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The Action of Paraquat and Diquat on the Respiration of Liver Cell Fractions

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1. Paraquat and diquat produce only a slight increase in the oxygen uptake of rat liver mitochondria, and it is likely that they do not penetrate the mitochondrial membrane. 2. In mitochondrial fragments inhibited by antimycin A or by Amytal, both substances stimulate oxygen uptake with NADH or β-hydroxybutyrate as substrate but not with succinate. The NADH dehydrogenase of the respiratory chain appears to be involved, at a site only partially inhibited by Amytal. 3. An NADPH oxidase activity is stimulated in rat liver microsomes by diquat, and to a smaller extent by paraquat; diquat also causes an NADH oxidase activity to develop. The effect is not inhibited by carbon monoxide or *p*-chloromercuribenzoate, and it is probable that a flavoprotein is involved by a mechanism not requiring thiol groups. 4. One molecule of oxygen can oxidize two molecules of NADPH in the stimulated microsomal system, the hydrogen peroxide produced being broken down by a catalase activity in the microsomes. 5. Diquat can stimulate NADH oxidase and NADPH oxidase activity in the postmicrosomal soluble fraction; the enzyme involved may be DT-diaphorase. 6. The mechanism of these reactions and their significance in relation to the toxicity of the dipyridilium compounds are discussed.

The dipyridilium compounds paraquat and diquat are now well established as herbicides (Calderbank & Crowdy, 1964), though the mechanism of their phytotoxic action has not yet been definitely established. They have been the subject of extensive toxicological investigations on experimental animals in these Laboratories (Clark, McElligott & Hurst, 1966; Daniel & Gage, 1966; Gage, 1968; D. G. Clark & E. W. Hurst, unpublished work). Their lethal action after high oral or parenteral doses is due to delayed effects as yet not clearly understood, though with paraquat proliferative changes in lung tissue are certainly an important factor. Their direct cytotoxic action is limited to exposed epithelial membranes, probably because of their poor penetration into tissues. During investigations to ascertain the mechanism of these local effects, the characteristic colours of the stable free radicals produced by reduction were observed when paraquat and diquat were incubated anaerobically with fresh rat liver homogenate. This effect was prevented by heating the homogenate or by dialysing it, and restored by adding NADH to the dialysed homogenate. As the coenzyme is itself unable to reduce these compounds to their free radicals, the intervention of a dehydrogenase seemed likely, and this investigation was undertaken to study the action of paraquat and diquat on liver cell oxidation–reduction systems.

MATERIALS AND METHODS

Reagents. Enzymes, coenzymes, ADP, glucose 6-phosphate, antimycin A and dicoumarol were purchased from Sigma (London) Chemical Co. Ltd. (London, S.W. 6). Amytal (Amylobarbitone B.P.) was obtained from Boots Pure Drug Co. Ltd. (Nottingham), and crystalline paraquat dichloride and diquat dichloride were supplied by Plant Protection Ltd. (Jealott's Hill, Berks.). EDTA was added as a stock solution of the disodium salt adjusted to pH 7·4.

Preparation of rat liver cell fractions. Male Alderley Park specific-pathogen-free albino rats were killed by a blow on the neck, and the livers were removed and rapidly chilled. All subsequent operations were performed at 0–4°. The liver was forced through a coarse nylon mesh and washed free from blood with 0·25 M-sucrose containing 0·1 mM-EDTA. A 10% (w/v) homogenate in the sucrose–EDTA medium was prepared by two strokes in a Potter–Elvehjem homogenizer fitted with a Fluon pestle. The homogenate was centrifuged for 10 min. at 1500*g* and the supernatant layer was removed and centrifuged for 8 min. at 10000*g*. The supernatant and loose upper layer were removed and the pellet was washed once in the same medium and centrifuged again; it was then dispersed in the medium

(1ml. of suspension for each 10g. of liver). For mitochondrial fragments the pellet was suspended in the same volume of 50mM-sodium phosphate buffer, pH7·4, containing 0·1mM-EDTA, and portions (2ml.) were subjected to two 15sec. bursts of an MSE 60 ultrasonic generator at maximal output.

