

Cytotoxicity of the Redox Cycling Compound Diquat in Isolated Hepatocytes: Involvement of Hydrogen Peroxide and Transition Metals

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Received April 8, 1987, and in revised form July 15, 1987

Diquat is a hepatotoxin whose toxicity *in vivo* and *in vitro* is mediated by redox cycling and greatly enhanced by pretreatment with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), an inhibitor of glutathione reductase. The mechanism by which redox cycling mediates diquat cytotoxicity is unclear, however. Here, we have attempted to examine the roles of three potential products of redox cycling, namely superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), in the toxicity of diquat to BCNU-treated isolated hepatocytes. Addition of high concentrations of catalase, but not superoxide dismutase, to the incubations provided some protection against the toxic effect of diquat, but much better protection was observed when catalase was added in combination with the iron chelator desferrioxamine. Addition of desferrioxamine alone also provided considerable protection, whereas the addition of copper ions enhanced diquat cytotoxicity. Taken together, these results indicate that both H_2O_2 and the transition metals iron and copper could play major roles in the cytotoxicity of diquat. The role of O_2^- remains less clear, however, but studies with diethylenetriaminepentaacetic acid indicate that O_2^- is unlikely to significantly contribute to the reduction of Fe^{3+} to Fe^{2+} . The hydroxyl radical or a related species seems the most likely ultimate toxic product of the H_2O_2/Fe^{2+} interaction, but hydroxyl radical scavengers afforded only minimal protection. © 1987 Academic Press, Inc.

Numerous foreign compounds are readily converted by one-electron reduction to free radical intermediates which then react with dioxygen. This reaction regenerates the parent compound and converts the dioxygen to superoxide anion radical (O_2^-)² (1). Thus, in the presence of a

supply of reducing equivalents, a small amount of such a chemical can generate large amounts of O_2^- by this redox cycling process (2). The toxicities of several redox cycling compounds, most notably the quinones menadione and adriamycin, have been studied extensively in isolated hepatocytes (3-6). Efforts to characterize the intracellular consequences of active oxygen generation in these studies have, however, been complicated by the fact that

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² Abbreviations used: O_2^- , superoxide anion radical; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GSH, reduced glutathione; GSSG, glutathione disulfide; $^{\bullet}OH$, hydroxyl radical; DEM, diethyl maleate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesul-

fonic acid; TBA, thiobarbituric acid; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid.

menadione and adriamycin participate in many other reactions, such as alkylation and conjugation. In the present study the action of another redox cycling compound, the herbicide 1,1'-ethylene-2,2'-bipyridylium ion (diquat), has been investigated in isolated hepatocytes. Unlike menadione or adriamycin, diquat does not participate in alkylation or conjugation reactions, and is therefore a valuable investigative tool for the study of oxidative damage resulting solely as a consequence of active oxygen generation (7).

We recently compared the relative abilities of diquat and other bipyridyl herbicides to generate active oxygen species in rat liver microsomes and found diquat to be the most potent (8). In addition, diquat was shown to be an effective inducer of oxidative stress in freshly isolated hepatocytes whose oxidative defenses had been compromised by pretreatment with low concentrations of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a relatively specific inhibitor of glutathione reductase (8). In BCNU-treated hepatocytes reduced glutathione (GSH) cannot be regenerated from the oxidized disulfide (GSSG), hence the ability of glutathione peroxidase to prevent the accumulation of hydrogen peroxide (H_2O_2) is impaired (8, 9). In BCNU-treated hepatocytes with inhibited glutathione reductase but normal glutathione levels, exposure to diquat results in rapid GSH oxidation, stimulation of lipid peroxidation, and loss of cell viability (8). This study extends this work and examines the importance of O_2^- , H_2O_2 , and the transition metals iron and copper in diquat cytotoxicity.

MATERIALS AND METHODS

Chemicals. Highly purified diquat (>99%) was a kind gift of Dr. L. L. Smith, I. C. I. plc (UK). BCNU was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, the National Cancer Institute (Bethesda, MD). Ebselen was a gift of A. Nattermann & Co. GmbH (Cologne, FRG). Desferrioxamine was obtained from Ciba-Geigy and manufactured by Ben Venue Labs (Bedford, OH) as deferoxamine mesylate. Collagenase, superoxide dismutase, and catalase were from Boehringer (Mannheim, FRG). All other chemicals were of the highest grade available from local suppliers.

