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Paraquat Toxicity: Proposed Mechanism of Action Involving Lipid Peroxidation

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The purpose of this study was to investigate the hypothesis that paraquant pulmonary toxicity results from cyclic reduction-oxidation of paraquat with sequential generation of superoxide radicals and singlet oxygen and initiation of lipid peroxidation. *In vitro* mouse lung microsomes catalyzed an NADPH-dependent, single-electron reduction of paraquat. Incubation of paraquant with NADPH, NADPH-cytochrome c reductase, and purified microsomal lipid increased malondialdehyde production in a concentration dependent manner. Addition of either superoxide dismutase or the singlet oxygen trapping agent 1,3-dipheylisobenzo furan inhibited paraquat-stimulated lipid peroxidation. *In vivo,* pretreatment of mice with phenobarbital decreased paraquat toxicity, possibly by competing for electrons which might otherwise reduce paraquat. In contrast, paraquat toxicity in mice was increased by exposure to 100% oxygen and by deficiencies of the antioxidants selenium, vitamin E, or reduced glutahione (GSH). Paraquat, given IP to mice, at 30 mg/kg, decreased concentrations of the water-soluble antioxidant GSH in liver and lipid soluble antioxidants in lung. Oxygen-tolerant rats, which have increased activities of pulmonary enzymes which combat lipid peroxidation, were also tolerant to lethal doses of paraquat as indicated by an increased paraquat LT_{50}. Furthermore, rats chronically exposed to 100 ppm paraquat in the water had elevated pulmonary activities of glucose-6-phosphate dehydrogenase and GSH reductase. These results were consistent with the hypothesis that lipid peroxidation is involved in the toxicity of paraquat.

Introduction

Paraquat (methyl viologen, 1,1'-dimethyl-4,4'-bypyridylium dichloride) is a widely used broad spectrum herbicide which is toxic to man *(1-3)* and laboratory animals *(4,5)*. Paraquat toxicity is characterized by delayed development of pulmonary lesions, expressed as initial pulmonary edema which progresses to interstitial fibrosis *(5,6)*. Examination of the ultrastructure of the pulmonary lesions in rats *(7,8)* and mice *(9)* revealed early damage to type I pneumocytes, increased numbers of fibroblasts, and a later proliferation of type II pneumocytes. The paraquat-induced pulmonary lesions have been observed to resemble those of oxygen toxicity *(10)*.

The specificity of paraquat for inducing pulmonary toxicity has been correlated by several investigators with a retention of paraquat in the lung relative to other organs *(11-13)*. Recently, *in vitro* experiments have demonstrated that rat lung slices accumulated paraquat by an apparent active transport process *(14)*. Thus, the retention of paraquat in lung tissue appears in part responsible for the development of pulmonary lesions.

Initial investigations into the mechanism of paraquat-induced tissue damage centered around the ability of paraquat to undergo a single-electron reduction to form the blue-colored paraquat radical [eq. (1)].

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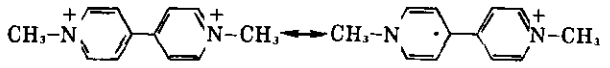

oxidized reduced

Under anaerobic conditions, paraquat has been demonstrated to be reduced to the radical form by both isolated plant chloroplasts (*15*) and rat liver microsomes (*16*). However, detection of the reduced paraquat radical is not possible under aerobic conditions because of immediate reoxidation by oxygen back to the parent compound. The oxidation of paraquat by molecular oxygen has been shown to generate superoxide (O_2^-) radicals (*17,18*), which result from the transfer of a single electron from reduced paraquat to oxygen.

Initiation of the membrane destructive process of lipid peroxidation is a possible result of the cyclic reduction-oxidation of paraquat in biological systems. Increased concentrations of malondialdehyde, a metabolic product of lipid peroxidation, have been demonstrated in plant leaves (*19*) and rat liver microsomal phospholipids (*16*) after *in vitro* incubation with paraquat. Thus, lipid peroxidation appears to be involved in both the plant and mammalian toxicity of paraquat.

