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RELEVANCE OF NADPH DEPLETION AND MIXED DISULPHIDE FORMATION IN RAT LUNG TO THE MECHANISM OF CELL DAMAGE FOLLOWING PARAQUAT ADMINISTRATION*

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Abstract—We have examined the possibility that the mechanism of paraquat toxicity in the lung involves both the formation of mixed disulphides (the amount of NPSH or GSH involved in protein disulphide formation) and the prolonged oxidation of NADPH, leading to NADPH depletion. We have compared the oxidation–reduction status of the lung, 2, 8 and 24 hr after dosing rats subcutaneously with 20 mg paraquat/kg (a dose which causes extensive lung damage 24 hr after dosing) or 20 mg diquat/kg (a chemically related bipyridyl which only causes very minimal lung damage 24 hr after dosing). Lung $NADP^+$ levels were not affected 2, 8 or 24 hr after dosing with either bipyridyl. However, although NADPH levels were unchanged 2 hr after dosing with paraquat, and 2, 8 and 24 hr after dosing with diquat, there was a significant decrease in NADPH levels by 8 and 24 hr after dosing with paraquat. The changes in NADPH levels were coincident with the lung damage (characterized previously) caused by these treatments. In contrast with these effects on NADPH levels, there was an increase in NPSH and GSH levels in the lung by 8 and 24 hr after dosing with paraquat or diquat. Thus, there was no simple relationship between lung NADPH levels and lung sulphydryl levels.

Lung mixed disulphide levels (the amount of NPSH or GSH involved in disulphide formation) were increased 2, 8 and 24 hr after dosing with paraquat or diquat, although oxidized glutathione levels remained normal. Thus, an early and persistent biochemical effect of paraquat and diquat in the lung involves an increase in mixed disulphide levels, which is probably a consequence of the lungs' response to an increase in the oxidation of NADPH and GSH. As suggested previously, the increase in mixed disulphide levels appears to be a mechanism for regulating the normal redox state of the lung. However, despite this regulatory mechanism, NADPH depletion occurs 8 and 24 hr after dosing with paraquat, but not diquat, coincident with the development of lung damage.

In conclusion, we suggest that mixed disulphide formation is not only a regulatory mechanism, but in some circumstances also may cause changes in essential biosynthetic and regulatory functions of the lung. This may ultimately lead to the drop in NADPH levels, which we propose is a critical biochemical event in the development of alveolar epithelial cell damage following the administration of paraquat.

Poisoning by the bipyridyl herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium) in both man and experimental animals can result in damage to several organs, although the lung is the most severely affected [1]. While it is generally agreed that the mechanism of paraquat toxicity involves its cyclical reduction and reoxidation, which results in the oxidation of NADPH‡ to $NADP^+$ and the production of oxygen free radicals [2–4], the precise biochemical events that lead to lung cell damage are not yet known. However, at least two hypotheses have been proposed. Firstly, the lipid peroxidation hypothesis, in which it is suggested that oxygen free radicals produced by paraquat react with membrane phospholipids and hence impair membrane structure and function [3]. Secondly, the NADPH oxidation hypothesis, in which it is argued that NADPH is oxidized both in the cyclical reduction and reoxidation of paraquat, and in the subsequent defence against the toxic effects of oxygen free radicals. If NADPH depletion occurs, this may cause cell death by disturbing vital physiological and biochemical functions [5].

Previous studies of the biochemical effects of paraquat on the lung have shown that the pentose phosphate pathway is stimulated [6–8] and lipid and fatty acid synthesis are reduced [6, 9]. In a previous paper, we extended these findings by showing that 2 hr after dosing rats subcutaneously with paraquat, there was an increased formation of mixed disulphides between protein and glutathione which was linearly related to the degree of stimulation of the pentose phosphate pathway and to the degree of reduction of fatty acid synthesis [10]. We suggested that these data indicate that mixed disulphide formation may be part of an important regulatory mech-

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‡ GSH, reduced glutathione; GSSG, oxidized glutathione; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; NPSH, non-protein sulphydryl.

anism in the lung which controls the oxidation/reduction of NADPH by regulating the activity of intermediary metabolism. The increase in mixed disulphides by paraquat may also be relevant to its mechanism of toxicity in the lung, because mixed disulphides may also be formed in those proteins whose enzymic and structural functions are needed for the integrity of the cell [11, 12].

