0026-895X/87/030417-06$02.00/0

MOLECULAR PHARMACOLOGY, 32:417-422

Evidence for Participation of Lipid Peroxidation and Iron in Diquat-Induced Hepatic Necrosis *In Vivo*

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Received January 23, 1987; Accepted June 2, 1987

SUMMARY

The hepatic necrosis produced in Fischer-344 rats by diquat appears to be mediated by redox cycling of diquat with generation of reactive oxygen species. We have now tested the hypothesis that chelates of iron are important in the cytotoxicity of reactive oxygen species, possibly through initiating the cleavage of peroxyl bonds. Pretreatment with the iron chelator desferrioxamine, 0.24 mmol/kg intraperitoneally, attenuated the hepatic damage produced by diquat. No additional protection was provided by a second dose of desferrioxamine 2 hr after diquat or by administration of the iron chelator by a different route of administration (subcutaneously). Ferrous sulfate (0.36 mmol/kg, intraperitoneally) alone produced no hepatic injury, but when given 15 min before diquat, it potentiated hepatic injury and animal mortality. In contrast, biliary excretion of glutathione disulfide in response to administration of diquat was neither potentiated by pretreatment with $FeSO_4$ nor diminished by pretreatment with desferrioxamine. The marked changes in hepatic injury produced by these pretreatments, without changes in glutathione disulfide production, indicate that shifts in thiol/disulfide equilibria are not likely to be initiating events in the pathogenesis of diquat-induced hepatic necrosis. Administration of a hepatotoxic dose of diquat quickly produced 5-fold stimulation of ethane and pentane expiration rates with return to control rates by 3 hr. Desferrioxamine markedly inhibited, and iron potentiated, hydrocarbon expiration in response to diquat. The parallel changes in diquat hepatic injury and ethane and pentane expiration rates in response to manipulation of iron availability suggest a possible causal role for Fenton chemistry and lipid peroxidation in diquat-generated, reactive oxygen-mediated hepatic injury *in vivo*.

Investigations of oxidative mechanisms of cytotoxicity have relied heavily on isolated cell systems, particularly isolated or cultured rat hepatocytes (1–4). Many compounds such as the bipyridylium herbicides, diquat and paraquat, produce substantial oxidative stress *in vivo* and *in vitro* and cause cell death *in vitro*, but cell death and necrosis were not observed with such compounds in our initial studies *in vivo* (5). Burk *et al.* (6) and Cagen and Gibson (7) have described hepatic damage in selenium deficient animals produced *in vivo* by diquat and paraquat administration, but characterization of the models was minimal. We subsequently found that hepatic necrosis is produced readily in male Fischer-344 rats treated with diquat (8).

Reactive oxygen species generated through redox cycling of diquat are thought to be the mediators of cellular damage, but it is unclear which modification of cellular molecules produced by these reactive intermediates is responsible for loss of cell viability. Certain biochemical parameters such as protein thiol oxidation were implicated as potential critical determinants of cell viability from studies in isolated cell systems (1, 2) but were found not to be altered similarly in association with initiation of cell death *in vivo* (8, 9). Our studies did not completely exclude selective or compartmentalized protein thiol oxidation as a critical target in diquat hepatotoxicity, rather that the extent of alteration identified by studies *in vitro* (1, 2) did not occur and therefore was not required for reactive oxygen-mediated hepatic necrosis.

An increase in LOH content of hepatic tissue produced by diquat was found, indicating that lipid peroxidation may contribute to cell killing but through mechanisms that do not lead to massive accumulation of LOHs (8). One such possibility is the degradation of lipid hydroperoxides by beta-scission mechanisms rather than reduction to LOHs. Because chemically reactive iron species are effective catalysts for the beta-scission of hydroperoxides, we therefore examined the effects of pretreatment with ferrous sulfate or with the iron chelator desferrioxamine on diquat-induced hepatic damage *in vivo* and on the expiration of ethane and pentane as well as on the biliary efflux of GSSG.

Materials and Methods

Male Fischer-344 rats were purchased from Harlan Sprague-Dawley (Houston, TX) and kept in an air conditioned room on a 12-hr light/

This work was supported by National Institutes of Health Grant GM 34120.

