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ROLE OF REDOX CYCLING AND LIPID PEROXIDATION IN BIPYRIDYL HERBICIDE CYTOTOXICITY

STUDIES WITH A COMPROMISED ISOLATED HEPATOCYTE MODEL SYSTEM

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Abstract—The role of active oxygen species and lipid peroxidation in the toxic effects of diquat, paraquat and other bipyridyl herbicides remains controversial. *In vitro* studies have shown that these compounds are potent generators of active oxygen species by redox cycling and that they stimulate lipid peroxidation. *In vivo* studies have failed, however, to show clear evidence of lipid peroxidation resulting from toxic exposures to these compounds. We have directly compared the abilities of three bipyridyl herbicides, diquat (DQ), paraquat (PQ) and benzyl viologen (BV), to generate superoxide anion radical ($O_2^{\cdot-}$) in rat liver microsomes and H_2O_2 in hepatocytes and correlated this with their relative toxicities to a compromised isolated hepatocyte system. DQ was the most potent generator of $O_2^{\cdot-}$ and H_2O_2, being slightly more potent than BV and much better than PQ. This ability of the bipyridyls to generate active oxygen was positively correlated with the ability to induce toxicity in hepatocytes pretreated with 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) to inhibit their glutathione reductase activity, i.e. DQ > BV > PQ. DQ caused a rapid depletion of cellular GSH and a concomitant increase in GSSG in this system. Toxicity, measured as loss of plasma membrane integrity, was pronounced after only 30–60 min of incubation and was accompanied by a significant increase in lipid peroxidation. The onset of lipid peroxidation could not be separated temporally from the expression of toxicity. However, the total inhibition of lipid peroxidation by the antioxidants Trolox C, promethazine and *N,N'*-diphenyl-*p*-phenylenediamine only delayed toxicity, indicating that, even though lipid peroxidation may play some role in enhancing bipyridyl herbicide toxicity, it is not essential for the toxicity to manifest itself.

The bipyridyl herbicides, which include paraquat (1,1'-dimethyl-4,4'-bipyridilium ion) and diquat (1,1'-ethylene-2,2'-bipyridilium ion), are widely used in the U.S. and other parts of the world. Exposure to high levels of these compounds produces lung, liver and kidney injury [1, 2]. For example, paraquat produces mainly lung injury due to its selective accumulation but also produces hepatic jaundice [2]. Diquat, on the other hand, is mainly hepatotoxic, especially in selenium-deficient animals [3]. Both paraquat and diquat, as well as other bipyridyls, are readily converted by one electron-reduction to free radicals which react very rapidly with dioxygen [4]. This reaction regenerates the native bipyridyl and converts the dioxygen to superoxide anion radical ($O_2^{\cdot-}$) [5]. Thus, in the presence of a good supply of reducing equivalents, a small amount of bipyridyl can generate large amounts of $O_2^{\cdot-}$ by this redox cycling process [5]. The importance of redox cycling in paraquat and diquat toxicity remains controversial, however, largely because one would expect the $O_2^{\cdot-}$ produced to stimulate lipid peroxidation, but some *in vivo* studies have failed to show increased rates of lipid peroxidation during paraquat toxicity (e.g. Ref. 6). Paraquat and diquat, however, do stimulate lipid peroxidation *in vitro*, notably in liver and lung microsomes [7, 8], and have also been shown to redox cycle *in vivo* [9].

1,3-bis(2-Chloroethyl)-1-nitrosourea (BCNU) is a relatively specific inhibitor of glutathione reductase [10, 11]. The addition of BCNU just prior to, or concurrently with, H_2O_2 and various redox cycling compounds, including Adriamycin and menadione, has been shown to produce synergistic toxic effects on freshly isolated hepatocytes [11], cultured hepatocytes [12], tumor cells [13, 14] and cultured endothelial cells [15]. BCNU does, however, deplete cellular reduced glutathione (GSH) in addition to inhibiting glutathione reductase, and a contribution by the former cannot be ruled out in the above studies. Recently, Eklow *et al.* [16] reported the development of a BCNU-treated isolated hepatocyte system with inhibited glutathione reductase but normal glutathione levels. Here we report the use of this compromised hepatocyte model with redox cycling bipyridyl herbicides, which do not alkylate cellular macromolecules, and demonstrate that redox cycling is essential for cytotoxicity but that lipid peroxidation plays a non-essential augmentative role.

