-OH· or ENZ-$Cu^{2+}$-O·. This intermediate species would then react with the imidazole ring of histidine, resulting in ring opening and subsequent release of the copper, which is required for enzyme activity. These investigators further postulated that the oxidized imidazole ring is a radical, in an electronically excited state, that could return to ground state by photon emission (chemiluminescence) or alternatively by interacting with suitable acceptor or target molecules. Thus, it is through this alternate utilization of the electronic excited energy that the rupture of the imidazole ring and subsequent inactivation of CuZn SOD would be prevented. The sequence of molecular events described for this "activation–inactivation" of CuZn SOD closely resembles the postulated molecular mechanism through which the antineoplastic agent, bleomycin, may mediate deoxyribose cleavage and subsequent base release from DNA. Bleomycin-mediated damage to DNA is believed to account not only for its cytotoxic action toward tumor cells [117] but toward lung cells as well [85, 188].

Bleomycin is a specialized molecule in that it has a moiety which recognizes and binds to specific regions of DNA and a separate and distinct region which binds metals [119]; however, the binding of bleomycin to DNA is not in itself sufficient to damage DNA. Studies in cell-free chemical systems have clearly demonstrated that bleomycin-induced DNA

damage is dependent on iron and oxygen as requisite co-factors [119]. Initially, it was proposed that the oxidation/reduction of adventitious iron bound to bleomycin resulted in the generation of "free" reactive oxygen species and that these species mediated the DNA damage [120, 121]. However, subsequent investigations by the Horwitz group at Albert Einstein have suggested that, in fact, "free" reactive oxygen species are not generated but that an activated ternary complex of bleomycin, iron and oxygen, possibly having the configuration of Blm-$Fe^{3+}$-OH·, mediates the DNA damage [122]. Moreover, if this complex is generated in the absence of target molecules (DNA), then the activated bleomycin decays spontaneously to a species which can no longer damage DNA; supposedly this inactivated bleomycin can no longer properly interact with iron [123].

According to this proposed model, the role that the bleomycin molecule plays in the damage to DNA is passive; that is, it functions to transport and selectively position iron in the DNA chain. However, the demonstration that the interaction of bleomycin with $Fe^{2+}$ results in the generation of chemiluminescence [124] and that the time course of this chemiluminescence coincides with the time course of bleomycin-mediated DNA damage in this chemical system suggests that following the formation of the bleomycin–iron–oxygen complex some portion of the bleomycin molecule itself may be activated to a labile, reactive intermediate in an electronically excited state. Moreover, such a bleomycin intermediate may be a radical, as has been suggested for the chemiluminescent compounds luminol [125] and luciferin [126]. Thus, like the "activated intermediate" of CuZn SOD, bleomycin utilizes electronic excited energy to initiate DNA damage, possibly through hydrogen abstraction as recently reported by Giloni *et al.* [127]. In support of this contention is the demonstration that hydrogen abstraction from DNA by acetone in an electronically excited state results in the production of alkali-sensitive sites [128, 129]. In accord with this hypothesis, bleomycin would then be an active, rather than a passive, participant in causing DNA damage. Moreover, these observations with CuZn SOD, bleomycin and acetone suggest that the activation of compounds to intermediates in an electronically excited state may be more widespread than we presently realize and may, in fact, represent an efficient mechanism by which compounds alter biomolecules. Indeed, activated intermediates in an electronically excited state have been proposed for benzo[*a*]pyrene [130, 131], 6-hydroxydopamine [132], imipramine [133, 134], retinoic acid [135] and spermine [136].