ABBREVIATIONS: *LOH*, lipid hydroxy acid; *GSSG*, glutathione disulfide; *GPT*, glutamate pyruvate transaminase; *GOT*, glutamate oxalacetate transaminase; *GSH*, glutathione; *BCNU*, 1,3,-bis(2-chloroethyl)-1-nitrosourea.

dark cycle. Food and tap water were available *ad libitum* throughout except during measurement of expired hydrocarbons. Diquat was provided generously by Dr. Ian Wyatt of Imperial Chemical Industries (Macclesfield, Cheshire, England). Desferrioxamine (Deferal, Ciba) was purchased from the college hospital pharmacy, and ferrous sulfate heptahydrate was bought from Fisher Scientific (Houston, TX). Other reagents were purchased from Sigma Chemical (St. Louis, MO) or Fisher.

Blood was obtained from animals anesthetized lightly with ether by retroorbital puncture with heparinized capillary tubes. Blood normally was collected 6 and 24 hr after diquat, but in some cases it was obtained earlier in animals that appeared to be dying. Plasma was obtained by centrifugation and used for measurement of GPT and GOT with kits purchased from Sigma.

For measurement of expired hydrocarbons, animals were treated with the appropriate drug or control solutions and placed in airtight stainless steel and Plexiglas containers with a raised stainless steel wire mesh floor. Ultra High Purity Zero Grade Air (Big Three Industries, Houston, TX) was passed through the chamber. This air was found to contain traces of methane but no measurable higher hydrocarbons. Air exiting the chamber was sampled essentially as described by Dillard *et al.* (10) with a six-port two-position Carle valve and a sample loop packed with activated alumina cooled in a liquid nitrogen-ethanol slush. Hydrocarbons were analyzed by gas chromatography with a 2 m column packed with alumina and were detected by flame ionization. The rates of hydrocarbon expiration were calculated from the chromatographic peak areas with standard curves obtained by injection of known quantities of ethane and pentane standards. Airflow rate, volume of air sampled, and animal body weight were incorporated to express expiration rates in picomoles per minute per kilogram animal.

Biliary GSSG efflux and plasma GPT and GOT were determined as we have previously described (8). Hepatic vitamin E was measured in 100 mg portions of liver cut from animals anesthetized with intraperitoneal pentobarbital (50 mg/kg). The tissue was frozen immediately in liquid nitrogen, weighed, homogenized, and tissue lipids extracted essentially by the method of Burton *et al.* (11) except that cyclohexane was employed as the hydrocarbon solvent and alpha-tocopherol acetate was added as an internal standard. Samples were analyzed by reverse phase high performance liquid chromatography on a Waters C_{18} μ-Bondapak column eluted with 989:10:1 MeOH:H_2O:HOAc at 1.5 ml/min. Fluorimetric detection on an Aminco-Bowman spectrophotofluorimeter with 290 nm excitation and 330 nm emission provided quantitation. The effects of diquat on hepatic ATP content were examined similarly but with enzymatic determination employing kits purchased from Sigma (technical bulletin 366-UV). In both the vitamin E studies and ATP determinations, control values were obtained by taking samples before administration of diquat and by parallel examination of animals treated with saline vehicle rather than with diquat.

Data are expressed as mean ± standard error, and hydrocarbon expiration rates are compared statistically with the unpaired Student's *t* test. Plasma transaminase activities are compared with the nonparametric Mann-Whitney rank sum test (12).

Results

Desferrioxamine, 0.24 mmol/kg, given 30 min before diquat, 0.1 mmol/kg, provided significant protection against hepatic damage (Table 1). Animal mortality was not altered measurably, and hepatic injury was not prevented entirely. Plasma GPT activities in the survivors of the animals given both desferrioxamine and diquat still exceeded 100 U/ml in five of the seven survivors at 6 hr and three of five survivors at 24 hr. Administration of desferrioxamine 30 min before and 2 hr after diquat provided similar but not improved protection (Table 2). Elevation of plasma GPT activities to greater than 100 U/ml was found in four of seven animals at 6 and 24 hr. In addition,

TABLE 1

Effect of desferrioxamine on diquat hepatic injury

Fed male Fischer-344 rats (Harlan) were given desferrioxamine intraperitoneally in saline solution 30 min before diquat, also administered intraperitoneally in saline solution. Control animals received equal volumes of saline as needed. Blood was obtained by retroorbital puncture of animals under light ether anesthesia with heparinized capillary tubes. Plasma was obtained by centrifugation and plasma transaminases determined with kits purchased from Sigma.