It was our intention in this study to (1) examine the possibility that the mechanism of paraquat toxicity involves NADPH depletion, and (2) to determine whether mixed disulphide formation by paraquat in the lung could be related to the development of lung damage and NADPH depletion. In order to do this, we determined the oxidation/reduction status of the lung by directly measuring NADPH and $NADP^+$ levels, non-protein sulphydryl and reduced and oxidized glutathione levels and mixed disulphide levels. We compared the oxidation/reduction status of the lung, 2, 8 and 24 hr after dosing rats subcutaneously with paraquat or the related bipyridyl herbicide diquat. Diquat was used in these studies because it has similar chemical and physical properties to paraquat, similar parenteral LD_{50} values, but only minimally damages the lung, whereas paraquat causes extensive lung damage [1, 13, 14]. This difference in lung toxicity may be due to the shorter half-life of diquat in the lung [15, 16], and may also be because only paraquat is accumulated and retained by the alveolar epithelial cells [17, 18].

MATERIALS AND METHODS

Materials. Paraquat (1,1′-dimethyl-4,4′-bipyridylium) dichloride and diquat (1,1′-ethylene-2,2′-bipyridylium) dichloride monohydrate (both 99.9% pure) were supplied by ICI, Plant Protection Division, Jealott's Hill Research Station, Bracknell, Berks, U.K. Halothane B.P. (FLUOTHANE*) was obtained from ICI, Pharmaceuticals Division, Macclesfield, Cheshire, U.K. All other chemicals were obtained from Sigma Chemical Company, London, U.K., or from BDH Chemicals Limited, Poole, Dorset, U.K.

Treatment of animals. Male, Wistar-derived, Alderley Park strain rats (190–230 g body wt) were used for all experiments. They were maintained in an air-conditioned animal room, with a 12 hr light–dark cycle at 21–25°, and had access to food and water until treated. The rats were dosed (2.0 ml/kg body wt) subcutaneously with either paraquat (20 mg paraquat cation/kg body wt) or diquat (20 mg diquat cation/kg body wt) dissolved in 0.9% saline. Control animals were dosed with saline only. At 2, 8 and 24 hr after dosing, the rats were deeply anaesthetized in a 3% halothane and air mixture. Whilst the animals were still breathing, the renal artery was cut and the lungs were rapidly perfused *in situ* through the pulmonary artery with ice-cold 0.9% NaCl.

Measurement of lung NADPH and $NADP^+$ levels and DNA content. The left lobe was quickly removed, bisected and each half was immediately frozen in liquid nitrogen. Pyridine nucleotides were extracted from the lung by the methods described by Slater *et al.* [19] and were assayed by the methods of Nisselbaum and Green [20]. The frozen top half of the left lobe was used for the measurement of NADPH levels whilst the bottom half of the left lobe was used for $NADP^+$. The samples were assayed on the same day as extraction, and an internal standard was assayed with each sample. The perchloric acid tissue precipitate (from $NADP^+$ determination) was used for DNA determination [21].

In order to validate the extraction/assay procedure for pyridine nucleotides, a known amount of $NADP^+$ or NADPH was added to the lungs prior to homogenization. Complete recovery of $NADP^+$ (102% ± 11 (4)) was recorded in the $NADP^+$ extract, with virtually no $NADP^+$ (3% ± 2 (4)) in the NADPH extract. Similarly, there was good recovery of NADPH (87% ± 7 (4)) in the NADPH extract and very little NADPH (0.3% ± 0.3 (4)) in the $NADP^+$ extract.

Measurement of lung mixed disulphide and non-protein sulphydryl levels. The left lobe was quickly removed and immediately frozen in liquid nitrogen. The frozen left lobe was weighed, and finally homogenized using a Polytron vortex homogenizer, in 4.75 ml ice-cold deoxygenated Tris–EDTA buffer (5 mM Tris-chloride, 5 mM EDTA and 0.15 M NaCl, pH 7.5). The homogenization was done in a nitrogen atmosphere using deoxygenated buffer: this was found to be essential to prevent autoxidation of sulphydryls to disulphides. Sulphydryls and disulphides were analysed on the same day as extraction.

