Biochemical Pharmacology, Vol. 25, pp. 2465–2468. Pergamon Press, 1976. Printed in Great Britain.

EARLY EFFECTS OF DIQUAT ON PLASMA CORTICOSTEROID CONCENTRATIONS IN RATS

HELEN C. CRABTREE and MICHAEL S. ROSE

Biochemical Mechanisms Unit, Imperial Chemical Industries Limited, Central Toxicology Laboratory, Alderley Park, Nr. Macclesfield, Cheshire, SK10 4TJ, England

(*Received* 23 *February* 1976; *accepted* 9 *June* 1976)

Abstract—Plasma corticosteroid concentrations were significantly elevated 15 min after intraperitoneal (i.p.) injection of 90 μmoles of diquat/kg body wt (LD_{50} dose) into phenobarbitone anaesthetised rats. Similar effects were noted after subcutaneous dosing but after oral administration there was a delay of approximately 1 hr before significantly elevated concentrations were observed. The magnitude of the increase after i.p. administration of diquat was dose-related up to 26 μmoles/kg body wt, as was the duration of the response. The increase in plasma corticosteroid concentration in diquat-treated rats could be prevented by pretreating animals with dexamethasone which also reduced adrenocorticotrophic hormone (ACTH) concentration in these animals. Experiments *in vivo* and *in vitro* indicated that diquat did not increase the steroidogenic response of adrenals to ACTH. It is concluded that the increased adrenal steroid synthesis observed at early times after diquat administration is caused by release of ACTH from the pituitary.

Diquat (1,1′-ethylene-2,2′-bipyridilium) is widely used as a non selective weed-killer [1]. It is moderately toxic to rats; the intraperitoneal (i.p.) and subcutaneous (s.c.) LD_{50} to rats being approximately 90 μmoles/kg body wt and the oral LD_{50} 900 μmoles/kg body wt [Parkinson, unpublished work].

The biochemical mechanism of diquat toxicity in mammals is not understood. In plants, the herbicidal activity is thought to involve peroxidation of lipid components of the cell membrane, causing disruption of the tonoplast [2]. This damage is probably related to the ability of the compound to form stable free radicals in aqueous solution [2]. Changes characteristic of a generalised lympholysis have been noted in the thymus and spleen of rats following diquat administration by both oral and parenteral routes [3, 4]. Glycogen synthesis by the liver is enhanced and a transient hyperglycaemia is observed. It has been suggested that these effects may be related to the increase in plasma corticosteroid concentration which occurs after dosing [5].

Significant increases in plasma corticosteroid concentrations are observed from 1 hr for up to 24 hr following i.p. administration of diquat. During the first 4 hr increased plasma corticosteroid concentrations are also observed in control animals injected i.p. with 0.9% NaCl [5]. Results presented in this paper show that the increase observed after saline injection can be prevented by phenobarbitone anaesthesia. Therefore, phenobarbitone anaesthetised rats have been used to investigate the early effects of diquat on adrenal steroid synthesis.

MATERIALS AND METHODS

Chemicals. Crystalline diquat dichloride monohydrate (Plant Protection Division, I.C.I. Ltd., Jealott's Hill Research Station, Berks, U.K.) was dissolved in sterile 0.9% NaCl for *in vivo* admininstration and in distilled water for *in vitro* purposes. Phenobarbitone sodium (Evans Medical Ltd., Speke, Liverpool, U.K.) and dexamethasone sodium phosphate (Merck, Sharp and Dohme Ltd., Hoddesdon, Herts., U.K.) were diluted with sterile 0.9% NaCl for injection. ACTH (HP ACTHAR-Gel, corticotrophin gelatin injection) was purchased from Armour Pharmaceuticals Co. Ltd., Eastbourne, Sussex, U.K., and diluted with 16% gelatin (Byco C, a gift from Croda Ltd., Widnes, Lancs., U.K.) to produce a slow release formulation for *in vivo* experiments. Purified ACTH (porcine, grade II) and corticosterone were purchased from Sigma (London) Chemical Co. Ltd., Kingston-on-Thames, Surrey, U.K. ACTH was dissolved in 1 mM HCl for all *in vitro* experiments.

Methods

(a) In vivo *experiments.* Male, Sprague–Dawley, specific-pathogen-free rats (150–300 g body wt), purchased from Charles River Ltd. were used for all experiments. Animals were starved 24 hr prior to oral administration of diquat; fed animals were used for all other experiments. They were anaesthetised by s.c. injection of phenobarbitone (150 or 200 mg/kg body wt) and 2 hr later were given 0.9% NaCl or diquat.