After the initial separation of the mitochondrial fraction the supernatant layer was centrifuged at $160000g_{av.}$ for 40min. to separate the microsomes. The supernatant layer was stored at −30° as the soluble fraction; the pellet was suspended in 50mM-potassium phosphate buffer, pH7·4, containing 0·1mM-EDTA (1·5ml. of suspension for each 1g. of liver), and the suspension was stirred gently overnight. This removed soluble protein and gave a more stable and reproducible preparation (Williams & Kamin, 1962). The microsomal pellet was centrifuged as before and suspended in the same medium (1ml. for each 1g. of liver). The suspension was divided into small portions and stored at −30°.

Measurements of oxygen uptake. An oxygen electrode (Rank Bros., Bottisham, Cambs.) was used, modified by the insertion of a piston sealed with rubber O-rings. The piston had a dished base and a central hole to permit the expulsion of air and the introduction of reagents. The measurements were made in air at 27°. In all experiments the final volume was 2ml. For mitochondria the medium was that described by Clark, Greenbaum & Slater (1965), with sodium DL-β-hydroxybutyrate as substrate and 0·1ml. of mitochondrial suspension. For the mitochondrial fragments (0·2ml.) the medium contained 100μmoles of potassium phosphate buffer, pH7·4, and the substrates were NADH (0·8μmole), sodium DL-β-hydroxybutyrate (8μmoles) with NAD^+ (0·1μmole), or succinate (8μmoles). For the microsomal suspension (0·1ml.), or the soluble fraction (0·3ml.), the medium contained 60μmoles of potassium phosphate buffer, pH7·4, and 1·0μmole of NADPH or NADH. The NADPH-generating system for microsomes contained potassium phosphate buffer, pH7·4 (100μmoles), glucose 6-phosphate (40μmoles), $NADP^+$ (1μmole), EDTA (0·2μmole) and glucose 6-phosphate dehydrogenase (2 units).

Reagents were added to the cell as the following solutions: ADP, 25mM; antimycin A, 5μg./ml. in aq. 10% (v/v) ethanol; dicoumarol, 20mM in ethanol; Amytal, 0·1M (with an equivalent amount of NaOH); diquat and paraquat, in aqueous solutions to give the required final concentration by the addition of 20μl.

Photometric measurements. The aerobic oxidation of NADPH and the anaerobic reduction of paraquat and diquat were studied with a Unicam SP.800 recording spectrophotometer, with 1cm. cells maintained at 27°. For the aerobic experiments the solutions were gassed with air and for the anaerobic with N_2. Extinctions of the free radicals were measured at wavelengths where there was no interference from NADPH or the parent compound, 600mμ for paraquat and 425mμ for diquat. The molecular extinction coefficients of the free radicals can be obtained by dithionite reduction of the parent dipyridilium compound (Zweig, Shavit & Avron, 1965), but, as this reagent tends to over-reduce, the extinction coefficients so obtained may be low. This error is not encountered when NADPH in the presence of microsomes is used as a reducing agent, but, as it is not possible to effect a complete conversion in this manner, the coefficients were derived from the accompanying disappearance of NADPH. The free-radical spectra overlap with that of NADPH, so the coefficients were calculated in the following manner. If a, b and c are the molar concentrations of NADPH, unchanged dipyridilium compound and free radical, and ϵ_A, ϵ_B and ϵ_C are their extinction coefficients at a particular wavelength, then the extinction E of the reaction mixture at that wavelength is given by the expression:

$$E = a\epsilon_A + b\epsilon_B + c\epsilon_C$$

As $a_0 = a + 0{\cdot}5c$ and $b_0 = b + c$, it follows that:

$$E = a_0\epsilon_A + b_0\epsilon_B + c(\epsilon_C - \epsilon_B - 0{\cdot}5\epsilon_A)$$

