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Biochemical Effects of Diquat and Paraquat

DISTURBANCE OF THE CONTROL OF CORTICOSTEROID SYNTHESIS IN RAT ADRENAL AND SUBSEQUENT EFFECTS ON THE CONTROL OF LIVER GLYCOGEN UTILIZATION

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1. Administration of diquat (*NN'*-ethylene-2,2'-bipyridilium) or paraquat (*NN'*-dimethyl-4,4'-bipyridilium) prevents the normal depletion of liver glycogen in starved rats. 2. There is an increase in blood glucose, which returns to normal values after approx. 7h. 3. After administration of diquat or paraquat, plasma corticosteroids increase to very high concentrations and remain high for at least 24h, but plasma ACTH (adrenocorticotrophin) is only increased for 4h. 4. Adrenal cyclic AMP is considerably increased after administration of diquat and remains significantly higher than control values for at least 24h. 5. It is suggested that diquat and paraquat increase the response of the adrenal cortex to ACTH.

Diquat (*NN'*-ethylene-2,2'-bipyridilium) and paraquat (*NN'*-dimethyl-4,4'-bipyridilium) salts are herbicidal and are widely used as non-selective weed killers (Calderbank, 1964). Their toxicity to mammals has been described (Clark & Hurst, 1970; Clark *et al.*, 1966). Animals given diquat die without any obvious histological damage that might account for death, although effects on the thymus, spleen and adrenals have been noted (Clark & Hurst, 1970). The major lesion seen with paraquat is in the lung, but changes have also been noticed in the kidney, adrenals and thymus (Clark *et al.*, 1966; Conning *et al.*, 1969; Vijeyaratnam & Corrin, 1971).

After oral administration, the compounds are poorly absorbed from the gastrointestinal tract (Daniel & Gage, 1966). After subcutaneous injection into rats, approx. 90% is excreted unchanged in the urine in the first 24h and there is no evidence of metabolism (Daniel & Gage, 1966). Whole-body radioautography in mice after intravenous injection of ^{14}C-labelled compounds shows that both diquat and paraquat are rapidly distributed throughout most tissues except for brain and spinal cord (Litchfield *et al.*, 1973). Loss of the compounds from tissues is rapid, but paraquat is lost more slowly from lung and skeletal muscle than from other tissues (Sharp *et al.*, 1972; Litchfield *et al.*, 1973).

The biochemical effects underlying the mammalian toxicity of diquat and paraquat are not understood. In plants, the herbicidal activity appears to be related to the ability of these compounds to form stable free radicals in aqueous solution, and lipid peroxidation in plant membranes has been detected (Calderbank, 1964; Dodge *et al.*, 1970). There is no evidence for involvement of free radicals in their effects on animals, and lipid peroxidation has not been detected in rat lung after paraquat administration (T. Green & J. Daniel, unpublished work). Free radicals, however, can be formed enzymically from these compounds *in vitro* by broken-cell preparations from rat liver (Gage, 1968). Inhibition of microsomal mixed-function oxidases *in vitro* has been reported (Krieger *et al.*, 1973), and this may well be related to free-radical generation, since the efficacy of the bipyridyls as inhibitors is related to their redox potentials (i.e. diquat > paraquat).

Paraquat has also been reported to diminish lung surfactant (Manktelow, 1967), but no effects on synthesis or breakdown of lung dipalmitoyl lecithin have been demonstrated (Fletcher & Wyatt, 1972). The present study of the biochemical effects occurring *in vivo* after diquat or paraquat administration has been undertaken in an attempt to understand some of the metabolic disturbances underlying the toxicity of the bipyridyls.

Experimental

Special materials

Crystalline paraquat dichloride and diquat dichloride were obtained from Plant Protection Limited, Jealott's Hill Research Station, Berks., U.K. They were dissolved in sterile 0.9% NaCl and dosed intraperitoneally at 20mg of cation/kg body wt. (LD_{50}). ACTH (adrenocorticotrophic hormone) for injection (Acthar–corticotropin, sterile) was purchased from Armour Pharmaceuticals Co. Ltd., Eastbourne, Sussex, U.K. Reserpine was purchased

from Ciba Laboratories, Horsham, Sussex, U.K. Phosphodiesterase (EC 3.1.4.1) from ox heart was purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Animals