In some experiments dexamethasone (250 μg/kg body wt) was injected s.c. 2 hr prior to phenobarbitone. Rats were killed rapidly by decapitation since this method causes the least increase in plasma corticosteroid concentrations [6]. Blood was collected from the trunk into heparinised beakers. Plasma corticosteroids were extracted and assayed fluorimetrically as described by Givner and Rochefort [7]. Dexamethasone does not interfere with the fluorimetric determination of corticosteroid in plasma [8]. ACTH in rat plasma was measured by radioimmunoassay (The Radiochemical Centre, Amersham, Bucks., U.K.) as described previously [5].

(b) In vitro *incubation of adrenal quarters.* The method used was modified from that described by Grahame-Smith *et al.* [9]. Animals were killed by

decapitation. Adrenals were removed, trimmed free of fat, weighed and quartered. These were preincubated (1 adrenal/flask) for 1.5 hr in a shaking water bath at 37°, under an atmosphere of 95% O_2, 5% CO_2. Incubations contained diquat or an equivalent volume of distilled water in 2.5 ml Krebs–Ringer bicarbonate [10] containing 11 mM glucose. Preincubation of adrenals in the presence of diquat ensured equilibration between the medium and the tissue at the start of the experimental period [11] [Crabtree unpublished work]. After 1.5 hr the tissue was transferred to fresh medium (2.5 ml) containing diquat and ACTH and incubated for a further 30 min. In control incubations, 10 μl distilled water or 1 mM HCl were added in place of diquat or ACTH, respectively. The reaction was stopped after 30 min by addition of 2.5 ml of an ethanol–water mixture (2:3 v/v) and the whole sample rapidly homogenised for 10 sec with a vortex homogeniser. Samples were assayed for corticosteroid by the method of Givner *et al.* [7].

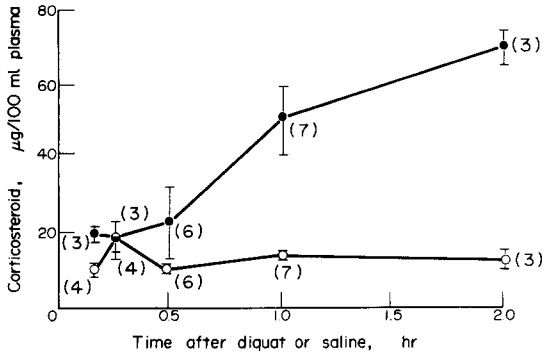

Fig. 2. Effects of orally administered diquat on plasma corticosteroid concentration in phenobarbitone-anaesthetised rats. Rats previously fasted for 24 hr were anaesthetised with subcutaneous phenobarbitone (200 mg/kg body wt) and 2 hr later they were given either 900 μmoles/kg body wt diquat [●] or 0.9% NaCl [○] by stomach tube. Plasma was assayed for corticosteroid as described in the materials and methods section. Points represent the mean ± S.E.M. with the number of animals in parenthesis.

RESULTS

Effect of in vivo *diquat administration into phenobarbitone-anaesthetised rats.* Plasma corticosteroid concentration in anaesthetised control animals injected i.p. with saline remained within the normal basal range over the 4-hr period studied (Fig. 1). A single i.p. injection of diquat (90 μmoles/kg body wt) into phenobarbitone-anaesthetised rats produced a significant increase in plasma corticosteroids by 15 min after dosing (Fig. 1). Maximum concentrations were reached by 30 min after dosing and remained at this level for at least 4 hr (Fig. 1). Subcutaneous injection of diquat produced a similar response. A maximal plasma concentration of 40 ± 8(4) μg corticosteroid/100 ml plasma was observed 15 min after s.c. dosing and was sustained for at least 1 hr. In contrast there was a delay of 1–2 hr before a maximum concentration of corticosteroid was observed following oral administration of an equitoxic dose (LD_{50} dose ≃ 900 μmoles/kg body wt) (Fig. 2).

The plasma corticosteroid concentration measured 1 hr after an i.p. injection increased with increasing dose of diquat to a maximum value (Fig. 3). Plasma corticosteroid concentration measured 4 hr after injecting 26 μmoles/kg body wt and 50 μmoles/kg body wt were not significantly different from control values, whereas maximal concentrations were still observed 4 hr after injecting 90 μmoles (LD_{50}) and 260 μmoles/kg body wt (Fig. 3).

