10. MacGillivray I, Buchanan TJ. Total exchangeable sodium and potassium in non-pregnant women and in normal and pre-eclamptic pregnancy. *Lancet* 1958; **ii:** 1090-1093.
11. Nanra RS, Kincaid-Smit P, Normal renal physiology and changes in pregnancy. In: Shearman RP, ed. *Human Reproductive Physiology*. Oxford: Alden & Mowbray, 1972: 594-625.
12. Lindheimer MD, Katz AI. Renal changes during pregnancy: their relevance to volume homeostasis. *Clin Obstet Gynecol* 1975; **2:** 345-364.
13. Paaby P. Changes in the water content of serum and plasma during pregnancy. *Acta Obstet Gynecol Scand* 1959; **38:** 297-314.
14. Robb CA, Davis JO, Johnsen JA, Blaine EH, Schneider EG, Banner JS. Mechanisms regulating the renal excretion of sodium during pregnancy. *J Clin Invest* 1970; **49:** 871-880.
15. Seelig MS. *Magnesium Deficiency in the Pathogenesis of Disease*. New York: Plenum Medical, 1980: 45.
16. Ashe JR, Schofield FA, Gram MR. The retention of calcium, iron, phosphate and magnesium during pregnancy, the adequacy of pre-natal diets with and without supplementation. *Am J Clin Nutr* 1979; **32:** 286-291.
17. Sims EAH. Renal function in normal pregnancy. *Clin Obstet Gynecol* 1968; **11:** 461-472.
18. Boyle JA, Campbell S, Duncan AM, Greig WR, Buchanan WW. Serum uric acid levels in normal pregnancy with observations on the renal excretion of urate in pregnancy. *J Clin Pathol* 1966; **19:** 501-503.
19. Thorling L. Jaundice in pregnancy: a clinical study. *Acta Med Scand (Suppl)* 1955; **151:** 1-123.
20. Studd J. The plasma proteins in pregnancy. *Clin Obstet Gynaecol* 1975; **2:** 285-300.
21. Paaby P. Changes in serum proteins during pregnancy. *J Obstet Gynaecol Br Emp* 1960; **67:** 43-55.
22. Honger PE. Albumin metabolism in pre-eclampsia. *Scand J Clin Lab Invest* 1968; **22:** 177-184.
23. Robertson GS. Serum protein and cholinesterase changes in association with contraceptive pills. *Lancet* 1967; **i:** 232-235.
24. Laurell CB, Kullander S, Thorell J. Effect of administration of combined oestrogen-progestogen contraceptive on the level of individual plasma proteins. *Scand J Clin Lab Invest* 1968; **21:** 337-341.

The effect of paraquat on the *in vitro* activity of cytosol, mitochondrial and microsomal enzyme systems

D. J. ROSSOUW, CAROL C. CHASE, F. M. ENGELBRECHT

Summary

Subcellular fractions (mitochondria, microsomes and cytosol) were prepared from the lungs of rabbits and rats to investigate the effects of paraquat (Aldrich Laboratories) on the activity of some cytosol and mitochondrial dehydrogenases and on the microsomal respiration and reduced pyridine nucleotide oxidation rate.

The normal basal oxygen consumption of rabbit lung microsomes was 1,9 ± 0,3 nmol O_2/mg microsomal protein/min, and the oxidation rates of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and reduced nicotinamide-adenine dinucleotide (NADH) were 4,29 ± 0,53 and 4,0 ± 0,55 nmol/mg microsomal protein/min respectively. One molecule of oxygen can therefore oxidize two molecules of NADPH or NADH, and the generated hydrogen peroxide is probably immediately broken down by the catalase activity of the normal lung microsomal preparation.

When Aldrich paraquat (1,0 mM) was added to microsomes metabolizing NADPH (0,5 - 0,75 mM), both the rate of oxygen consumption and the generation of nicotinamide-adenine dinucleotide phosphate (NADP) were significantly ($P<0,001$) stimulated over the first 5 minutes, and thereafter returned to within basal limits. When microsomes were pre-incubated with 1,0 mM paraquat before NADPH was added, the oxygen consumption was substantially lower (10,01 ± 1,01 nmol oxygen/mg microsomal protein/min), while the NADPH oxidation rate was almost similar to the basal rate in the absence of paraquat. This resulted in a striking dissociation in the H/O ratio under these circumstances. The addition of potassium cyanide (KCN) (5,0 mM) prior to paraquat pre-incubation and followed by the addition of NADPH restored the stimulatory effect of paraquat on microsomal respiration and on NADPH oxidation rate.

Paraquat (0,01 mM) had no effect on the reaction rates of the following enzyme systems, glucose-6-phosphate dehydrogenase (G-6-PD), glyceraldehyde-3-phosphate dehydrogenase (GAPD), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and isocitrate dehydrogenase (IDH). However, 0,1 mM paraquat slightly inhibited the mitochondrial IDH system, and 1,0 mM paraquat significantly inhibited all the enzymes tested except for mitrochondrial and cytosol MDH.

The addition of KCN 5,0 mM led to a total inhibition of the LDH and MDH enzyme systems *in vitro*, but did not affect the IDH, GAPD and G-6-PD systems. However, when KCN was added before or after the addition of 1,0 mM paraquat to the test systems for IDH, GAPD or G-6-PD the inhibitory effect of paraquat was reversed and the reaction rates returned to normal or almost normal.

Paraquat (1,0 mM) had no effect on the nicotinamide-adenine dinucleotide-dependent microsomal respiration, and no basic differences were noted between the responses of rat and rabbit lung microsomes exposed to paraquat *in vitro*.

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Although the biochemical mechanism of paraquat toxicity is not known, its pulmonary specificity is well documented.[1] In experimentally induced paraquat poisoning it has been shown that paraquat accumulates in the lung,[2] presumably by an energy-dependent uptake mechanism.[3] Previous reports[4–7] from this laboratory indicated that paraquat interferes with the aerobic metabolism of lung cells, probably by affecting some enzyme/co-enzyme system in the pentose phosphate pathway, the mitochondria or the cyanide-insensitive microsomal metabolic pathways.

Similarly, results in the literature show that paraquat has an effect on a variety of enzyme systems, i.e. mitochondrial dehydrogenases,[8] reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases and cytochrome P-450,[9] microsomal fatty acid desaturase,[10] prolyl hydroxylase,[11] lysosomal enzymes (acid phosphatase, cathepsin D and ß-*N*-acetylglucosaminidase) and cytosolic superoxide dismutase, catalase, glutathione peroxidase and reductase systems, glucose-6-phosphate dehydrogenase (G-6-PD) and non-protein sulphydryl (SH) levels in lung homogenate.[12] However, no one had studied the reduced nicotinamide-adenine dinucleotide (NADH)- or NADPH-dependent cytosolic, mitochondrial and microsomal dehydrogenases of rabbit lung. We therefore decided to investigate the *in vitro* effect of paraquat on different cytoplasmic, microsomal and mitochondrial enzyme systems which play a central and strategic role in metabolic pathways which may be influenced by paraquat.

