Biochemical Pharmacology, Vol. 42, No. 2, pp. 303–309, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

COMPARISON OF ONE-ELECTRON REDUCTION ACTIVITY AGAINST THE BIPYRIDYLIUM HERBICIDES, PARAQUAT AND DIQUAT, IN MICROSOMAL AND MITOCHONDRIAL FRACTIONS OF LIVER, LUNG AND KIDNEY (*IN VITRO*)

MASAFUMI TOMITA*
Department of Forensic Toxicology, Kawasaki Medical School, Kurashiki, Japan

(*Received* 12 *November* 1990; *accepted* 12 *March* 1991)

Abstract—The first one-electron reduction steps of paraquat and diquat were compared using microsomal and mitochondrial fractions of rat liver, lung and kidney. Both fractions reduced each herbicide effectively, with the order of the V_{max} values in microsomes and mitochondria being liver > lung > kidney and kidney > liver > lung, respectively. Although similar V_{max} values were obtained from the liver and lung with the two subcellular fractions, the affinity of mitochondrial enzymes was lower, suggesting that the reduction of both herbicides in a microsomal site would be dominant in these two organs. The V_{max} values for radical formation of paraquat were higher than those of diquat in all the endogenous one-electron reducing systems. The apparent K_m values for diquat, however, were lower than those for paraquat in both subcellular fractions from the three tissues, indicating the superiority of the reduction for diquat to that for paraquat at low concentrations. This difference in the K_m values supported the finding that the reduction velocity for diquat was significantly higher than that for paraquat at 1 mM concentration. Thus, at low concentrations, diquat would be reduced more easily than paraquat. In addition, tissue enzymatic specificity for paraquat was not obtained. From these data, it seems reasonable to conclude that the tissue-selective accumulation of paraquat previously proposed determines its toxicity.

Various exogenous compounds are supposed to exert their toxic effects on biological systems via oxygen reduction, and then by peroxidative damage to unsaturated fatty acids, on the constituents of cell membranes [1]. The toxicities of the bipyridylium herbicides paraquat and diquat have also been attributed to their redox properties and lipid peroxidation has been suggested as the molecular mechanism [2–6]. Although there are conflicting observations regarding the toxic mechanism [7–11], it is certain that these compounds must first be reduced by intracellular enzymes to have a highly toxic effect. Therefore, study of the first reduction step, one-electron reduction, is surely one of the keys to clarifying the toxicosis caused by these herbicides. Baldwin *et al.* [12], after comparing the velocity of radical formation of the bipyridylium herbicides in homogenates, concluded that the order among herbicides was morfamquat > diquat > paraquat and the order among tissues was liver > lung > kidney. Paraquat is, however, more toxic than diquat and in addition produces severe pulmonary damage while diquat does not.

In this study, I have attempted to define more precisely the first step by which these herbicides are reduced by intracellular enzymes. In order to clarify the difference in the toxicities of paraquat and diquat their reducing activities in rat microsomal and mitochondrial fractions from the liver, lung and kidney were compared and discussed using kinetic parameters. In addition, whether the pulmonary damage produced by paraquat poisoning depends on only the selective accumulation of the poison or on the specificity of enzymes was also discussed.

* Address correspondence to: M. Tomita, Department of Forensic Toxicology, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-01, Japan.

MATERIALS AND METHODS

Chemicals. Paraquat dichloride (methyl viologen), NADPH and NADH were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Diquat dibromide was a kind gift from ICI (Japan). All other reagents were of the highest purity available. Twice distilled and deionized water was used throughout.

