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LIPID PEROXIDATION AND PARAQUAT TOXICITY*

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Abstract—Paraquat, added *in vitro*, stimulated lipid peroxidation in lung microsomes obtained from mouse lung but not from rat lung. Pretreatment of mice with *N,N'*-diphenyl-*p*-phenylene diamine, an antioxidant, or a high carbohydrate diet prevented the stimulatory effects of paraquat on lipid peroxidation but did not protect the animals against the lethal effects of paraquat. Conjugated dienes were not elevated *in vivo* after a dose of paraquat in mice equivalent to twice the LD_{50}. Plasma and lung concentrations and edematogenic activities of paraquat and diquat were measured in rats after subcutaneous injections of these chemicals. At equimolar doses, diquat was less edematogenic and found at lower concentrations in lung than was paraquat, but, considering the greater efficacy of diquat in stimulating lipid peroxidation *in vitro* (R. Talcott, H. Shu and E. Wei, [24], these results indicate that the effects of bipyridinium herbicides *in vitro* may not be related to their mechanisms of toxicity *in vivo*.

Paraquat (1,1'-dimethyl-4,4'-bipyridinium ion), a widely used herbicide, is toxic to the lungs of man and most experimental species. Bus *et al.* [1] have proposed that paraquat damages the lung by catalyzing the peroxidation of lung lipids because paraquat, in the presence of appropriate cofactors, stimulates the formation of peroxidized lipids *in vitro*. In additional studies, Bus *et al.* [2, 3] showed that paraquat decreases lipid-soluble antioxidant levels in lungs, that mice deficient in antioxidants are more susceptible to the lethal effects of paraquat, and that the onset of paraquat lethality is delayed in rats tolerant to hyperbaric oxygen.

While it is generally accepted that the herbicidal actions of paraquat and its congener, diquat (1,1'-ethylene-2,2'-bipyridylium ion), are most likely due to lipid peroxidation [4], evidence for lipid peroxidation as a mechanism for the mammalian toxicity is more ambiguous. No effective antidotes of paraquat toxicity in mammals have been found, although antioxidants have been tested [5]. Antioxidants, however, are effective against oxidizing gases which have been proposed to damage the lung by a lipid peroxidative mechanism [6, 7].

Any attempt to explain the mechanism of paraquat toxicity must also consider the actions of diquat. Diquat, which is chemically similar to paraquat, produced little lung damage in rodents [8], although on a molar basis, it is more potent than paraquat in stimulating lipid peroxidation *in vitro* [24]. Diquat is retained to a lesser degree by lung than is paraquat [9, 10] and this factor may account, in part, for its lack of toxic effect on the lungs. In this study, the significance of tissue concentrations of paraquat and diquat and of antioxidants in paraquat toxicity are re-examined in light of the proposal that paraquat produces lung damage by a lipid peroxidative mechanism.

MATERIALS AND METHODS

Experiments were conducted on male Sprague–Dawley rats weighing 200–300 g and on male Swiss–Webster mice weighing 30–40 g.

Lipid peroxidation studies. Formation of malondialdehyde and conjugated dienes was used as an index of lipid peroxidation [11]. Microsomes were isolated from pooled lungs of four rats and incubated (0.75 mg protein/ml) in 50 μM ferric pyrophosphate, 50 mM Tris hydrochloride, pH 7.4, and various concentrations of paraquat dichloride (Sigma, St. Louis, MO) at 37°. Lipid peroxidation reactions were initiated by adding NADPH, 0.5 mM, to the incubation mixture. At appropriate intervals, samples were removed and assayed for malondialdehyde by the thiobarbituric acid method [11, 12]. Absorbance readings at 535 nm were converted to malondialdehyde concentration with an extinction coefficient of 156 A/mM [12].