Paraquat markedly stimulates the pentose phosphate pathway[7] and thereby induces an increase in the activity of G-6-PD to enable the lung to maintain adequate tissue concentrations of NADPH.[12,13] We previously reported[7] that paraquat caused an initial and consistent increase in the 6-$^{14}CO_2$ production of lung slices, but gradually inhibited 6-^{14}C-glucose oxidation over a period of 3 hours. Glyceraldehyde-3-phosphate dehydrogenase (GAPD) was chosen from the glycolytic enzyme systems because the enzyme molecule is biochemically a natural tetramer and contains an SH group in the active site of the molecule.[14] In view of the highly significant increase in oxygen consumption induced by paraquat exposure *in vitro*, to such an extent that intracellular hypoxia may arise, we decided to investigate the effect of paraquat on lactate dehydrogenase (LDH) because the reoxidation of NADH via lactate formation allows glycolysis to proceed in the absence of oxygen by regenerating sufficient nicotinamide-adenine dinucleotide (NAD^+) for the reaction catalysed by GAPD.[14]

The two citric acid cycle enzymes, isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH), were selected because both enzymes operate in the mitochondrial and cytosolic fractions of the cell. Furthermore, different pyridine nucleotide IDH enzyme systems are known to exist, i.e. a NAD-dependent mitochondrial enzyme and a nicotinamide-adenine dinucleotide phosphate (NADP)-dependent mitochondrial and cytosolic system. Both systems need manganese ions (Mn^{++}) as an essential component in the decarboxylation reaction, and it is believed that the intermediary product, oxalosuccinate, remains bound to the enzyme as an intermediate in the reaction.[15] While MDH may catalyse similar reactions on the inside and outside of mitochondria, the two enzymes may not in fact be the same protein[15] and therefore may not necessarily be similarly affected by paraquat.

Furthermore, in view of the contradictory results regarding the species differences in paraquat toxicity,[4] as well as the fact that the microsomal drug-metabolizing activity of rat lung is relatively low in comparison with that of rabbit lung,[9] we decided to investigate the effects of paraquat on the oxygen consumption and pyridine nucleotide metabolism of both rat and rabbit lung microsomes.

Because paraquat is actively accumulated by rat lung, a wide range of extracellular concentrations could, over different periods of time, result in similar intracellular effects. Relatively high and potentially lethal concentrations of paraquat (0,1 and 1,0 mM) were chosen for the present study in order to obtain maximal changes *in vitro* over a short time period. In addition, we investigated the effect of different types of commercially available paraquat in order to establish whether differences in potency might account for the conflicting results from various laboratories.

Material and methods

Male New Zealand White rabbits weighing 1 500 - 2 500 g and male Long-Evans rats weighing 180 - 220 g were used. Tissue slices (1 mm) were obtained from perfused lungs,[5] and mitochondrial, microsomal and cytosol preparations were prepared as follows: the lung slices were homogenized in a medium containing 150 mM sucrose, 150 mM mannitol, 1 mM *tris*-HCl (pH 7,4) and 1 mM ethylenediamine tetra-acetic acid (EDTA) with a glass-Teflon homogenizer (0,15 mm clearance), using two strokes of the pestle. The homogenates were then filtered through a single layer of cheesecloth and the filtrate was centrifuged at 1 000 *g* for 10 minutes in a precooled bench centrifuge (IEC HN-S centrifuge) at 4°C, in order to pellet all the remaining cell debris and nuclei.

To isolate mitochondria, the 1 000 *g* supernatant was centrifuged at 9 000 *g* for 10 minutes (J-21 B centrifuge; JA 20 rotor; Beckman). This pellet was resuspended and recentrifuged at 7 000 *g* for another 10 minutes to get rid of some of the contaminating microsomal and lysosomal elements. This final mitochondrial pellet was then resuspended in 3,0 ml of a hypotonic *tris*-$MgSO_4$ solution, and sonicated for two 15-second periods in a Biosonik IV ultrasonicator (Bronwill Scientific Inc.) at 30 kilocycles/s.

The 9 000 *g* supernatant was centrifuged at 105 000 *g* for 60 minutes at 4°C (Spinco L2 ultracentrifuge; type 50 titanium rotor; Beckman). This supernatant was used as the cytosol preparation. The microsomal pellet was resuspended in *tris*-KCl buffer (0,15M KCl, 0,02M *tris*-HCl, 0,1 mM EDTA, pH = 7,4) and recentrifuged at 105 000 *g* at 4°C for another 45 minutes. This final microsomal pellet was again resuspended in *tris*-KCl medium to remove most of the contaminating haemoglobin. A cytosol preparation and microsomes were also prepared by homogenizing lung slices in a *tris*-KCl medium and starting with an initial spin of 15 000 *g* for 15 minutes to pellet cell debris, nuclei and mitochondria. Thereafter the supernatant was treated in a similar way as described above. No basic differences were noted in specific activity of cytosol and microsomal enzymes.

The protein content of the cytosol, sonicated mitochondrial and microsomal preparations was determined by the method of Lowry *et al.*[16] on the same day on fresh preparations using crystalline bovine serum albumin (Cohn fraction V; Koch-Light Laboratories) as standard.

Different types of commercially available paraquat (supplied by Aldrich Laboratories, Wisconsin, and by Sigma Chemicals, London) were analysed on a mass spectrophotometer by Professor K. L. van der Merwe of the Department of Biochemistry at the University of Stellenbosch, but no biochemical differences were detected between paraquat bought from these two companies.[1] (K. L. van der Merwe — personal communication.)

The oxygen consumption of lung microsomes was measured polarographically at 30°C using an oxygraph (model K-IC; Gilson Medical Electronics) equipped with a Clark-type oxygen electrode. The reactions were carried out in a 2,0 ml reaction chamber containing the incubation medium (*tris*-KCl buffer) saturated with room air and maintained at 30°C in a water bath. Lung microsomes (0,25 - 0,5 mg microsomal protein/ml) were

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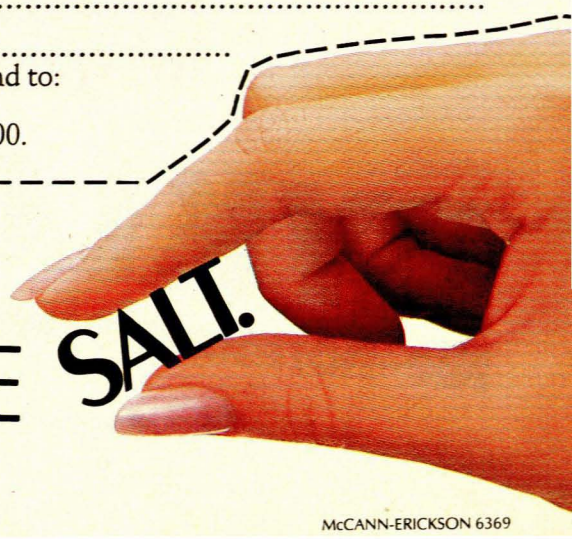

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allowed to equilibrate for 5 minutes in the buffer, and the various reagents were then added via the inlet channel through the glass stopper to yield the following final concentrations: NADH or NADPH (Miles Laboratories) 0,5 - 0,75 mM, potassium cyanide (KCN) (Merck) 5,0 mM, and paraquat (Aldrich Laboratories) 1,0 mM, 0,1 mM and 0,01 mM. Aqueous solutions of all reagents were prepared to give the required final concentration by the addition of not more than 100 μl to the reaction chamber. The results were expressed in terms of nmol oxygen consumed/mg microsomal protein/min.

The aerobic oxidation of NADH or NADPH at 30°C was measured with a Zeiss PM6 spectrophotometer with a programmed XP2 printer at 340 nm. Lung microsomes (0,25 - 0,5 mg microsomal protein/ml), NADH or NADPH (0,5 - 0,75 mM), KCN (5,0 mM) and paraquat (1,0 mM; 0,1 mM and 0,01 mM) and *tris*-KCl medium respectively were added in a specific sequence to obtain a final volume of 2,0 ml. The oxidation rates of the reduced pyridine nucleotides were calculated from the slopes of the curves (see Figs 4 and 5) and converted into nmol NADH/NADPH oxidized/mg microsomal protein/min.