Mitochondrial and microsomal preparations. Male Wistar rats weighing 270–300 g were fed commercial rat chow and water *ad lib.* and then fasted overnight prior to being killed by bleeding under anesthesia. The liver, lung and kidney were removed rapidly and placed into ice-cold sucrose–Tris buffer (0.25 M sucrose, 5 mM Tris–HCl, pH 7.4). The parenchyma of the lung was separated from the visible bronchia and the pelvis of the kidney was removed. These tissues were washed several times until essentially blood-free, then minced with stainless steel scissors. After being washed thoroughly they were homogenized in four volumes of the same buffer using a motor-driven Teflon–glass homogenizer. Mitochondrial and microsomal fractions were obtained by differential centrifugation in the usual way. Briefly, the homogenate was centrifuged at 900 *g* for 10 min, and the resultant supernatant was

Table 1. The proportion of enzyme activities in subcellular fractions

	NADPH-cytochrome *c* reductase			Cytochrome oxidase		
	Liver	Lung	Kidney	Liver	Lung	Kidney
Microsomes	100.0	39.6	32.1	3.2	6.8	27.8
Mitochondria	7.1	8.6	3.5	100.0	49.2	124.0
Cytosol	1.7	3.2	1.7	0.3	0.1	1.0

NADPH-cytochrome *c* reductase and cytochrome oxidase were determined to be the marker enzymes for microsomes and mitochondria, respectively.

The results are expressed as the proportion of the activity when the enzyme activity of each marker in liver tissues was 100.

Table 2. Reduction rate activity for paraquat (Pq) and diquat (Dq) in microsomal and mitochondrial fractions

	NADPH			NADH		
	Liver	Lung	Kidney	Liver	Lung	Kidney
Microsomes						
Pq	75.7 ± 14.1*	33.4 ± 4.7*	26.2 ± 5.8	1.8 ± 0.8	0.8 ± 0.5	17.5 ± 2.3
Dq	121.1 ± 8.6*‡	54.9 ± 6.5*‡	38.7 ± 2.5†‡	6.5 ± 2.7‖	3.4 ± 0.2‡	25.3 ± 4.2§
Mitochondria						
Pq	0.3 ± 0.3	2.3 ± 0.4	2.2 ± 0.2	14.4 ± 2.9	11.7 ± 2.4	27.9 ± 6.7
Dq	4.4 ± 0.3‡	5.1 ± 0.3‡	1.7 ± 0.2§	25.3 ± 2.9‡	30.4 ± 5.1‡	46.9 ± 6.9§

The concentration of each herbicide incubated was 1 mM. The specific rates of appearance of the radical cations were calculated from the rate of increase in absorbance and the absorption coefficients for each radical. They are given in μmol/L/min/mg protein (mean ± SD; N = 5–6).

* $P < 0.001$ and † $P < 0.05$ compared to each value obtained from mitochondrial fractions and NADH.

‡ $P < 0.001$, § $P < 0.01$ and ‖ $P < 0.05$ compared to each value of Pq.

recentrifuged at 13,000 *g* for 15 min. The pellet was resuspended with the same buffer and a mitochondrial pellet was obtained by repetition of the centrifugations mentioned above. The supernatant obtained by the first centrifugation of 13,000 *g* for 15 min was recentrifuged at 13,000 *g* for 20 min. A microsomal pellet was obtained from the supernatant by centrifugation at 100,000 *g* for 60 min. Each pellet was washed once and resuspended in the same buffer. The final protein concentrations were 16–20 mg/mL for the liver, about 5 mg/mL for the lung and 10–13 mg/mL for the kidney. All procedures were performed at 4°. Protein concentrations were determined by the method of Lowry *et al.* [13] using bovine serum albumin as the standard.

Enzymatic assays. NADPH-cytochrome *c* reductase was assayed by measuring the rate of reduction of yeast cytochrome *c* by NADPH [14]. The increase in the optical density at 550 nm with time was measured by a Gilford model 2400-2 spectrophotometer. The activity of cytochrome oxidase was assayed by measuring oxidation of reduced cytochrome *c* [15]. The reduced cytochrome *c* was prepared by adding sodium dithionite to cytochrome *c* solution and the excess dithionite and its degradation products were removed by passing them through a Sephadex G-25 column. The oxidation was recorded as the decrease in optical density at 550 nm with time using a Gilford model 2400-2 spectrophotometer.