Male Alderley Park (Wistar derived) specific-pathogen-free rats (body wt. 180–220g) and male Alderley Park specific-pathogen-free mice (body wt. 30–40g) were used. Hypophysectomized rats (Sprague–Dawley CFY) were purchased from Carworth Europe, Huntingdon, U.K. Hypophysectomized and adrenalectomized Alderley Park rats were prepared by Dr. D. M. Ferguson in our laboratories. Hypophysectomized rats were used 24h after operation and were not given any supportive therapy, whereas adrenalectomized rats were maintained for at least 3 days on daily doses (at 16.00h) of 0.1mg of aldosterone/rat (Aldocorten; Ciba Laboratories). This dose of aldosterone kept blood glucose and liver glycogen concentrations within the normal range, as has been demonstrated by Pores (1961).

Methods

Glycogen. Liver glycogen was extracted and measured as described by Hassid & Abraham (1957). Anthrone was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and was recrystallized before use. Glycogen (Sigma type II) for use as a standard was purchased from Sigma (London) Chemical Co.

Glucose incorporation into liver glycogen. [U-^{14}C]-Glucose (3mCi/mmol, obtained from The Radiochemical Centre, Amersham, Bucks., U.K.) was injected intravenously into rats as described in the legend to Fig. 1. Liver glycogen was isolated and dissolved in water. A portion was then counted for radioactivity in a liquid-scintillation spectrometer with a scintillator composed of 100g of naphthalene, 10g of 2,5-diphenyloxazole, 0.25g of 1,4-bis-(5-phenyloxazol-2-yl) benzene in 1 litre of dioxan and containing 4% (w/v) silica. Counting efficiency was measured by the addition of internal standards.

Glucose. Glucose was measured in whole blood by using the glucose oxidase method as described by Meites (1965). Glucose oxidase (EC 1.1.3.4, type II), peroxidase (EC 1.11.1.7, crude RZ 0.3) and *o*-dianisidine (purified) were obtained from Sigma (London) Chemical Co. Ltd.

Corticosteroids. Plasma corticosteroids were assayed fluorimetrically as described by Givner & Rockefort (1965). Corticosterone for use as a standard was purchased from Sigma (London) Chemical Co. Ltd.

ACTH. ACTH in rat plasma was measured with an immunoassay kit purchased from The Radiochemical Centre. The kit contains human ACTH as a standard and uses antiserum to human ACTH which has been shown to cross-react with rat ACTH (Matsuyama *et al.*, 1971). The values given for ACTH are therefore measured in terms of pg of human ACTH. ^{125}I radioactivity was measured in a gamma-scintillation spectrometer.

Cyclic AMP. Adrenal homogenates in 30mM-HCl were centrifuged and to a portion (0.5ml) of the supernatant was added 25μl of 2.5M-Tris–HCl, pH8.0 at 20°C, containing 0.2M-$MgCl_2$. After centrifugation, unwanted nucleotides and P_i were precipitated by using the $Ba(OH)_2$–$ZnSO_4$ treatment described by Krishna *et al.* (1968). The cyclic AMP content of the supernatant after centrifugation was measured by using a binding protein (Brown *et al.*, 1971). Some samples were divided into halves, one of which was treated with phosphodiesterase before assay to check specificity for cyclic AMP. [8-^{3}H]Adenosine 3′:5′-cyclic monophosphate, ammonium salt (20.7Ci/mmol) was purchased from The Radiochemical Centre and cyclic AMP, sodium salt, was purchased from Sigma (London) Chemical Co. Ltd. The cyclic [^{3}H]AMP was counted for radioactivity in a liquid-scintillation spectrometer by using a scintillator consisting of 16g of butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (Ciba Laboratories) and 201g of naphthalene in 2 litres of dioxan.

Catecholamines. Anhydrous Na_2SO_4 was added to 1.0ml of adrenal homogenate until a thick paste was formed. This was extracted with 5×5ml of butan-1-ol and the butanol extracts were pooled and centrifuged. A portion (15ml) of the butanol extract was added to a mixture of 15ml of light petroleum (heptane fraction; general-purpose reagent from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) and 2.0ml of 0.5M-potassium phosphate buffer, pH7.3. After shaking for 10min the phases were separated by centrifugation and 0.5ml of the aqueous phase was used for measurement of catecholamines as described by Welch & Welch (1969). L-Adrenaline and L-noradrenaline (free base) for use as standards were purchased from Sigma (London) Chemical Co. Ltd.

