Biochemical Pharmacology, Vol. 19, pp. 2461–2467. Pergamon Press. 1970. Printed in Great Britain

# EFFECT OF ADRENALECTOMY AND CORTISONE ADMINISTRATION ON COMPONENTS OF THE LIVER MICROSOMAL MIXED FUNCTION OXYGENASE SYSTEM OF MALE RATS WHICH CATALYZES ETHYLMORPHINE METABOLISM*

J. A. CASTRO,† F. E. GREENE,‡ P. GIGON,§ H. SASAME and J. R. GILLETTE

Laboratory of Chemical Pharmacology, National Heart Institute
National Institutes of Health, Bethesda, Md. 20014, U.S.A.

(*Received* 14 *November* 1969; *accepted* 23 *February* 1970)

**Abstract**—The effects of adrenalectomy and cortisone administration on components of the mixed function oxygenase system of liver microsomes from adult male rats were studied. The *N*-demethylation of ethylmorphine (EM) as measured by formaldehyde production was used as an index of the overall activity of this system. EM demethylase activity in liver microsomes from adrenalectomized rats was 56 per cent of the value obtained with sham-operated controls, while cytochrome P-450 content fell by only 19 per cent. NADPH cytochrome c reductase and cytochrome P-450 reductase activities were decreased 68 and 69 per cent, respectively, in liver microsomes from adrenalectomized animals, while the apparent affinity constant ($K_{sp}$) of EM for cytochrome P-450 spectral change was not altered. The decrease in maximal spectral change caused by adrenalectomy corresponded to the decrease in cytochrome P-450 content. Cortisone acetate administration (5 mg/kg/day for 8 days) to adrenalectomized rats prevented the decrease in EM demethylase activity. Accordingly, the steroid treatment increased the NADPH cytochrome c reductase and cytochrome P-450 reductase activities to 113 and 140 per cent, respectively, of the values obtained with the sham-operated controls, without significantly altering the cytochrome P-450 content or the kinetic constants for the spectral changes. These data suggest that the well known decrease in drug-metabolizing activity seen in liver microsomes of adrenalectomized animals is not related to changes in cytochrome P-450 content or to a decrease in the ability of cytochrome P-450 to bind drug substrates. However, there appears to be a relationship between the reductase activities and the adrenal function, because the changes in activities in NADPH cytochrome c reductase and cytochrome P-450 reductase in liver microsomes from adrenalectomized and cortisone-treated adrenalectomized rats paralleled the changes in EM demethylase activity.

* A preliminary report of this work has been presented (*Fedn Proc.* **27**, 350, 1968).

† Fellow of The Consejo Nacional de Investigaciones Cientificas y Tecnicas, Republica Argentina. Present address: Laboratorio de Quimica-Bio-Toxicologica, 3 deFebrero 1250, San Martin, Pvcia de Buenos Aires, Argentina.

‡ Supported by Special Fellowship 9 FO3-GM-37751-02A1, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Md. 20014. Present address: Department of Pharmacology, M. S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pa. 17033.

§ Sponsored by The Swiss Academy of Medical Sciences. Present address: Pharmazeutisches Institut der Universitaet, 10 Sahlistrasse, 3000 Bern, Switzerland.

IN 1958 REMMER showed that adrenalectomy lowered the activity of the rat liver microsomal enzymes which catalyze the *N*-demethylation of monomethyl 4-aminoantipyrine and the oxidation of hexobarbital[1, 2] and that the decrease was prevented by the administration of prednisolone.[2] Extending these findings, Kato and Gillette[3] found a sex difference in the effect of adrenalectomy of rats; whereas it impairs the metabolism of aminopyrine and hexobarbital without affecting the metabolism of aniline and zoxazolamine in male rats, it does not decrease the metabolism of any of these substances in female rats.