Reduction of paraquat and diquat. All assays were conducted anaerobically in a Thunberg cuvette (1 cm path length) at 37°. Each subcellular fraction and the herbicide aqueous solution were put into the main chamber and NADPH or NADH was placed in the side bulb of the cuvette. Oxygen-free nitrogen gases were introduced into the cuvette, after which the cuvette was degassed by an aspirator and then gassed again. This procedure was repeated several times for 10 min per incubation. After preincubation for 5 min at 37°, the reaction was started by the addition of 0.5 mM NADPH or 1.0 mM NADH from the side bulb. Incubation was done in 0.17 mM phosphate buffer (pH 7.5) and the final volume of the incubation was 2.7 mL. The amounts of microsomal and mitochondrial proteins of the liver, lung and kidney used per incubation in this study were approximately 0.5, 0.3 and 0.5 mg, respectively. Changes in absorbance with time were measured with a spectrophotometer (ULTROSPEC II, LKB) connected to a personal computer (PC-8001, NEC, Japan). The absorbance of the paraquat and diquat radicals was measured at 395 and 377 nm, respectively. The absorption coefficients obtained for these radicals were $38.3 \times 10^3\ M^{-1}\ cm^{-1}$ for the paraquat radical and $47.4 \times 10^3\ M^{-1}\ cm^{-1}$ for the diquat radical.

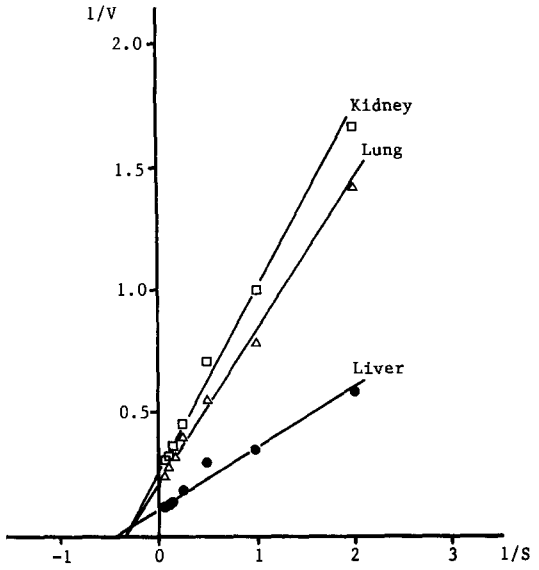

Fig. 1. Lineweaver–Burk plot of the paraquat concentration vs the rate of its radical formation by the NADPH-induced system in microsomal fractions from liver, lung and kidney (mean of 4–6 animals). Paraquat, at concentrations of 0.5–13.3 mM, was incubated with NADPH and each microsomal fraction under the anaerobic conditions described in Materials and Methods. The rate of radical formation was measured as an increase in absorbance at 395 nm. The velocity is given as ΔA/min/mg protein and the substrate concentration is mM.

RESULTS AND DISCUSSION

Table 1 presents data on the distribution of two enzymes, NADPH-cytochrome *c* reductase and cytochrome oxidase, in the subcellular fractions. These were determined to be the marker enzymes for microsomes and mitochondria, respectively. Slight contamination was found between microsomal and mitochondrial fractions, and contamination of the microsomal fraction of the kidney by mitochondrial components was conspicuous. Table 2 shows the specific rates of appearance of the radical cations in both fractions under a constant concentration (1 mM) of each herbicide. Both herbicides were reduced more effectively by each microsomal particle from the three tissues and NADPH than by these particles and NADH. The order of the rates obtained among the tissues with NADPH was liver > lung > kidney, which was consistent with the activity of NADPH-cytochrome *c* reductase. Thus, the redox cycling process in microsomes is thought to be mediated by NADPH-cytochrome *c* reductase, as indicated by earlier studies [2, 16, 17]. Kidney microsomes also effectively reduced both herbicides with NADH but this may have been affected by contamination by mitochondrial components. Mitochondrial particles effectively reduced the herbicides with NADH. The highest activity for both herbicides was obtained in the kidney mitochondria. A comparison of the reduction activities in microsomal (NADPH) and mitochondrial (NADH) fractions showed those in the microsomal fractions from liver and lung to be