Preparation of isolated rat hepatocytes. Male Sprague-Dawley rats weighing 200–240 g were allowed food and water *ad lib*. All rats received sodium phenobarbital (1 mM) in their drinking water for 5–10 days prior to use. Hepatocytes were isolated by collagenase perfusion as described by Moldeus *et al.* (10), yielding $240\text{--}300 \times 10^6$ cells per liver. Viability, as measured by the exclusion of 0.2% (w/v) trypan blue (10) was routinely 95–99% in freshly isolated preparations.

Pretreatment with BCNU and diethyl maleate (DEM). Freshly isolated hepatocytes were pretreated with 50 μ M BCNU for 20 min and then allowed to recover GSH levels in methionine (1 mM)-supplemented medium, essentially as described previously (8, 11). Glutathione reductase inhibition, assayed according to Roos *et al.* (12) was 90–95% following BCNU pretreatment, and viability was decreased to approximately 75–80% postrecovery. Cellular GSH was depleted in some experiments by pretreating freshly isolated hepatocytes with 0.3 mM DEM for 30 min. DEM pretreatment lowered cellular GSH to approximately 20% of control values.

Hepatocyte incubations. All incubations were performed at 10^6 cells/ml in rotating round-bottom flasks at 37°C under an atmosphere of 95% O_2 /5% CO_2 in equilibrated Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (10, 13).

Biochemical assays. Total levels of GSH and GSSG were determined in 1-ml samples of cell incubate by HPLC following derivatization with iodoacetic acid and Sanger's reagent, as described in (14). Alternatively, GSH levels were determined as acid-soluble thiols by the method of Saville (15). Lipid peroxidation was assayed as thiobarbituric acid (TBA)-reactive products as previously described (8, 16).

RESULTS

Diquat undergoes rapid redox cycling in isolated hepatocytes, and creates a condition of oxidative stress within the cell. Cytotoxicity is not readily manifested, however, unless a primary defense against oxidative stress, the glutathione peroxidase/reductase system, has been compromised. This is illustrated in Table I where the addition of 1.5 mM diquat to control (uncompromised) hepatocytes failed to significantly affect either cellular GSH levels or viability, as assessed at 15 and 60 min, respectively. Pretreatment of hepatocytes with DEM lowered cellular GSH to 20% of control levels, but did not significantly alter the time course of diquat cytotoxicity (Table I). However, when hepa-

TABLE I

EFFECT OF DIQUAT ON GSH CONTENT AND VIABILITY OF CONTROL, DEM-PRETREATED, AND BCNU-PRETREATED HEPATOCYTES

Treatment	No. expt.	GSH ^a (nmol/10 ⁶ cells)		Toxicity (% trypan blue uptake)	
		0 min	15 min	0 min	60 min
Control ^b	5	50 ± 4 ^f	46 ± 5	8 ± 6 ^f	16 ± 5
Control + diquat ^e	5	55 ± 5	49 ± 10	8 ± 6	25 ± 10
DEM ^c	3	10 ± 2	11 ± 2	11 ± 4	32 ± 1
DEM + diquat ^e	3	10 ± 2	14 ± 3	11 ± 4	27 ± 4
BCNU ^d	15	42 ± 2	40 ± 3	18 ± 4	25 ± 5
BCNU + diquat ^e	15	40 ± 2	7 ± 3	18 ± 4	67 ± 11

^a Reduced glutathione was determined by HPLC after derivatization with iodoacetic acid and Sanger's reagent as described in (14).

^b Cells were isolated by collagenase perfusion and incubated in Krebs-Henseleit buffer (see Materials and Methods).

^c Upon isolation, cells were pretreated with 0.3 mM DEM for 30 min (see Materials and Methods).

^d Upon isolation, cells were pretreated with 50 μ M BCNU for 20 min, followed by recovery of GSH levels (see Materials and Methods).

^e 1.5 mM diquat.

^f Values represent $\bar{x} \pm$ SD of n experiments.

tocytes were pretreated with BCNU, an irreversible inhibitor of glutathione reductase, significant toxicity was manifest within 60 min (Table I). As previously reported (8), this loss of viability was dependent upon the redox cycling of diquat, and was preceded by a rapid depletion of cellular GSH (Fig. 1) and an equally rapid increase in GSSG.