Proposed Mechanism of Paraquat Toxicity.

The studies of Pederson and Aust (*20*) into the mechanism of xanthine oxidase-promoted lipid peroxidation provided a mechanism whereby paraquat might initiate lipid peroxidation. Utilizing superoxide dismutase, an enzyme which scavenges superoxide radicals (*21*), and a singlet oxygen-trapping agent, 1,3-diphenylisobenzofuran (*22*), *in vitro*, they concluded that xanthine oxidase-induced lipid peroxidation was mediated by superoxide radicals. Highly reactive singlet oxygen was formed nonenzymatically from superoxide radicals with subsequent reaction with unsaturated fatty acids to form fatty acid hydroperoxides and initiation of lipid peroxidation.

Because paraquat is reduced *in vitro* by rat liver microsomes (*16*) and superoxide radicals are a demonstrated product of the cyclic reduction-oxidation of paraquat (*17*), a hypothesis based upon the work of Pederson and Aust (*20*) was constructed as a possible mechanism for the mammalian toxicity of paraquat (Fig. 1). Paraquat was proposed to undergo a single-electron reduction with NADPH as a source of electrons. Upon aerobic oxidation of reduced paraquat by molecular oxygen, superoxide radicals are formed which may nonenzymatically dismutate to singlet oxygen. Singlet oxygen then reacts with unsaturated lipids associated with cell membranes to produce lipid hydroperoxides, which spontaneously decompose in the presence of trace amounts of transition metal ions to lipid free radicals (*23*). The lipid free radicals then begin the chain reaction process of lipid peroxidation which is damaging to cell membranes.

The mechanism depicted in Figure 1 illustrates three defense mechanisms present in mammalian organisms which combat the toxic effects of oxidant agents. First, superoxide dismutase rapidly scavenges toxic superoxide radicals (*21*). Second, endogenous antioxidants such as vitamin E terminate the free-radical chain reaction of lipid peroxidation (*24*). Third, glutathione (GSH) peroxidase enzymatically reduces the unstable lipid hydroperoxides to stable lipid alcohols and thus prevents further formation of free radicals (*25*). Recently, Rotrunck et al. (*26*) demonstrated that selenium was required for GSH peroxidase activity, which may account for the antioxidant activity attributed to selenium (*27*). In addition, GSH peroxidase along with GSH reductase and glucose-6-phosphate (G-6-P) dehydrogenase, which generate reducing equivalents for GSH peroxidase activity, were induced in rats on exposure to the oxidant gas ozone (*28*). The induction of the three enzymes, termed the GSH peroxidase system enzymes (*28*), was proposed to be a response to increased concentrations of lipid hydroperoxides generated by ozone.

Thus, the mechanism proposed for paraquat toxicity, in addition to known biological defenses against lipid peroxidation, suggested both *in vitro* and *in vivo* experiments to examine the involvement of lipid peroxidation in paraquat toxicity.

In Vitro Studies of the Paraquat Mechanism

The first requirement for the proposed mechanism was that paraquat be reduced to the free radical by mammalian tissue. In 1968, Gage (*16*) demonstrated the anaerobic reduction of paraquat *in vitro* by rat liver microsomes and NADPH. However, as the lung is the most severely affected organ in paraquat toxicity, Bus et al. (*29*) investigated whether lung tissue could also reduce paraquat. Anaerobic incubation of paraquat with mouse lung microsomes and NADPH produced the blue-colored paraquat radical in a concentration-dependent manner (Fig. 2) Furthermore, addition of antibody to NADPH-cytochrome c reductase inhibited the reduction of paraquat, which indicated that NADPH-cytochrome c reductase was the microsomal enzyme