The activities of the following enzyme systems, in which the reactions involved led to the reduction of NAD(P) or oxidation of NAD(P)H and a change in the light absorption in the ultraviolet region with a maximum at 340 nm, were determined spectrophotometrically acccording to the methods described in the annotated references: G-6-PD,[17] GAPD,[18] and LDH[19] in the cytosol preparation, MDH[20] and IDH[21] in both the cytosol and mitochondrial preparations, and the microsomal NAD(P)H oxidase systems as described earlier.[8]

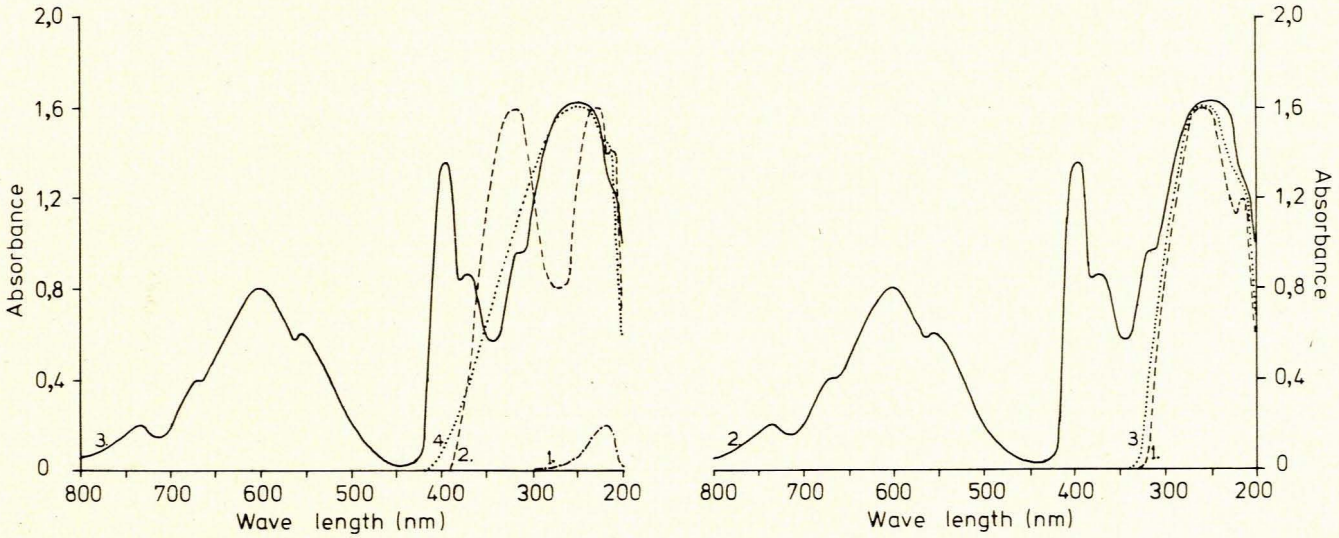
Conditions were standardized so that the reaction rates of the different enzyme systems at 30°C were linear with time over a minimum of a 10-minute incubation period. Calibration curves for the rate of oxidation of NADH or the rate of reduction of NADP were obtained before the start of each test system. These values were then used together with the calculations from the initial reaction rates of the enzyme activities to express the results as nmol NAD or NADPH generated/mg protein/min by the cytosol, mitochondrial or microsomal preparations respectively.

In view of the observed effects of KCN 5,0 mM on the NADPH-dependent microsomal enzymes, as well as the reports by Colowick *et al.*[22] that KCN might interfere with the spectrophotometric determination of some dehydrogenase enzymes, a control reaction rate was calculated for all the enzyme systems with or without paraquat in the presence or absence of 5,0 mM KCN. Continuous spectral analyses (220 - 800 nm; SP 800 Unicam) of mixtures of oxidized and reduced pyridine nucleotides, paraquat and KCN at different pH values in various media were performed to investigate the possibility of the formation of cyanide complexes which could interfere with the absorbance at 340 nm.

Results

Figs 1, 2 and 3 illustrate the absorption spectra of *in vitro* oxidized and reduced paraquat and the effect of KCN and paraquat on the rate of oxidation of NADPH by lung microsomes in a *tris*-KCl buffer (pH 7,4) at 30°C. Oxidized paraquat showed a maximum absorption in the ultraviolet region (258 nm), while reduced paraquat (in the presence of sodium dithionite ($Na_2S_2O_4$)) exhibited two absorption maxima at 394 nm and 600 nm respectively. The presence of KCN neither altered the absorption spectra of oxidized or reduced paraquat nor affected the normal absorption spectrum (340 nm) of NADPH or NADH in the presence or absence of paraquat at pH 7,4.

When 1,0 mM oxidized paraquat (PQ^{2+}) was added to a mixture of lung microsomes and NADPH in a *tris*-KCl buffer (pH 7,4), continuous spectral analyses between 220 nm and 800 nm showed a constant absorption peak at 258 nm (i.e. that of paraquat), a decreasing absorbance peak at 340 nm (probably due to the oxidation of NADPH), and no sign of absorbance in the wavelength of 600 nm. Furthermore, the addition of KCN



1. Rc: medium. Sc: medium + $Na_2S_2O_4$ crystals
2. Rc: medium. Sc: medium + dissolved $Na_2S_2O_4$ (colourless)
3. Rc: medium. Sc: medium + $Na_2S_2O_4$ + Paraquat (blue)
4. Rc: medium. Sc: medium + $Na_2S_2O_4$ + PQ + O_2 (colourless)

1. Rc: medium. Sc: medium + Paraquat
2. Rc: medium. Sc: medium + PQ + $Na_2S_2O_4$ (blue)
3. Rc: medium. Sc: medium + PQ + $Na_2S_2O_4$ + O_2 (colourless)

(Rc = Reference cuvette. Sc = Sample cuvette)

Fig. 1. Continuous absolute absorption spectra (200 - 800 nm) to illustrate some physicochemical characteristics of oxidized (PQ^{2+}) and cation radical ($PQ^{+\cdot}$) paraquat *in vitro*.