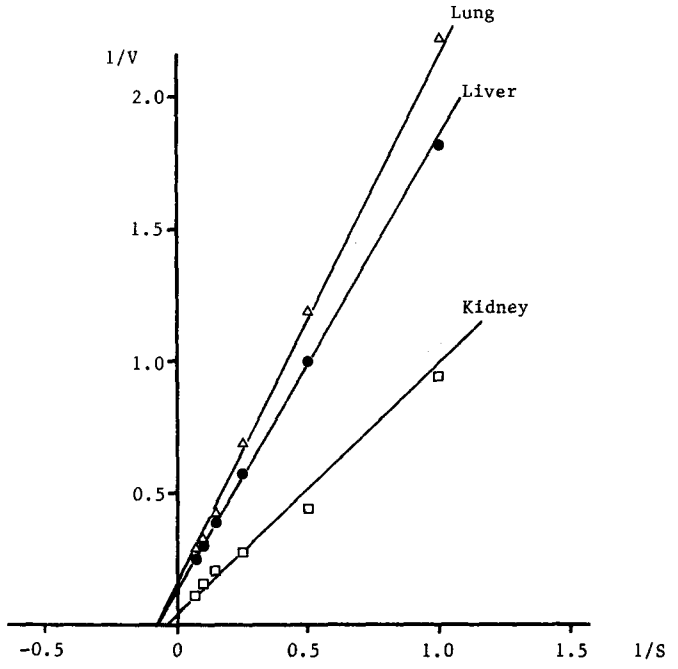

Fig. 2. Lineweaver–Burk plot of the paraquat concentration vs the rate of its radical formation by the NADH-induced system in mitochondrial fractions from liver, lung and kidney (mean of 4–6 animals). Paraquat, at concentrations of 1.0–13.3 mM, was incubated with NADH and each mitochondrial fraction under the anaerobic conditions described in Materials and Methods. The rate of radical formation was measured as an increase in absorbance at 395 nm. The velocity is given as ΔA/min/mg protein and the substrate concentration is mM.