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MATERIALS AND METHODS

Chemicals. Highly pure (>99%) paraquat and diquat were gifts of Dr. L. L. Smith, CTL, I.C.I. (U.K.) plc. Benzyl viologen and promethazine were obtained from Sigma (St. Louis, MO). BCNU was supplied by the NationalCancer Institute (Bethesda, MD). *N,N'*-Diphenyl-*p*-phenylenediamine was from Eastman Kodak (Rochester, NY). Collagenase, superoxide dismutase and NADPH were from Boehringer (Mannheim, FRG). Trolox C was from Aldrich (Milwaukee, WI). Succinoylated cytochrome *c* was prepared as described in Ref. 17. All other chemicals were of the highest purity available.

Preparation of microsomes and isolated hepatocytes from rat liver. Male Sprague–Dawley rats weighing 200–240 g were allowed food and water *ad lib*. Some of the rats received sodium phenobarbital (1 mM) in their drinking water for 5–10 days prior to use. For the preparation of hepatic microsomes, the rats were killed by cervical dislocation, and the livers were perfused with saline and homogenized with a polytron. Microsomes were prepared from the homogenate as described by Ernster *et al.* [18]. Isolated hepatocytes were prepared by collagenase perfusion [19]. This isolation technique routinely yields 240–300 × 10^6 cells, that are 95–99% viable. Viability was measured by the exclusion of 0.2% (w/v) trypan blue [19].

BCNU pretreatment. The procedures used were essentially those originally described by Eklow *et al.* [16] with only minor modifications. Freshly isolated hepatocytes were resuspended in pH 7.4 Dulbecco's MEM (Minimal Eagle Medium, Gibco) supplemented with 1 mM methionine at 10^6 cells/ml, and placed in rotating round bottom flasks at 37° under 95% O_2/5% CO_2. BCNU was suspended in ethanol and added to a final concentration of 50 μM. After 20 min of incubation in the presence of BCNU, the cells were centrifuged at 50 *g*, the MEM/BCNU medium was removed, and the cells were resuspended in fresh MEM containing 1 mM methionine. This methionine-supplemented MEM enhances the recovery of glutathione levels in the hepatocytes. The recovery was allowed to continue for 75 min. The cells were then centrifuged again at 50 *g*, the MEM/methionine medium was removed, and the cells were resuspended in Krebs–Henseleit buffer. Typically, 40–50% of the cells was lost during the above treatment, and viability was decreased to approximately 75–80%. Glutathione reductase activity in the BCNU-treated cells was inhibited >90%. Reduced glutathione levels in the treated cells, following recovery in the presence of methionine, were normal, i.e. approximately 50 nmoles GSH/10^6 cells. Surface morphology was normal, and the cells remained viable for an additional 5 hr in Krebs buffer.

Hepatocyte incubations. All incubations were performed at 10^6 cells/ml in rotating round bottom flasks at 37° 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-piperazine-ethanesulfonic acid (HEPES) [19, 20]. One set of experiments was performed at 4° by placing the rotating flasks in iced water.