In order to examine the involvement of O_2^- and H_2O_2 in diquat cytotoxicity, superoxide dismutase (SOD) and catalase were added to BCNU-compromised hepatocyte incubations. SOD, added at 733 U/ml, provided no protection against diquat cytotoxicity (Fig. 1). Addition of catalase at 650 U/ml also had no protective effect, although addition at 1300 U/ml did delay both GSH depletion and the expression of cytotoxicity (Fig. 1). The addition of boiled catalase had no protective effect (data not shown). Interestingly, the addition of SOD (733 U/ml) in combination with the lower concentration of catalase (650 U/ml) afforded greater protection against GSH loss than that provided by the higher catalase concentration alone. The expression

of cytotoxicity was, however, only briefly delayed by the coaddition of SOD and catalase (Fig. 1).

The seleno-organic compound 2-phenyl-1,2-benzisoselenazol-3(*H*)-one (ebselen; PZ51) has been demonstrated to possess both glutathione peroxidase-like activity and a separate antioxidant activity in

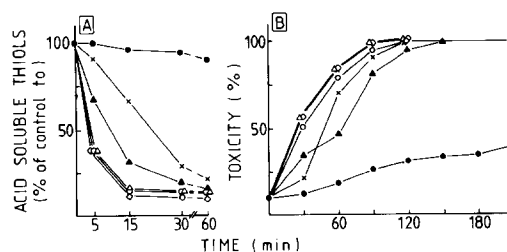


FIG. 1. Effect of SOD and catalase on diquat-induced depletion of GSH (A) and cytotoxicity (B). BCNU-pretreated hepatocytes, prepared as described under Materials and Methods, were incubated with no additions (●), or in the presence of 1.5 mM diquat, either alone (○), or with 733 U/ml SOD (◇), 650 U/ml catalase (Δ), 1300 U/ml catalase (▲), or 733 U/ml SOD and 650 U/ml catalase (×). Results shown are from one experiment typical of three.

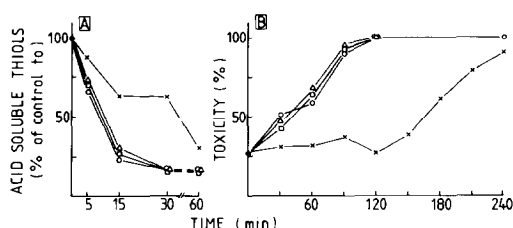


FIG. 2. Protective effect of ebselen and GSH on diquat-induced depletion of GSH (A) and cytotoxicity (B). BCNU-pretreated hepatocytes, prepared as described under Materials and Methods, were incubated in the presence of 1.5 mM diquat, with either no further additions (○), 50 μM ebselen (□), 1 mM GSH with further 0.5 mM additions at 30, 60, and 90 min (Δ), or 50 μM ebselen and 1 mM GSH with further 0.5 mM additions at 30-min intervals (×). Results shown are from one experiment typical of three.

studies with rat liver microsomes and hepatocytes (17, 18). As shown in Fig. 2, the addition of ebselen in combination with exogenously added GSH provided significant protection against both diquat-induced GSH loss and cytotoxicity in BCNU-compromised cells. When ebselen was added in the absence of exogenous GSH no protection was observed; similarly, GSH added alone also had no protective effect (Fig. 2).

Formation of the short-lived, but highly reactive and potentially damaging $\cdot\text{OH}$ may result from either a direct interaction between O_2^- and H_2O_2 or a transition metal-catalyzed reaction (19). The possible involvement of $\cdot\text{OH}$ in diquat cytotoxicity was investigated initially by adding two $\cdot\text{OH}$ scavengers, mannitol and di-

methyl sulfoxide (DMSO), to diquat-treated hepatocytes. Mannitol addition at either 0.2 or 0.05 M did not provide significant protection against diquat cytotoxicity (results not shown). Cells incubated in the presence of DMSO (1.75%), on the other hand, were protected somewhat against diquat cytotoxicity during the initial hour of incubation (Fig. 3B), but neither GSH depletion, nor lipid peroxidation were affected by DMSO addition (Figs. 3A, 3C).