Effect of dexamethasone pretreatment. The increase in plasma corticosteroid observed 1 hr after various doses of i.p. administered diquat was prevented by prior treatment of rats with dexamethasone (Fig. 4). Plasma ACTH concentrations in the same animals were significantly reduced (Table 1).

Response to injected ACTH. Rats were dosed with dexamethasone prior to injection of either saline or diquat. Thirty min later they were injected s.c. with a solution of ACTH in gelatin. There was no significant difference in plasma corticosteroid concentration between the group given saline and that given diquat (Fig. 5).

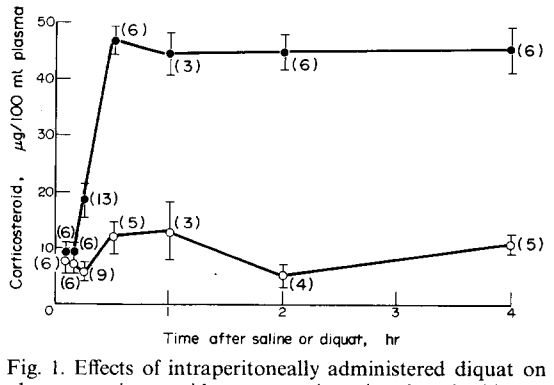

Fig. 1. Effects of intraperitoneally administered diquat on plasma corticosteroid concentrations in phenobarbitone-anaesthetised rats. Rats were anaesthetised with 150 or 200 mg/kg phenobarbitone and 2 hr later were given either 0.9% NaCl [○] or 90 μmoles/kg body wt diquat [●] intraperitoneally. Plasma was assayed for corticosteroid as described in the methods section. Points are the mean ± S.E.M. with the number of animals in parenthesis.

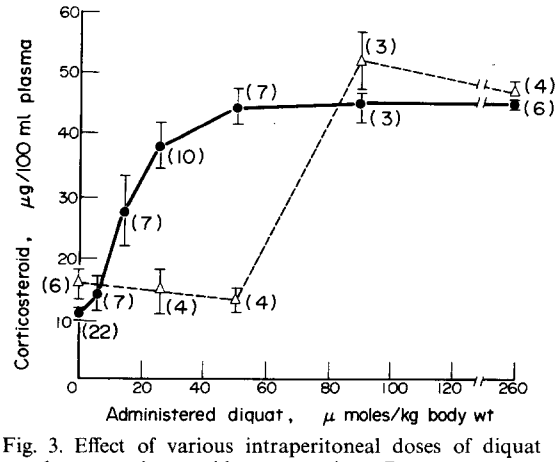

Fig. 3. Effect of various intraperitoneal doses of diquat on plasma corticosteroid concentrations. Rats were anaesthetised with 200 mg/kg phenobarbitone 2 hr prior to intraperitoneal injection of either 0.9% NaCl or various amounts of diquat. Animals were killed 1 hr [●] and 4 hr [△] after dosing. Plasma was assayed for corticosteroid as described in the methods section. Points represent the mean ± S.E.M. with the number of animals in parenthesis.

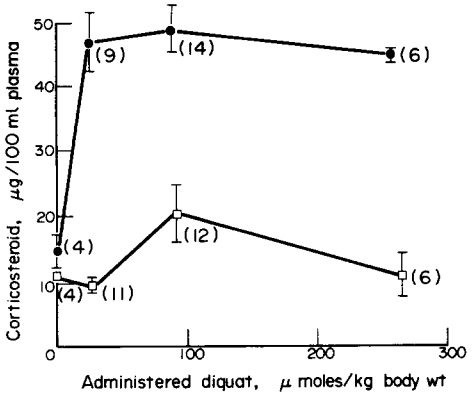

Fig. 4. Effect of dexamethasone on plasma corticosteroid concentrations observed 1 hr after intraperitoneal administration. Rats were injected subcutaneously with 250 μg/kg dexamethasone [□] or an equivalent volume of 0.9% NaCl [●] 2 hr before anaesthetising with 200 mg/kg phenobarbitone. After a further 2 hr diquat was administered by intraperitoneal injection to both groups of rats. They were killed 1 hr later. Corticosteroid in the plasma was measured as described in the methods section. Points represent the mean ± S.E.M. with the number of aninals in parenthesis.