At a wavelength where E remains constant as c increases:

$$c(\epsilon_C - \epsilon_B - 0{\cdot}5\epsilon_A) = 0$$

or

$$\epsilon_C = \epsilon_B + 0{\cdot}5\epsilon_A$$

The spectra were repeatedly scanned during the progress of the reaction, and an isosbestic point was observed at 328mμ with paraquat and at 340mμ with diquat (Fig. 1). From the known extinction coefficients of NADPH and the dipyridilium compounds at these wavelengths, the extinction coefficients of paraquat and diquat free radicals were calculated to be $2{\cdot}93 \times 10^3$ and $3{\cdot}16 \times 10^3$. The spectra of the free-radical solutions obtained by partial reduction of the parent compound with hydrogen and platinized asbestos were then measured, and a correction for interference arising from over-reduction was obtained from the extinction after aeration. From these spectra the extinction ratios at 600mμ/328mμ for paraquat and at 425mμ/340mμ for diquat were found to be 4·7 and 1·49 respectively. Hence the extinction coefficients were calculated to be $1{\cdot}38 \times 10^4$ at 600mμ for paraquat and $4{\cdot}7 \times 10^3$ at 425mμ for diquat.

RESULTS

Mitochondria. The mitochondrial preparation was under respiratory control by ADP; the addition

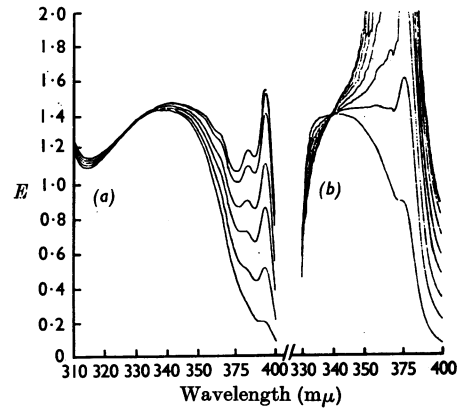

Fig. 1. Determination of the extinction coefficients of paraquat and diquat free radicals formed by reduction with NADPH in the presence of microsomes. The spectra of the anaerobic preparations described in the Materials and Methods section were scanned repeatedly every few minutes: (*a*) with 1mM-paraquat; (*b*) with 1mM-diquat.

of 125nmoles of ADP was attended by an oxygen consumption of 29nmoles, a P/O ratio 2·25. The resting respiration rate was 12nmoles/min.; the addition of diquat or paraquat to give a final concentration of 1·0mM resulted in a slight increase of about 3nmoles/min. This increase could not be inhibited by the addition of 2μmoles of Amytal, nor by the prior addition of the same amount. Antimycin A (0·1μg.) also had no effect on the increased respiration. In the absence of ADP and inorganic phosphate, 1·0mM-diquat or -paraquat failed to increase oxygen uptake; under these conditions 0·01mM-2,4-dinitrophenol in the absence of paraquat and diquat had a marked stimulant action.

Mitochondrial fragments. When the respiration of this preparation with β-hydroxybutyrate or NADH as substrates was inhibited by antimycin A, it could be stimulated with both diquat and paraquat; the former was rather the more active, but with both an effect was observed at 0·1mM and not at 0·01mM (Table 1). With a preparation inhibited by Amytal, the stimulated oxygen uptake was slower. Neither paraquat nor diquat had any effect with succinate as substrate, after inhibition by antimycin A. EDTA had no effect on this preparation, and the addition of catalase after stimulated respiration had been allowed to continue for several minutes did not result in liberation of oxygen.

Microsomes. The stimulation of respiration by paraquat and diquat, with NADPH, NADH or a glucose 6-phosphate NADPH-generating system as substrate, is shown in Table 2. The increased oxygen uptake was not diminished by prior gassing with carbon monoxide–oxygen (4:1), nor by the addition of 10μM-*p*-chloromercuribenzoate, 0·1mM-EDTA or catalase (400 units). There was no liberation of oxygen when catalase was added to a preparation after respiration had been allowed to continue for several minutes. The catalase activity of the microsomal preparation (0·1ml. with 100μmoles of potassium phosphate buffer, pH 7·4, and 0·2μmole of EDTA) was demonstrated in the oxygen-electrode cell by adding 0·25μmole of hydrogen peroxide; there was an immediate evolution of

Table 1. *Effects of paraquat and diquat on mitochondrial fragments*

Experimental details are given in the Materials and Methods section. Initial uptakes of O_2, before and after the addition of inhibitor, are average values for all experiments with that substrate and inhibitor. —, Component not included in an incubation.