Biochemical assays. The bipyridyl-stimulated generation of $O_2^{\cdot-}$ in rat liver microsomes was determined using succinoylated cytochrome *c*, NADPH and superoxide dismutase as described in Ref. 21. Total levels of GSH and GSSG were determined in samples of cell incubate as described in Ref. 22. Briefly, samples containing 10^6 cells were treated with perchloric acid to precipitate protein, and the supernatant fraction containing acid soluble thiols was neutralized, derivatized with iodoacetic acid and Sanger's reagent, and analyzed by high performance liquid chromatography (HPLC) [22]. For simplicity, in some experiments GSH levels were determined as acid soluble thiols by the method of Saville [23]. Protein was determined according to Lowry *et al.* [24]. Lipid peroxidation was assayed as thiobarbituric acid (TBA)-reactive products [25]. Briefly, 0.5-ml aliquots of cell incubate (10^6 cells/ml) were added to 0.5 ml of 30% (w/v) trichloroacetic acid containing 1 mM butylated hydroxytoluene. The samples were kept on ice for 30 min and centrifuged at 1000 *g*; aliquots of the supernatant fraction were heated to 100° with equal volumes of TBA (182 mg/25 ml) for 15 min. The samples were then cooled, and absorbance was measured at 535 nm. The amount of TBA-reactive products formed was determined using an extinction coefficient of 156 mM^{-1} cm^{-1} [26].

RESULTS

Comparative studies with paraquat, diquat and benzyl viologen. The structures of the three bipyridyls, paraquat (PQ), diquat (DQ) and benzyl viologen (BV), are shown in Fig. 1. The relative abilities of these three bipyridyls to redox cycle and generate $O_2^{\cdot-}$ in rat liver microsomes are shown in Fig. 2. These results show that DQ was by far the best generator of $O_2^{\cdot-}$ under these conditions, being almost eight times better than PQ. BV was intermediate between these two, being three times better at generating $O_2^{\cdot-}$ than PQ (Fig. 2). The results for PQ and DQ are in close agreement with those of Gage [4], who measured free radical generation.

In BCNU-treated hepatocytes, the formation of GSSG is a measure of oxidative challenge to the cells. The major mechanism of bipyridyl-induced oxidative challenge is the generation of $O_2^{\cdot-}$ and its subsequent dismutation to H_2O_2. The removal of H_2O_2 via glutathione peroxidase is at the expense of the conversion of GSH to GSSG [27], but in BCNU-treated cells the catalytic cycle regenerating GSH

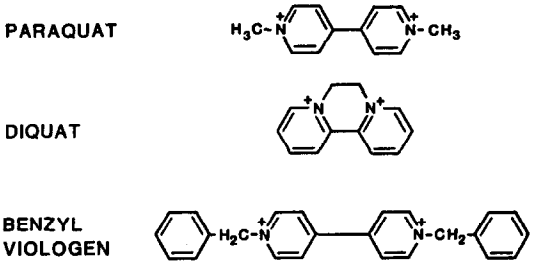

Fig. 1. Bipyridyl structures.

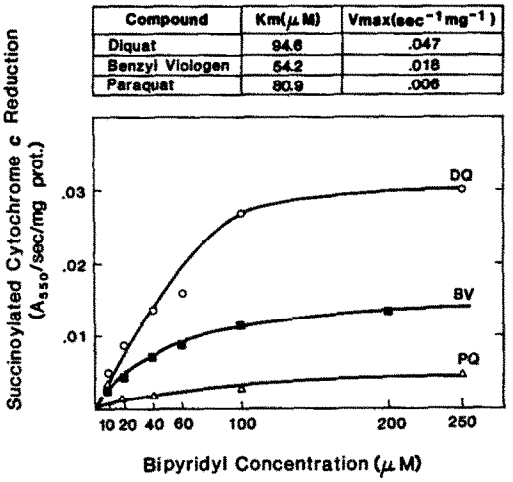

Fig. 2. Bipyridyl-stimulated generation of superoxide anion radical in rat liver microsomes. Superoxide generation was measured spectrophotometrically at 550 nm as the rate of reduction of succinoylated cytochrome *c*. Specificity of the reaction was checked by addition of 0.2 mg/ml superoxide dismutase (SOD). Various concentrations of the bipyridyls diquat (○), benzyl viologen (■) and paraquat (△) were added to cuvettes containing 1 ml of the reaction mixture (0.1 M Tris–HCl buffer at pH 7.4 and 25°, containing 50 mM HCl, 1 mM NADPH, 0.2 mg/ml succinoylated *c* and 0.1 mg/ml microsomal protein).