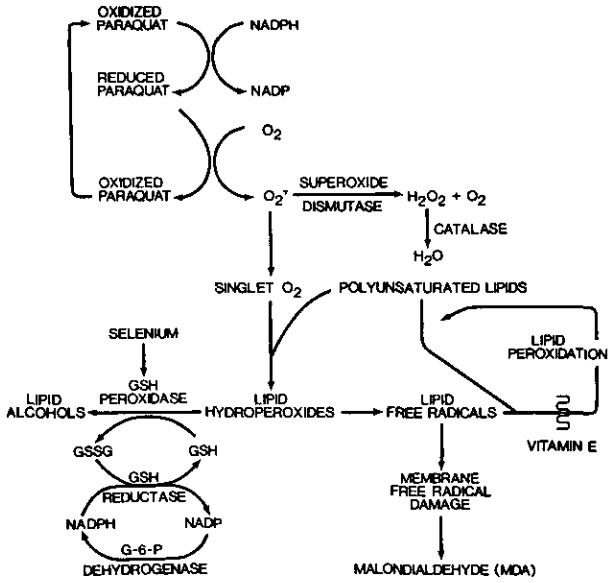

FIGURE 1. Proposed mechanism for paraquat toxicity.

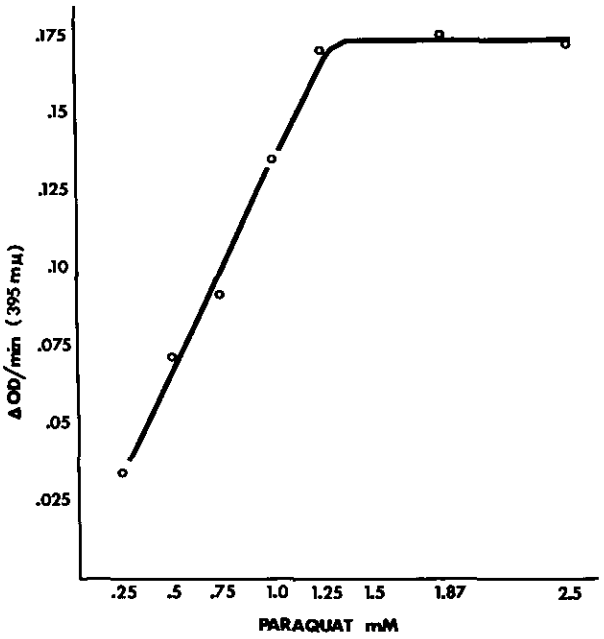

FIGURE 2. Anaerobic reduction of paraquat in the presence of 0.06 mg/ml mouse lung microsomal protein and 0.1 *mM* NADPH.

which catalyzed paraquat reduction *(29)*. Therefore, lung tissue, which is a site of the paraquat lesion *in vivo*, was capable of reducing paraquat to the free-radical form.

The second requirement of the proposed mechanism was that the cyclic reduction-oxidation of paraquat initiate lipid peroxidation through subsequent superoxide radical and singlet oxygen intermediates. To examine this, paraquat was incubated aerobically with NADPH, as source of electrons for paraquat reduction, NADPH-cytochrome c reductase, which catalyzed the transfer of electrons from NADPH to paraquat, and purified rat liver microsomal lipid, which was the substrate for lipid peroxidation *(29)*. Paraquat, incubated in a system which promoted its reduction-oxidation, increased malondialdehyde formation (an indicator of lipid peroxidation) in a concentration-dependent manner (Table 1). Addition of either superoxide dismutase or the singlet oxygen-trapping agent 1,3-diphenylisobenzofuran inhibited paraquat-stimulated malondialdehyde formation (Table 2). The synergistic inhibition of lipid peroxidation when both agents were incubated together further indicated that superoxide radicals and singlet oxygen might be sequential intermediates in paraquat-stimulated lipid peroxidation. The results of these experiments indicated that paraquat stimulated lipid peroxidation *in vitro*.