Other authors[4] have pointed out the relationship between the pituitary-adrenal system and drug-metabolizing activity by showing that hind limb ligation shortened the duration of action of pentobarbital, hexobarbital, zoxazolamine and meprobamate in intact rats but not in adrenalectomized animals. Moreover, the sleeping time of rats receiving hexobarbital was shortened by treatment of hypophysectomized rats with either corticosterone or ACTH or by treatment of adrenalectomized rats with corticosterone. Despite this continued interest, however, the mechanism by which the pituitary-adrenal system regulates drug metabolism has not been determined.

During the past few years, considerable knowledge has been gained about the mixed function oxygenase system that hydroxylates drugs and other foreign compounds. In the current view, hydroxylation is thought to proceed through a series of steps. With certain drug substrates such as ethylmorphine, the first step is binding of the drug to oxidized cytochrome P-450. This complex is then reduced by NADPH cytochrome c reductase either directly or indirectly through an unidentified electron carrier. The reduced complex then reacts with oxygen to form an active oxygen complex which rearranges to form the hydroxylated drug and oxidized cytochrome P-450. (This mechanism is discussed extensively in reference 5.)

A number of studies suggest that steroids are hydroxylated by the same microsomal enzyme systems which metabolize drugs: (1) both drug and steroid hydroxylation by liver microsomes require NADPH and oxygen[6] and are inhibited by carbon monoxide;[7] (2) both drugs and steroids interact with microsomes to produce spectral changes;[8] and (3) steroids competitively inhibit drug hydroxylation.[9]

Thus there are at least two possible mechanisms by which steroid hormones may influence drug metabolism by the liver microsomal system: (1) by combining with cytochrome P-450, thereby altering the apparent affinity of the enzyme system for drugs; and (2) by altering the amount or the activity of one or more of the components in the electron transport system. In this study we have attempted to evaluate these possibilities by studying the various components of the electron transport system in liver microsomes from adrenalectomized and cortisone-treated rats.

## MATERIALS AND METHODS

*Chemicals.* $NADP^+$ was obtained from Sigma Chemical Company and trisodium isocitrate from Calbiochem. Isocitric acid dehydrogenase from porcine heart was purchased from Calbiochem as a 50% glycerol solution having an activity of 2·5 enzyme units/mg at 30°. Cortisone acetate suspension was supplied by Merck, Sharp & Dohme Inc. (Cortone acetate) and diluted with saline before use. Carbon monoxide, having less than 25 ppm oxygen, was purchased from Matheson Company and further deoxygenated by bubbling it through a solution consisting of 0·05% 2-anthraquinonesulfonic acid sodium salt and 0·5% $Na_2S_2O_4$ in 0·1 N NaOH.

*Animals and animal treatments.* Male Sprague–Dawley rats, weighing 120–140 g, were obtained from Hormone Assay Laboratories, Inc., Chicago, Ill., and were received 2 days after either adrenalectomy or sham adrenalectomy. Since anorexia and weight loss, commonly seen after adrenalectomy, may produce a starvation-like state which in itself affects drug metabolism,[10] all rats used for these experiments were kept as nearly as possible in an identical nutritional condition by oral force feeding. Accordingly, the postoperative oral feeding schedule with diet no. 4370 described by Scow[11] was begun on the day the animals arrived. Each rat was given an equal amount of the diet. The diet of the adrenalectomized rats was suspended in saline, whereas that of the sham-operated controls was suspended in water. The adrenalectomized rats were also given saline to drink.

One group of adrenalectomized rats was treated with a saline suspension of cortisone acetate, 5 mg/kg, subcutaneously once a day for 6 days before sacrifice. The sham-operated rats were injected with an equal amount of saline.

*Enzyme preparation.* The livers were homogenized in 4 vol. of an ice-cold solution of Tris-KCl (1·15% KCl containing 0·05 M Tris-HCl, pH 7·4) with a motor driven Teflon-glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min in a Servall refrigerated centrifuge. The supernatant fraction was centrifuged for 1 hr at 30,000 rpm (78,000 *g*, av.) in a no. 30 rotor with a Spinco model L preparative ultracentrifuge. The microsomal pellet was resuspended in a volume of the Tris-KCl buffer equivalent to the original volume of 9000 *g* supernatant. All operations were performed at 0–4°.