from GSSG via glutathione reductase is blocked. The GSSG generated in BCNU pretreated cells is therefore a reflection of the amount of H_2O_2 produced by redox cycling [21]. Measurement of GSSG formation in samples of BCNU-treated cells incubated in the presence of equimolar concentrations of the three bipyridyls indicated that DQ, BV and PQ redox cycle in intact hepatocytes at relative rates similar to those observed in microsomes, i.e. DQ > BV > PQ (Fig. 3B). Intracellular concentrations of the different bipyridyls cannot be assumed to be equivalent, however, and structural considerations suggest that BV, with its hydrophobic benzyl substituents, may cross cell membranes to a greater extent than DQ or PQ. Nevertheless, both the relative toxicity (DQ ≥ BV > PQ) (Fig. 3C) and the relative amounts of lipid peroxidation (DQ ≥ BV > PQ) (Fig. 3D) produced by equimolar doses of the bipyridyls in BCNU-treated hepatocytes correlate with the abilities of the compounds to redox cycle and generate active oxygen species in this model hepatocyte system.

Further studies on the role of redox cycling. Figure 4 shows that exposures to increasing concentrations of diquat caused correspondingly greater amounts of cytotoxicity, lipid peroxidation and GSH loss in BCNU-treated hepatocytes. These results further point to a significant role for GSH depletion and redox cycling in diquat cytotoxicity, as do the data presented in Fig. 5. These data show that, if BCNU-treated hepatocytes were incubated at 4° and exposed to diquat, no significant toxicity or GSH loss was observed. Thus, GSH loss is not the result of a direct interaction of diquat with GSH, but rather an event which requires a metabolically active system. Moreover, when hepatocytes were incubated at 37° under a nitrogen atmosphere, very little GSH loss was observed compared to that observed under an oxy-

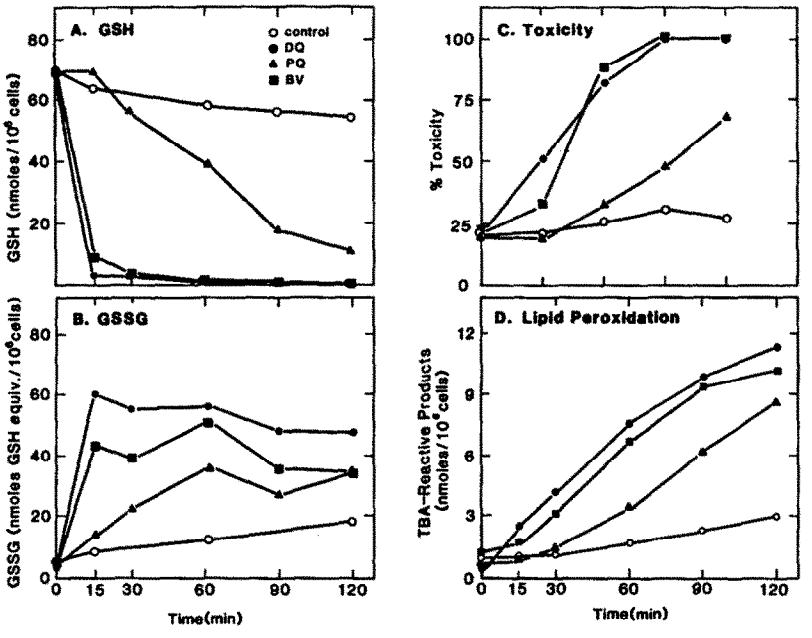

Fig. 3. Effects of different bipyridyls on GSH depletion (A), GSSG formation (B), toxicity (C) and lipid peroxidation (D) in BCNU-treated hepatocytes. Hepatocytes were isolated from phenobarbital-induced rats and treated with BCNU as described in Materials and Methods. They were then incubated at 10^6 cells/ml in Krebs–Henseleit buffer, pH 7.4, with no additions (○), 1.5 mM diquat (●), 1.5 mM paraquat (▲) or 1.5 mM benzyl viologen (■). Levels of reduced (GSH) and oxidised (GSSG) glutathione were measured by HPLC. Results shown are from one experiment, typical of three.