The potential for an interaction between active oxygen species and transition metals in diquat cytotoxicity was then investigated by addition of the iron chelator desferrioxamine. As shown in Fig. 4A, 100 μM desferrioxamine did not protect against GSH depletion and 1 mM desferrioxamine only slightly delayed the depletion of GSH. Both concentrations of desferrioxamine did, however, inhibit lipid peroxidation, and both significantly delayed the expression of diquat-induced cytotoxicity (Figs. 4B, 4C). Desferrioxamine at 1 mM afforded no more protection against cytotoxicity than it did at 100 μM.

Two other iron chelators, diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA), did not offer any significant protection against diquat cytotoxicity (Fig. 5). DTPA (1 mM) only slightly delayed diquat-induced GSH depletion, cytotoxicity, and lipid peroxidation. The addition of EDTA (1 mM) failed to protect against either GSH depletion or lipid peroxidation, and diquat-induced cytotoxicity was actually

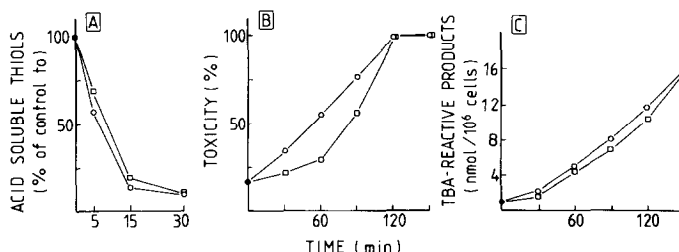


FIG. 3. Effect of DMSO on diquat-induced depletion of GSH (A), cytotoxicity (B), and accumulation of TBA-reactive products (C). BCNU-pretreated hepatocytes, prepared as described under Materials and Methods, were incubated with 1.5 mM diquat in the absence (○) or presence (□) of DMSO (final concentration 1.75%). Results shown are from one experiment typical of three.

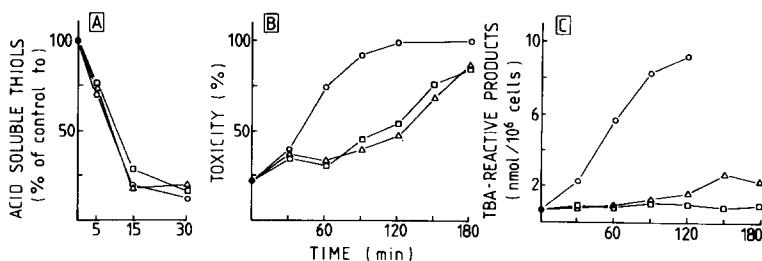


FIG. 4. Protective effect of the iron chelator desferrioxamine against diquat-induced depletion of GSH (A), cytotoxicity (B), and accumulation of TBA-reactive products (C). BCNU-pretreated hepatocytes, prepared as described under Materials and Methods, were incubated in the presence of 1.5 mM diquat, with either no further additions (○), 100 μM desferrioxamine (Δ), or 1 mM desferrioxamine (□). Results shown are from one experiment typical of three.

slightly enhanced by its inclusion. Addition of an Fe^{2+} /DTPA complex (150 μM FeSO_4 /150 μM DTPA) also enhanced cytotoxicity, but interestingly, GSH depletion was delayed to almost the same extent as in the presence of 1 mM DTPA.

The possible interaction of another transition metal, copper, with diquat-generated active oxygen species was then investigated. Copper (10 μM) was added as CuSO_4 to BCNU-compromised hepatocytes incubated in the presence of a lower and less toxic concentration (0.5 mM) of diquat. As shown in Fig. 6, the addition of 10 μM Cu^{2+} dramatically enhanced the cytotoxicity of diquat, killing 80% of the hepatocytes within the first 30 min of incubation, whereas only 40% of the cells were dead after 30 min in the absence of Cu^{2+} . Diquat-induced toxicity was enhanced by copper addition even in the presence of the previously protective iron chelator, des-

ferrioxamine (Fig. 6). Although copper itself is cytotoxic to hepatocytes, the loss of cell viability following the addition of 10 μM CuSO_4 to control incubations was not expressed during the first 90 min of incubation (results not shown).