	Uptake of O_2 (nmoles/min.)							
Addition	None	Antimycin A (0·2μg.)	Amytal (4μmoles)	Paraquat		Diquat		
Substrate				(1·0mM)	(0·1mM)	(1·0mM)	(0·1mM)	(0·01mM)
NADH	19	2·5	—	10	—	32·5	7·5	—
	22	—	0	7·5	2·5	20	5	2·5
β-Hydroxybutyrate	20	2·5	—	10	—	30	10	5
	20	—	2·5	7·5	2·5	15	7·5	2·5
Succinate	38	3	—	3	—	3	—	—

Table 2. *Effects of paraquat and diquat on microsomal respiration and NADPH oxidase*

Details are given in the Materials and Methods section. The NADPH oxidation was calculated from the initial slopes of the curves in Fig. 2, and converted into a rate/2ml. for comparison.

Concn. of paraquat (mM)	Concn. of diquat (mM)	Uptake of O_2 (nmoles/min.) NADPH	NADPH-generating system	NADH	NADPH oxidation (nmoles/min./2ml.)
1·0	—	22, 23, 25	15	4	42
0·1	—	13, 15	8·5		32, 29
0·01	—	6, 5			5
—	1·0	25, 27·5	17·5	10, 11	50
—	0·1	23, 25	16	2	42
—	0·01	20, 20	17		32
—	0·001	11, 7·5	10		
—	0·0001	4, 5			

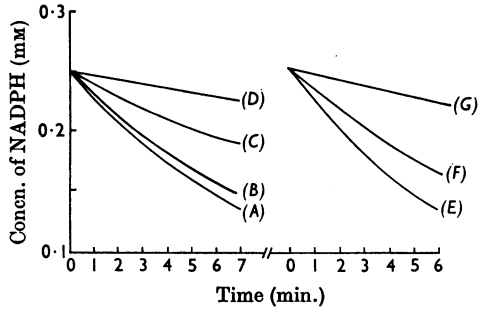

Fig. 2. Aerobic oxidation of NADPH in the presence of microsomes and paraquat or diquat. Concn. of diquat (mM): (*A*) 1·0; (*B*) 0·1; (*C*) 0·01; (*D*) 0·001. Concn. of paraquat (mM): (*E*) 1·0; (*F*) 0·1; (*G*) 0·01.

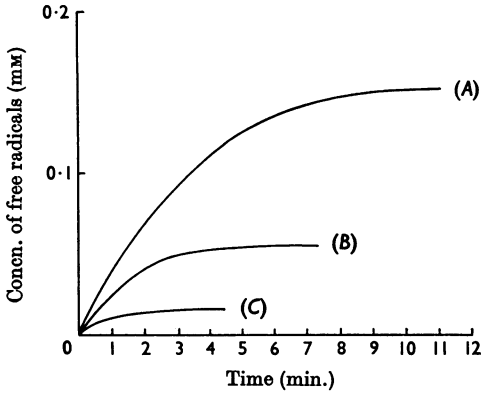

Fig. 3. Anaerobic production of free radicals from paraquat and diquat in the presence of microsomes and NADPH. (*A*) 1·0mM-Diquat; (*B*) 0·1mM-diquat; (*C*) 1·0mM-paraquat.

oxygen. The same experiment without microsomes gave only a slow oxygen output. Fig. 2 shows the aerobic NADPH oxidation in the presence of paraquat and diquat, and Fig. 3 the anaerobic production of free radicals by the same system. The initial slopes of the graphs in Fig. 2 are included in Table 2.

Soluble fraction. Diquat increased the oxygen uptake of the soluble fraction to about the same extent with NADH or NADPH as substrate, 7nmoles/min. at 1mM and 2·5nmoles/min. at 0·1mM. Paraquat had a negligible activity at these concentrations with both substrates. Dicoumarol had only a slight inhibitory action at 0·2mM, and no effect at 0·033mM.