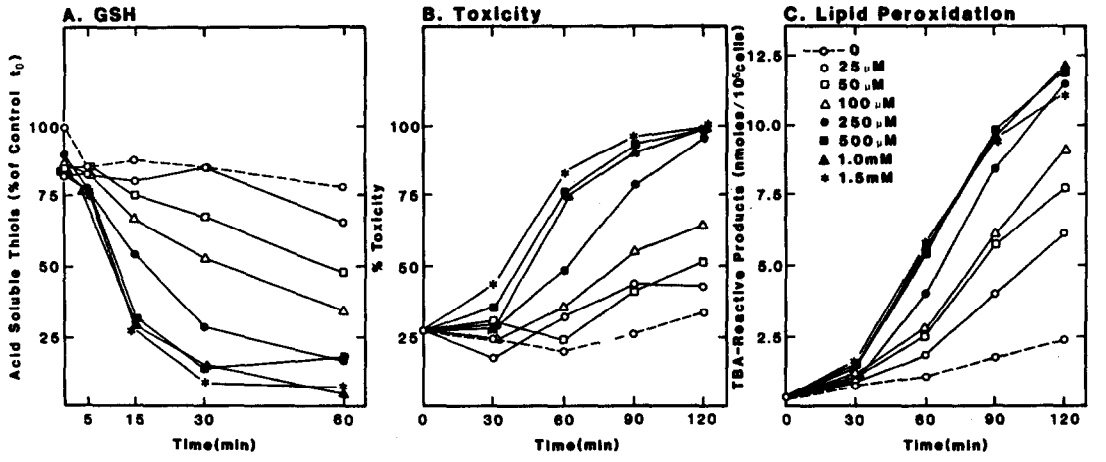

Fig. 4. Effect of various concentrations of diquat on GSH depletion (A), toxicity (B) and lipid peroxidation (C) in BCNU-treated hepatocytes. Hepatocytes were isolated from phenobarbital-induced rats and treated with BCNU as described in Materials and Methods. They were then incubated at 10^6 cells/ml in Krebs–Henseleit buffer, pH 7.4, with 0 μM (--○--), 25 μM (—○—), 50 μM (—□—), 100 μM (△), 250 μM (●), 500 μM (■), 1 mM (▲) and 1.5 mM (—*—) diquat. GSH levels were measured as acid soluble thiols and expressed as a percentage of control levels determined immediately prior to incubation with diquat, i.e. t_0. Results shown are representative of a typical experiment.

gen containing atmosphere (Fig. 5). This further points to the importance of redox cycling and active oxygen in causing the diquat-induced GSH loss and cytotoxicity.

Importance of glutathione reductase in protecting against diquat cytotoxicity. Incubation of control hepatocytes from phenobarbital-treated rats with 1.5 mM diquat induced no signficant increase in GSH loss, GSSG formation or toxicity (Fig. 6). If, however, hepatocytes were pretreated with BCNU and allowed to recover their GSH, they became extremely susceptible to the toxic effects of diquat, losing most of their GSH within 15 min. From then on, they began dying at a rapid rate so that they were nearly all dead within 90 min (Fig. 6). These results demonstrate the importance of glutathione reductase in maintaining normal thiol homeostasis and viability in hepatocytes following exposure to diquat. Furthermore, they suggest that DQ is being cytotoxic via oxidative mechanisms, such as the generation of O_2^- and its dismutation to H_2O_2, since BCNU inhibition of glutathione reductase also causes inhibition of glutathione peroxidase activity, the main defense of the hepatocyte against H_2O_2 formed in the cytoplasm [27].