Presentation of results. All results are expressed where possible as mean ±S.E.M. (number of determinations). In some figures, the number of determinations is also included in parentheses next to the point on the graph. Student's *t* test was used to assess the significance of differences between means.

Results

Effects of diquat and paraquat on liver glycogen and blood glucose concentrations

After 24h starvation, liver glycogen in rats normally falls from approx. 70 to less than 1mg/g wet wt.

Table 1. *Liver glycogen concentrations after administration of diquat or paraquat*

Rats were given an intraperitoneal injection of 0.9% NaCl or a bipyridyl, kept with or without food but with access to water, and killed either 24 or 48h later. Liver glycogen was determined as described in the Experimental section. Results are expressed as means ±S.E.M. (number of determinations).

Treatment	Glycogen (mg/g wet wt. of liver)
Saline control, fed (24h)	67.2±3.3 (4)
Saline control, starved (24h)	0.8±0.2 (8)
Saline control, starved (48h)	0.7±0.1 (4)
Diquat, starved (24h)	27.7±6.7 (4)
Paraquat, starved (24h)	17.5±3.2 (8)
Paraquat, starved (48h)	12.8±3.9 (8)

Table 2. *Effect of diquat on liver glycogen concentrations in starved mice*

Three groups of mice were starved for 24h and one group was then killed. The second group was given an intraperitoneal injection of 0.9% NaCl and the third was given an intraperitoneal injection of diquat. Both groups were then killed 4h later. Liver glycogen was determined as described in the Experimental section. Results are expressed as means ±S.E.M. (number of determinations).

Treatment	Glycogen (mg/g wet wt. of liver)
Control, 24h starved	4.6±1.4 (8)
Saline injected, 28h starved	4.8±1.1 (8)
Diquat injected, 28h starved	11.5±2.4 (6)*

* Significantly different from saline injected, $P<0.02$.

of liver (Table 1). When rats were given an LD_{50} dose of diquat or paraquat, starvation for 24h did not deplete liver glycogen to the same extent (Table 1). Starvation for 48h after paraquat administration still did not decrease liver glycogen concentrations to those of 0.9%-NaCl-injected starved controls (Table 1).

Mice were starved for 24h to deplete their liver glycogen and they were then given intraperitoneal 0.9% NaCl or diquat and killed 4h later. Glycogen concentrations in the diquat-treated mice were significantly higher than in 0.9%-NaCl-injected controls (Table 2). Thus not only is glycogen utilization impaired but net synthesis can occur after diquat administration.

[U-^{14}C]Glucose incorporation into liver glycogen was stimulated after administration of paraquat (Fig. 1) over a period when there was no significant difference in the size of the glycogen pool, indicating that actual synthesis of glycogen must be stimulated.

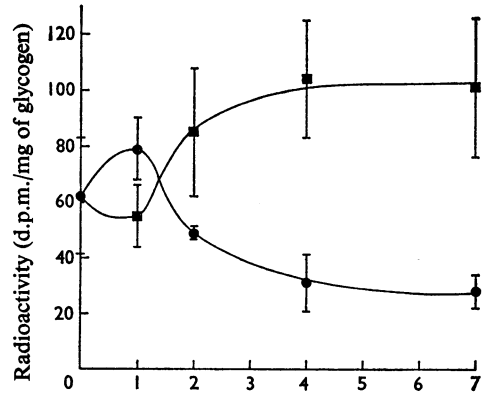

Fig. 1. *Effect of paraquat on the incorporation of [U-^{14}C]-glucose into rat liver glycogen*

Fed rats were given either 0.9% NaCl (●) or paraquat (■) and then starved. They were killed at various times after injection and were given 2μCi of [U-^{14}C]glucose intravenously 1h before killing. Liver glycogen was isolated and radioactivity measured as described in the Experimental section. The points are the means ±S.E.M. from three animals.