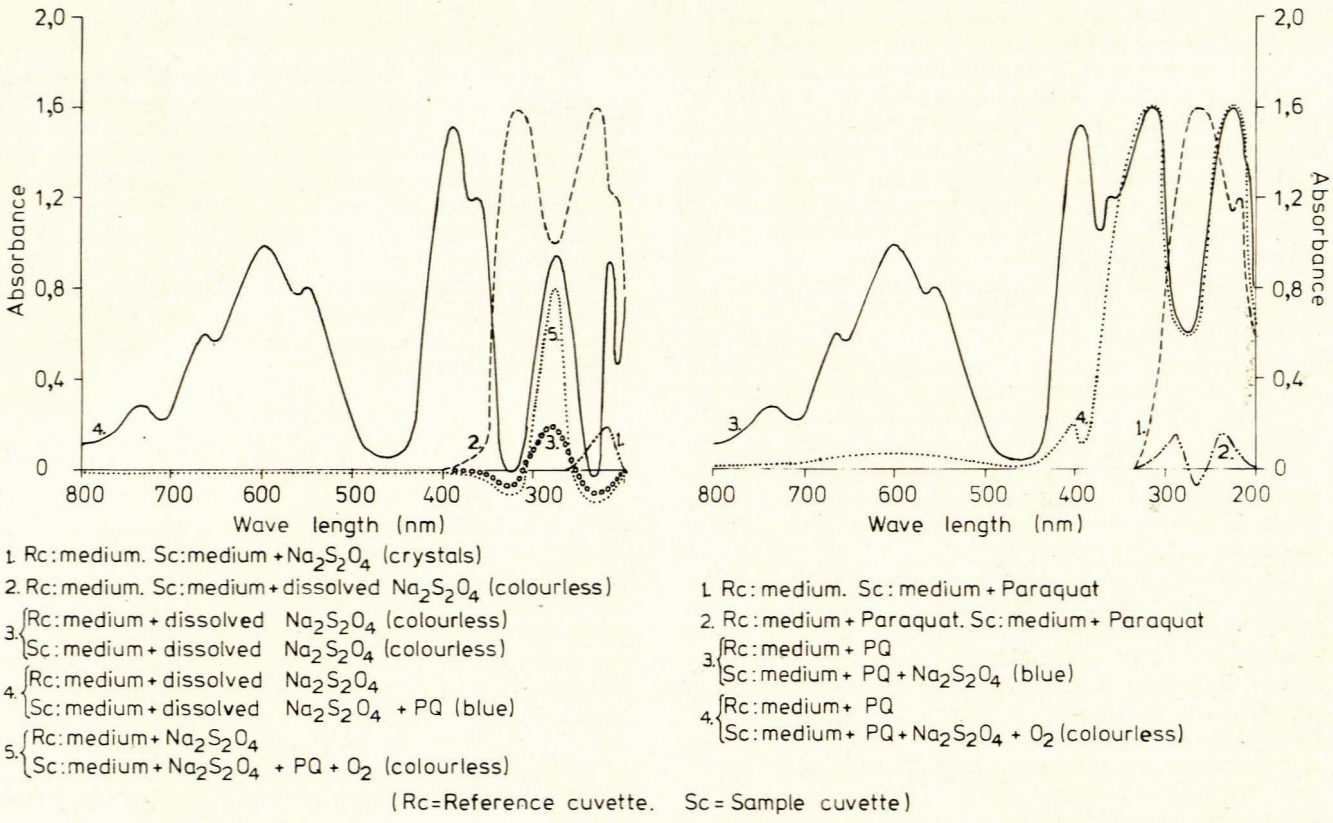


Fig. 2. Continuous differential absorption spectra (200 - 800 nm) to illustrate some physicochemical characteristics of oxidized (PQ^{2+}) and cation radical ($PQ^{+\cdot}$) paraquat *in vitro*.

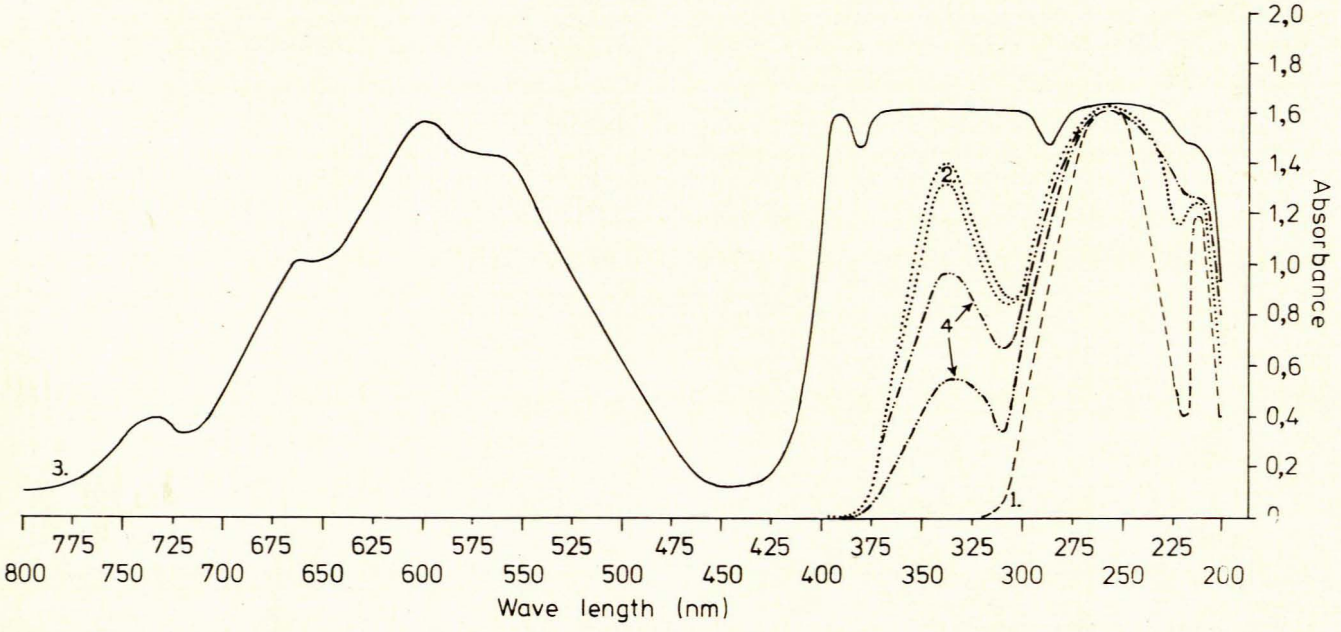


Fig. 3. Continuous absorption spectra (200 - 800 nm) to illustrate the effect of cation radical paraquat on the microsomal oxidation of NADPH at 30°C. KCN did not interfere with the absorption maxima of any component.

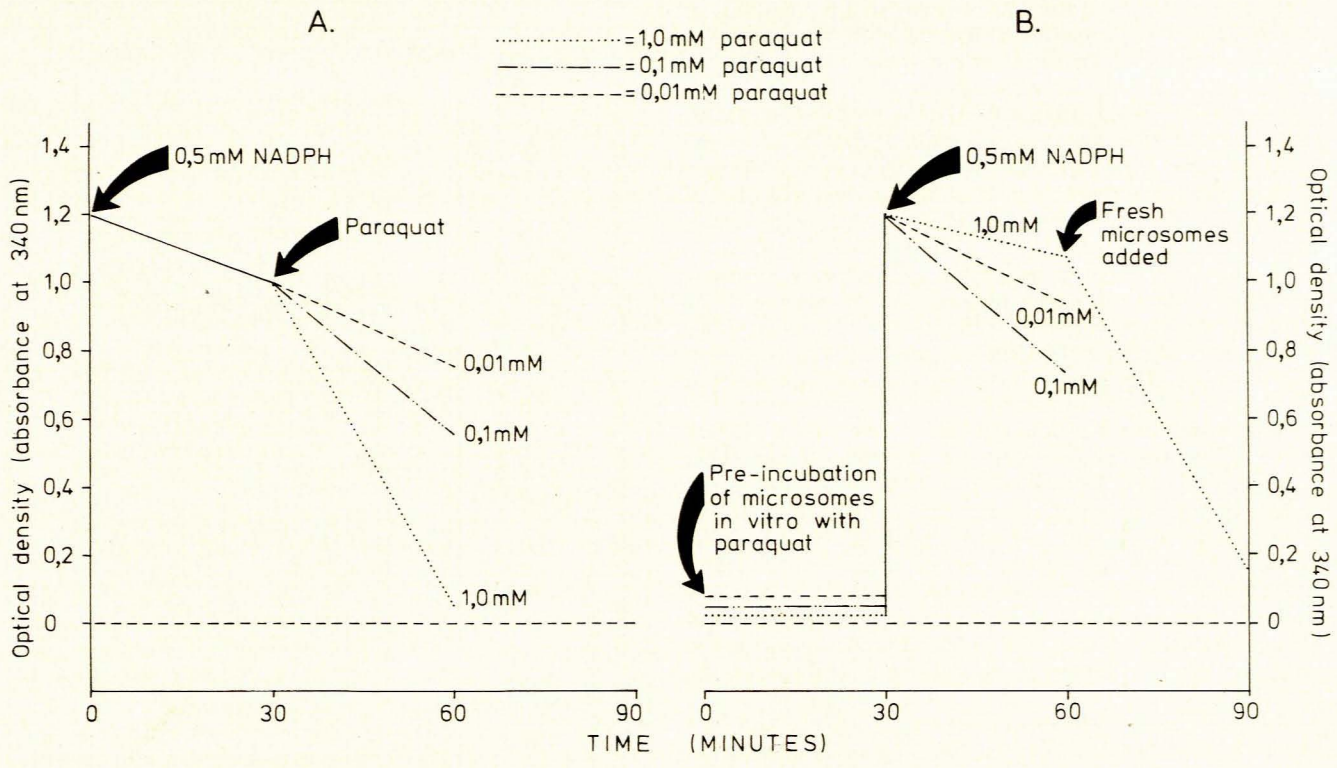


Fig. 4. Typical spectrophotometric recordings (340 nm) to show the effect of various concentrations of paraquat on the NADPH oxidation rate of lung microsomes (A — without pre-incubation; B — after pre-incubation with paraquat).