Using reduced glutathione (GSH) as a standard, non-protein sulphydryl (NPSH) levels were measured as GSH equivalents according to Sedlak and Lindsay [22] and GSH levels were measured according to Hissin and Hilf [23]. Mixed disulphide levels were measured as the amounts of NPSH and GSH involved in forming disulphide bonds. This was calculated as the difference in NPSH or GSH levels before and after reduction with sodium borohydride according to DeLucia *et al.* [24]. In recovery studies in which a known amount of GSH or GSSG standard was added to the lung homogenates, there was good recovery of GSH in the free NPSH assay (84% ± 3 (5)) and of GSSG in the mixed disulphide assay (72% ± 1 (5)). As expected, there was little recovery of GSSG in the free NPSH assay (11% ± 1 (5)) and zero recovery of GSH in the mixed disulphide assay (−2% ± 10 (5)).

Measurement of lung oxidized glutathione levels. The left lobe was quickly removed, weighed, immediately homogenized (Polytron vortex homogenizer) in 4.75 ml ice-cold 0.1 M phosphate–5 mM EDTA buffer (pH 8.0) containing 1 ml 25% metaphosphoric acid and finally centrifuged at 100,000 *g* for 30 min at 4°. Oxidized glutathione (GSSG) was measured according to Hissin and Hilf† [23].

* FLUOTHANE is a registered trademark of Imperial Chemical Industries plc.

† An analysis of this method [25] has shown that in normal rat liver, GSSG represents only a small fraction of the total *o*-phthalaldehyde-reactive compounds. We have similarly found this to be the case for rat lung. Therefore this method cannot be used to determine the absolute GSSG levels in the lung. Instead, we have used the method here in order to estimate any increase in GSSG concentration in the lungs of rats dosed with paraquat, compared with control animals dosed with saline.

RESULTS

Lung DNA content

Although paraquat can cause lung oedema [1], we have previously shown that this does not develop by 24 hr after dosing with 20 mg paraquat/kg or 20 mg diquat/kg [14]. In order to further validate these findings, we measured lung DNA content, and found that the amount of DNA per g wet wt was not altered by any of the treatments (data not presented). This is important since the data presented in this paper are expressed as per g wet wt. Thus any changes in lung pyridine nucleotide, non-protein sulphydryl, or mixed disulphide content (per g wet wt) were not due to changes in lung wet weight.

Lung NADPH and NADP$^+$ levels

The total amount of NADPH and NADP$^+$ found in the lung (38.8–42.5 nmole NADPH + NADP$^+$/g lung wet wt—Table 1) was similar to that obtained by other workers (10.8 nmole/g wet wt [26], 32.5 nmole/g wet wt [27]; 55.0 nmole/g wet wt [28]; 35.3 nmole/g wet wt [13]). Furthermore, the ratio of reduced to oxidized pyridine nucleotide in the lung was approximately 4.0, which was similar to that reported by Witschi *et al.* [13].

Two hours after dosing, the amount of NADPH or NADP$^+$ in lung tissue taken from rats dosed with paraquat or diquat was not different from controls (Table 1). However, by 8 hr after dosing with 20 mg paraquat/kg the NADPH levels were significantly lower (about 10 nmole/g wet wt) than controls (Table 1). By 24 hr after dosing, the drop in NADPH levels was even more pronounced (about 12 nmole/g wet wt—Table 1). When lung NADPH levels were reduced (8 and 24 hr after dosing), there was no increase in NADP$^+$ levels (Table 1); thus the total pyridine nucleotide content in the lung of these rats was reduced (Table 1). In contrast with these effects, NADPH levels were not reduced 8 and 24 hr after dosing with diquat (Table 1). In all three treatment groups there was a drop in NADP$^+$ levels with time (Table 1), which may be because the rats were starved after treatment.

Lung non-protein sulphydryl levels

The amount of free NPSH and GSH found in the lung (1.78 μmole NPSH/g wet wt and 1.53 μmole GSH/g wet wt—Table 2) was similar to that reported by other workers (1.6 μmole NPSH/g wet wt and 1.5 μmole GSH/g wet wt [24]; 1.59 μmole NPSH/g wet wt [29]; 1.85 μmole NPSH/g wet wt [30]).