Desferrioxamine	Diquat	Time	Survival	GPT	GOT
mmol/kg		*h*		*U/ml*	
0	0.10	6	9/10	1674 ± 757	2514 ± 1058
		24	6/10	2851 ± 1341	3445 ± 1685
0.24	0.10	6	7/10	162 ± 35[a]	271 ± 47[a]
		24	5/10	155 ± 60[a]	201 ± 62[a]
0	0	6	4/4	54 ± 5	143 ± 18
		24	4/4	53 ± 5	139 ± 8

[a] Different from corresponding values obtained from animals not receiving desferrioxamine by Mann-Whitney rank sum test, $p < 0.05$.

TABLE 2

Effect of multiple dose desferrioxamine on diquat hepatotoxicity

Fed male Fischer-344 rats (Harlan) were given desferrioxamine in saline or an equal volume of saline intraperitoneally (or subcutaneously as indicated) 0.5 h before and 2 h after diquat, also given intraperitoneally as a saline solution. Blood was obtained from animals anesthetized lightly with ether by retroorbital puncture with heparinized capillary tubes. Plasma was provided by centrifugation and transaminase activities determined with kits purchased from Sigma.

Desferrioxamine	Diquat	Time	Survival	GPT	GOT
mmol/kg		*h*		*U/ml*	
0 × 2	0.1	6	8/9	1010 ± 295	1442 ± 449
		24	8/9	1134 ± 369	1574 ± 707
0.24 × 2	0.1	6	7/10	114 ± 19[a]	203 ± 20[a]
		24	7/10	207 ± 76[a]	252 ± 69[a]
0.24 × 2 s.c.	0.1	6	4/4	165 ± 42	266 ± 28
		24	4/4	101 ± 13	185 ± 28

[a] Different from animals not given desferrioxamine by rank sum test, $p < 0.05$.

administration of desferrioxamine by subcutaneous injection provided similar protection, with three of four animals at 6 hr and two of four at 24 hr showing plasma GPT > 100 U/ml. Animals given higher doses of diquat, 0.15 and 0.20 mmol/kg, also showed smaller increases in plasma transaminase activities when treated with desferrioxamine, but hepatoprotection was not complete and no change was produced in the extensive animal mortality at these doses (data not shown). Desferrioxamine did not protect against increases in plasma transaminase activity produced in mice and rats by administration of acetaminophen (13; C.V. Smith, unpublished data). The effects on diquat-treated animals therefore are not the result of a nonspecific protection against all hepatotoxins, nor is inhibition of the transaminases by desferrioxamine or its metabolites a possibility.

In striking contrast, ferrous sulfate given 15 min before administration of diquat markedly potentiated both hepatotoxicity and animal mortality (Table 3). Although neither saline plus 0.05 mmol/kg diquat nor 0.36 mmol/kg ferrous sulfate plus saline produced any hepatic injury, the administration of both ferrous sulfate and diquat in these doses produced a marked and rapid rise in plasma transaminase activities and extensive animal mortality at 7–10 hr after diquat. Three of the 15 animals studied survived 24 hr and showed normal plasma transaminase activities at both 6 and 24 hr. We have observed a similar bimodal response in other studies where most animals sustain massive injury, but a few show no response, with no intermediate responses observed. We were concerned that the unintentional injection of diquat into the

TABLE 3

Effect of iron on diquat hepatotoxicity

Fed male Fischer-344 rats (Harlan) were given ferrous sulfate in saline or saline alone, intraperitoneally, 15 min before intraperitoneal administration of diquat in saline solution. Plasma was obtained and transaminase activities determined as described in Materials and Methods.

$FeSO_4$	Diquat	Time	Survival	GPT	GOT
mmol/kg		*h*		*U/ml*	
0	0.05	6	9/9	53 ± 15	99 ± 16
		24	9/9	71 ± 37	173 ± 69
0.36	0	6	6/6	46 ± 3	162 ± 28
		24	5/6	46 ± 4	128 ± 12
0.36	0.05	6	11/15	5911 ± 1853[a]	8335 ± 2494[a]
		24	3/15	41 ± 22	138 ± 66
0.36	0.10	1.5	4/4	643 ± 297	605 ± 257
		2	0/4		

[a] Different from animals receiving iron alone or diquat alone by rank sum test, $p < 0.05$.