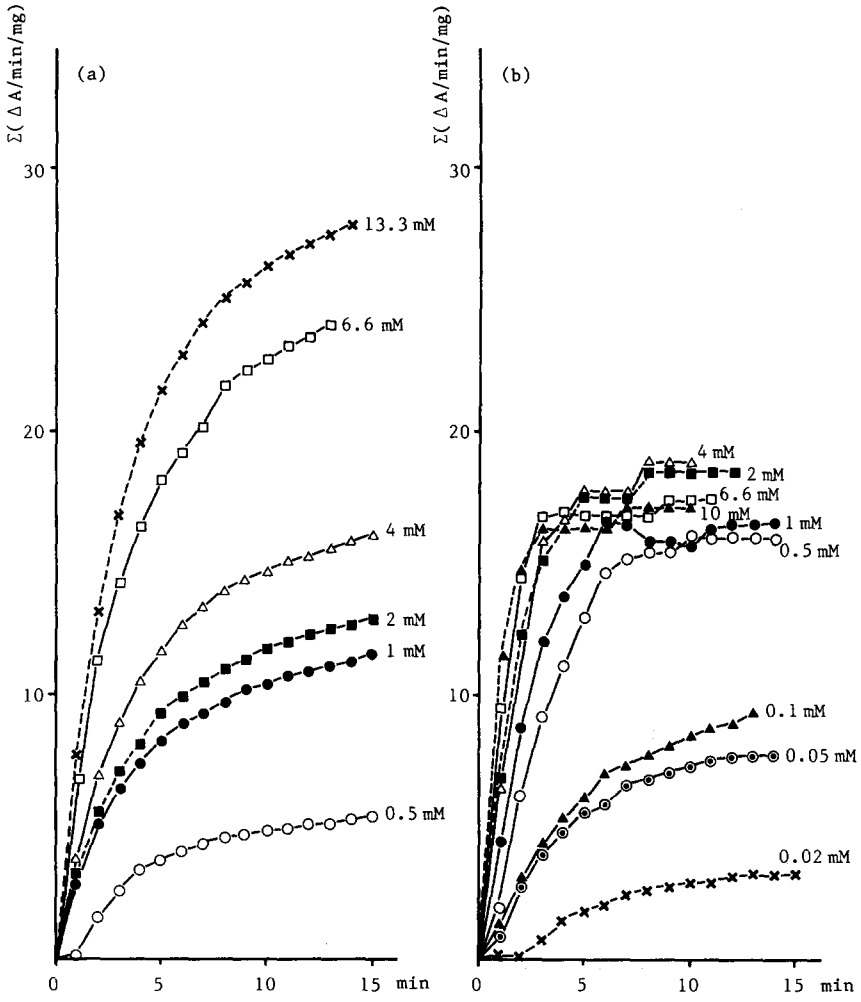

Fig. 3. Increase in absorbance of the paraquat radical (395 nm) and diquat radical (377 nm) at different concentrations: (a) paraquat, (b) diquat. Paraquat (0.5–13.3 mM) or diquat (0.02–10 mM) was incubated with NADPH and liver microsomal fractions.

significantly higher than those in mitochondrial ones. Kidney mitochondria, however, showed similar or higher activities. The reduction velocity of diquat was significantly faster than that of paraquat in all fractions examined. Zychlinski *et al.* [18], using rat lung microsomes, reported that diquat stimulated NADPH oxidation more actively than paraquat. Baldwin *et al.* [12] concluded that the rate of diquat radical formation was faster than that of paraquat radical formation. My findings were essentially similar to these results.

However, it is well-known that paraquat is more toxic than diquat. Therefore, the kinetic parameters of each reduction activity for paraquat and diquat were further examined. Figures 1 and 2 show the microsomal and mitochondrial double-reciprocal plots, respectively, of the reduction velocity against paraquat concentrations. Diquat also exhibited similar Lineweaver–Burk plots. These and other kinetic parameters are summarized in Table 3. Although high concentrations were examined under these experimental conditions these results support the results obtained at a 1 mM concentration of each herbicide (see Table 2). In microsomes and NADPH the order of the V_{max} value obtained for both herbicides was liver > lung > kidney and the K_m values were the same for the three tissues. Although the V_{max} values for paraquat were about two-fold higher than those for diquat the apparent K_m values for diquat were significantly lower than those for paraquat indicating that the microsomal enzymes involved in the reduction processes had a higher affinity for diquat than paraquat. In mitochondria and NADH the order of the V_{max} values of the three tissues for both herbicides were the same as seen in cytochrome oxidase activity; i.e. kidney > liver > lung. As seen in microsomes, the V_{max} values for paraquat were higher than those for diquat but the apparent K_m values for diquat were four- to five-fold lower than those for paraquat. A comparison of the kinetic parameters between the liver and lung showed similar V_{max} values for microsomes and mitochondria but the apparent K_m values for paraquat and diquat in microsomes were lower than