## SELECTIVE CELL AND ORGAN TOXICITY DUE TO FREE RADICALS: IMPLICATIONS FOR CANCER CHEMOTHERAPY

Many of the compounds that form free radicals demonstrate selective toxicity for certain organs and even specific cell types within these organs. As shown in Table 1, such selectivity is determined by a number of factors, both tissue and compound related, including: the relative potential for a compound to be activated to a radical intermediate; the presence of enzyme systems capable of activating the compound; the presence and relative activities of biochemical and enzyme defense mechanisms; and the capacity to repair xenobiotic-mediated damage. In addition to these factors, the degree of cell oxygenation could play an important role in determining the mechanism of cell damage. Many of these concepts which explain the selectivity for xenobiotic-induced toxicity are best exemplified by neoplastic cells. It is interesting to note that many of the drugs currently being used in cancer chemotherapy are activated to radical intermediates, including adriamycin, mitomycin C, misonidazole and bleomycin.

In comparison to host cells, neoplastic cells have been shown to be relatively deficient in the CuZn and Mn forms of superoxide dismutase [137], glutathione [138] and glutathione peroxidase [138]. Thus, by their very nature, neoplastic cells would appear to be lacking in many defense mechanisms and, hence, inherently more susceptible to the cytotoxic effects initiated by radical generating compounds. Interestingly, Oberley *et al.* [139] have proposed an intriguing hypothesis, suggesting that this inherent deficiency in superoxide dismutase could be an integral component in the development of the neoplastic state. Moreover, based on this concept, they have proposed that synthetic compounds with superoxide dismutase-like activity may be utilized to treat neoplasms.

Solid tumors contain both well-oxygenated and hypoxic regions [140], and, given that redox cycling compounds can initiate damage to biomolecules by several modes (Fig. 2), the mechanism of radical-mediated cytotoxicity could vary. Indeed, drugs such as bleomycin, adriamycin and mitomycin C demonstrate a cytotoxic selectivity for either well-oxygenated or hypoxic cells [141]. Therefore, the possibility exists that, for some drugs, one radical-initiated mechanism may account for its chemotherapeutic effect and another mechanism for its selective and oftentimes dose-limiting toxicity to host tissues. For example, mitomycin C is reductively metabolized under anaerobic conditions by the mixed-function oxidase system to a bifunctional DNA alkylating agent [142], but in an aerobic environment the same enzyme system catalyzes the redox cycling of mitomycin C, resulting in reactive oxygen-dependent lipid peroxidation [105]. In addition, other redox cycling compounds, such as the nitroheterocyclic compound misonidazole, are being developed not so much for their cytotoxic effects, but rather because they increase the sensitivity of hypoxic tumor cells to ionizing radiation [143].

Hypoxic cells are relatively resistant to the cytotoxic effects of ionizing radiation although the generation of ·OH and ·H from water is not oxygen dependent. Besides interacting with biomolecules, the ·OH and ·H can recombine to form water, a reaction which is minimized in the presence of oxygen [22]. This process accounts, in part, for the enhancing effect of oxygen on radiation-initiated cell damage. More important perhaps is the demonstration that $O_2$ can react with hydroxyl radical-induced radical sites on DNA to form non-repairable peroxides [144]; however, under reduced oxygen tension, these radical sites on DNA can be repaired

Table 1. Compounds which exhibit selective cell and tissue toxicity

| Compound | Target Organ/Tissue | Possible Factors Contributing to Selective Toxicity | |
|---|---|---|---|
| | | Tissue Related | Mechanism |
| Adriamycin | Heart, Tumors | Deficiency in cellular defenses; decreased ability to metabolize adriamycin to aglycone | NADPH-cytochrome P450 reductase-dependent formation of semiquinone radical; generation of reactive oxygen metabolites |
| Alloxan | Pancreas | Selective uptake and accumulation in beta cells | Autoxidation of reduced form of alloxan, dialuric acid; generation of reactive oxygen metabolites |
| Bleomycin | Lung, Sensitive Tumors | Low levels of bleomycin hydrolase; high levels of parent compound | Interaction with iron; microsomal-mediated conversion to an activated complex which damages DNA |
| Diethylstilbestrol | Kidney (Syrian hamster), Uterus (Mouse) | Presence of peroxidase | Peroxidase-mediated metabolism to quinones and catechols; covalent binding of metabolites to proteins and DNA; potential for metabolites to redox cycle |
| γ-L-Glutaminyl-4-Hydroxybenzene | Melanocarcinomas | High levels of tyrosinase | Tyrosinase-mediated metabolism to quinones; covalent binding of metabolites to biomolecules; potential for metabolites to redox cycle |
| 6-Hydroxydopamine | Neuronal | Selective uptake and accumulation in peripheral adrenergic and central catecholaminergic neurons | Autoxidation to quinones; covalent binding to biomolecules; generation of reactive oxygen metabolites |
| Paraquat | Lung | Selective uptake and accumulation in target cells | NADPH-cytochrome P450 reductase-dependent formation of bipyridinium cation radical; generation of reactive oxygen metabolites |