Free non-protein sulphydryl (NPSH) or reduced glutathione (GSH) levels in lung were not affected 2 hr after dosing rats with paraquat or diquat (Table 2). However, by 8 hr after dosing, free NPSH and GSH levels were significantly increased (Table 2). Twenty-four hours after dosing with paraquat, free NPSH and GSH levels were still significantly increased (Table 2). Free NPSH or GSH levels were also higher than controls 24 hr after dosing with diquat but this effect was not statistically significant.

Lung mixed disulphide levels

When non-protein sulphydryls (NPSH), such as reduced glutathione (GSH) or cysteine (CSH), react with protein disulphide (ProtSSProt) groups, mixed disulphides (NPSSProt) are produced:

$$\text{NPSH} + \text{ProtSSProt} \leftrightarrow \text{NPSSProt} + \text{ProtSH}.$$

Similarly, mixed disulphides are also produced when protein sulphydryls (ProtSH) react with non-protein disulphides (NPSSNP) such as oxidized glutathione (GSSG) or cystine (CSSC):

$$\text{NPSSNP} + \text{ProtSH} \leftrightarrow \text{NPSSProt} + \text{NPSH}.$$

Table 1. Effect of paraquat or diquat on pyridine nucleotide content of rat lung

Treatment	Pyridine nucleotide concentration in lung nmole NADP$^+$/g wet wt 2 hr	8 hr	24 hr
Control	11.56 ± 1.85 (5)	8.52 ± 1.59 (5)	7.04 ± 1.55 (5)
20 mg paraquat/kg	11.04 ± 0.98 (5)	7.53 ± 1.28 (5)	7.25 ± 1.25 (5)
20 mg diquat/kg	10.06 ± 0.84 (5)	8.51 ± 1.97 (3)	7.77 ± 1.91 (4)
	nmole NADPH/g wet wt		
Control	30.57 ± 1.18 (9)	34.01 ± 2.07 (5)	31.76 ± 2.30 (5)
20 mg paraquat/kg	33.37 ± 1.36 (10)	24.28 ± 2.07 (5)*†	19.70 ± 2.28 (5)*†
20 mg diquat/kg	29.11 ± 2.10 (9)	33.20 ± 3.91 (3)	26.97 ± 1.05 (4)
	nmole NADPH + NADP$^+$/g wet wt		
Control	40.76 ± 4.10 (5)	42.53 ± 1.99 (5)	38.80 ± 2.23 (5)
20 mg paraquat/kg	44.41 ± 2.22 (5)	31.82 ± 3.11 (5)*	26.94 ± 2.43 (5)*†
20 mg diquat/kg	39.84 ± 4.20 (5)	43.57 ± 10.41 (3)	34.74 ± 1.99 (4)

Rats were dosed s.c. with paraquat or diquat and 2, 8 and 24 hr after dosing were anaesthetized in 3% halothane. The lungs were perfused *in situ* with ice-cold saline, and the left lobe was frozen in liquid nitrogen. NADP$^+$ was determined in the supernatant fraction of a perchloric acid tissue homogenate. NADPH was determined in the supernatant (62,000 *g*, 30 min) fraction of a sodium hydroxide tissue digest. Results are expressed as means ± S.E. Number of animals per determination in parentheses.

* Significantly different from control ($P < 0.05$).

† Significantly different from diquat-dosed animals ($P < 0.05$).

Table 2. Effect of paraquat or diquat on non-protein sulphydryl levels of rat lung

Treatment	Free NPSH levels in lung nmole NPSH/g wet wt 2 hr	8 hr	24 hr
Control	1784 ± 48 (17)	1763 ± 36 (20)	1726 ± 59 (19)
20 mg paraquat/kg	1863 ± 39 (18)	2045 ± 41 (20)*	1909 ± 43 (15)*
20 mg diquat/kg	1634 ± 64 (10)	2019 ± 47 (19)*	1889 ± 55 (11)
	Free GSH levels in lung nmole GSH/g wet wt		
Control	1527 ± 48 (12)	1412 ± 51 (20)	1417 ± 29 (18)
20 mg paraquat/kg	1544 ± 17 (14)	1619 ± 45 (20)*	1519 ± 42 (15)*
20 mg diquat/kg	1494 ± 36 (10)	1611 ± 48 (19)*	1481 ± 49 (11)

Rats were dosed s.c. with paraquat or diquat and 2, 8 and 24 hr after dosing were anaesthetized in 3% halothane. The lungs were perfused *in situ* with ice-cold saline, and the left lobe was frozen in liquid nitrogen. Free NPSH or GSH levels were determined in the supernatant fraction of a trichloroacetic acid treated tissue homogenate. Results are expressed as means ± S.E. Number of animals per determination in parentheses.