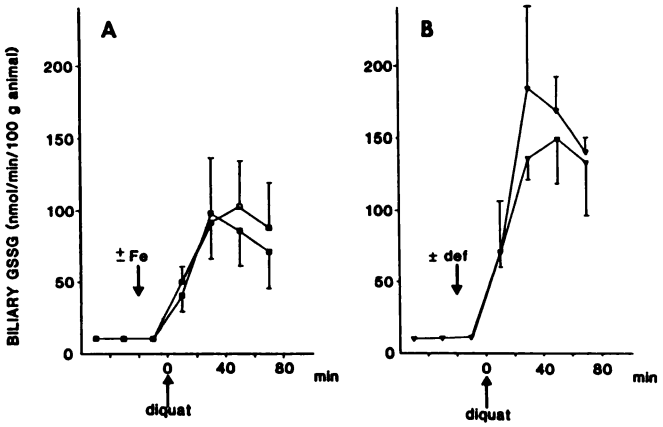

Fig. 1. Effect of ferrous sulfate (*A*) and desferrioxamine (def) (*B*) on diquat-induced increases in biliary efflux of GSSG. Male Fischer-344 rats were anesthetized with pentobarbital and their bile ducts cannulated. Aliquots (20 min) of bile were collected, and GSSG content was measured enzymatically. *A*, animals were given either 0.36 mmol/kg $FeSO_4$ (■, Fe^+, $n = 6$) or an equal volume of saline (□, Fe^-, $n = 6$) intraperitoneally 20 min before both groups received 0.05 mmol/kg of diquat also intraperitoneally. *B*, animals were given 0.24 mmol/kg desferrioxamine mesylate (▼, Def^+, $n = 4$) or an equal volume of saline (▽, Def^-, $n = 3$) intraperitoneally 20 min before both groups received 0.1 mmol/kg of diquat also intraperitoneally. Data are given as means ± standard error and are not different statistically by Student's unpaired *t* test.

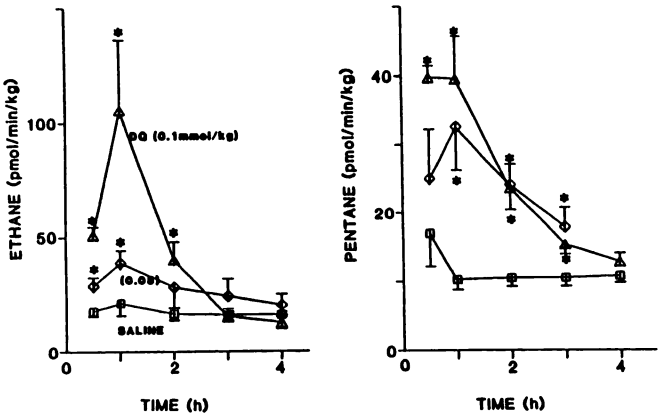

Fig. 2. Effect of diquat (DQ) on expiration of ethane and pentane. Diquat was administered intraperitoneally in saline solution to fed male Fischer-344 rats and expired hydrocarbons measured. Doses (in mmol/kg): 0 (□) saline control, 0.05 (◇), and 0.1 (△). Data are given as means ± standard error for four to six animals per dose group. *Different from saline-treated controls by unpaired Student's *t* test, $p < 0.05$.