*Incubation procedures.* The final composition of the incubation mixtures was: Tris-HCl buffer, pH 7·4, 50 mM, sodium isocitrate, 8 mM; NADP, 0·33 mM; isocitric acid dehydrogenase, 0·36 unit per ml; microsomal protein, about 2 mg/ml; and substrate in appropriate concentrations. The final volume was 3·0 ml. Nicotinamide was omitted from the incubation mixture because it has been shown to inhibit the metabolism of various drugs.[12] In the kinetic experiments, ten different concentrations of substrate were used, ranging from 0·1 to 1·0 mM. In other experiments 1 mM ethylmorphine was used. The mixtures were incubated in air for 10 min at 37° in a Dubnoff metabolic incubator and the amount of formaldehyde formed was measured according to the method of Nash.[13]

*Spectral methods and reductase assays.* The spectral dissociation constants ($K_{sp}$) and maximal spectral changes for ethylmorphine were determined as described by Schenkman *et al.*[8]

Microsomal P-450 content was estimated in a Shimadzu MPS-50 L recording spectrophotometer and expressed as millimicromoles of cytochrome P-450 per milligram of microsomal protein with the use of the extinction coefficient of 91 $mM^{-1}cm^{-1}$.[14]

Cytochrome P-450 reductase activity was determined as described by Gigon *et al.*,[15] and NADPH cytochrome c reductase was measured according to the method of Phillips and Langdon.[16]

The reductase assays were done with a Beckman DU spectrophotometer equipped with a Gilford 222 photometric unit and a Gilford model 2000 multiple sample recorder. The cell block was maintained at 37°.

*Protein concentrations.* Protein concentrations were estimated by the method of Lowry *et al.*[17] using bovine serum albumin as the standard.

*Statistics.* The Michaelis constants ($K_m$), maximum velocities ($V_{max}$), the spectral dissociation constants ($K_{sp}$) and maximum spectral changes were calculated by computer as described by Davies *et al.*[18] Individual values for $V_{max}$ and $K_m$ in which the standard error was greater than 15 per cent were not considered valid, and therefore were omitted. The Student's *t*-test was used as a test for the significance of differences between the groups of animals.

## RESULTS

*Effect of adrenalectomy on ethylmorphine* N-*demethylase kinetics.* Adrenalectomy produces quantitative as well as qualitative changes in microsomal enzymes (Table 1). The decrease in drug metabolism usually seen after adrenalectomy results from a decrease in $V_{max}$ and a small but statistically significant increase in $K_m$.

TABLE 1. KINETIC CONSTANTS FOR THE *N*-DEMETHYLATION OF ETHYLMORPHINE BY LIVER MICROSOMES FROM SHAM-OPERATED AND ADRENALECTOMIZED RATS*

| Rats | $K_m$ (mM) | % Change | $V_{max}$ (mμmoles HCHO formed/10 min/mg protein) | % Change |
|---|---|---|---|---|
| Sham | 0·27 ± 0·02 (6) | | 91 ± 7 (6) | |
| Adrenalectomized | 0·36 ± 0·02 (10)† | + 33·3 | 53 ± 4 (10)† | − 41·7 |

* EM at ten different concentrations over a range of 0·1 to 1·0 mM was incubated for 10 min at 37° with liver microsomes from either sham-operated or adrenalectomized animals, as described in Methods. Values represent the mean ± S.E.M. The numbers in parentheses are animals per group.
† $P < 0·01$.

*Oral feeding.* No differences were found in either the body or liver weights of the sham-operated and adrenalectomized rats, showing that the orally administered diet adequately maintained a nutritional balance between the two groups. As reported by Bellamy *et al.*,[19] however, cortisone treatment caused an increase in liver weight and a decrease in body weight. In none of the groups was the weight gain equivalent to that in animals fed *ad lib.*, but any nutritional effects on microsomal enzymes were presumably the same for all groups. Morevoer, the finding that the $K_m$ value of the ethylmorphine *N*-demethylation in sham adrenalectomized rats (Table 1) was similar to that of well fed animals[18] also suggests that the diet was adequate, because Gigon and Gram (see reference 20) have found that 3-day starvation of rats tends to increase both the $K_m$ and $V_{max}$ of ethylmorphine *N*-demethylation.