To inhibit lipid peroxidation, mice were pretreated with *N,N'*-diphenyl-*p*-phenylene diamine (DPPD) or fed a high carbohydrate diet. DPPD, obtained from Pfaltz & Bauer, Stamford, CT, was suspended in corn oil and injected 600 mg/kg, i.p., once daily for 2 days [13]. Controls received corn oil or saline only. The high carbohydrate diet (Fat-free Test diet, ICN Pharmaceuticals, Cleveland, OH) was fed to mice for 2 weeks in order to reduce or eliminate peroxidation-susceptible lipids from the endoplasmic reticula [14]. Controls were maintained on Purina lab chow. Microsomes were isolated from the pooled lungs of four to six mice from each group and incubated in the medium described above, with or without supplementation with 200 μM paraquat. Aliquots removed were assayed for the presence of malondialdehyde by the thiobarbituric acid method [11, 12].

Conjugated dienes were measured in mice injected with paraquat dichloride, 68 mg/kg, i.p. Microsomes

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isolated from the lungs of these mice at 3, 24 and 48 hr after paraquat injection were diluted to 0.75 mg protein/ml with 50 mM Tris–hydrochloride, pH 7.4. The lipids were extracted with chloroform–methanol (2:1) and the extract was concentrated to dryness, weighed, and redissolved in methanol (0.25 mg/ml). The methanol solutions of the extracted lipids were scanned in the ultraviolet for the presence of conjugated dienes [11, 15].

Lethality studies. The LD_{50} of paraquat was determined in control and experimental mice by injecting, i.p., paraquat dichloride dissolved in 0.9% saline at four dosage levels—23.0, 27.6, 33.1 and 39.7 mg/kg—with eight to ten mice in each dosage group. The number of dead mice was recorded daily for 2 weeks. All deaths occurred in the first week. The LD_{50}, slope, and 95 per cent confidence limits were calculated according to Litchfield and Wilcoxon [16].

Tissue concentrations of paraquat and diquat. [^{14}C]-paraquat dichloride (30 mCi/m-mole) and [^{14}C]-diquat dibromide (29 mCi/m-mole) labeled in the methylene and ethylene carbons, respectively, were obtained from Amersham Searle, Arlington Heights, IL, and were diluted with unlabeled paraquat or diquat in saline. Paraquat or diquat was injected subcutaneously in rats. At appropriate intervals, the animals were anesthetized with ether and exsanguinated via the abdominal aorta. The heart and lungs were then excised as a unit and the lungs were perfused via the left ventricle five times successively with 10 ml each of 0.9% saline. The lungs were then dissected from the heart and vessels, minced with scissors, homogenized for 15 sec with a Polytron homogenizer, and brought to a volume of 7 ml with 0.9% saline.

Duplicate samples of 0.4 ml lung homogenate from each rat were digested with Protosol (New England Nuclear, Boston, MA) at 55°, and then mixed with 15 ml of scintillation fluid containing 0.8% 2,5-diphenyloxazole, 0.01% *p*-bis-(2,5-phenyloxazoyl) benzene and 37.5% anhydrous methanol in toluene. Radioactivity was measured using a Nuclear Chicago scintillation counter with efficiency determined individually for each sample with ^{14}C internal standards. Paraquat and diquat levels per lung were calculated from the specific activity of the injected solution after correcting for background and efficiency.

Determination of lung edema with [^{125}I]*albumin.* The method of Alpert *et al.* [17] for measuring alveolar lung edema was used with minor modifications in these studies. ^{125}I-bovine serum albumin (New England Nuclear, Boston, MA) was diluted in 0.9% saline and injected (80 μCi/kg) into the femoral vein of ether-anesthetized rats. Saline, paraquat or diquat was injected subcutaneously immediately thereafter. The animals quickly recovered from the anesthetic and were returned to their cages. At 24 or 48 hr after injection, animals were anesthetized again with ether, blood was collected from the abdominal aorta, the thorax was exposed, and the trachea was cannulated and tied to a lavage syringe.