Table 1. Paraquat-induced *in vitro* lipid peroxidation.[a]

Paraquat concentration of incubation mixture, *M*	Malondialdehyde formed nmole/min/ml [b]	Increase in malondialdehyde formed, %
0	0.37 ±0.01	0
10^{-6}	0.43 ±0.03	16.1
10^{-5}	0.60 ±0.02 [c]	62.2
10^{-4}	1.21 ±0.09 [c]	227.0

[a] Incubation mixtures contained 0.25*M* NaCl, 2.0 *mM* ADP, 0.12*mM* $Fe(NH_4)_2(SO_4)_2$, 0.5 mole/ml lipid phosphorus, 0.2 *mM* NADPH, 60 μg/ml liver microsomal NADPH-cytochrome c reductase, and paraquat in 0.25*M* Tris buffer, pH 6.8.

[b] Mean ±S.E. of three determinations.

[c] Significantly different from no paraquat, $p<0.05$.

In Vivo Studies of the Paraquat Mechanism

The previous *in vitro* experiments had demonstrated that microsomal NADPH-cytochrome c reductase catalyzed paraquat reduction *(29)*, which was the first requirement in the proposed mechanism of paraquat toxicity. The possible *in vivo* role of this enzyme in paraquat reduction was investigated in mice pretreated with phenobarbital *(30)*. The toxicity of paraquat, as measured by the paraquat single dose 7-day intraperitoneal LD_{50}, was significantly decreased in mice pretreated with phenobarbital in the drinking water for 10 days and with continued phenobarbital treatment after paraquat injection (Table 3). However, the paraquat LD_{50} was not

Table 2. Inhibition of paraquat-induced *in vitro* lipid peroxidation by superoxide dismutase and 1,3-diphenylisobenzofuran.[a]

Incubation medium	Malondialdehyde formed, nmole/min/ml [b]	Relative peroxidation, % [c]
Paraquat, $10^{-4}M$	0.84 ±0.09	
Paraquat plus superoxide dismutase		
$20\mu M$	0.60 ±0.06	71.4
$60\mu M$	0.28 ±0.04	33.3
Paraquat plus 1,3-diphenylisobenzofuran		
$2.0\mu M$	0.72 ±0.04	85.7
$10.0\mu M$	0.45 ±0.03 [d]	53.6
Paraquat plus superoxide dismutase ($20\mu M$) and 1,3-diphenylisobenzofuran ($10.0\mu M$)	0.11 ±0.02 [e]	13.1

[a] Incubation conditions were identical to those given in Table 1 except as described above.
[b] Mean ± S.E. of three determinations corrected for no paraquat control.
[c] As percentage of peroxidation with $10^{-4}M$ paraquat.
[d] Significantly different from $0^{-4}M$ paraquat, $p<0.05$.
[e] Not significantly different from basal rate, $p>0.05$.

Table 3. Effect of phenobarbital (PB) pretreatment on the paraquat single-dose 7-day intraperitoneal LD_{50}.[a]

Treatment	Paraquat LD_{50}, mg/kg	95% Confidence limits	Potency ratio
Control	30.0	26.3-34.2	
PB 10 days prior and continued after paraquat	46.0	41.4-51.4	1.53 [b]
PB 10 days prior plus 1 day tap water	39.0	33.0-46.0	1.30
PB, 50 mg/kg IP, 30 min before paraquat	26.8	22.7-31.6	0.89

[a] Phenobarbital as 0.1% solution in water.
[b] Significantly different from control, $p<0.05$.

significantly different from control when 10-day phenobarbital pretreatment was followed by one day of tap water exposure before paraquat injection with continued tap water after paraquat treatment. A single injection of phenobarbital 30 min before paraquat administration also did not alter the paraquat LD_{50} (Table 3).

Phenobarbital pretreatment, coupled with continued post-injection treatment, may have raised and maintained the body concentration of phenobarbital such that the barbiturate, during its own metabolism, could compete for electrons supplied by NADPH which might otherwise be utilized in paraquat reduction (*30*). If the reduction of paraquat was decreased in such a way, less superoxide radicals would be generated with a resultant decrease in toxicity. The possible microsomal competition for electrons is further supported from *in vitro* experiments in which the microsomal metabolism of aldrin (*31*) and bromobenzene (*32*) was inhibited by paraquat, presumably by competing for electrons in the microsomal electron transport system. The failure of the other two phenobarbital pretreatments to protect against paraquat toxicity (Table 3) indicated the tissue concentration of phenobarbital was not high enough or sustained enough to provide protection.