As shown in Figs. 1 and 4, neither the addition of 100 μM desferrioxamine nor the addition of catalase at 650 U/ml had any significant effect on diquat-induced GSH depletion. However, when catalase and desferrioxamine were added together at these concentrations, a dramatic protection against the depletion of GSH was observed (Fig. 7). The extent to which GSH depletion was delayed was similar to that observed following the coaddition of SOD and catalase (Fig. 1). Although SOD/catalase did not provide prolonged protection against cytotoxicity, coaddition of desferrioxamine and catalase delayed diquat-induced cytotoxicity to a greater

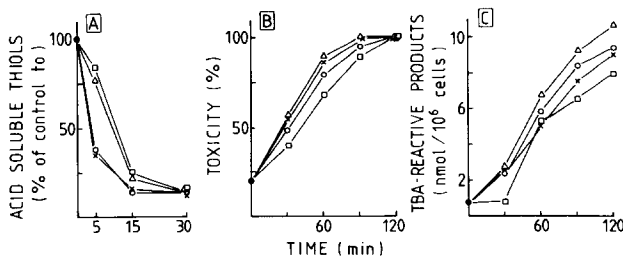


FIG. 5. Effect of DTPA and EDTA on diquat-induced depletion of GSH (A), cytotoxicity (B), and accumulation of TBA-reactive products (C). BCNU-pretreated hepatocytes, prepared as described under Materials and Methods, were incubated in the presence of 1.5 mM diquat, with either no further additions (○), 1 mM DTPA (□), 150 μM DTPA and 150 μM FeSO_4 (Δ), or 1 mM EDTA (×). Results shown are from one experiment typical of three.

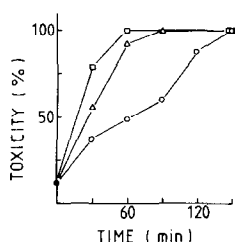


FIG. 6. Addition of the transition metal copper enhances diquat-induced cytotoxicity. BCNU-pretreated hepatocytes, prepared as described under Materials and Methods, were incubated in the presence of 0.5 mM diquat, with either no further additions (○), 10 μ M CuSO_4 (□), or 10 μ M CuSO_4 and 100 μ M desferrioxamine (Δ). Results shown are from one experiment typical of three.

extent than the single addition of either desferrioxamine or catalase (Fig. 7).

DISCUSSION

Diquat was employed here as a model redox cycling compound capable of generating large amounts of O_2^- and H_2O_2 within cells, and thus creating a condition of oxidative stress, without participating in alkylation or conjugation reactions. Isolated rat hepatocytes are well protected against oxidative stress, however, and diquat-induced cytotoxicity is not readily expressed unless cellular defenses against active oxygen are compromised. The pretreatment of hepatocytes with DEM diminished glutathione levels to approximately 20% of normal cellular GSH levels, but these cells were far less susceptible to diquat-induced injury than BCNU-pretreated cells possessing normal levels of GSH but only trace amounts of glutathione reductase activity. In control and DEM-pretreated cells the ability of glutathione reductase to regenerate GSH from the oxidized form (GSSG) is apparently so great that merely catalytic amounts of glutathione are sufficient to detoxify H_2O_2 and other hydroperoxides via glutathione peroxidase. The demonstrated importance of this GSH-dependent detoxification system in protecting hepatocytes against diquat-induced cytotoxicity implicates H_2O_2 as a major mediator of diquat toxicity within this *in*

vitro model system. Recent studies by Eklöv-Låstbom *et al.* (20) confirm the importance of GSH in protecting against diquat cytotoxicity in hepatocytes.

The relative importance of the redox cycling products O_2^- and H_2O_2 in mediating diquat-induced cytotoxicity was studied by adding SOD and catalase to hepatocyte incubations. Although the efficacy of exogenously added SOD and catalase was limited by the inability of either enzyme to pass through the hepatocyte plasma membrane, extracellular enzyme activity would provide protection against active oxygen species present in the extracellular environment. Indeed, the ability of both the protonated form of O_2^- , $\text{HOO}^\bullet$, and H_2O_2 to readily diffuse across membranes suggests that a component of the cytotoxic injury observed in our compromised hepatocyte system may be extracellular. The lack of protection afforded by exogenous SOD suggested that extracellular O_2^- does not contribute significantly to diquat cytotoxicity, although the possibility exists that added SOD was inactivated by H_2O_2 , thus masking any protection. The small protection observed with the addition of catalase at the higher concentration (1300 U/ml) suggested that some H_2O_2 is released from the cell and does contribute to cell death. A protective effect of extracellular catalase has previously been demonstrated in other studies performed with