Role of lipid peroxidation in diquat cytotoxicity. If redox cycling is important for the toxicity of the bipyridyl herbicides, one would also expect that the

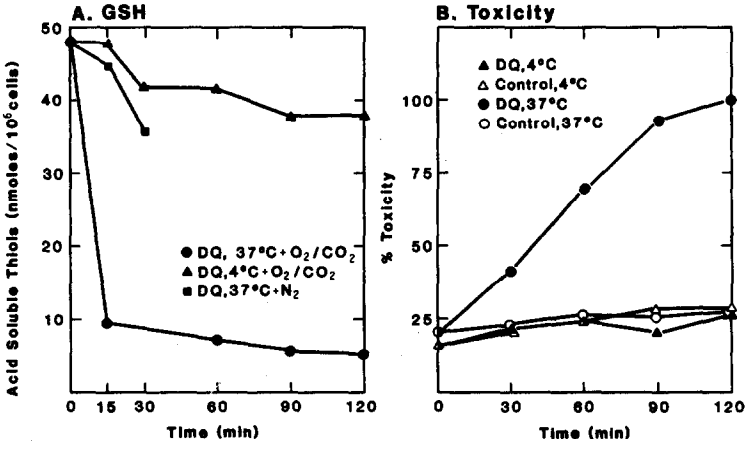

Fig. 5. Importance of redox cycling in diquat-induced GSH loss (A) and toxicity (B) to BCNU-treated hepatocytes. Hepatocytes were isolated from phenobarbital-induced rats and treated with BCNU as described in Materials and Methods. They were then incubated at 10^6 cells/ml in Krebs–Henseleit buffer, pH 7.4, under an O_2/CO_2 (19:1, v/v) atmosphere at 37° (○, ●) or at 4°C (△, ▲) or under a nitrogen atmosphere at 37° (■). Hepatocytes were incubated with 0 mM (○, △) or 1.5 mM (●, ▲, ■) diquat. GSH levels were determined as acid soluble thiols.

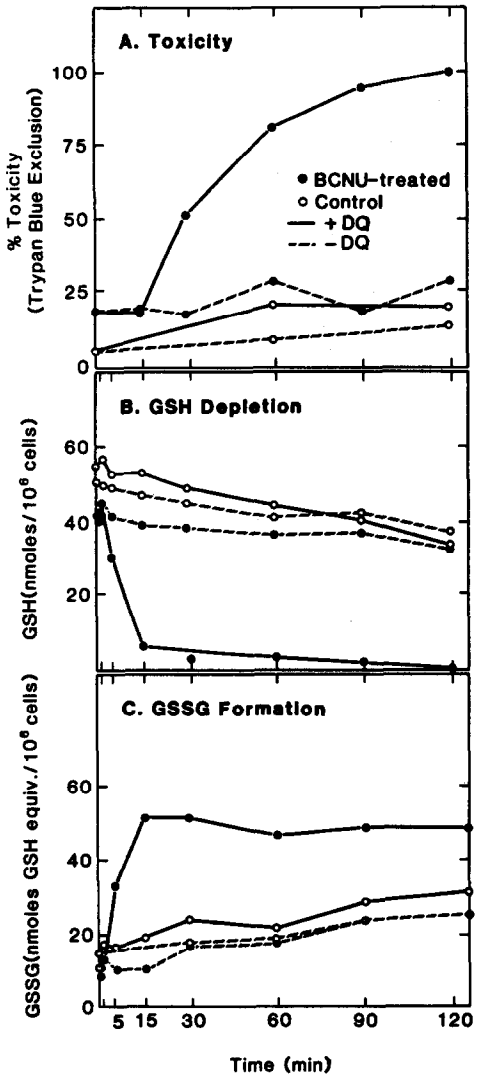

Fig. 6. Effect of BCNU treatment on diquat-induced toxicity (A), GSH depletion (B) and GSSG formation (C) in isolated hepatocytes. Hepatocytes isolated from phenobarbital-induced rats were treated with either 0 μM (○) or 50 μM (●) BCNU as described previously. Hepatocytes (10^6 cells/ml) were then incubated in Krebs–Henseleit buffer, pH 7.4, with 0 mM (---) or 1.5 mM (——) diquat. GSH and GSSG levels were measured by HPLC. Results shown are from one experiment, typical of three.