The increased paraquat LD_{50} with phenobarbital pretreatment apparently was not due to a shift in the tissue distribution of paraquat, since the elimination of paraquat from mouse plasma, lung, liver and kidney was similar in both phenobarbital-pretreated and control mice (*30*). However, the general depressant effect of phenobarbital on cellular oxidative metabolism may have interfered with *in vivo* reduction and thus accounted for the protective effect of phenobarbital.

The *in vivo* reduction-oxidation of paraquat was proposed to generate increased concentrations of superoxide radicals (Fig. 1). Evidence that molecular oxygen is reduced to superoxide with the oxidation of reduced paraquat was provided in experiments which examined the interaction of oxygen exposure with paraquat toxicity (*33*). In these experiments, 42-day-old mice exposed chronically to 50 and 100 ppm paraquat in the drinking water were placed in a 100% oxygen environment and the LT_{50}, or calculated median time to death, determined (Table 4). The oxygen LT_{50} for both 50 and 100 ppm paraquat-treated mice was significantly decreased compared to the control LT_{50}, thus indicating an enhanced sensitivity to oxygen toxicity in the presence of paraquat. Fisher et al. (*34*) have also reported that oxygen enhanced the acute toxicity of paraquat in rats, and there is some evidence in mice that de-

Table 4. Effect of paraquat in the water from day 8 of gestation to 42 days postnatally on the survival of mice exposed to 100% oxygen.

Paraquat treatment, ppm	Paraquat LT_{50}, hr	95% Confidence limits	Potency ratio
0 (Control)	160	126-203	—
50	108	81-144	1.5 [a]
100	40	30-54	4.0 [a]

[a] Significantly different from respective control, $p<0.05$.

creased oxygen concentrations (below 21%) protect against paraquat toxicity (*35*). Recently, Autor (*36*) demonstrated that administration of superoxide dismutase delayed paraquat-induced lethality in rats. This observation, coupled with the observations that paraquat toxicity was enhanced by elevated oxygen tension (*33,34*), strengthened the argument that superoxide radicals are formed *in vivo* in paraquat toxicity.

Paraquat-induced lipid peroxidation *in vitro* was determined by assaying for malondialdehyde generation (*29*). However, the measurement of malondialdehyde is not a reliable indicator of lipid peroxidation *in vivo*, as it is rapidly metabolized in animal tissues (*37*). Consequently, the *in vivo* involvement of lipid peroxidation in paraquat toxicity was investigated by determining the relationship of paraquat toxicity to both enzymatic and biochemical defenses against lipid peroxidation (*38,39*).

The mechanism outlined in Figure 1 predicts that animals deficient in selenium, which is required for GSH peroxidase activity, or the endogenous antioxidant vitamin E should be sensitive to paraquat toxicity because both agents protect against lipid peroxidation. In mice deficient in either selenium or vitamin E (*38*), sensitivity to paraquat toxicity, as measured by the single-dose 7-day intraperitoneal LD_{50}, was significantly increased in both deficient groups (Table 5). Supplementation of the respective deficient diets with selenium or vitamin E returned the paraquat LD_{50} to the control value. However,

Table 5. Alteration of paraquat single dose 7-day intraperitoneal LD_{50} in mice by various selenium or vitamin E diets and diethyl maleate pretreatment.

Treatment	Paraquat LD_{50}, mg/kg	95% Confidence limits	Potency ratio
Control	30.0	26.5-35.1	—
Selenium-deficient[a]	10.4	8.9-12.2	2.88[b]
Selenium, 0.1 ppm[c]	27.3	24.8-30.0	1.10
Selenium, 2.0 ppm[c]	25.5	22.2-29.3	1.18
Diethyl maleate[d]	9.4	6.5-13.5	3.20[b]
Vitamin E-deficient[a]	9.2	6.3-13.3	3.26[b]
Vitamin E, 1500 mg/kg[c]	29.0	23.8-35.4	1.03

[a] Deficient diet, 5 week exposure prior to paraquat treatment.
[b] Significantly different from control, $p < 0.05$.
[c] Basal deficient diet supplemented with selenium or vitamin E.
[d] 1.2 ml/kg, IP, 30 min before paraquat.