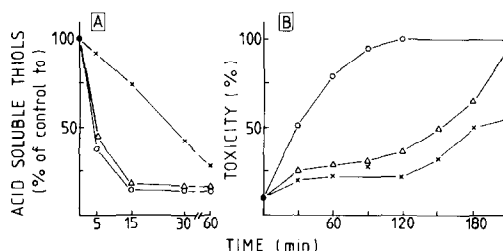


FIG. 7. Enhanced protection against diquat-induced depletion of GSH (A) and cytotoxicity (B) by coaddition of desferrioxamine and catalase. BCNU-pretreated hepatocytes, prepared as described under Materials and Methods, were incubated in the presence of 1.5 mM diquat, with either no further additions (○), 100 μ M desferrioxamine (Δ), or 100 μ M desferrioxamine and 650 U/ml catalase (X). Results shown are from one experiment typical of three.

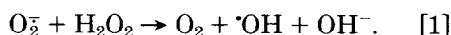
cultured cells exposed to an active oxygen challenge (21, 22).

In addition to delaying the onset of diquat-induced cytotoxicity, high levels of catalase also delayed depletion of intracellular GSH. This sparing effect on GSH suggested that extracellular catalase activity favors H_2O_2 efflux from the cell, effectively lowering the intracellular H_2O_2 concentrations and thus the demand for GSH (Fig. 8). Although addition of catalase at a lower concentration (650 U/ml) had no effect upon diquat-induced GSH loss or cytotoxicity, coaddition of 650 U/ml catalase with SOD reduced the rate of GSH depletion and briefly delayed the expression of cytotoxicity. The mechanism by which this catalase/SOD combination so dramatically delayed the oxidation of cellular glutathione is unknown but the following scenario seems most likely. The activity of extracellular SOD would favor O_2^- efflux as $\text{HOO}^\cdot$, thereby reducing the amount of O_2^- present within the cell. Such a reduction in intracellular O_2^- content would limit H_2O_2 formation within the cell, yet generation of H_2O_2 outside the cell would be enhanced by the activity of extracellular SOD. Diffusion of this extracellular H_2O_2 across the plasma membrane and into the cell would still result in a rapid loss of cellular GSH, unless this H_2O_2 influx was either prevented or diminished. Extracellular catalase activity could result in just such a diminution in H_2O_2 influx. Although the initial maintenance of GSH by coaddition of catalase

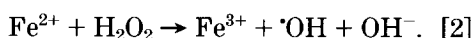
and SOD may explain the 30 min delay observed in the expression of diquat cytotoxicity, cellular GSH was nevertheless depleted during that 30 min period, and unabated cytotoxicity was expressed soon afterward (Fig. 1).

Addition of ebselen, a synthetic compound possessing GSH peroxidase-like activity (17), protected against diquat cytotoxicity when present in conjunction with a supply of extracellular GSH. Coaddition of ebselen and GSH also delayed the diquat-induced depletion of cellular GSH, while no protection was observed with the addition of exogenous GSH in the absence of ebselen. These protective effects of ebselen suggest that H_2O_2 is involved in diquat cytotoxicity, and have been further explored in Ref. (23).

H_2O_2 can react with O_2^- in a Haber-Weiss reaction (24) (Eq. [1]) to yield the highly reactive $^\cdot\text{OH}$, which may be the most damaging product of redox cycling (25, 26):



Alternatively, H_2O_2 may participate in a transition metal-catalyzed Fenton reaction (Eq. [2]), also yielding $^\cdot\text{OH}$:



Although hydroxyl radical scavengers are not regarded as being either very efficient or specific, we tested the effects of mannitol and DMSO on diquat cytotoxicity. Only the addition of DMSO provided any protection against toxicity. The mini-