To obtain an estimate of the amount of albumin in the alveolar space, the lungs were lavaged five times with 5 ml of 0.9% saline and the lavage fluid collected, diluted, and counted for ^{125}I radioactivity. Of the 25 ml of saline used in lavage, more than 23 ml was consistently recovered from each animal. This lavage procedure yields at least 80 per cent of the recoverable radioactivity [17]. The amount of ^{125}I bound to albumin was estimated also. For the injected solution, 95 per cent of the ^{125}I was precipitable by 10% trichloroacetic acid, a value in agreement with that of Alpert *et al.* [17]. In plasma and lavage samples obtained 48 hr after injection, the respective percentages were 81 and 70. These values show that this method measures labeled albumin and principally reflects transfer of serum proteins from the vascular to the alveolar space.

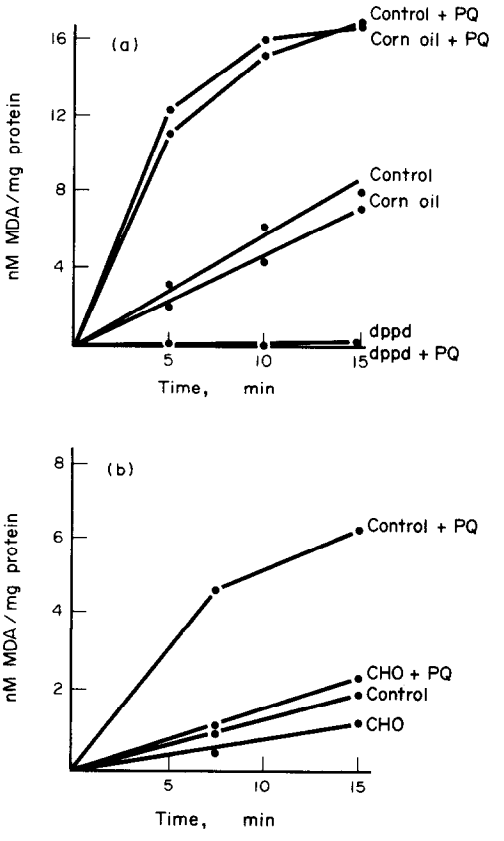

Fig. 1. Inhibition of malondialdehyde production by DPPD or a high carbohydrate, fat-free diet in mouse lung microsomes. Mice (N = 6) were injected with DPPD, 600 mg/kg i.p. suspended in corn oil, once daily for 2 days [13]; control animals received corn oil or saline only (Fig. 1A). Another group of mice were fed a high carbohydrate, fat-free diet for 2 weeks. [14]; controls were maintained on laboratory chow (Purina) (Fig. 1B). Microsomes were isolated from the pooled lungs of each group and were incubated in the presence and absence of 200 μM paraquat (PQ). Malondialdehyde content of removed aliquots was assayed [11, 12].

RESULTS

Paraquat added *in vitro* stimulated the formation of malondialdehyde in mouse lung microsomes (Fig. 1) but not in rat lung microsomes (Fig. 2). Pretreatment of mice with DPPD or a high carbohydrate, fat-free diet reduced the basal rate of lipid peroxidation and effectively attenuated the stimulatory effects of paraquat on malondialdehyde production (Fig. 1).