Table 6. Liver and lung reduced glutathione (GSH) after acute paraquat treatment in mice.[a]

Time after paraquat, hr	GSH, mg/g wet weight[b] Liver	Lung
Control	4.67 ±0.27	1.85 ±0.02
12	3.71 ±0.33	1.88 ±0.11
24	3.21 ±0.17[c]	1.94 ±0.09
36	3.20 ±0.45[c]	2.08 ±0.17
48	3.86 ±0.88	1.83 ±0.09

[a] Paraquat, 30 mg/kg, IP.
[b] Mean ± S.E. of four determinations.
[c] Significantly different from control, $p < .05$.

no protection against paraquat toxicity was observed. In other experiments, pretreatment of mice with diethyl maleate, which depletes tissue reduced glutathione (GSH), also significantly reduced the paraquat LD_{50} (Table 5). The increased paraquat toxicity may have been caused by removal of reducing equivalents (GSH) necessary for GSH peroxidase activity or possibly by the loss of the intrinsic antioxidant activity of GSH. Thus, these experiments agreed with the proposed mechanism, in that mice deficient in the antioxidants selenium, vitamin E, and GSH were sensitized to paraquat toxicity.

Recently, DiLuzio (*40*) reported that lipid peroxidation induces a reduction in tissue antioxidants, presumably because these agents are utilized in terminating lipid free-radical chain reactions. Therefore paraquat, which was proposed to act through lipid peroxidation and whose toxicity had been demonstrated to be significantly enhanced by antioxidant deficiency states (*38*), was examined for its effect on tissue antioxidant concentrations (*39*).

Paraquat, administered at the IP LD_{50} dose of 30 mg/kg to mice, significantly reduced the concentrations of the water-soluble antioxidant GSH in liver but not in lung tissue (Table 6). The decrease in liver GSH may have resulted from increased utilization of GSH by GSH peroxidase in detoxifying lipid hydroperoxides (Fig. 1) or possibly from the direct utilization of GSH as an antioxidant in terminating free radical reactions. The effect of paraquat on lipid-soluble antioxidants, which primarily consist of the tocopherols (*40*), was the reverse of that seen with GSH. Paraquat, 30 mg/kg IP, significantly decreased lipid-soluble antioxidant concentrations in the

Table 7. Lung and liver lipid-soluble antioxidants after an acute dose of paraquat.[a]

Time after paraquat, hr	Lipid-soluble antioxidants % of control[b]	
	Liver	Lung
1	72.4 ±4.4[c]	64.0 ±2.8[c]
4	95.4 ±7.9	73.6 ±10.1[c]
12	103.7 ±5.8	52.7 ±6.0[c]
24	94.7 ±2.8	72.7 ±6.6[c]
48	103.7 ±5.0	56.4 ±4.4[c]
96	87.0 ±5.6	59.4 ±4.5[c]
193	88.9 ±9.5	90.7 ±6.5

[a]Paraquat, 30 mg/kg, IP.
[b]Mean ±S.E. of four determinations.
[c]Significantly different from control, $p < 0.05$.

lung but not in liver (Table 7). In addition, paraquat-induced depression in lung lipid-soluble antioxidants was very prolonged, with recovery to control concentrations not reached until 193 hr after paraquat administration. This observation suggested either a continual slow rate of lipid peroxidation in lung tissue or possibly an initial rapid decrease in antioxidant with a slow rate of replenishment. The differential organ effects of paraquat on GSH and lipid-soluble antioxidants indicated possible differences in the cellular site(s) of paraquat toxicity or available pools of antioxidant in liver and lung tissue.