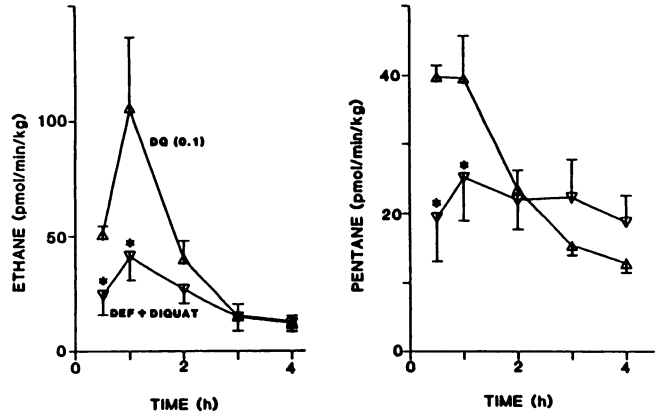

Fig. 3. Effect of desferrioxamine on expiration of ethane and pentane from rats in response to diquat. Fed male Fischer-344 rats were treated intraperitoneally with 0.36 mmol/kg desferrioxamine (▽) or an equal volume of saline (△) 30 min before 0.1 mmol/kg diquat in saline. Expired hydrocarbons were measured and are presented as means ± standard error of six animals per group. *Different from animals not receiving desferrioxamine by unpaired Student's *t* test, $p < 0.05$.

intestine rather than into the intraperitoneal cavity might be responsible for the absence of toxicity. However, injection of diquat into the stomach, small intestine, or colon of rats anesthetized and opened by midline incision to permit visual verification of locus of drug administration showed that 0.1 mmol/kg diquat was hepatotoxic by all three sites of administration (data not shown). The reasons for occasional animal resistance to diquat are unknown at this time.

The increase in biliary efflux of GSSG produced by administration of 0.05 mmol/kg diquat was not potentiated by pretreatment with 0.36 mmol/kg ferrous sulfate (Fig. 1A), in contrast with the dramatic potentiation of hepatic injury (Table 3). Similarly, 0.24 mmol/kg desferrioxamine did not decrease biliary GSSG excretion in rats given 0.1 mmol/kg diquat (Fig. 1B) despite decreased hepatic injury (Table 1) observed in other animals treated with these doses.

Saline-treated Fischer-344 rats expired ethane and pentane at rates in the range of 10–20 pmol/min/kg animal (Fig. 2). Treatment with a hepatotoxic dose of diquat, 0.1 mmol/kg, produced a rapid rise in hydrocarbon production that peaked at four to five times control by 1 hr and returned to control levels by 3–4 hr. Significant increases in ethane and pentane expiration were observed also in animals given 0.05 mmol/kg diquat, a dose that does not produce hepatic necrosis. Desferrioxamine diminished the amount of ethane and pentane expired in response to 0.1 mmol/kg diquat (Fig. 3). As seen with the effects on hepatic damage, increased ethane and pentane production was not prevented entirely, but the responses were diminished significantly. Similarly, ferrous sulfate alone did not increase significantly the expiration of ethane and pentane but when administered before 0.05 mmol/kg diquat, it produced marked stimulation of hydrocarbon exhalation (Fig. 4). Ferrous sulfate treatment before 0.075 mmol/kg diquat produced even higher rates of hydrocarbon expiration and rapid and extensive animal death.

We were concerned about a possible role for tissue anoxia or for vitamin E depletion in diquat-induced necrosis. The rapid redox cycling metabolism of diquat might consume hepatic oxygen fast enough to produce some degree of anoxia, but this did not seem to be the case, inasmuch as hepatic ATP content

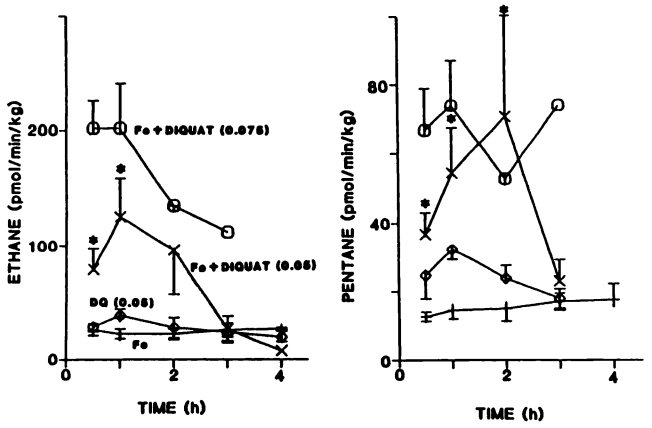

Fig. 4. Effect of ferrous sulfate on expiration of ethane and pentane in response to diquat (DQ). Fed male Fischer-344 rats were treated intraperitoneally with saline or 0.24 mmol/kg ferrous sulfate in saline 15 min before administration of diquat or saline control and expired hydrocarbons measured. Treatment groups: ◊ saline and 0.05 mmol/kg diquat; + 0.24 mmol/kg ferrous sulfate and saline; × ferrous sulfate and 0.05 mmol/kg diquat; ○ ferrous sulfate and 0.075 mmol/kg diquat. Data are presented as means ± standard error of four to six animals per group. Statistical difference evaluated by unpaired Student's *t* test: *Different from animals receiving saline and diquat; **Different from animals receiving saline and ferrous sulfate, $p < 0.05$. Def, desferrioxamine.