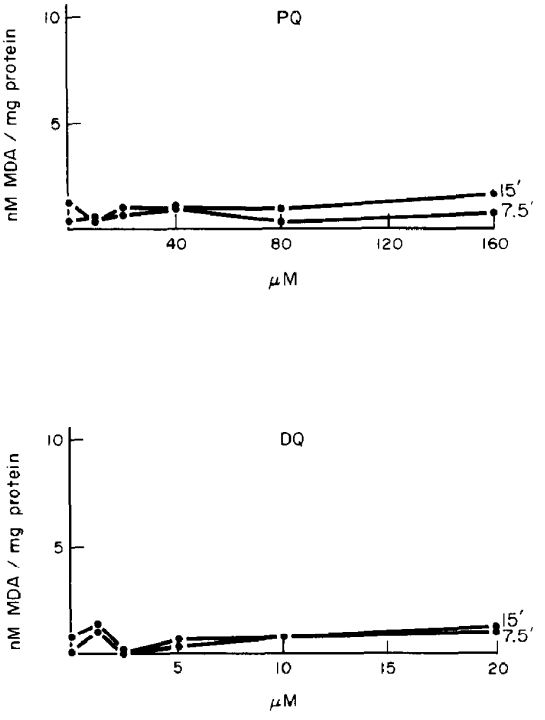

Fig. 2. Paraquat-stimulated malondialdehyde production by rat lung microsomes. Microsomes obtained from rat lungs (N = 4) were incubated in different paraquat or diquat concentrations, as indicated on the abscissae. Aliquots were removed at 7.5 and 15 min and the malondialdehyde content was determined by the thiobarbituric acid method [11, 12].

Pretreatment with DPPD or a high carbohydrate, fat-free diet did not, however, protect these mice against the lethal effects of paraquat (Table 1). The LD_{50} of paraquat in mice pretreated with DPPD was actually lowered (Table 1), although the ability of paraquat to stimulate lipid peroxidation in these animals was blocked (Fig. 1).

Although paraquat stimulated lipid peroxidation in mouse lung microsomes *in vitro*, we found no evidence for increased levels of conjugated dienes in the lung microsomes of mice treated with a dose of paraquat equivalent to twice the LD_{50} dose (Fig. 3).

Table 1. Effect of DPPD or a high carbohydrate diet on paraquat LD_{50}*

Treatment	Paraquat LD_{50} and 95 per cent confidence limits (mg/kg)	Slope and 95 per cent confidence limits
DPPD	24.6 (21.8–27.8)†	1.28 (1.10–1.48)
Corn oil	34.6 (29.2–40.7)	1.28 (1.02–1.60)
High carbohydrate diet	40.0 (34.2–46.8)	1.38 (1.06–1.79)
Control diet	39.0 (33.3–45.6)	1.26 (1.04–1.52)

*Groups of eight to ten mice per dosage group were treated with DPPD, cork oil, a fat-free, high carbohydrate diet, or with a standard laboratory diet as described in Materials and Methods. After the pretreatment, paraquat was administered to the various dosage groups within each treatment group and the LD_{50} values, with slope and confidence intervals, were determined from the numbers of dead mice. All deaths that occurred, occurred within 1 week after paraquat treatment.

†Significantly lower than control value ($P < 0.05$).

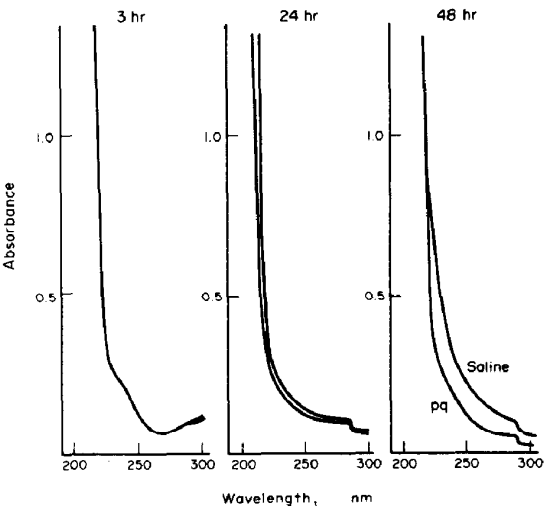

Fig. 3. Conjugated dienes in microsomes of mouse lung after paraquat poisoning. Mice (N = 6) were injected with saline or paraquat, 68 mg/kg i.p., and lipids were extracted from microsomes obtained from the lungs with chloroform–methanol (2:1). The extracts were concentrated to dryness with nitrogen, redissolved in methanol, and scanned for the presence of conjugated dienes, which absorb maximally in the 230–249 nm region [11, 15]. At 3 hr, the curves are superimposable.