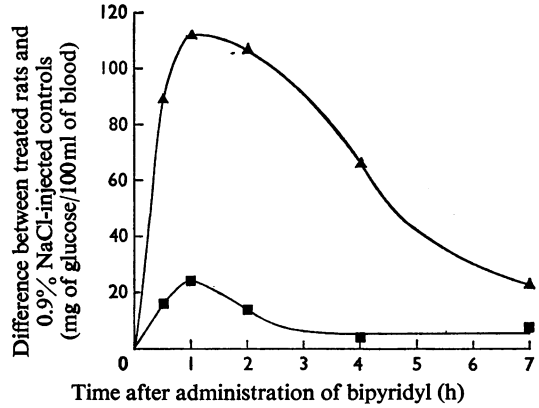

Fig. 2. *Effect of diquat (▲) and paraquat (■) on blood glucose concentrations in rats*

Fed rats were given either 0.9% NaCl or bipyridyl and then starved. Blood (20μl) was removed at intervals by cutting the tip off the tail and glucose was determined as described in the Experimental section. Each point plotted is the difference between the mean values of four 0.9%-NaCl-injected controls and four treated animals. The control blood glucose concentration at the start of the experiment was 117±2 (6) mg/100ml.

After injection of diquat or paraquat, blood glucose rose rapidly to a maximum at about 1h and then fell to control values at 7h (Fig. 2). The increase after diquat administration was much greater than after

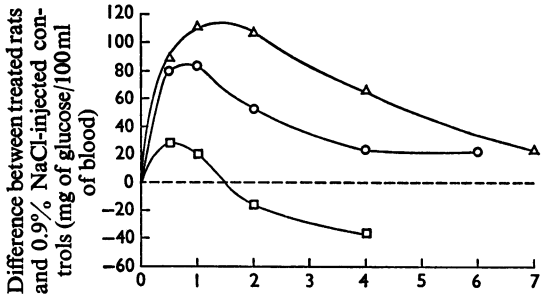

Fig. 3. *Effect of hypophysectomy and adrenalectomy on the increase in blood glucose after diquat administration*

The methods used were as described in the legend to Fig. 2. Each point is the difference between the mean of four 0.9%-NaCl-injected controls and four treated animals. △, Normal animals; ○, hypophysectomized; □, adrenalectomized. The blood glucose concentrations at the start of the experiments in the control groups were: adrenalectomized, 117±6 (4) mg/100ml; hypophysectomized, 88±5 (4) mg/100ml.

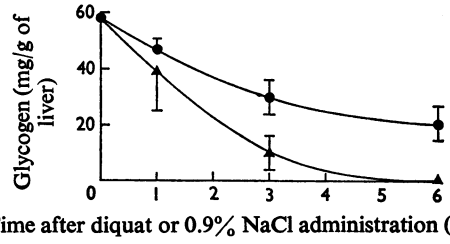

Fig. 4. *Effect of diquat on liver glycogen concentrations in adrenalectomized rats*

Adrenalectomized rats were given either 0.9% NaCl (●) or diquat (▲) at 09:00h and then starved. They were killed at intervals during the next 6h and liver glycogen was measured as described in the Experimental section. Each point is the mean±S.E.M. of three animals.

paraquat treatment but when a $2 \times LD_{50}$ of paraquat was administered, a bigger response was obtained (blood glucose of 95mg/100ml 4h after dosing).

When adrenalectomized rats were given diquat, a much smaller increase in blood glucose occurred within 30min of injection followed by a hypoglycaemic phase (Fig. 3) and usually death with in 6h. The increase in blood glucose after diquat administration in hypophysectomized rats was smaller than in intact animals, but the overall response was very similar.

A rapid loss of liver glycogen was seen in adrenalectomized rats given diquat (Fig. 4). Thus the slower loss of glycogen in normal animals after diquat administration (Table 1) is not seen in adrenalectomized rats.

Effects of diquat and paraquat on adrenal catecholamines and plasma corticosteroids

There was no significant difference in adrenal concentrations of adrenaline and noradrenaline between rats given diquat and those given 0.9% NaCl (Table 3). It was possible, however, to detect the expected depletion of catecholamines 24h after an injection of reserpine (Table 3).

After injection of diquat, plasma corticosteroids rose to maximum concentrations by 30min and remained high for at least 24h, whereas after injection of 0.9% NaCl plasma corticosteroids rose to a maximum and then declined to a resting value by 4h (Fig. 5). Paraquat has a similar long-lasting effect: values of plasma corticosteroids 24h after administration were 47.6±10 (6) μg/100ml.