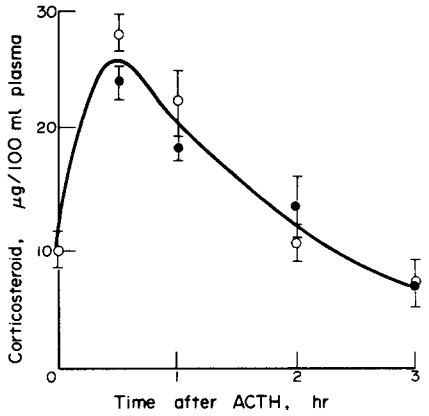

Fig. 5. The steroidogenic effect of 100 mU ACTH in dexamethasone-pretreated animals after 90 μmoles/kg body wt diquat. Rats were injected subcutaneously with 250 μg/kg dexamethasone and after a further 2 hr were anaesthetised with 200 mg/kg phenobarbitone. One hour later animals were dosed intraperitoneally with 0.9% NaCl [○] or 90 μmoles/kg diquat [●] followed by subcutaneous administration of 100 mU ACTH a further 30 minutes later. Animals were killed at various times after the ACTH injection. Corticosteroid was measured as described in the methods section. Points represent the mean ± S.E.M. for groups of 4 animals.

In vitro *response of adrenals to ACTH*. Diquat had no significant effect on the amount of steroid synthesised by adrenal quarters in the absence of ACTH (Table 2). In the presence of 1 mU ACTH, a concentration which produces sub maximal stimulation, there was a significant inhibition of corticosteroid synthesis when 25 or 50 μM diquat was included in the incubation medium (Table 2). Lower concentrations of diquat had no significant effect (Table 2).

DISCUSSION

The concentration of corticosteroid in rat plasma increased after oral, i.p. and s.c. routes of administration (Figs. 1 and 2). Previous work has shown that the rate of corticosteroid metabolism and excretion from the plasma of diquat-dosed animals is unchanged over this early period [5]. It may, therefore, be concluded that the increased concentration observed in the plasma is a consequence of increased synthesis of steroid by the adrenal cortex.

Increased glucocorticoid synthesis is a frequently observed response of animals to noxious stimuli [12]. Many seemingly unrelated phenomena such as hypoglycaemia, starvation, injection of histamine or endotoxin, inhalation of ether, or exposure to cold, light or noise have this effect. All these stimuli have in common the ability to stimulate ACTH release from the pituitary and on this basis can be defined as "stress" inducing agents [13].

The rate of corticosteroid synthesis by the adrenal is normally controlled by the plasma concentration

Table 1. Plasma ACTH concentrations in control and dexamethasone-pretreated rats 1 hr after intraperitoneal injection of diquat

Diquat administered (μmoles/kg body weight)	ACTH 1 hr after injection (pg/ml plasma)	
	Diquat alone	Diquat + dexamethasone
26	982 ± 93 (5)	203 ± 15 (7)*
90	1301 ± 160 (6)	696 ± 324 (4)*
260	1505 ± 448 (4)	339 ± 61 (6)*

Rats were injected s.c. with 250 μg/kg dexamethasone or an equivalent volume of 0.9% NaCl. Two hr later they were anaesthetised with 200 mg/kg phenobarbitone and after a further 2 hr diquat was injected i.p. Animals were killed 1 hr later. Plasma ACTH was measured as described in the methods section. Results are quoted as mean ± S.E.M. (no. of determinations).

* Significantly less than "diquat alone", $P < 0.05$.

Table 2. Response of adrenal quarters to ACTH: the effect of diquat *in vitro* on corticosteroid synthesis

Concn DQ (μM)	Steroid (μg/g adrenal wet wt)	
	No ACTH	1 mU ACTH
0	60.8 ± 6.6 (8)	101.4 ± 17.4 (8)
5	—	102.7 ± 24.4 (4)
10	68.8 ± 11.2 (4)	71.4 ± 4.7 (4)
25	—	50.2 ± 5.7 (4)*
50	64.3 ± 22.9 (4)	57.8 ± 10.8 (4)*

* Significantly less than in the absence of diquat, $P < 0.02$.

Adrenal quarters were pre-incubated with or without diquat in Krebs–Ringer bicarbonate medium containing 11 mM glucose for 1½ hr before transferring to identical fresh medium. ACTH (1 or 500 mU) or an equivalent volume of 1 mM HCl was added to the flasks and these were incubated for a further 30 min before the reaction was stopped. Samples assayed for corticosteroid as described in the methods section. Values are quoted as mean ± S.E.M. (no. of determinations). Amount of steroid produced during a 30-min incubation in the presence of 500 mU ACTH was 211.3 ± 21.0 (8).

of ACTH [14]. Hypophysectomy abolishes the diquat-induced rise in plasma corticosteroid concentration [5], indicating that the response to diquat is ACTH-dependent. Two hypotheses are consistent with the data; (a) that the increased concentration of steroid in the plasma is caused by increased ACTH release from the pituitary or (b) that diquat has increased the sensitivity of the adrenal cortex to ACTH (i.e. a potentiation of the normal adrenal response).