active oxygen species produced would stimulate lipid peroxidation. Whilst this has been observed in microsomal fractions *in vitro* [7, 8], some *in vivo* studies have failed to show increased lipid peroxidation during paraquat toxicity [6]. Figure 3D shows that lipid peroxidation certainly accompanied the toxicity of DQ, PQ and BV in BCNU-treated hepatocytes, but its relative importance cannot be ascertained because it came neither well before nor well after measurable toxicity, unlike peroxidation produced by some other agents [25, 28]. The role of lipid peroxidation in DQ cytotoxicity was therefore investigated by using three very potent antioxidants, namely DPPD, Trolox C and promethazine. DPPD and Trolox C did not affect DQ-induced GSH loss (Fig. 7A) but totally blocked lipid peroxidation (Fig. 7D) in BCNU-treated hepatocytes at the concentrations shown. Both antioxidants delayed, but did not prevent, DQ cytotoxicity (Fig. 7C). Promethazine did have some effect on DQ-induced GSH loss, possibly by interacting with the diquat cation radical or O_2^- directly, and it also totally blocked lipid peroxidation (Fig. 7, A and D). Promethazine was therefore slightly more effective at delaying DQ cytotoxicity than DPPD or Trolox C (Fig. 7C), but it did not completely prevent toxicity. None of the antioxidants had any toxic effects of their own on hepatocytes at the concentration used. Taken together, these results suggest that lipid peroxidation plays only an augmentative role in DQ cytotoxicity, rather than being absolutely essential to produce toxic effects.

DISCUSSION

By slightly modifying the procedures originally described by Eklow *et al.* [16], an isolated hepatocyte model with compromised defenses against oxidative stress has been further refined. This model is based upon pretreatment of the hepatocytes with BCNU and incubation to allow recovery of GSH homeostasis. Given the relatively specific effect of BCNU on glutathione reductase [10], the marked potentiation of diquat cytotoxicity by BCNU pretreatment shown here demonstrates the importance of this enzyme and the glutathione redox state in general, in protecting against diquat and active oxygen cytotoxicity. This marked potentiation further suggests that H_2O_2, or a reactive product(s) derived therefrom, is playing a central role in diquat and other bipyridyl herbicide cytotoxicity.

Using the compromised isolated hepatocyte model, it has also been demonstrated here that the rate of redox cycling of either different bipyridyls or different concentrations of diquat correlates well with both GSH loss and cytotoxic effects. The cyclic single electron reduction/oxidation of the parent molecule, therefore, seems to be a critical mechanistic event in bipyridyl herbicide cytotoxicity. Further evidence for this was provided by experiments performed at 4° and under an N_2 atmosphere, where conditions unfavourable for redox cycling resulted in an inhibition of the toxic effects of diquat.

The important role of redox cycling in bipyridyl herbicide cytotoxicity demonstrated here and previous findings which show that exposure to elevated oxygen concentrations increases the lethality of paraquat to both rats and mice [29–31] clearly suggest that active oxygen is involved in diquat and paraquat cytotoxicity. However, the toxicological consequences of redox cycling and active oxygen production are far less clear. One effect of active oxygen could be the peroxidation of membrane lipids, as has been demonstrated in numerous studies using subcellular fractions (e.g. Refs. 7 and 8). The role of lipid peroxidation in bipyridyl herbicide cytotoxicity remains controversial, however, with some workers suggesting that it plays a critical role and others contesting that it has little or no role whatsoever. This is largely because attempts to directly quantitate *in vivo* lipid peroxidation during bipyridyl herbicide