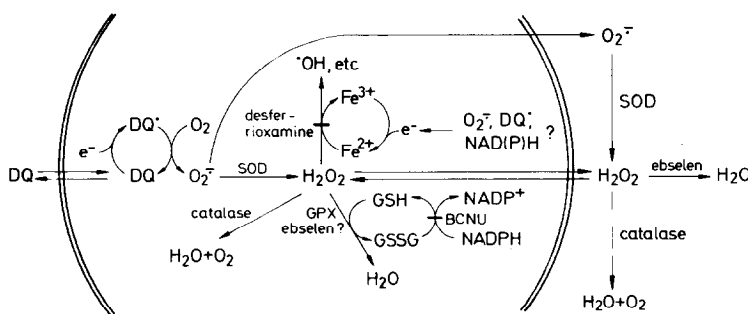


FIG. 8. Scheme of postulated interactions between diquat, diquat-derived active oxygen species, internal and external enzymes, and transition metals. DQ, diquat; DQ $^\cdot$, diquat radical; GPX, glutathione peroxidase.

mal protection afforded by DMSO does not preclude a role for $\cdot\text{OH}$ in the toxicity of diquat, however, since the scavenging action of DMSO may result in the formation of a methyl radical which could also contribute to cell damage, lipid peroxidation, and cytotoxicity (27). The site of formation of $\cdot\text{OH}$ within the cell may also have a significant effect upon the accessibility of $\cdot\text{OH}$ to scavengers, and the subsequent efficacy of those scavengers (28).

The dramatic protection observed following desferrioxamine addition provided further evidence for the involvement of $\cdot\text{OH}$ or a closely related species in diquat toxicity. Desferrioxamine chelates intracellular iron in the Fe^{3+} state with an affinity constant of 10^{31} (29), effectively blocking reduction (mediated by O_2^- , the diquat radical, or other cellular reductants) and thus preventing the participation of iron in the Fenton reaction (Eq. [2]). EDTA, which chelates iron but does not prevent its reduction, can actually stimulate the Fenton reaction (30). The enhancement of cytotoxicity observed following EDTA addition further supports a role for Fenton-type reactions in diquat toxicity.

The marked effect of copper ion addition upon diquat-induced toxicity further established the importance of transition metals in this *in vitro* hepatocyte model. Cytotoxicity was greatly enhanced in the presence of added Cu^{2+} . Not surprisingly, this effect was only slightly diminished by the addition of desferrioxamine, since desferrioxamine does not block the reduction of copper from Cu^{2+} to Cu^+ . This suggests that desferrioxamine's principal protective action is indeed the chelation of iron, and not the inhibition of lipid peroxidation or the direct scavenging of $\cdot\text{OH}$ (31).

In conclusion, these results indicate that H_2O_2 and iron play important roles in the *in vitro* cytotoxicity of diquat, and suggest that $\cdot\text{OH}$ may be the ultimate toxin. The continued production of $\cdot\text{OH}$ via the Fenton reaction requires an adequate supply of both H_2O_2 and transition metals in the reduced state (e.g., Fe^{2+} , Cu^+). Continuous transition metal reduction is therefore es-

sential. O_2^- can reduce Fe^{3+} to Fe^{2+} in chemical systems (32), but Fe^{3+} reduction via O_2^- does not appear to occur to any significant extent in diquat-treated hepatocytes, since the addition of DTPA to chelate iron and slow the reduction of Fe^{3+} by O_2^- (19) provided little protection against diquat cytotoxicity. Although DTPA-chelated Fe^{3+} is relatively unreactive with O_2^- , reaction with other reductants (e.g., ascorbic acid, reducing equivalents from NADPH-cytochrome *P*-450 reductase, or the diquat radical) to form the ferrous chelate can still occur. O_2^- may still play a role in the release of iron from cellular stores, however, and contribute to $\cdot\text{OH}$ generation indirectly (33, 34), although it has recently been shown that bipyridylum radicals can also do this (35). Further work is therefore needed to fully characterize the possible contributions of O_2^- to the cytotoxicity of the bipyridyl herbicide diquat, as well as for its congener paraquat.

ACKNOWLEDGMENTS

This work was supported by the National Foundation for Cancer Research, the Northern California Occupational Health Center, I.C.I. plc (UK), and the Swedish Medical Research Council. We thank Dr. D. Di Monte and Ms. P. Doane-Setzer for their assistance in some of the experiments.

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