Diquat has no effect on the metabolism and excretion of corticosteroids, since the rate of disappearance of corticoids from the plasma of hypophysectomized rats after the administration of ACTH was the same after treatment with 0.9% NaCl or diquat (Fig. 6).

Effects of diquat on plasma ACTH

After diquat injection, plasma ACTH was only significantly higher than plasma ACTH in 0.9%-NaCl-injected controls for the first 4h and was within the range of values obtained with unstressed controls by 24h (Fig. 7).

Table 3. *Effect of diquat on catecholamine concentrations in rat adrenals*

Rats were given either 0.9% NaCl or 5mg of reserpine/kg body wt. or diquat (20mg of cation/kg body wt.) intraperitoneally and then starved. At various times after injection the animals were killed by cervical dislocation. The adrenals were removed and each pair homogenized in a medium consisting of 0.01M-HCl (5.0ml) and 10% (w/v) EDTA (0.1ml) at 4°C. Catecholamines in the homogenate were measured as described in the Experimental section. Values are the means of two experiments.

	Concn. (% of 0.9% NaCl-injected control)	
Treatment	Adrenaline	Noradrenaline
1h after diquat	113	105
2h after diquat	111	97
24h after diquat	114	89
24h after reserpine	48	43

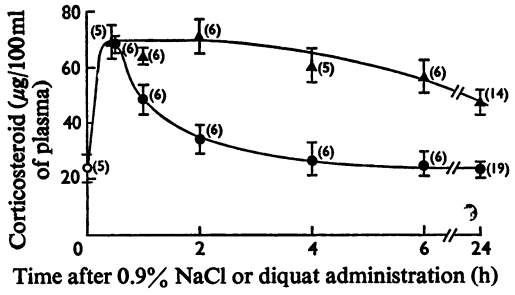

Fig. 5. *Effect of diquat on plasma corticosteroid concentrations*

Fed rats were given either 0.9% NaCl (●) or diquat (▲) at 09:00h and then starved. They were kept in a quiet, warm environment and killed with minimum stress by decapitation at various times during the next 24h. Blood was collected from the trunk into a heparinized beaker. Corticosteroid in the plasma was measured as described in the Experimental section. Points are the means±S.E.M. with the numbers of animals in parentheses.

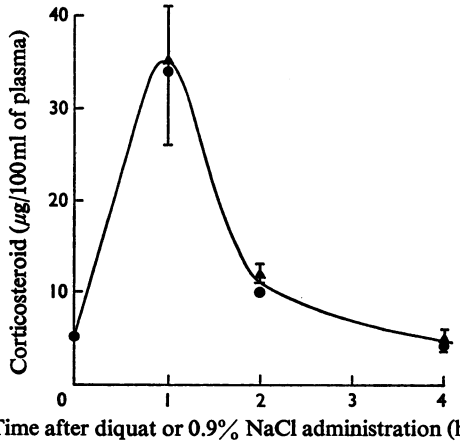

Fig. 6. *Effect of diquat on the removal of corticosteroids from plasma*

Rats were hypophysectomized and kept for 24h before use. They were then given ACTH (2i.u./rat) intraperitoneally and 5min later either 0.9% NaCl (●) or diquat (▲) by intraperitoneal injection. After killing by decapitation, blood was collected from the trunk and corticosteroid concentrations were measured in the plasma as described in the Experimental section.

Effects of diquat on plasma corticosteroids in hypophysectomized rats

Injection of diquat or 0.9% NaCl had no effect on plasma corticosteroids of hypophysectomized rats [0.9%-NaCl-injected rats, 5.3±0.2 (6) μg/100ml;