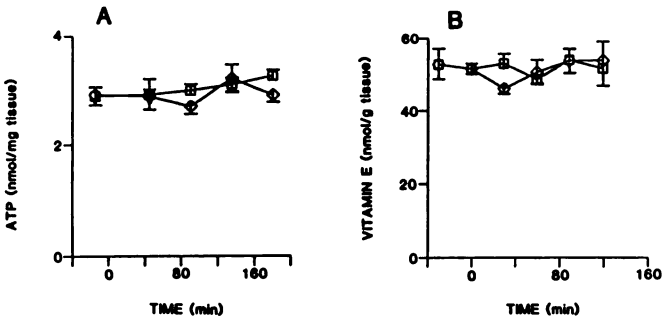

Fig. 5. Effect of diquat on hepatic ATP and vitamin E content. Data are given as means ± standard error ($n = 4$) at selected times after diquat administration (0.1 mmol/kg, intraperitoneally) at 0 min and are not different at any time by either Student's unpaired *t* test or Mann-Whitney nonparametric rank sum test.

was not diminished by a hepatotoxic dose of diquat (Fig. 5), and ATP depletion is a characteristic result of tissue anoxia (14). The hydrophilic nature of the presumed diquat-derived oxidant species, H_2O_2 or $Fe(O)O^{n+}$, suggested to us that depletion of the lipophilic antioxidant vitamin E would not be a feature of diquat-induced hepatic damage, because the hydrophilic antioxidants GSH and ascorbate are not depleted substantially by hepatotoxic doses of diquat (8). Indeed, as shown in Fig. 5, a hepatotoxic dose of diquat did not deplete hepatic vitamin E content; similarly, we have found that hepatotoxic doses of CCl_4 also fail to deplete hepatic vitamin E (C. Smith, unpublished observations).

Discussion

The simplest explanation of the present data is that metabolic redox cycling of diquat produces superoxide and hydrogen peroxide and that the extent of damage produced depends additionally on the availability of chemically reactive iron chelates, presumably to cleave peroxyl bonds with formation of the more reactive hydroxyl, alkoxyl, or related radical species. The massive biliary efflux of GSSG in response to diquat documents the oxidative stress (8), and potentiation of diquat-induced hepatic damage by decreased hepatic activities of glutathione peroxidase (6, 7) or glutathione reductase (15) indicates a causal role for reactive oxygen species such as hydrogen peroxide. The present data demonstrating amelioration of diquat-induced hepatic damage by treatment with desferrioxamine and potentiation of diquat hepatotoxicity by treatment with ferrous sulfate indicate a role for iron complexes in the development of the hepatic lesion.

Studies *in vitro* have suggested that shifts in cellular thiol/disulfide status could be critical in the initiation of cell death by oxidative toxins, possibly through the S-thiolation of proteins and inhibition of membrane pumps essential for the maintenance of calcium homeostasis (1, 2, 16). Our initial studies *in vivo* were not entirely inconsistent with this hypothesis in that Fischer rats responded to 0.1 mmol/kg diquat with biliary efflux rates four times those observed in Sprague-Dawley rats, which paralleled the susceptibility of the Fischer rats and the resistance of the Sprague-Dawley rats to diquat-induced hepatic necrosis (8). However, these same studies showed that despite huge increases in hepatic production of GSSG in response to hepatotoxic doses of diquat, protein thiol content was not decreased. Protein thiol oxidation still may be a contributing causal mechanism in diquat-induced hepatic necrosis, but the extent of protein thiol oxidation clearly is much less than that required to initiate cell death in isolated hepatocytes exposed to menadione (1). Compartmentation of protein thiol oxidation cannot be completely excluded, but animals given 0.05 mmol/kg diquat excrete comparable amounts of GSSG into the bile whether pretreated with saline or ferrous sulfate (Fig. 1A) despite the enormous difference in hepatic injury (Table 3). Similarly, the hepatoprotective effects of desferrioxamine (Tables 1 and 2 are not accompanied by a decrease in biliary GSSG efflux (Fig. 1B).