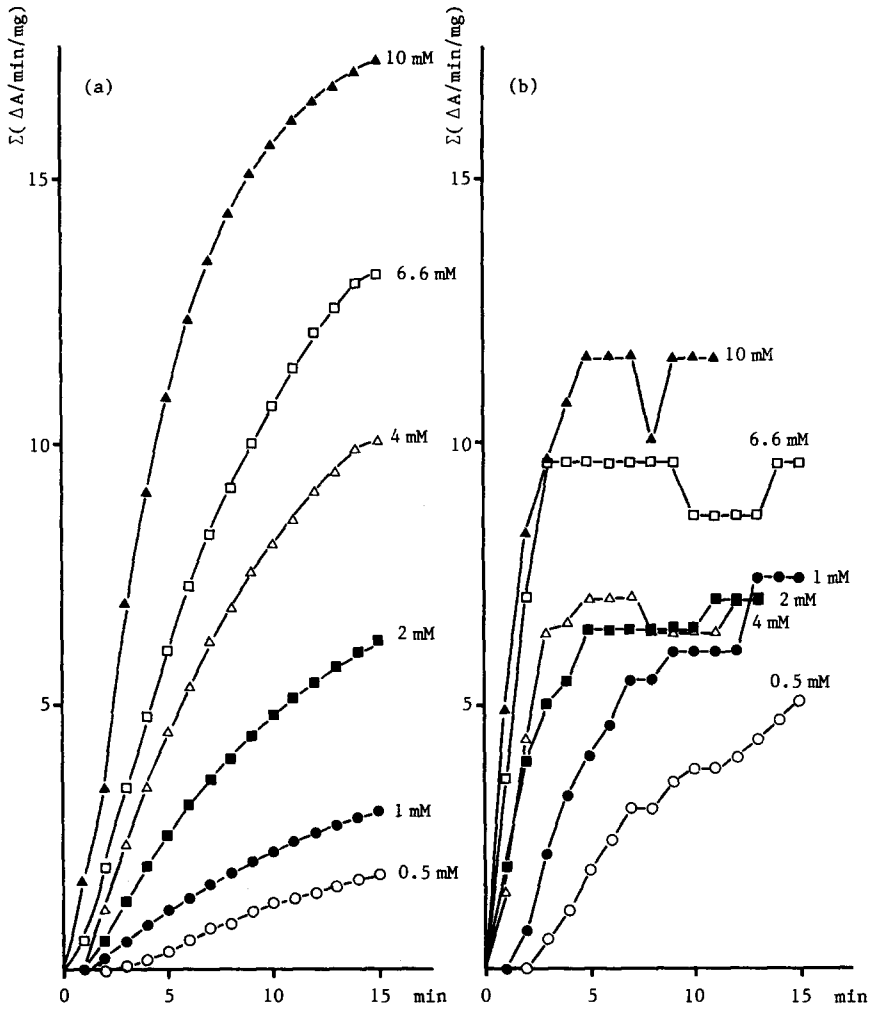

Fig. 4. Increase in absorbance of the paraquat radical (395 nm) and diquat radical (377 nm) at different concentrations: (a) paraquat, (b) diquat. Paraquat (0.5–10 mM) or diquat (0.5–10 mM) was incubated with NADH and liver mitochondrial fractions.

Table 3. Kinetic parameters of microsomal (NADPH) and mitochondrial (NADH) fractions for one-electron reduction of paraquat (Pq) and diquat (Dq)

	Pq		Dq	
	V_{max}	K_m	V_{max}	K_m
Microsomes (NADPH)				
Liver	245.2	2.0	123.8	0.09
Lung	130.4	2.3	74.8	0.13
Kidney	89.8	2.3	45.9	0.13
Mitochondria (NADH)				
Liver	181.6	11.7	116.2	3.3
Lung	138.9	10.0	83.8	2.1
Kidney	447.0	14.6	199.1	3.2

Microsomal and mitochondrial fractions were incubated with NADPH and NADH, respectively. Experimental values were obtained from Lineweaver–Burk plots of the reaction velocity against the concentration of each herbicide. V_{max} is given in μmol/L/min/mg protein and K_m is mM (N = 4–6).