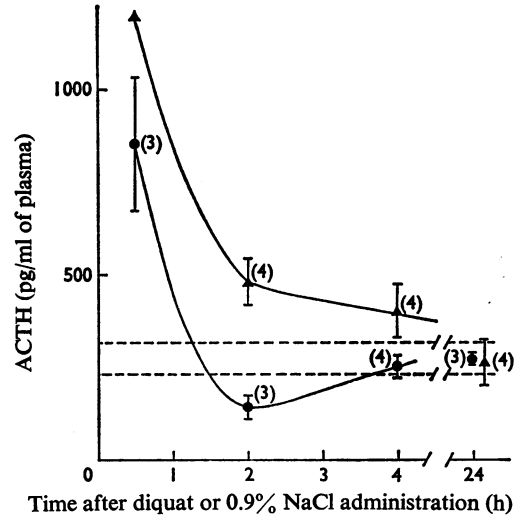

Fig. 7. *Effect of diquat on plasma ACTH concentrations*

Fed rats were treated as described in the legend to Fig. 5. After decapitation, blood was collected from the trunk into heparinized beakers. ACTH was measured in the plasma as described in the Experimental section. Each point is the mean±S.E.M. for four animals. ----, Mean values±S.E.M. of ACTH found in four animals that had been kept under the same experimental conditions but not injected. ●, 0.9%-NaCl-treated rats; ▲, diquat-treated rats.

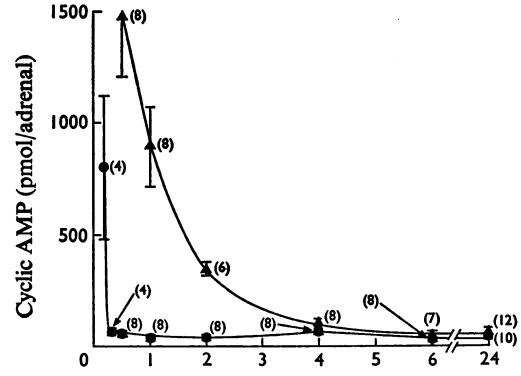

Fig. 8. *Effect of diquat on the cyclic AMP content of rat adrenals*

Fed rats were given either 0.9% NaCl (●) or diquat (▲) and then starved. They were kept in a quiet, warm environment and killed by decapitation at various times after injection. The adrenals were rapidly dissected out (within min) and dropped into liquid N_2. Each adrenal was homogenized in 30mM-HCl (1.0ml) and assayed for cyclic AMP as described in the Experimental section. Points are the means±S.E.M. with the numbers of determinations in parentheses.

diquat-injected rats, 4.3±0.4 (5) μg/100ml]. These values were lower than those of intact animals and are in the expected range (Guillemin *et al.*, 1958).

Effect of diquat on adrenal cyclic AMP

The amount of cyclic AMP in adrenals of rats given diquat was extremely high over the first few hours of poisoning and significantly higher than in adrenals from 0.9%-NaCl-injected controls over the whole of the 24h period (Fig. 8). The 24h values were: 0.9%-NaCl-injected controls, 41±4 (10) pmol/adrenal; diquat-treated rats, 55±6 (12) pmol/adrenal (significantly different at $P<0.01$). There was no significant increase in adrenal wet wt. during this period.

There was no increase in adrenal cyclic AMP in hypophysectomized rats 10min after an injection of either 0.9% NaCl or diquat [0.9%-NaCl-injected rats, 28±3 (4) pmol/adrenal; diquat-injected rats, 22±5 (4) pmol/adrenal].

Discussion

Role of catecholamines and corticosteroids in the effects of bipyridyls

After the administration of diquat or paraquat, there was increased synthesis of liver glycogen (Tables 1 and 2 and Fig. 1) and an increase in blood glucose (Fig. 2). These effects appear to be mediated by the adrenal, since adrenalectomy prevented them (Figs. 3 and 4).

Both adrenal-medullary secretion (catecholamines) and cortical secretion (corticosteroids) have the effect of raising blood glucose (Weiner, 1964; Landau, 1965). Of these, only corticosteroids would also have the effect of increasing glycogen synthesis (Landau, 1965). No loss of catecholamines was observed from medullary stores in diquat-treated rats (Table 3). However, catecholamines may be involved in the hyperglycaemic effect, since an increase in blood glucose was seen in diquat-treated hypophysectomized rats (Fig. 3), in which there were no increases in plasma corticosteroids. Therefore catecholamine release after diquat treatment must be small and cannot be compared with that contributing to hyperglycaemia in stress, where there is a detectable loss of adrenal catecholamines at 2 and 24h after bilateral hindlimb ischaemia (Stoner & Westerholm, 1969).

In normal rats, the hyperglycaemia which follows the administration of the bipyridyls is probably due to both catecholamine release and the large increase in plasma corticosteroids (Fig. 5). The effects on liver glycogen, however, must be mainly due to the high circulating concentration of corticosteroids.