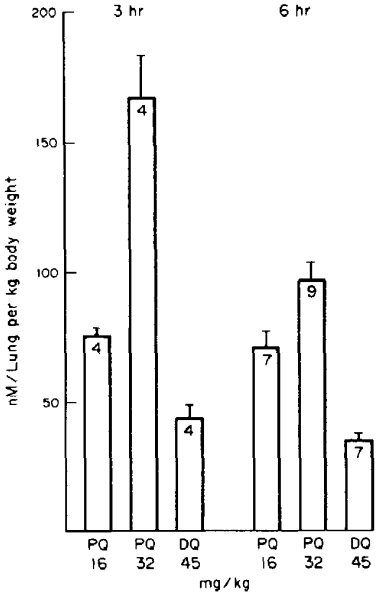

Fig. 4. Paraquat and diquat levels in rat lung after subcutaneous injection. Paraquat or diquat was administered with radioactive tracers at the doses indicated to rats, which were killed at either 3 or 6 hr after dosing. Radioactivity was determined in aliquots of lung homogenate. Values represent the mean ± S. E.; the number of the bar graph represents the number of rats used. Diquat dibromide, 45 mg/kg, is equimolar to paraquat dichloride, 32 mg/kg. DQ = diquat; PQ = paraquat.

The concentrations of paraquat and diquat in lung tissues of rats after subcutaneous injection of these substances are shown in Fig. 4. Paraquat and diquat

Table 2. Effect of paraquat on [^{125}I]albumin distribution*

Time (hr)	Paraquat (mg/kg)	Cpm/ml plasma/kg body wt ($\times 10^{-6}$)	Cpm lavage/kg body wt ($\times 10^{-6}$)	Cpm lavage/cpm/ml plasma
24	0	1.177 ± 0.033	0.082 ± 0.016	0.070
	8.0	1.197 ± 0.053	0.109 ± 0.016	0.091
	11.3	1.133 ± 0.046	0.053 ± 0.009	0.047
	16.0	0.951 ± 0.067	0.068 ± 0.003	0.071
	22.6	1.181 ± 0.103	0.207 ± 0.068†	0.175†
	32.0	1.142 ± 0.066	0.217 ± 0.051†	0.190†
48	0	0.346 ± 0.012	0.023 ± 0.003	0.067
	8.0	0.339 ± 0.020	0.021 ± 0.001	0.061
	11.3	0.353 ± 0.022	0.032 ± 0.006	0.091
	16.0	0.380 ± 0.016	0.066 ± 0.022†	0.175†
	22.6	0.458 ± 0.042†	0.348 ± 0.142†	0.761†
	32.0	0.489 ± 0.046†	0.761 ± 0.133†	1.557†

*Rats (N = 6–11) were injected with [^{125}I]albumin, i.v., and paraquat was injected s.c. Twenty-four and 48 hr later, ^{125}I levels in the plasma and lung lavage fluid were determined [17]. Values represent mean ± S. E.

†$P < 0.01$, with respect to saline control values.

were present in lung at higher concentrations at 3 hr than at 6 hr after injection, an observation not altogether consistent with those of other workers, who had used much higher doses of paraquat [9] and/or different routes of administration [9, 18]. Differences in experimental protocol preclude quantitative comparisons of the results of these various studies on paraquat lung disposition. Qualitatively, our results (Fig. 4) agree with those of previous reports [9, 10] regarding the specificity of the lung for paraquat: 3 hr after an equimolar dose of paraquat and diquat, the concentration of paraquat in the lung exceeded that of diquat by 3.9-fold. At 6 hr, paraquat was still present at three times the concentration of diquat. (Fig. 4). At these time points, paraquat lung damage was not extant and, therefore, the lung perfusion was not compromised by hemorrhage. Thus, the results shown in Fig. 4 are not attributable to blood contamination of the lung homogenates.

Paraquat produced a dose-dependent increase in the albumin content in the alveolar space (Table 2). At 24 hr, the two higher doses, 22.6 and 32.0 mg/kg, the latter approximately equal to an LD_{50} dose, elevated the albumin content by 3-fold above saline control levels. At 48 hr, 16.0, 22.6 and 32.0 mg/kg of paraquat increased alveolar albumin content by 3-, 15- and 33-fold respectively. The plasma radioactivity at 48 hr is also slightly elevated, suggesting that the clearance of albumin had been affected by paraquat (Table 2).