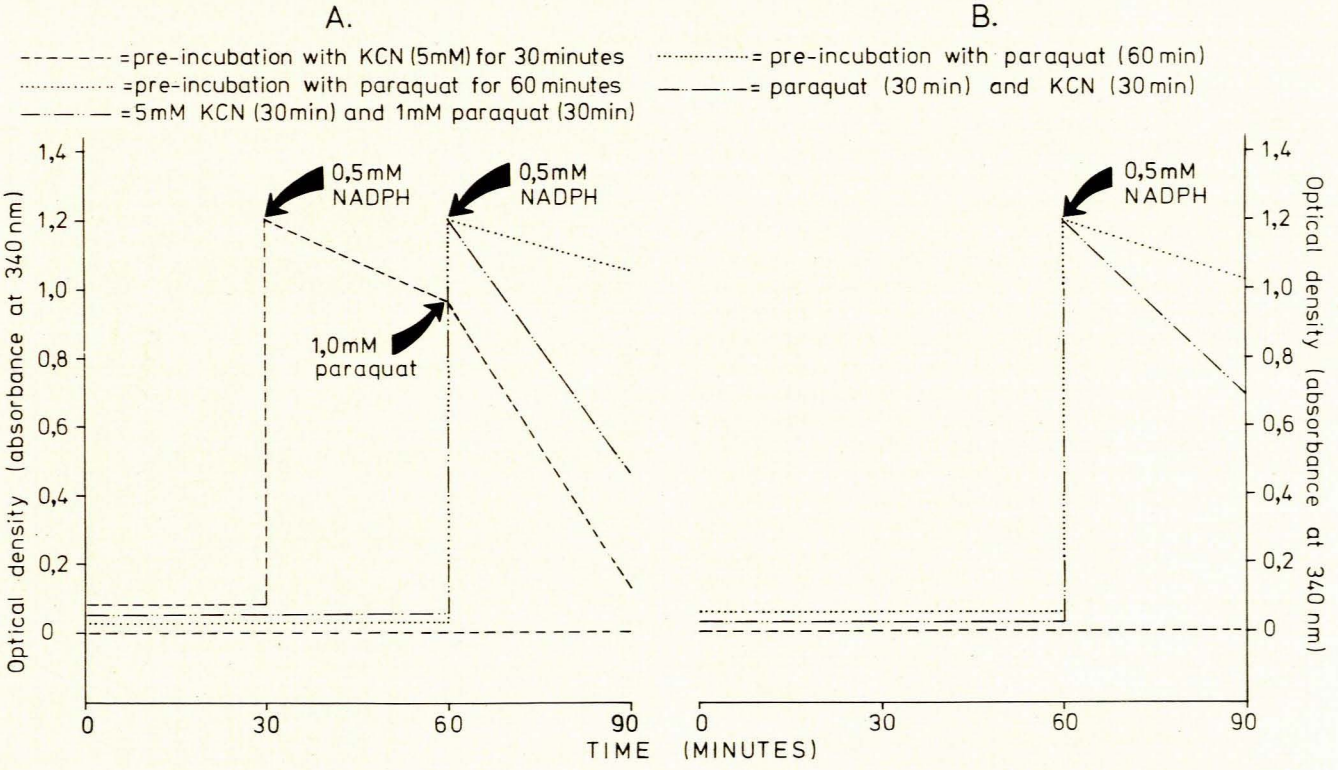


Fig. 5. Typical spectrophotometric recordings (340 nm) to show the effect of KCN on the paraquat (1,0 mM)-induced changes in the microsomal NADPH oxidation pathways (A — pre-incubation with KCN; B — the addition of KCN after paraquat pre-incubation).

to the buffer (pH 7,4) containing lung microsomes, NADPH or NADH and paraquat neither interfered with the normal absorption maxima of the various components nor induced the appearance of any additional absorption peaks (Fig. 3).

The endogenous oxygen uptake and the NADPH or NADH oxidation rate of rabbit lung microsomes (Figs 4 and 5) are given in Table I. One molecule of oxygen (1,90 ± 0,34 nmol O_2/mg microsomal protein/min) can oxidize two molecules of NADPH (4,29 ± 0,53 nmol NADPH → NADP/mg microsomal protein/min) or NADH (4,01 ± 0,5 nmol NADH → NAD/mg microsomal protein/min). When some of these experiments were repeated 6 months later (to investigate the effect of paraquat bought from Sigma Chemicals), mean values of 2,24 nmol O_2/mg microsomal protein/min and 3,86 nmol NADPH → NADP/mg microsomal protein/min were obtained (Table I).

Paraquat (1,0 mM) had no significant effect on the NADH dependent microsomal systems in the rabbit lung. When added simultaneously with 500 - 700 μM NADPH to the microsomes (0,25 - 1,0 mg microsomal protein/ml), it stimulated both the oxygen consumption and rate of oxidation of NADPH significantly (Table I). Calculations of the initial reaction rates showed an approximate ratio of oxygen consumed to NADPH oxidized (H/O_2 ratio) of 2:1. There was neither a liberation of oxygen when catalase was added to the reaction mixture in the oxygraph nor any change in absorbance at 340 nm in the spectrophotometer.

When microsomes were pre-incubated with 1,0 mM paraquat (Aldrich Laboratories) *in vitro* for 10 - 30 minutes prior to the addition of NADPH, the initial oxygen uptake was substantially lower (10,1 ± 1,01 nmol O_2/mg protein/min v. 24,4 ± 2,34 nmol O_2/mg protein/min), while the oxidation rate of NADPH (4,58 ± 1,4 nmol/mg protein/min) was almost similar to the endogenous control value of 4,29 ± 0,53 nmol/mg protein/min (Figs 4 and 5). This resulted in bizarre H/O_2 ratios, which were only found when Aldrich paraquat was used. Paraquat bought from Sigma Chemicals (London), the British Drug Houses (BDH) or Imperial Chemical Industries (ICI, England), despite having identical absorption spectra to Aldrich paraquat on mass spectrophotometry, (K. L. van der Merwe — personal communication) did not show a similar effect (Table I).

The influence of KCN 5,0 mM on endogenous microsomal metabolism and on the paraquat-induced changes in the respiration and NADPH oxidation rate of rabbit lung microsomes is shown in Table I. It seems as if KCN does not affect the endogenous respiration or basal rate of NADPH and NADH oxidation by lung microsomes. However, addition of KCN before pre-incubation with paraquat (Aldrich Laboratories) (Fig. 4, A) restored the highly significant increase in microsomal respiration and NADPH oxidation rate (Table I). Furthermore, when lung microsomes were exposed to Aldrich paraquat in the presence of 5,0 mM KCN, the H/O_2 ratio returned to a 2:1 value. When microsomes were exposed to Sigma paraquat, the H/O_2 ratios calculated from the mean values for respiration and NADPH oxidase activity were slightly different when KCN was omitted from or added to the reaction mixture. Because of the relatively high SEM values, however, no statistically significant difference could be demonstrated between these values.