In other studies, we have shown that although BCNU pretreatment, which inhibited hepatic glutathione reductase activities by 75%, potentiates both the biliary efflux of GSSG and the hepatic damage produced by diquat in Fischer rats, Sprague-Dawley rats pretreated with BCNU remained relatively resistant to hepatic necrosis despite biliary GSSG efflux rates five times those produced by hepatotoxic doses of diquat in the Fischer rats (15). Further, we have shown that hepatic microsomes isolated from diquat-treated Fischer rats show an increased permeability to calcium (17), similar to that observed in microsomes and plasma membrane vesicles isolated from CCl_4-treated rats (18). Although altered calcium homeostasis may be critical in the initiation of cell death (19), the increased calcium permeability of microsomes from diquat-treated Fischer rats shows a molecular lesion that survives preparation of the subcellular fractions, whereas protein S-thiolation might be partially reversed by thiol/disulfide exchange reactions.

In contrast to these thiol data, the present results are consistent with lipid peroxidation as a working hypothesis for a mechanism contributing to diquat-induced hepatic necrosis. First, diquat increases ethane and pentane expiration early, as would be expected from a cause, rather than late as would be expected for a result of the hepatic damage (20). The significant, but much smaller, increases in hydrocarbon exhalation produced by the nonhepatotoxic dose of 0.05 mmol/kg diquat (Fig. 2) further suggest an event that precedes the damage, as op-

posed to one that arises from the damage. In addition, the decrease in hydrocarbon expiration in parallel with hepatoprotection by desferrioxamine and the converse potentiation of hepatic damage and ethane and pentane production by administration of ferrous sulfate demonstrate a striking correlation. The effects of iron and desferrioxamine pretreatment on diquat-induced changes in hepatic LOH content are unknown now but are being examined; however, unlike hepatotoxins such as acetaminophen where LOH formation and alkane expiration are not correlated (20), the two indexes of lipid peroxidation are increased significantly in animals given diquat alone. The importance of using both the alkanes and LOHs as indexes of lipid peroxidation is illustrated by the scheme in Fig. 6. Although lipid peroxidation and the effects of reactive iron chelates are much more complex than the simplified scheme presented in Fig. 6, for a given amount of fatty acid hydroperoxide produced, the iron-catalyzed beta-scission pathway competes with reduction of the lipid hydroperoxide to LOH.

Most tissue iron is stored in the ferric form by ferritin. Reduction to the ferrous form leads to release of stored iron, a reaction that can be produced by exposure of ferritin to superoxide (21). Iron complexes that contain at least one exchangeable coordination site can stimulate peroxyl bond cleavage, but desferrioxamine and other agents that provide tightly bound hexacoordinate complexes block this activity, and desferrioxamine has been shown to inhibit lipid peroxidation *in vitro* (21–23). Thus, superoxide generated by the metabolic redox cycling of diquat not only appears to produce hydrogen peroxide but also may increase the availability of chemically reactive iron species through reduction of ferritin-bound ferric iron. The released iron thus becomes available to stimulate production of hydroxyl and/or alkoxyl radicals (hence the destruction of the lipid hydroperoxide) through reductive cleavage of the peroxide (Figure 6). Thomas and Aust (24) have reported that the NADPH-cytochrome P-450 reductase-catalyzed reduction of paraquat and diquat promote the release of iron from ferritin. Under aerobic conditions, the release of iron is inhibited only partially by superoxide dismutase and under anerobic conditions the release of iron is very rapid. Thus, paraquat and diquat can transfer directly reducing equivalents to ferritin, and hypoxia created by the rapid redox cycling reduction of