those in mitochondria. These results, as shown also in Table 2, indicate that the reduction activity for both herbicides in microsomes was dominant in liver and lung tissues. On the other hand, the high V_{max} value and high rate of reduction activity at a 1 mM concentration in kidney mitochondria raised the possibility that these herbicides cause lesions of kidney. In a previous study, the highest concentrations of paraquat and diquat were maintained in the kidney for at least 30 hr after oral administration of 680 μmol/kg [19]. Although the detailed observations have not been evaluated, a degeneration of mitochondria was electron microscopically observed at renal tubular cells of rats orally injected with 240 mg/kg paraquat for 6–72 hr. The kidney mitochondria were swollen and the density of the matrix was decreased. In addition, numerous lipid droplets were observed in the cytoplasm along the basal lamina.

Figures 3 and 4 illustrate the time course of the increase in absorbance for both herbicides as they were reduced by liver microsomes with NADPH and by liver mitochondria with NADH, respectively. The total amount of the increase in absorbance, which is given as Σ (Δ A/min/mg protein), is comparable with accumulation of the radical. Thus, the overall extent of accumulation represents the balance between the rate of production of the transient radical and the rate of its decay. Under these experimental conditions, paraquat showed the time- and concentration-dependent increase in absorbance seen in both figures. On the other hand, the first rate of reduction for diquat was faster than that for paraquat and it reached its maximum level within 5–10 min in both systems. However, the diquat radical did not continue to accumulate indefinitely. These *in vitro* studies have suggested that the diquat radical may be more fragile than the paraquat radical. Microsomes and mitochondria derived from the kidney and lung also showed the same results (data not shown).

Paraquat is more toxic than diquat and paraquat poisoning commonly leads to death resulting most often from a progressive proliferative pulmonary fibrosis [20–22]. The V_{max} values for paraquat obtained in the present study were higher than those for diquat in all assay systems, while the apparent K_m values for diquat were lower than those for paraquat. The reduction velocity for diquat was significantly higher than that for paraquat at 1 mM in the three tissues examined. Therefore, in the case of the much lower concentrations found in *in vivo* studies diquat may be reduced faster than paraquat. In addition, enzyme specificity in lung tissues was not observed. Therefore, paraquat toxicity and its organ specificity would depend on its selective accumulation in the target organ [19, 23–27].

Acknowledgements—The author would like to thank Dr Satoshi Kawai for his helpful discussions during the preparation of this manuscript and Miss Toshiko Okuyama for her excellent technical assistance.