The effects of various concentrations of Aldrich paraquat on the rate of oxidation of NADPH by rabbit and rat lung microsomes *in vitro* are given in Table II. The normal NADPH oxidation rate of rabbit lung microsomes (4,10 ± 1,9 nmol/mg protein/min) corresponded well with earlier experiments (Table I). Paraquat in concentrations of 0,01 mM, 0,1 mM and 1,0 mM increased the NADPH oxidation rates progressively. When microsomes were pre-incubated with increasing concentrations of paraquat *in vitro* and NADPH was added thereafter, no stimulation of the NADPH oxidation rate was observed with 1,0 mM paraquat. However, with exposure to 0,1 mM and 0,01

TABLE I. INFLUENCE OF 1 mM PARAQUAT (ALDRICH LABORATORIES AND SIGMA CHEMICALS) ON THE RESPIRATION AND NADPH OR NADH OXIDATION RATE OF RABBIT LUNG MICROSOMES IN THE PRESENCE AND ABSENCE OF 5 mM KCN*

Treatment of microsomes	Paraquat (Aldrich Laboratories, Wisconsin)				Paraquat (Sigma Chemicals, London)			
	Oxygen consumption (nmol O_2/mg protein/min)		NAD(P)H oxidation rate (nmol co-enzyme/mg protein/min)		Oxygen consumption (nmol O_2/mg protein/min)		NAD(P)H oxidation rate (nmol co-enzyme/mg protein/min)	
	Mean ± SE	No. of experiments	Mean ± SE	No. of experiments	Mean ± SE	No. of experiments	Mean ± SE	No. of experiments
Microsomes + NADPH	**1,9 ± 0,34**	**10**	**4,29 ± 0,53**	**10**	**2,24 ± 0,37**	**5**	**3,86 ± 0,46**	**5**
Microsomes + NADH	**1,9 ± 0,2**	**5**	**4,01 ± 0,5**	**5**				
Microsomes + NADPH + KCN	**2,04 ± 0,64**	**5**	**3,74 ± 0,36**	**5**	**1,76 ± 0,94**	**5**	**3,86 ± 0,06**	**5**
Microsomes + NADH + KCN	**1,6 ± 0,4**	**5**	**3,28 ± 1,6**	**5**				
Microsomes + NADPH + PQ (1 mM)	**24,4 ± 2,34**	**10**	**41,37 ± 7,81**	**10**	**23,18 ± 1,01**	**5**	**32,4 ± 0,61**	**5**
Microsomes + NADH + PQ (1 mM)	**2,0 ± 0,4**	**5**	**2,86 ± 0,6**	**5**				
Microsomes + NADPH + KCN + PQ (1 mM)	**28,8 ± 3,13**	**5**	**42,73 ± 9,51**	**5**	**27,82 ± 6,88**	**5**	**33,8 ± 4,6**	**5**
Microsomes + NADH + KCN + PQ (1 mM)	**2,14 ± 0,46**	**5**	**3,14 ± 0,81**	**5**				
Microsomes + PQ (1 mM) + NADPH	**10,01 ± 1,01**	**10**	**4,58 ± 1,4**	**10**	**22,38 ± 1,55**	**5**	**30,52 ± 0,8**	**5**
Microsomes + PQ (1 mM) + NADH	**2,0 ± 0,5**	**5**	**1,72 ± 0,4**	**5**				
Microsomes + KCN + PQ (1 mM) + NADPH	**28,44 ± 3,53**	**5**	**40,18 ± 10,06**	**5**	**30,65 ± 7,41**	**5**	**34,44 ± 3,4**	**5**
Microsomes + KCN + PQ (1 mM) + NADH	**2,2 ± 0,3**	**5**	**2,40 ± 0,46**	**5**				

* Each value represents the mean (± SE) of triplicate determinations on the number of animals used in each experiment.
PQ = paraquat.

TABLE II. THE EFFECT OF VARIOUS CONCENTRATIONS OF PARAQUAT ON THE NADPH OXIDATION RATE OF RABBIT AND RAT LUNG MICROSOMES*

Treatment of microsomes	NADPH oxidation rate (initial reaction rate) (nmol NADPH → NADP/mg microsomal protein/min)			
	Rabbit		Rat	
	Mean ± SE	No. of experiments	Mean ± SE	No. of experiments
Microsomes + NADPH:	**4,10 ± 1,9**	**15**	**2,21 ± 0,5**	**15**
+ PQ (0,01 mM)	**10,70 ± 4,0**	**5**	**5,36 ± 1,1**	**5**
+ PQ (0,10 mM)	**27,83 ± 6,4**	**5**	**10,74 ± 3,7**	**5**
+ PQ (1,0 mM)	**42,61 ± 7,8**	**5**	**17,01 ± 5,2**	**5**
Microsomes + PQ + NADPH:				
+ PQ (0,01 mM)	**7,0 ± 2,0**	**5**	**4,9 ± 2,3**	**5**
+ PQ (0,10 mM)	**20,6 ± 6,5**	**5**	**9,2 ± 2,3**	**5**
+ PQ (1,0 mM)	**4,3 ± 0,3**	**5**	**1,3 ± 0,4**	**5**

* Each value represents the mean (± SE) of triplicate determinations on the number of animals used in each experiment.
PQ = paraquat.

mM paraquat increases comparable with those obtained when paraquat was added after NADPH were found.

As illustrated in Table II, rat lung microsomes showed an endogenous NADPH oxidation rate of 2,21 ± 0,5 nmol/mg protein/min, and responded in a very similar way to the rabbit lung microsomes to pre- and post-incubation with various concentrations of paraquat *in vitro* (Table II).

Table III summarizes the effects of potassium cyanide and various concentrations of Aldrich paraquat on the *in vitro* activity of some pyridine nucleotide dependent dehydrogenases in the mitochondrial and cytosol fractions from rabbit lung. The presence of 5,0 mM KCN in the different test systems did not affect the *in vitro* activity of G-6-PD, GAPD and IDH, but significantly ($P<0,001$) inhibited the baseline or basal values for LDH and MDH activities. Although 0,1 mM and lower concentrations of paraquat showed no statistically significant effects on any of the enzymes tested, 1,0 mM and higher concentrations resulted in total inhibition of the activity of GAPD, a highly significant inhibition of G-6-PD, LDH and IDH, and no effect on both cytosol and mitochondrial MDH activities.

The addition of 5,0 mM KCN to the test systems, either before the start of the experiment or halfway through the 10-minute period of registration, almost completely reversed the inhibitory effects of paraquat on G-6-PD and IDH enzyme

TABLE III. THE EFFECT OF POTASSIUM CYANIDE (5 mM) AND 1 mM and 0,1 mM CONCENTRATIONS OF PARAQUAT ON THE *IN VITRO* ACTIVITY OF SOME PYRIDINE NUCLEOTIDE-DEPENDENT DEHYDROGENASES IN SUBCELLULAR FRACTIONS FROM RABBIT LUNG*