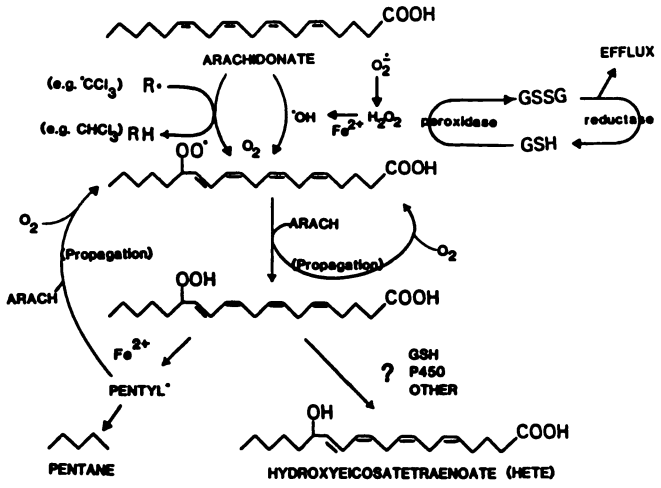

Fig. 6. A scheme of lipid peroxidation illustrating a highly simplified outline of probable sites that reactive iron chelates may influence the formation and/or degradation of lipid hydroperoxides.

oxygen would not stop the release of iron but would be expected to promote the rate of release. Although desferrioxamine chelates Fe^{3+} preferentially and both the iron released from ferritin and the iron administered exogenously are in the ferrous form, the redox status of the reactive iron chelates will be shifted in response to the redox status of the surrounding biological matrix. Therefore a decrease in the availability of Fe^{2+} by desferrioxamine chelation of Fe^{3+} through the redox equilibration of the two forms would be expected. Moreover, the recent report of Minotti and Aust (25) indicates that both Fe^{2+} and Fe^{3+} are important in lipid peroxidation so that chelation of Fe^{3+} may suppress lipid peroxidation independently of possible decreases in Fe^{2+}.

Hepatotoxic doses of diquat do not deplete hepatic ATP (Fig. 5), indicating that diquat metabolism does not deplete tissue oxygen availability, because tissue ATP concentrations decrease rapidly during ischemia or anoxia (14). Our concern was not that depletion of ATP was responsible for cell death, but that depletion of tissue oxygen might be contributing to the hepatic damage or to the increased expiration of ethane and pentane. The former can be excluded since Farber previously showed that hepatic ATP can be depleted *in vivo* by 80% for 36–48 hr without compromising viability (14). However, alteration of ethane and pentane expiration by tissue anoxia is possible. Oxygen is an effective radical trap and can compete for ethyl and pentyl radicals, inhibiting the formation of ethane and pentane, in a manner analogous to the reaction of the lipid-derived free radical to form its corresponding lipid peroxyl radical. Of course, localized hypoxia created by the redox cycling of diquat cannot be excluded on the basis of the ATP data, but insofar as tissue ATP content reflects O_2 availability, such effects would appear to be limited.

In summary, the marked changes in hepatic injury and the lack of changes in biliary GSSG efflux in response to diquat produced by the pretreatments in these studies suggest that shifts in thiol/disulfide equilibria are not responsible for diquat-induced hepatic necrosis *in vivo*. The parallel changes in diquat-initiated hepatic injury and ethane and pentane expiration rates strongly support a possible causal role for lipid peroxidation and Fenton chemistry in diquat-generated, reactive oxygen-mediated hepatic injury. Although other chemical or physiological factors may prove to be important, the probable mechanism for the participation of iron chelates in diquat-induced hepatic damage is through the cleavage of peroxyl bonds. Nevertheless, it is recognized that correlations between alkane expiration and hepatic injury induced by diquat and the effects of administration of ferrous sulfate or desferrioxamine on alkanes and damage do not definitively establish a causal relationship, and additional work is needed to clarify the important roles of iron chemistry and lipid peroxidation in diquat-induced hepatic necrosis.

Consistent with the present work, other investigators (26) have noted a stimulation of ethane expiration in animals treated with both ferrous sulfate and diquat. Interestingly, these investigators observed no increase in ethane production in response to diquat alone, but it would seem likely that this occurred because the mouse species studied by them is insensitive to diquat-induced hepatic necrosis. We have found that male ICR mice are resistant to diquat hepatotoxicity but show mild hepatotoxicity when treated with diquat and ferrous sulfate (C.V. Smith, unpublished data).

Acknowledgments

The support and advice of Dr. J. R. Mitchell is gratefully acknowledged.

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