* Significantly different from control ($P < 0.05$).

In this paper we measured the amount of GSH or NPSH involved in disulphide formation as the difference in the levels of GSH or NPSH in lung homogenates before and after reduction with borohydride [24]. We define and refer to the amount of GSH involved in disulphide formation as the mixed (glutathione) disulphide pool, and to the amount of NPSH involved in disulphide formation as the mixed (total) disulphide pool.

The amount of mixed (total) disulphide (379 nmole/g wet wt—Table 3) was similar to that reported by DeLucia *et al.* [24] (350 nmole/g wet wt). To our knowledge the amount of mixed (glutathione) disulphides (92 nmole/g wet wt—Table 3) has not been reported previously. It is of interest that the mixed (glutathione) disulphide pool represented only a small proportion (about 24%) of the mixed (total) disulphide pool. Thus, about 76% of the lung mixed (total) disulphide pool must occur as mixed (non-glutathione) disulphides such as cysteine SSProt.

Two hours after dosing with paraquat or diquat,

Table 3. Effect of paraquat or diquat on disulphide levels of rat lung

Treatment	†Mixed (total) disulphide levels in lung nmole/g wet wt 2 hr	8 hr	24 hr
Control	379 ± 27 (17)	383 ± 55 (10)	357 ± 30 (19)
20 mg paraquat/kg	460 ± 29 (18)*	453 ± 31 (10)*	429 ± 29 (15)
20 mg diquat/kg	602 ± 82 (10)*	506 ± 37 (10)*	482 ± 46 (11)*
	†Mixed (glutathione) disulphide levels in lung nmole/g wet wt		
Control	92 ± 44 (12)	96 ± 21 (10)	164 ± 31 (18)
20 mg paraquat/kg	174 ± 42 (14)	171 ± 23 (10)*	186 ± 43 (15)
20 mg diquat/kg	151 ± 35 (9)	210 ± 21 (10)*	240 ± 65 (11)
	Oxidized glutathione levels in lung nmole/g wet wt		
Control	25 ± 3 (5)	29 ± 11 (5)	13 ± 2 (5)
20 mg paraquat/kg	23 ± 1 (5)	48 ± 11 (5)	29 ± 6 (5)
20 mg diquat/kg	25 ± 5 (5)	47 ± 11 (5)	24 ± −(2)

Rats were dosed s.c. with paraquat or diquat and 2, 8 and 24 hr after dosing were anaesthetized in 3% halothane. The lungs were perfused *in situ* with ice-cold saline, and the left lobe was frozen in liquid nitrogen. Mixed disulphide levels were determined after reduction with sodium borohydride, in the supernatant fraction of a trichloroacetic acid treated tissue homogenate. Oxidized glutathione levels were determined in the supernatant fraction of a metaphosphoric acid treated tissue homogenate. Results are expressed as means ± S.E. Number of animals per determination in parentheses.

* Significantly different from control ($P < 0.05$).

† Amount of NPSH or GSH involved in disulphide formation (the difference between the levels of NPSH or GSH in lung homogenate before and after reduction with borohydride).

mixed (total) disulphide levels were significantly increased in the lung, although the increase was greater for the diquat-dosed rats (Table 3). Quantitatively, the increase caused by diquat (about 220 nmole/g wet wt) was not matched by a similar change in mixed (glutathione) disulphides (about 60 nmole/g wet wt), although the effects of paraquat were quantitatively similar (about 80 nmole/g wet wt), whether expressed as total disulphides or glutathione disulphides (Table 3). Eight and twenty-four hours after dosing with paraquat or diquat, mixed disulphide levels were still increased (about 70 and 120 nmole/g wet wt respectively), but the effects were not as great as at 2 hr (Table 3). Unlike the results at 2 hr, both bipyridyls affected mixed (glutathione) disulphides and (total) disulphides to a similar extent (Table 3).