Isolated enzyme system	Subcellular fraction	Pyridine nucleotide involved	Enzyme activity (nmol NADPH or NADH/mg protein/min)				
			Control value (without KCN)	Control value (+ 5 mM KCN)	Paraquat 0,1 mM	Paraquat 1.0 mM	Paraquat 1 mM + KCN 5 mM
G-6-PD	**Cytosol**	**NAPD → NADPH**	**40,5 ± 1,1 (N = 9)**	**42,2 ± 2,9 (N = 4)**	**39,6 ± 1,7 (N = 8)**	**24,4 ± 1,5 (N = 8)**	**35,9 ± 3,9 (N = 4)**
GAPD	**Cytosol**	**NAD → NADH**	**100,3 ± 6,9 (N = 7)**	**122,8 ± 8,8 (N = 7)**	**89,0 ± 23,6 (N = 6)**	**—**	**45,5 ± 6,8 (N = 7)**
LDH	**Cytosol**	**NADH → NAD**	**493,8 ± 15,7 (N = 8)**	**94,5 ± 31,5 (N = 4)**	**472,7 ± 24,7 (N = 6)**	**35,8 ± 13,2 (N = 6)**	**4,8 ± 1,7 (N = 4)**
IDH	**Cytosol**	**NADP → NADPH**	**57,2 ± 1,7 (N = 9)**	**57,0 ± 2,5 (N = 7)**	**55,0 ± 2,2 (N = 6)**	**1,8 ± 0,5 (N = 8)**	**54,2 ± 2,7 (N = 7)**
IDH	**Mitochondrial**	**NADP → NADPH**	**199,4 ± 6,2 (N = 9)**	**182,4 ± 5,8 (N = 6)**	**187,9 ± 6,4 (N = 9)**	**12,9 ± 3,4 (N = 9)**	**174,1 ± 7,0 (N = 6)**
MDH	**Cytosol**	**NADH → NAD**	**1 109,1 ± 57,5 (N = 11)**	**246,3 ± 9,4 (N = 7)**	**1 076,3 ± 83,4 (N = 7)**	**1 054,8 ± 82,5 (N = 7)**	**243,3 ± 10,4 (N = 7)**
MDH	**Mitochondrial**	**NADH → NAD**	**1 506,0 ± 159,5 (N = 7)**	**369,0 ± 19,5 (N = 4)**	**1 540,3 ± 186,7 (N = 7)**	**1 538,7 ± 165,7 (N = 7)**	**369,3 ± 26,4 (N = 4)**

* Each value represents the mean (± SE) of triplicate determinations on the number of animals used in each experiment.
N = number of animals.

activities. Furthermore, the presence of KCN partially cancelled the total inhibition of 1,0 mM paraquat on the GAPD system caused an additional inhibition of the LDH activity, and depressed the MDH activities to values more or less in line with control values in the presence of KCN alone (Table III).

Under the experimental conditions relevant to our *in vitro* systems, no complexes interfering with KCN were produced at pH 7,4. When the pH of the buffer was deliberately increased by the addition of 0,1M NaOH such interfering complexes were found between cyanide and NADP and NAD at 340 nm, but only when the pH of the medium exceeded 9,0.

When paraquat preparations supplied by Sigma Chemical Company, London, were investigated in our *in vitro* systems for the determination of enzyme activity, paraquat concentrations up to 1 mM had no effect.

Discussion

The mechanisms whereby paraquat causes pulmonary toxicity are not well understood. Recent studies have been focused on oxidation-reduction cycles of paraquat, oxygen radical formation and superoxide production.[23] These processes have been investigated in plants, and it is tempting to suggest that the hypothesis for the toxic action of paraquat in plants also applies to mammals. However, far less is known about the biochemistry of paraquat toxicity in mammalian cells than in plant cells. Any satisfactory theory must explain not only the origin of cellular damage but also its selective action on the lung. The suggestion that a process of cyclic oxidation and reduction of paraquat is involved in its toxicity seems to be a good working hypothesis.[23]

Paraquat is reduced to the radical cation by lung microsomes, and under aerobic conditions the reduced paraquat radical is immediately re-oxidized by molecular oxygen.[23] The possible generation of hydrogen peroxide (H_2O_2) and highly reactive oxygen species (superoxide anions = $O_2^{\cdot-}$ and/or singlet oxygen = Δ g O_2) in these reactions *in vivo*[10,24] are supported by our observations that the toxicity of paraquat is increased by exposure to high oxygen tensions and decreased by administration of superoxide dismutase.[25]

Previous reports indicated that about 14% of the total endogenous oxidative metabolism of rabbit lung homogenates was not inhibited by KCN.[5] This probably corresponds to the basal rate of microsomal respiration (1,9 $\pm$ 0,34 nmol O_2/mg protein/min) obtained in the present investigation. Rabbit lung microsomes incubated aerobically with NADPH resulted in a NADPH oxidation rate which constituted a H/O_2 ratio of 2:1.

The highly significant increase in the respiration rate and rate of NADPH oxidation induced by 1,0 mM paraquat resulted in H/O ratios which implied the formation of H_2O_2 under such circumstances. The magnitude of the increase in microsomal metabolism appeared to be directly related to the concentration of paraquat, and the generated H_2O_2 is probably immediately broken down by endogenous microsomal catalase.[8] This may explain the lack of any polarographic deviation when exogenous catalase was added to the reaction chamber while monitoring microsomal respiration.

Because no reaction was observed when paraquat and NADPH were incubated without microsomes, the increase in microsomal oxidative metabolism most certainly involved an enzyme-dependent reaction. The concomitant appearance of reduced paraquat ($PQ^{+\cdot}$) — illustrated by the absorption peaks at 394 nm and 600 nm — and the aerobic reoxidation via transfer of a single electron from cation radical paraquat to oxygen may mediate the formation of superoxide or other highly reactive oxygen radicals.[26]

These findings are supported by results in the literature[23] which indicated that peroxidation of membrane lipids and possibly also surfactant lipids occurred, and thereby constitute a biochemical mechanism for the pulmonary toxicity of paraquat in mammals as well. There are, however, indications that paraquat or the reactive radicals may affect certain enzyme systems[8–12], such as mitochondrial dehydrogenases, NADPH oxidases, microsomal fatty acid desaturase and cytochrome P-450 in the rat. Whatever the exact molecular target may prove to be, it seems likely that the toxicity of paraquat in mammals, as in plants, is related to its cyclic oxidation and reduction within cells, possibly in conjunction with the synthesis of NADPH and its subsequent oxidation.[27]

The time- and concentration-dependent cyanide-reversible effect of paraquat, also reversible by addition of fresh microsomes, posed a very fascinating problem. Whether this is simply an 'artefact', indicative of some cyanide-sensitive factor in the microsomes which play a role in paraquat toxicity, remains to be investigated.

Several investigators have described some species differences between the rat and rabbit regarding *in vivo* or *in vitro* reactions to paraquat. It has been shown that paraquat caused a decrease in the concentrations of cytochrome P-450 in the rat, but did not affect the concentration of this co-enzyme in the rabbit.[9] There is also evidence that the microsomal drug-metabolizing activity as well as the production of H_2O_2 is lower in the rat than in the rabbit. However, the production of superoxide radicals is higher in the rat.[24] The activity of the energy-dependent paraquat pump system also seemed to be higher in the rat, while on the other hand paraquat is removed much faster from the lungs in the rabbit than in the rat.[9] Reports from this laboratory showed that there is a stimulation of protein synthesis and an inhibition of lipid synthesis in the rat under certain experimental conditions, but that paraquat had no effect on the synthesis of proteins and lipids under similar circumstances in the rabbit.[28] However, the present investigation showed that no species differences exist between rat and rabbit lung microsomes as far as the rate of microsomal respiration and the rate of NADPH oxidation are concerned.

There seemed to be no common mechanistic denominator among the enzymes (G-6-PD, IDH, LDH and GAPD) which are totally or subtotally inhibited by 1,0 mM paraquat. The mechanism(s) of inhibition of these enzymes by paraquat still remains an enigma, although the reversal of the inhibition by the addition of KCN may provide an important clue in further investigations. Similarly, the inability of high concentrations of paraquat to influence both the MDH enzyme systems may point to a very interesting mode of action.