through hydrogen-donation from thiols such as glutathione. It has been proposed that hypoxic cell radiosensitizers such as misonidazole may function much like $O_2$ by forming a drug–DNA complex that may not be repairable [22]. In addition, incubation of cells with misonidazole significantly reduces the intracellular levels of glutathione [22] possibly through the interaction of glutathione with the nitro anion radical intermediate of misonidazole [40].

While neoplastic cells may be more sensitive to radical-forming drugs, unfortunately cancer patients themselves are likely to be predisposed to the organ-specific host toxicities elicited by such drugs. Two possible factors are the age of the patients and their nutritional status; activities of radical defense mechanisms are known to decline with age [145] and cancer patients suffer nutritional deficiencies as a result of cachexia and as a side-effect of chemotherapy. Cellular levels of α-tocopherol, ascorbic acid and the selenium for glutathione peroxidase are maintained through the diet. In addition, previous chemotherapy may alter cellular defense mechanisms and increase the potential for radical-initiated reactions by either subsequent administration of the same drug or a different radical-forming compound [56]. In this regard also, prior irradiation to the thorax has been shown to increase the potential for adriamycin-induced cardiotoxicity and bleomycin-induced pulmonary toxicity [146].

To minimize the potential for such life-threatening complications, it is imperative that we have a thorough understanding of the interactions which may contribute to the toxicity elicited by these compounds. Moreover, through the careful characterization and understanding of factors, both biological and chemical, which account for the selectivity of

radical-generating antineoplastic drugs to both host and neoplastic tissues, rational pharmacologic approaches may then be developed to increase the therapeutic effectiveness of these compounds and at the same time reduce the potential for undesirable toxic effects. Similarly, through such research efforts it should be possible to rationally design and synthesize compounds to exploit those particular characteristics of neoplastic cells which facilitate the interaction between radicals and biomolecules, with the goal of increasing selective cytotoxic effectiveness.

## CONCLUDING REMARKS

Intermediary metabolism is based on the orderly channeling of electrons; concomitantly, the various flavo- and heme-proteins involved in cellular metabolism "leak" electrons resulting in the formation of potentially toxic oxygen radicals. Fortunately, aerobic cells have evolved a number of protective mechanisms to ensure their survival in an oxygen atmosphere. Thus, because of these defense mechanisms, the cellular generation of oxygen free radicals may not be acutely harmful; however, it has been hypothesized that the long-term, low-level generation of free radicals and any cumulative, non-repaired damage which results may be the natural chemical basis of ageing, mutagenesis and carcinogenesis [147, 148]. Clearly, the activation of chemicals to reactive metabolites and, in particular, to radical intermediates is a burgeoning area within biochemical pharmacology. As discussed in this commentary, any number of diverse compounds can interact with various enzyme systems resulting in the enhanced generation of radicals; depending on the nature of the cell such actions can be viewed as beneficial or toxic (Fig. 1). Because of the potential for a beneficial therapeutic action of radical-forming compounds, the challenge facing researchers in various disciplines is to learn to control and manipulate this process.

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