Lung oxidized glutathione levels

Oxidized glutathione (GSSG) levels in the lung (13–29 nmole/g wet wt—Table 3) were lower than that reported by DeLucia *et al.* [24] (120 nmole/g wet wt). Furthermore, in agreement with Beutler and West [25], we found that the fluorometric assay of Hissin and Hilf [23] overestimates the amount of GSSG in the lung (see Materials and Methods). Thus lung GSSG levels are probably lower than 13 nmole/g wet wt. The amount of GSSG in lung was not significantly affected 2, 8 or 24 hr after dosing rats with paraquat or diquat (Table 3). Thus the changes in mixed disulphide levels described above were apparently not the result of an increase in GSSG levels.

DISCUSSION

NADPH depletion and non-protein sulphydryl levels

In agreement with the suggestion that the mechanism of paraquat toxicity involves pulmonary NADPH depletion, we found that paraquat caused a drop in NADPH levels 8 and 24 hr after dosing, whereas diquat did not (Table 1). Thus, as cell damage develops by 8 and 24 hr after dosing with paraquat but not diquat [14], NADPH depletion appears to be related to the development of lung cell damage. These results contrast with those of Witschi *et al.* [13], who did not observe a relationship between a drop in the ratio of NADPH to $NADP^+$ and cell death. In their studies, both paraquat and diquat produced a sharp drop in the ratio of NADPH to $NADP^+$ within 1 hr of intravenous administration. The studies of Witschi *et al.* differ from ours in two respects. Firstly, we dosed the animals subcutaneously, whereas Witschi *et al.* dosed intravenously. Secondly, our methods of analysis of NADPH were different. When measuring the lung pyridine nucleotide content, Witschi *et al.* [13] used the assay technique described by Slater *et al.* [19] with 2,6-DCPIP (dichlorophenol indophenol) as the terminal electron acceptor of an enzyme cycling assay. This assay has recently been criticized on the basis of its non-sensitivity, non-linearity and, most importantly, the spontaneous reaction of 2,6-DCPIP with thiols present in cell extracts [31]. We used a more sensitive enzyme cycling assay which has thiazolyl blue as the terminal electron acceptor in place of 2,6-DCPIP, and overcomes technical problems [20, 32]. Our findings indicate that NADPH depletion may be a critical biochemical event in the development of lung cell damage caused by paraquat. This NADPH depletion may be the result of a loss or destruction of NADPH, since the drop in NADPH levels did not coincide with a corresponding increase in $NADP^+$ levels (Table 1). We believe that this is the first time that a relationship has been demonstrated between NADPH depletion and the development of lung cell damage. However, as pointed out by Witschi *et al.* [13], several attempts to do this in other systems have yielded equivocal results. Thus, although NADPH depletion may be a critical biochemical event in the toxicity of paraquat to the lung, this may not necessarily be the only toxic event that leads to cell damage.

Despite the drop in NADPH levels by 8 and 24 hr after dosing (Table 1) we could find no similar changes in free NPSH or GSH levels (Table 2). Indeed, when NADPH depletion and cell damage had occurred, both NPSH and GSH levels in the lung were significantly increased (about 20%). This increase in sulphydryl levels has been reported previously [33], although other workers were unable to detect an increase in NPSH levels up to 2 days after dosing [3]. As pointed out by Omaye and Reddy [33], the increase in sulphydryl levels may be due to a multiplicity of factors which may be initiated by the cell damage caused by paraquat. However, since diquat also produced similar changes, in NPSH and GSH levels, the effects seem more likely to be attributable to some other systemic effect of the bipyridyls on the animals, such as disturbing plasma corticosteroid levels [34].

Formation of mixed disulphides

Several investigators have suggested that the cyclical and NADPH-dependent reduction and reoxidation of paraquat [2, 3], which results in superoxide anion production [4], leads to a prolonged increase in cellular demand for NADPH [5–7]. In an earlier paper we showed that 2 hr after dosing with paraquat, the lung compensates for this redox stress by increasing lung mixed disulphide levels and simultaneously regulating the activity of the pentose phosphate pathway and fatty acid synthesis [10]. Furthermore, in agreement with other authors [11, 12, 35], we suggested that mixed disulphide formation may be part of an important regulatory mechanism by which the lung controls its intermediary metabolism [10]. This may explain why lung NADPH and $NADP^+$ levels were normal 2 hr after dosing (Table 1). In the present paper we have extended these findings by demonstrating that lung mixed disulphide levels were increased 2 hr after dosing with 20 mg diquat/kg (Table 3). Diquat also reduces fatty acid synthesis and increases pentose phosphate pathway activity to a similar extent to 20 mg paraquat/kg (Keeling and Smith, unpublished). Thus mixed disulphide formation may be important in regulating intermediary metabolism following diquat poisoning, as well as following paraquat poisoning. This is consistent with the possibility that mixed disulphide formation is a result of NADPH and

glutathione oxidation because both bipyridyls are cyclically reduced and reoxidized in the lung [36].