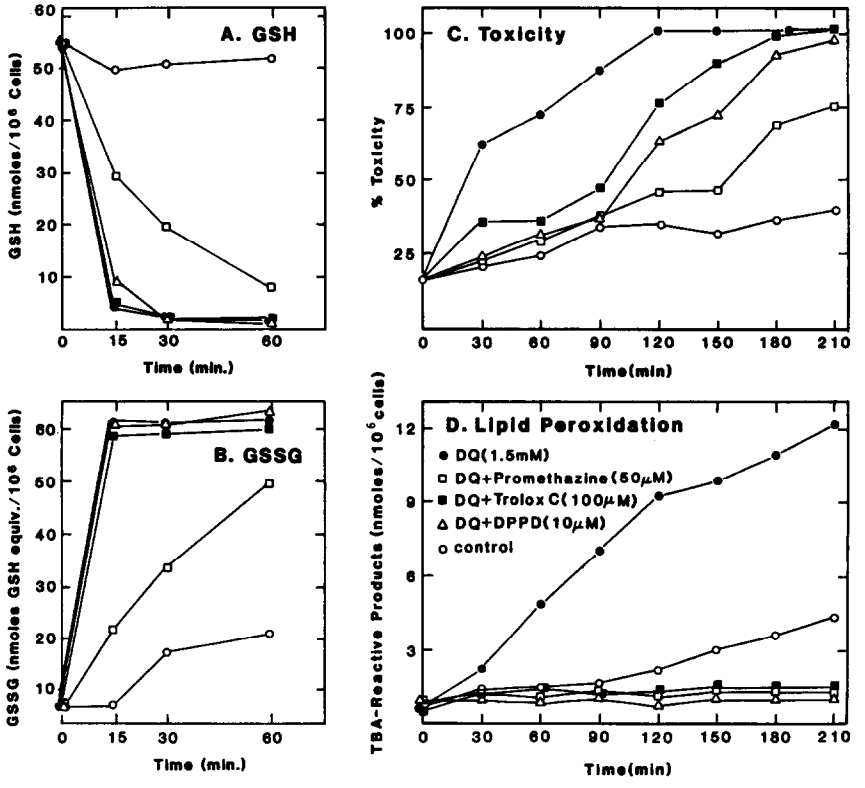

Fig. 7. Effects of three different antioxidants on diquat-induced GSH depletion (A), GSSG formation (B), toxicity (C) and lipid peroxidation (D) in BCNU-treated hepatocytes. Hepatocytes were isolated from phenobarbital-induced rats and treated with BCNU as described in Materials and Methods. They were incubated at 10^6 cells/ml in Krebs–Henseleit buffer, pH 7.4, with either no additions (○), 1.5 mM diquat (●), 1.5 mM diquat and 50 μM promethazine (□), 1.5 mM diquat and 10 μM DPPD (△), or 1.5 mM diquat and 100 μM Trolox C (■). GSH levels were measured as acid soluble thiols and expressed as a percentage of control thiol levels determined immediately prior to incubation with diquat, i.e. t_0. Results shown are from one experiment, typical of three.

toxicity have met with mixed results. Reddy and coworkers [32] demonstrated a 2-fold increase in ethane expiration, a known index of *in vivo* lipid peroxidation [33], 2 hr after paraquat treatment, and a subsequent study [3] showed a 4-fold increase in ethane expiration in rats during the 6-hr period immediately after treatment with an LD_{50} dose of paraquat. Steffen *et al.* [6], however, observed only a 26% increase in ethane expiration 4 hr after treatment of rats with a lethal dose of paraquat. Moreover, Shu *et al.* [34] failed to demonstrate any increase in conjugated dienes in the lungs of mice receiving a dose of paraquat equal to twice the LD_{50}. These workers also found that pretreatment of the mice with DPPD or a high carbohydrate diet prevented *in vitro* paraquat stimulation of lipid peroxidation but failed to protect against paraquat toxicity [34].

These conflicting results can now be compared with our data from the compromised isolated hepatocyte model. Using this model we have shown that DPPD and another antioxidant, Trolox C, totally prevented diquat-induced lipid peroxidation but only delayed its toxicity. These results, in agreement with those of Shu *et al.* [34], demonstrate that antioxidants do not prevent the toxic effects of bipyridyl herbicides. Our studies do not, however, totally rule out a role for lipid peroxidation. They do, in fact, suggest that lipid peroxidation plays an augmentative, but non-essential, role in bipyridyl herbicide cytotoxicity. Thus, if lipid peroxidation is not the critical toxicological consequence of redox cycling and active oxygen production, the question remains as to what is the true nature of the toxic lesion. Further studies are in progress in our laboratory to determine this.

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