REFERENCES

1. Kappus H and Sies H, Toxic drug effects associated with oxygen metabolism; redox cycling and lipid peroxidation. *Experientia* **37**: 1233–1241, 1981.
2. Bus JS, Aust SD and Gibson JE, Superoxide and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat toxicity. *Biochem Biophys Res Commun* **58**: 749–755, 1974.
3. Bus JS, Aust SD and Gibson JE, Lipid peroxidation: a possible mechanism for paraquat toxicity. *Res Commun Chem Pathol Pharmacol* **11**: 31–38, 1975.
4. Talcott RE, Shu H and Wei ET, Dissociation of microsomal oxygen reduction and lipid peroxidation with the electron acceptors, paraquat and menadione. *Biochem Pharmacol* **28**: 665–671, 1979.
5. Trush MA, Mimnaugh EG, Ginsburg E and Gram TE, *In vitro* stimulation by paraquat of reactive oxygen-mediated lipid peroxidation in rat lung microsomes. *Toxicol Appl Pharmacol* **60**: 279–286, 1981.
6. Aldrich TK, Fisher AB, Cadenas E and Chance B, Evidence for lipid peroxidation by paraquat in the perfused rat lung. *J Lab Clin Med* **101**: 66–73, 1983.
7. Ilett KE, Stripp G, Menard RH, Reid WD and Gillette JR, Studies on the mechanism of the lung toxicity of paraquat. *Toxicol Appl Pharmacol* **28**: 216–226, 1974.
8. Steffen C and Netter KJ, On the mechanism of paraquat action in microsomal oxygen reduction and its relation to lipid peroxidation. *Toxicol Appl Pharmacol* **47**: 593–602, 1979.
9. Kornbrust DJ and Mavis RD, The effect of paraquat on microsomal lipid peroxidation *in vitro* and *in vivo*. *Toxicol Appl Pharmacol* **53**: 323–332, 1980.
10. Keeling PL and Smith LL, Relevance of NADPH depletion and mixed disulfide formation in rat lung to the mechanism of cell damage following paraquat administration. *Biochem Pharmacol* **31**: 3243–3249, 1982.
11. Sandy MS, Moldeus P, Ross O and Smith MT, Role of redox cycling and lipid peroxidation in bipyridyl herbicide toxicity. Studies with a compromised isolated hepatocyte model system. *Biochem Pharmacol* **35**: 3095–3101, 1986.
12. Baldwin RC, Pasi A, MacGregor JT and Hine CH, The rates of radical formation from the dipyridilium herbicides. Paraquat, diquat and morfamquat in homogenates of rat lung, kidney and liver: an inhibitory effect of carbon monoxide. *Toxicol Appl Pharmacol* **32**: 298–304, 1975.
13. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
14. Omura T and Takesue S, A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome c reductase from rat liver microsomes. *J Biochem* **67**: 249–257, 1970.
15. Orii Y and Okunuki K, Studies on cytochrome *a*. XV. Cytochrome oxidase activity of the Okunuki preparation and its activation by heat, alkali and detergent treatments. *J Biochem* **58**: 561–568, 1965.
16. Boyd MR, Stiko AW and Sasame HA, Metabolic activation of nitrofurantoin—possible implications for carcinogenesis. *Biochem Pharmacol* **28**: 601–606, 1979.
17. Bachur NR, Gordon SL, Gee MV and Kon H, NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. *Proc Natl Acad Sci USA* **76**: 954–957, 1979.
18. Zychlinski L, Raska-Emery P and Montgomery MR, Selective inhibition of bipyridyl-stimulated NADPH oxidation by ascorbic acid. *Toxicology* **47**: 285–294, 1987.
19. Rose MS, Lock EA, Smith LL and Wyatt I, Paraquat accumulation: tissue and species specificity. *Biochem Pharmacol* **25**: 419–423, 1976.
20. Sykes BI, Purchase IFH and Smith LL, Pulmonary

ultrastructure after oral and intravenous dosage of paraquat to rats. *J Pathol* **121**: 233–241, 1977.
21. Dearden LC, Fairshter RD, McRae DM, Smith WR, Glauser FL and Wilson AF, Pulmonary ultrastructure of the late aspects of human paraquat poisoning. *Am J Pathol* **93**: 667–680, 1978.
22. Torre C, Pavanelli PL, Varetto L and Belliardo F, Lung morphology in experimental acute paraquat poisoning. *Am J Forensic Med Pathol* **5**: 125–129, 1984.
23. Sharp CW, Ottolenghi A and Posner HS, Correlation of paraquat toxicity with tissue concentrations and weight loss of the rat. *Toxicol Appl Pharmacol* **22**: 241–251, 1972.
24. Rose MS, Smith LL and Wyatt I, Evidence for energy-dependent accumulation of paraquat into rat lung. *Nature* **252**: 314–315, 1974.
25. Rose MS and Smith LL, Tissue uptake of paraquat and diquat. *Gen Pharmac* **8**: 173–176, 1977.
26. Smith LL and Wyatt I, The accumulation of putrescine into slices of rat lung and brain and its relationship to the accumulation of paraquat. *Biochem Pharmacol* **30**: 1053–1058, 1981.
27. Wright AF, Green TP, Robson RT, Niewola Z, Wyatt I and Smith LL, Specific polyclonal and monoclonal antibody prevents paraquat accumulation into rat lung slices. *Biochem Pharmacol* **36**: 1325–1331, 1987.