Corticosteroid synthesis in diquat-poisoned rats

The adrenal cortex stores very little corticosteroid (Smith, 1972). The rate of synthesis is normally controlled by the plasma concentration of ACTH (Garren *et al.*, 1969; Pitot & Yatvin, 1973) and appears to be related to increased concentrations of cyclic AMP in the adrenal (Haynes, 1958; Grahame-Smith *et al.*, 1967; Grahame-Smith, 1970; Kumar *et al.*, 1972). Only very small increases in adrenal cyclic AMP appear to be necessary for quite significant increases in ACTH-stimulated corticosteroid synthesis (Grahame-Smith *et al.*, 1967; Nakamura *et al.*, 1972; Kumar *et al.*, 1972).

The high plasma concentrations of corticosteroid observed after diquat or paraquat administration could be due to increased synthesis, decreased breakdown and excretion, or both. The loss of corticosteroid from plasma of hypophysectomized rats after administration of a single dose of ACTH was unaffected by diquat (Fig. 6). Thus there is no significant inhibition of breakdown and excretion, and the high concentration of plasma corticosteroids in intact animals given diquat must be due to stimulation of synthesis. The increase in adrenal cyclic AMP (Fig. 8) supports this.

Although plasma corticosteroid concentrations remained very high for at least 24h (Fig. 5) and adrenal cyclic AMP was significantly increased for 24h (Fig. 8), plasma ACTH in diquat-treated rats reached stressed concentrations [>1000pg/ml of plasma (Matsuyama *et al.*, 1971; Rees *et al.*, 1971)] in the first hour and then fell towards values found for 'unstressed' controls (Fig. 7). After 24h the plasma concentration of ACTH was identical with that of 0.9%-NaCl-injected controls and that of uninjected control rats (Fig. 7). Both injected and uninjected controls had plasma concentrations of corticosteroids of approx. 25μg/100ml, whereas diquat-treated rats had plasma concentrations of corticosteroids of approx. 48μg/100ml (Fig. 5). Thus apparently the prolonged stimulation of the adrenals after diquat treatment is not mediated by ACTH. However, since there was no increase in plasma corticosteroids or adrenal cyclic AMP in hypophysectomized rats after diquat injection, there must be a requirement for ACTH in the steroidogenic effect. This apparent paradox would be resolved if diquat made the adrenal cortex more sensitive to ACTH. That this might be the case is suggested by preliminary experiments which indicate that adrenals from diquat-poisoned rats synthesize more corticosteroid in response to a suboptimal concentration of ACTH *in vitro* than do adrenals from 0.9%-NaCl-injected controls.

The stimulation of adrenal corticosteroid synthesis by diquat cannot be ascribed to stress, since it is not mediated simply by ACTH. Also, stress after injury is characterized by catecholamine and corticosteroid

release and results in hyperglycaemia as a result of glycogen breakdown (Stoner & Heath, 1973). The response to diquat gives rise to hyperglycaemia, but mobilization of liver glycogen is not the source of this glucose.

The effects of diquat and paraquat on adrenals are unlikely to be related to the free-radical-generating properties of the bipyridyls. Similar effects on liver glycogen utilization are seen with 2,2'-bipyridilium [after 24h starvation, liver glycogen was 30.4±7.8 (4) mg/g wet wt.] and 4,4'-bipyridilium [after 24h starvation, liver glycogen was 16.8±4.2 (4) mg/g wet wt.] which do not form stable free radicals at all readily. It should therefore be borne in mind that other effects of the bipyridyls in mammals may not be in any way related to free-radical generation.

Relevance of the effects of diquat and paraquat on the adrenal to their toxicology

The phenomena described in the present study are common to both diquat and paraquat and cannot therefore have direct relevance to the lung lesion that develops only after paraquat poisoning. The high circulating corticosteroid concentration after the unusual adrenal stimulation may, however, go some way to account for some of the unexplained histological findings with bipyridyl poisoning, including adrenal necrosis after paraquat poisoning in man (Nagi, 1970), atrophy of lymphoid tissue in rabbit after paraquat (Butler & Kleinerman, 1971) and changes in thymus, spleen and adrenals in rats after both diquat and paraquat (Clark *et al.*, 1966; Clark & Hurst, 1970).

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