DISCUSSION

When the ability of mitochondrial fragments to respire in the presence of NADH or β-hydroxybutyrate is inhibited by antimycin A, the addition of paraquat or diquat markedly stimulates oxygen uptake, but no such effect is observed with succinate as substrate (Table 1). This indicates that these compounds can intervene by interaction with an NADH dehydrogenase, the most likely candidate being the flavoprotein of the respiratory chain. This effect with diquat and paraquat is less marked if the basic respiration is suppressed with Amytal, so the site of action has a limited sensitivity to this inhibitor. Paraquat and diquat produce only a slight stimulation of respiration with intact rat liver mitochondria, not subject to inhibition by Amytal or antimycin A. It is not certain whether this effect can be attributed to a slight action of the dipyridilium compounds on intact mitochondria, analogous to the non-specific increase in adenosine triphosphatase activity from mechanical or thermal shock, or whether the small proportion of damaged mitochondria, usually to be found in such a preparation, is responsible. It seems probable that paraquat and diquat are unable to penetrate the mitochondrial membrane.

With microsomes, paraquat and diquat produce a stimulation of oxygen uptake that is associated with an increase in NADPH oxidase activity; both effects have a maximal rate with paraquat at 1·0mM and with diquat at 0·1mM. As carbon monoxide does not inhibit this reaction, these compounds probably exert an action on microsomal NADPH dehydrogenase similar to that which occurs with mitochondrial flavoprotein. *p*-Chloromercuribenzoate also does not inhibit, and hence the dipyridilium compounds, unlike neotetrazolium (Orrenius, 1965), must intervene at a site not involving thiol groups. Under anaerobic conditions NADPH reduces the dipyridilium compounds to their free radicals in the presence of microsomes, the reaction proceeding until an equilibrium free-radical concentration is reached. The actual interaction is between paraquat and the reduced enzyme and, as the effect of concentration indicates that a ternary reaction is unlikely, there is probably a sequential attack of two molecules on the enzyme. The aerobic oxidation of NADPH involves a cyclic reduction and reoxidation of the dipyridilium compound; a similar mechanism probably accounts for the stimulation of microsomal NADPH oxidation by methylene blue reported by Gillette, Brodie & La Du (1957).

The initial rate of stimulated oxygen uptake is almost one-half of the initial rate of disappearance of NADPH (Table 2), indicating that one molecule of oxygen oxidizes two molecules of NADPH. Though it is known that hydrogen peroxide is produced from the oxidation of dipyridilium compounds with molecular oxygen, these results indicate that hydrogen peroxide is not produced in

the microsomal preparation, and this is confirmed by the inactivity of added catalase. It appears that microsomes have sufficient catalase activity to prevent the accumulation of hydrogen peroxide under these conditions.

The difference between the effect of paraquat and diquat on the microsomal and postmicrosomal fractions is evidence that the activity in the post-microsomal fraction is not due to residual microsomes. The slight inhibition by dicoumarol suggests the involvement of DT-diaphorase (Ernster, Danielson & Ljunggren, 1962), but this needs to be confirmed by studies on the purified enzyme.

As these dipyridilium compounds do not apparently penetrate the mitochondrial membrane, the limited cytotoxic action that they exhibit in animals cannot be due to a by-passing of the mitochondrial electron-transport chain by an alternative NADH oxidase mechanism, leading to a depletion of ATP. An attack on microsomal or soluble flavoprotein is more likely; in plants, Davenport (1963) and Naik & Nicholas (1967) showed that paraquat and diquat react with NADH- or NADPH-dependent dehydrogenases, and Zweig *et al.* (1965) suggested that the attendant cyclic reduction and reoxidation of diquat enables it to promote photophosphorylation. The mechanism proposed by Mees (1960) attributes the phytotoxicity of the dipyridilium compounds to the hydrogen peroxide liberated when free radicals are oxidized by molecular oxygen, but, if microsomes exhibit *in vivo* their catalase activity *in vitro*, such a mechanism would not operate in animal cells. It seems more likely that transient free radicals arising from the reduction of oxygen are involved; preliminary experiments indicate that the cyclic reduction and reoxidation of the dipyridilium compounds is associated with aromatic hydroxylation and an increase in thiobarbituric acid-reacting material in phospholipids, reactions more associated with free radicals than with hydrogen peroxide.

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