The relationship of enzymatic defenses against lipid peroxidation to paraquat toxicity *in vivo* was further investigated in rat experiments (*39*). The pulmonary GSH peroxidase system enzymes in rats have been observed to be induced in response to the oxidant gas ozone (*28*). Thus paraquat, which was proposed to act by oxidant attack of unsaturated lipids, was studied for its effect on pulmonary GSH peroxidase system enzyme activity. Chronic exposure of rats to 100 ppm paraquat resulted in significant increases in pulmonary G-6-P dehydrogenase and GSH reductase activity (Table 8). Recently, lung G-6-P dehydrogenase has also been shown to be induced in rats after acute paraquat treatment (*41*). The lack of any GSH peroxidase induction with chronic paraquat treatment may have been caused by sacrificing the rats at a time when the enzyme had not yet been induced by paraquat.

Pretreatment of rats with 85% oxygen for 7 days induces tolerance to subsequent 100% oxygen exposure (*42*). Pulmonary superoxide dismutase (*42*) and G-6-P dehydrogenase (*43*) activities are elevated in oxygen tolerant rats, which may in part be responsible for tolerance development. When oxygen-tolerant rats were treated with a toxic dose of paraquat (*39*), the time to death indicated by the LT_{50} was increased (Table 9). The increased survival time after paraquat treatment would be expected in oxygen-tolerant rats, which have increased enzymatic defenses against lipid peroxidation. Furthermore, lung GSH peroxidase activity was also elevated in oxygen-tolerant rats (*39*), which may provide protection against paraquat toxicity. These observations, therefore, are consistent with the hypothesis that lipid peroxidation is involved in paraquat toxicity.

Table 8. Activity of glutathione peroxidase system enzymes in rats after 100 ppm paraquat in the drinking water for 3 weeks.

	Lung enzyme activity[a]		
	GSH peroxidase, μmole NADPH oxidized/min-mg protein	GSH reductase, nmole NADPH oxidized/min-mg protein	Glucose-6-phosphate dehydrogenase, μmole NADPH reduced/min-mg protein
Control	1.48 ±0.14	36 ±5	47.0 ± 2.1
Paraquat	1.53 ±0.05	68 ±1	78.2 ±11.7[b]

[a] Mean ±S.E. of four determinations.
[b] Significantly different from control, $p < 0.05$.

Table 9. Effect of 7-day 85% oxygen pretreatment on survival of rats after a toxic dose of paraquat.[a]

Treatment	LT_{50}, hr	95% Confidence limits	Potency ratio
Control	26.0	21.0-32.2	
Pretreatment with 85% oxygen for 7 days[b]	50.0	38.5-65.0	1.92[c]

[a] Paraquat, 45 mg/kg IP.
[b] Paraquat administered immediately after removal from 85% oxygen.
[c] Significantly different from control, $p<0.05$.

Summary and Conclusions

These studies have demonstrated that the *in vivo* toxicity of paraquat may be mediated through lipid peroxidation of cell membranes. Paraquat toxicity appeared to result from cyclic reduction-oxidation of the herbicide with subsequent generation of superoxide radicals. Superoxide radicals may nonenzymatically dismutate to singlet oxygen, which reacts with unsaturated lipids in cell membranes to form lipid hydroperoxides. The spontaneous decomposition of lipid hydroperoxides to lipid free radicals initiates the membrane destructive process of lipid peroxidation.

The significance of suggesting lipid peroxidation as a mechanism for paraquat toxicity is of threefold importance. First, the elucidation of a mechanism provides a basis for predicting possible interactions of paraquat with environmental agents. This is particularly useful in the assessment of paraquat as an environmental hazard. Second, a defined mechanism for paraquat toxicity provides useful information for implementation of a rational therapeutic approach in treating victims of paraquat poisoning. Finally, and perhaps most important, these studies have provided *in vivo* evidence for lipid peroxidation as a cause of tissue damage. Thus, the mechanism outlined for paraquat toxicity may serve as a model toxic mechanism for the investigation of the toxicity of other drugs and environmental agents.

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