Although these findings illustrate that mixed disulphide formation may function as an important regulatory mechanism in the lung, it is also possible that this biochemical change could also produce loss of both enzymic and structural functions needed for the integrity of the cell [12]. If this were to be the case, there should be differences between the effects of paraquat and diquat in the lung. For example, the effects of paraquat, with its long half-life in the lung [15, 16], might be more persistent than those of diquat. However, contrary to this expectation, both bipyridyls produced a persistent increase in mixed disulphide levels (Table 3). Thus there was no simple or obvious relationship between the increase in mixed disulphide levels and the development of NADPH depletion and lung cell damage by paraquat, but not diquat. While this may indicate that mixed disulphide formation is not involved in the development of lung cell damage following paraquat poisoning, this possibility should not be excluded. For example, the effects of paraquat may be compartmentalized in different cell types to that of diquat. Thus, although both bipyridyls cause redox stress as a result of NADPH and glutathione oxidation, the effects of paraquat may be specific for the alveolar epithelial cells, whereas the effects of diquat may be more generally distributed throughout all the lung cell types. This latter possibility is consistent with the proposal that paraquat is selectively accumulated and retained by the alveolar epithelial cells, whereas diquat is not [17, 18].

Relevance to the mechanism of cell damage

The findings that lung sulphydryl levels were not depleted following paraquat poisoning may explain why lipid peroxidation *in vivo* has not been conclusively demonstrated [37–40], because lipid peroxidation is not induced in liver perfused with lipid hydroperoxides, or *in vivo* by agents known to conjugate glutathione, until NADPH and GSH levels are depleted [41, 42]. Thus, while reduced glutathione levels are maintained, it seems unlikely that lipid peroxidation is the only and/or basic mechanism of paraquat toxicity. It seems more likely that the prolonged redox stress in the target cells results in an increase in NADPH and glutathione oxidation. This is detected as an increased leakage of GSSG through cell membranes [43], and also by the formation of mixed (glutathione) disulphides reported here. The prolonged increase in mixed disulphides has important regulatory functions by controlling NADPH oxidation/reduction [10], and also by sparing protein sulphydryls from irreversible oxidation [11]. However, it is also possible that mixed disulphides are formed in those proteins whose enzymic and structural functions are needed for the integrity of the cell [12]. Such changes in essential biosynthetic and regulatory functions as a consequence of mixed disulphide formation, together with an inhibition of amino acid and pyridine nucleotide biosynthesis [44, 45], and an inhibition of reduction of $NADP^+$ to NADPH [46], may ultimately cause the NADPH depletion reported here, and a cascade of biochemical events which result in cell damage. This proposed mechanism of toxicity is summarized schematically in Fig. 1.

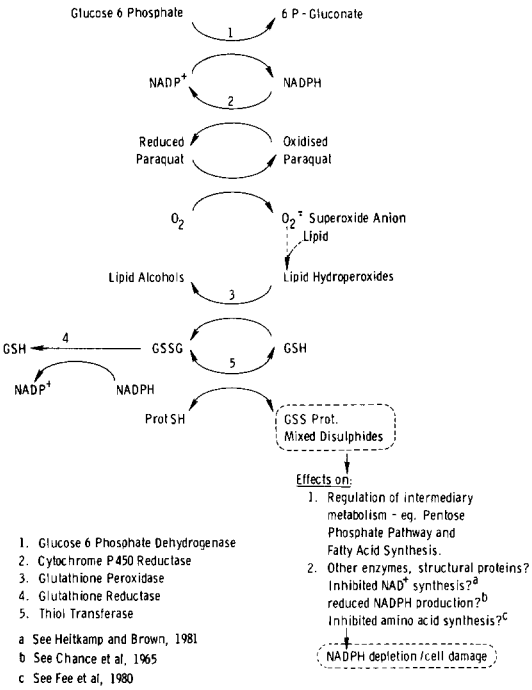

Fig. 1.

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