The addition of KCN to the different enzyme assays, where the change in absorbance at 340 nm was caused by the reduction of the pyridine nucleotide involved, showed no interference with the *in vitro* enzyme activitiy. However, in the LDH and MDH assays, where the reduced pyridine nucleotide was oxidized in the reaction used, KCN caused a marked inhibition of the enzyme activity. This inhibition was by no means an artefact[22] because no cyanide complexes could be demonstrated under these experimental conditions.

The subtotal and/or total inhibitory effect of paraquat on certain key enzymes in the metabolism of carbohydrates may be meaningful as an additional mechanism of paraquat toxicity. It has been shown that paraquat is concentrated in lung cells by an energy-dependent pump system,[3] which means that the intracellular concentration of paraquat may reach relatively high levels. In most of the metabolic reactions studied in the present investigations[4–7] paraquat caused an initial stimulation of aerobic processes. In the later stages, however, a gradual inhibition of metabolic pathways which may coincide with the increasing intracellular concentrations of paraquat was noted. This may induce an inhibition of the G-6-PD system, with a consequent decrease in the NADPH generation. According to Forman *et al.*[27] the one electron reduction of paraquat is dependent on an

optimal concentration of NADPH, and a decrease in its concentration would lead to an additional increase in the intracellular concentration of paraquat. The inhibition of other glycolytic enzymes by paraquat, as well as the inhibitory effect of different substrates and intermediary products accumulating intracellularly, may further contribute to a disordered metabolic system.

Although the hypothesis of oxidation-reduction cycles of paraquat with the production of active radicals may still be an important membrane-damaging process in the acute stages of paraquat toxicity, the inhibition of enzyme systems may be instrumental in the destruction of organelles and cells, which may then release fibrogenetic factors initiating fibrogenesis. Because fibroblasts were shown to be far less sensitive to paraquat than alveolar macrophages, for example,[6] an irreversible and progressive interstitial fibrosis may follow.

REFERENCES

1. Haley TJ. Review of the toxicology of paraquat 1,1'-dimethyl-4,4'-bipyridinium chloride. *Clin Toxicol* 1979; **14:** 1-46.
2. Rose MS, Lock EA, Smith LL, Wyatt I. Paraquat accumulation: tissue and species specificity. *Biochem Pharmacol* 1976; **25:** 419-423.
3. Rose MS, Smith LL, Wyatt I. Evidence for energy-dependent accumulation of paraquat into rat lung. *Nature* 1974; **252:** 314-315.
4. Rossouw DJ, Engelbrecht FM. The influence of paraquat on the *in vitro* oxygen consumption of rabbit lung. *S Afr Med J* 1978; **54:** 199-201.
5. Rossouw DJ, Engelbrecht FM. The effect of paraquat on the respiration of lung cell fractions. *S Afr Med J* 1978; **54:** 1101-1104.
6. Rossouw DJ, Engelbrecht FM. The effect of paraquat on the aerobic metabolism of rabbit alveolar macrophages and lung fibroblasts. *S Afr Med J* 1979; **55:** 20-23.
7. Rossouw DJ, Engelbrecht FM. The effect of oxygen and paraquat on the ^{14}C-Glucose oxidation of rabbit alveolar macrophages and lung slices. *S Afr Med J* 1979; **55:** 558-560.
8. Gage JC. The action of paraquat and diquat on the respiration of liver cell fractions. *Biochem J* 1968; **109:** 757-761.
9. Ilett KF, Stripp B, Menard RH, Reid WD, Gilette JR. Studies on the mechanism of the lung toxicity of paraquat: comparison of tissue distribution and some biochemical parameters in rats and rabbits. *Toxicol Appl Pharmacol* 1974; **28:** 216-226.
10. Montgomery MR. Interaction of paraquat with the pulmonary microsomal fatty acid desaturase system. *Toxicol Appl Pharmacol* 1976; **36:** 543-554.
11. Hollinger MA, Chavapil M. Effect of paraquat on rat lung prolyl hydroxylase. *Res Commun Chem Pathol Pharmacol* 1977; **16:** 159-162.
12. Omaye ST, Reddy AK. Early and delayed biochemical effects of paraquat toxicity on rat lung. *Exp Mol Pathol* 1980; **33:** 84-89.
13. Witschi H-P, Hirai K-I, Côté MG. Primary events in lung following exposure to toxic chemicals. In: Autor AP, ed. *Biochemical Mechanisms of Paraquat Toxicity*. New York: Academic Press, 1977: 1-20.
14. Mayes PA. Metabolism of carbohydrate. In: Harper HA, Rodwell VW, Mayes PA, eds. *Review of Physiological Chemistry*. Los Altos, Calif: Lange Medical Publications, 1979; 294-320.
15. Mayes PA. The citric acid cycle. In: Harper HA, Rodwell VW, Mayes PA, eds. *Review of Physiological Chemistry*. Los Altos, Calif: Lange Medical Publications, 1979: 285-293.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. *J Biol Chem* 1951; **193:** 265-275.
17. Lohr GW, Waller HD. Glucose-6-phosphate dehydrogenase. In: Bergmeyer HU, Gawehn K, eds. *Methods of Enzymatic Analysis*, vol 2. 2nd English ed. New York: Academic Press, 1974: 636-643.
18. Bergmeyer HU, Gawehn K, Grassl M. Glyceraldehyde-3-phosphate dehydrogenase. In: Bergmeyer HU, Gawehn K, eds. *Methods of Enzymatic Analysis*, vol 1. 2nd English ed. New York: Academic Press, 1974: 466-467.
19. Bergmeyer HU, Bernt E. Lactate dehydrogenase. In: Bergmeyer HU, Gawehn K, eds. *Methods of Enzymatic Analysis,* vol 2. 2nd English ed. New York: Academic Press, 1974: 574-578.
20. Bergmeyer HU, Bernt E. Malate dehydrogenase. In: Bergmeyer HU, Gawehn K, eds. *Methods of Enzymatic Analysis,* vol 2. 2nd English ed. New York: Academic Press, 1974: 613-617.
21. Bergmeyer HU, Bernt E. Isocitrate dehydrogenase. In: Bergmeyer HU, Gawehn K, eds. *Methods of Enzymatic Analysis,* vol 2. 2nd English ed. New York: Academic Press, 1974: 624-627.
22. Colowick SP, Kaplan NO, Ciotti MM. The reaction of pyridine nucleotide with cyanide and its analytical use. *J Biol Chem* 1951; **191:** 447-459.
23. Bus JS, Aust SD, Gibson JE. Lipid peroxidation: a possible mechanism for paraquat toxicity. *Res Commun Chem Path Pharmacol* 1975; **11:** 31-38.
24. Montgomery MR. Paraquat toxicity and pulmonary superoxide dismutase: an enzymic deficiency of lung microsomes. *Res Commun Chem Path Pharmacol* 1977; **16:** 155-158.
25. Autor AP. Reduction of paraquat toxicity by superoxide dismutase. *Life Sci* 1974; **14:** 1309-1319.
26. Baldwin RC, Pasi A, MacGregor JT, Hine CH. The rates of radical formation from the dipyridylium herbicides paraquat, diquat and morfamquat in homogenates of rat lung, kidney and liver: an inhibitory effect of carbon monoxide. *Toxicol Appl Pharmacol* 1975; **32:** 298-304.
27. Forman HJ, Nelson J, Fisher AB. Rat alveolar macrophages require NADPH for superoxide production in the respiratory burst: effect of NADPH depletion by paraquat. *J Biol Chem* 1980; **255:** 9879-9883.
28. Engelbrecht FM, Rossouw DJ, Nienaber MWP. The effect of paraquat on the incorporation of ^{14}C-leucine and ^{14}C-palmitate into lung proteins and lung lipids of rats and rabbits. *S Afr Med J* 1981; **59:** 953-956.