Diquat also produced an elevation of alveolar albumin content (Table 3). However, the magnitude of this response is small compared to that of paraquat: at 24 hr there was a 1.6-fold increase, and at 48 hr there was a 2.1-fold increase over controls. A lower dose of diquat, 32 mg/kg, was used for the 48-hr measurement because several rats died at 45 mg/kg. Diquat, like paraquat, produced a slight increase in the plasma level of radioactivity. The elevation of alveolar albumin content by diquat, however, is no longer significant when the ratio of alveolar to plasma albumin is compared to the control ratio (Table 3).

DISCUSSION

In recent years it has been proposed for a number of chemicals that a common mechanism of cellular injury is lipid peroxidation [19]. Bus *et al.* observed that paraquat stimulates lipid peroxidation in isolated microsomes [1] and they postulated that this action is responsible for the toxic effects of paraquat on the lung, *in vivo* [2, 3]. This study has shown, however, that it is possible to prevent paraquat-stimulated lipid peroxidation without decreasing paraquat toxicity. Furthermore, no evidence of lipid peroxidation, as measured by conjugated diene accumulation, could be found in mouse lung after a toxic dose of paraquat. Also, paraquat-stimulated lipid peroxidation could not be demonstrated in lung microsomes isolated from the rats used in these studies, yet

Table 3. Effect of diquat on ^{125}I-distribution*

Time (hr)	Diquat (mg/kg)	Cpm/ml plasma/kg body wt ($\times 10^{-6}$)	Cpm lavage/kg body wt ($\times 10^{-6}$)	Cpm lavage/cpm/ml plasma
24	0	1.03 ± 0.041	0.057 ± 0.0052	0.056 ± 0.006
	45.0	1.26 ± 0.038†	0.093 ± 0.011†	0.074 ± 0.008
48	0	0.43 ± 0.045	0.037 ± 0.0048	0.088 ± 0.013
	32.0	0.68 ± 0.051†	0.080 ± 0.0071†	0.122 ± 0.013

*Rats (N = 6–11) were injected with [^{125}I]albumin, i.v., and diquat was injected s.c. Twenty-four and 48 hr later, ^{125}I levels in the plasma and lung lavage fluids were determined [17]. Values represent the mean ± S. E.

†$P < 0.05$, with respect to control value.

paraquat produced edema in the lungs of these rats. These results are not consistent with a lipid peroxidation mechanism of toxicity and, together with the report of Ilett *et al.* [20], raise questions that should be addressed before such a mechanism can be accepted.

An interesting aspect of bipyridinium herbicide toxicity is the lack of effect of diquat on rodent lung, in contrast to the toxic effects of paraquat. The concentration of diquat required for half-maximal stimulation of lipid peroxidation in lung microsomes is approximately ten times lower than that of paraquat [24]. At equimolar doses *in vivo*, the maximal lung concentrations of diquat are but four times lower than those attained with paraquat. These quantitative differences again suggest that the causative mechanism of lung toxicity cannot, as yet, be confidently attributed to lipid peroxidation. It remains to be shown if the low edematogenic activity of diquat can be attributed solely to lower uptake by lung or to intrinsic differences in the mechanisms of toxicity between paraquat and diquat.

The cellular distribution of paraquat in the lung is not known. Histological evidence indicates that the type I cells of the alveolar epithelium and the capillary endothelial cells are damaged by paraquat, and the type II cells are unaffected [21]. However, the type II cells and the Clara cells (also not reported to be damaged by paraquat) are the probable sources of lung microsomes [22, 23]. If paraquat, *in vivo*, interacts with endoplasmic reticula to cause peroxidative destruction, it remains to be explained why the principal cellular sources of endoplasmic reticula are not damaged by paraquat, whereas the cells not known to have endoplasmic reticula are damaged. Perhaps as better techniques for separating and isolating specific lung cell types become available, the question of the cellular specificity of paraquat can be better addressed.

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