Previous measurement of ACTH concentrations in the plasma of conscious rats 24 hr after dosing suggested that there was insufficient ACTH present to account for the observed increase in plasma corticosteroid concentration observed at this time [5]. This led us to favour the potentiation hypothesis. This hypothesis can be tested using dexamethasone-treated rats since prior treatment with this steroid inhibits stress induced increases in plasma corticosteroids by preventing ACTH release from the pituitary [15, 16]. The adrenals of rats treated with dexamethasone have been shown to respond normally to ACTH [15, 16].

In dexamethasone-treated rats, there was no significant difference in the amount of steroid observed in the plasma after injection of 100 mU ACTH into diquat or saline treated animals (Fig. 5). Therefore, diquat does not appear to potentiate the response of the adrenal to ACTH. This conclusion is supported by experiments using quartered adrenal glands *in vitro*, where incubation with various concentrations of diquat did not give rise to any increased synthesis. On the contrary corticosteroid synthesis was significantly inhibited in the presence of 25 and 50 μM diquat (Table 2). The concentrations of diquat used in the *in vitro* experiments were selected on the basis of observed *in vivo* concentrations in the adrenal over the first 24 hr after dosing with an i.p. LD_{50} dose (Rose unpublished work).

Together these experiments make the possibility of a potentiated response of the adrenal to ACTH after diquat administration unlikely and thus support the alternative hypothesis of a pituitary-mediated effect. This conclusion is supported by the observation that pretreatment of rats with dexamethasone, at concentrations which have been reported to inhibit stress induced release of ACTH [15] abolished the diquat-induced rise in plasma corticosteroid (Fig. 5) and decreased plasma ACTH concentrations in these animals (Table 1).

Acknowledgements—We should like to thank Mrs. Eileen Gore for her valuable technical assistance.

REFERENCES

1. A. Calderbank, in *Advances of Pest Control Research*, Vol. 8 (Ed. R. L. Metcalf). Interscience (1968).
2. A. D. Dodge, N. Harris and B. C. Baldwin, *Biochem. J.* **118,** 43P (1970).
3. D. M. Conning, K. Fletcher and A. A. B. Swan, *Br. med. Bull.* **25,** 245 (1969).
4. D. G. Clark and E. W. Hurst, *Br. J. Indust. Med.* **27,** 51 (1970).
5. M. S. Rose, H. C. Crabtree, K. Fletcher and I. Wyatt, *Biochem. J.* **138,** 437 (1974).
6. J. A. Carney and B. L. Walker, *Lab. Animal Sci.* **23,** 675 (1973).
7. M. L. Givner and J. G. Rochefort, *Steroid* **6,** 485 (1965).
8. G. Giuliani and A. Maffei-Faccioli, *Atti Accad. med. Lombarda* **15,** 45 (1960).
9. D. G. Grahame-Smith, R. W. Butcher, R. L. Ney and E. W. Sutherland, *J. biol. Chem.* **242,** 5535 (1967).
10. W. W. Umbreit, R. H. Burris and J. F. Stauffer, in *Manometric Techniques* p. 132, 4th edn. Burgess Publishing Company, Minneapolis (1964).
11. M. S. Rose, E. A. Lock, L. L. Smith and I. Wyatt, *Biochem. Pharmac.* **25,** 419 (1976).
12. J. P. Allen, C. F. Allen, M. A. Greer and J. J. Jacobs, in *Brain–Pituitary–Adrenal Interrelationships* pp. 99–127. Karger, Basel (1973).
13. H. Selye, *Science* **122,** 625 (1955).
14. L. D. Garren, W. W. Davis, G. N. Gill, H. L. Moses, R. L. Ney and R. M. Crocco, *Progr. Endocrinol int. Symp. Endocrinol.* 3rd, pp. 102–114 (1969).
15. M. F. Dallman and F. E. Yates, *Mem. Soc. Endocrinol.* **17,** 39 (1968).
16. P. N. Cheifetz, *Acta endocrinol.* **70,** 56 (1972).