*Effects of adrenalectomy and cortisone administration.* In another experiment adrenalectomy resulted in a similar decrease in cytochrome P-450 reductase and NADPH cytochrome c reductase activities, while ethylmorphine demethylase was decreased to a slightly greater extent (Table 2). Cortisone administration to adrenalectomized rats prevented the decrease in ethylmorphine *N*-demethylation and NADPH cytochrome c reductase activities, but increased cytochrome P-450 reductase activity. Thus cytochrome P-450 reductase is more responsive to cortisone treatment than is NADPH cytochrome c reductase.

*Cytochrome P-450 and spectral binding constants.* Although Table 2 shows that adrenalectomy causes a decrease in microsomal cytochrome P-450 content, this effect

TABLE 2. EFFECT OF CORTISONE TREATMENT ON THE CHANGES PRODUCED BY ADRENALECTOMY*

| Animals | EM demethylation (mμmoles HCHO formed/mg protein/10 min)† | Cytochrome P-450 (mμmoles mg protein) | NADPH cytoc reductase (mμmoles reduced/min/mg protein) | Cyto P-450 reductase (mμmoles reduced/min/mg protein) | EM spectral dissociation constant ($K_{sp}$) (mM) | EM maximum spectral change (ΔO.D./mg) × $10^3$ | Maximal spectral change (mμmoles cyto P-450) × $10^3$ |
|---|---|---|---|---|---|---|---|
| Sham-operated | 45 ± 1 | 1 27 ± 0 04 | 113 ± 5 | 7·36 ± 0·40 | 0·05 ± 0 004 | 9·0 ± 0·6 | 7·1 ± 0·36 |
| Adrenalectomized | 24 ± 3 (56)‖ | 1·03 ± 0·06‡ (81) | 78 ± 3‡ (69) | 4·97 ± 0·34‡ (68) | 0·05 ± 0·003 (100) | 6·6 ± 0·3§ (73) | 6·6 ± 0 42 (93) |
| Adrenalectomized + cortisone | 53 ± 4 (124) | 0·96 ± 0·05‡ (76) | 127 ± 9 (113) | 10·30 ± 0·08‡ (140) | 0·055 ± 0·01 (110) | 6·8 ± 0·7§ (76) | 7·0 ± 0·53 (99) |

* Results are expressed as the mean ± S.E.M. obtained from five animals.
† $P < 0·01$ as compared with sham-operated controls.
‡ Numbers in parentheses are per cent of control.
§ $P < 0·05$ as compared with sham-operated controls.
‖ Ethylmorphine (EM), 1 mM, was incubated for 10 min with liver microsomes as described in Methods. Details of other procedures are given in Methods.

was not always reproducible. In some experiments no change in cytochrome P-450 content was observed after adrenalectomy. Moreover, the administration of cortisone to adrenalectomized rats did not prevent the apparent decrease in the cytochrome P-450 content shown in Table 2.

The spectral dissociation constant ($K_{sp}$) was not significantly affected by adrenalectomy or by cortisone administration to adrenalectomized rats. However, the maximal spectral change was significantly lower in microsomes from adrenalectomized animals than in those from sham-operated rats and remained low in those from adrenalectomized rats receiving cortisone. This decrease in the maximal spectral change produced by the interaction of ethylmorphine with microsomes paralleled the decrease in cytochrome P-450 content (last column, Table 2).

## DISCUSSION

In agreement with the findings of Remmer,[1, 2] this study demonstrates the decrease in drug metabolism by adrenalectomy and its reversal by a glucocorticoid. Kinetic analysis of ethylmorphine demethylase activity (Table 1) revealed that adrenalectomy causes both a decrease in $V_{max}$ and a small increase in $K_m$.

The decrease in ethylmorphine demethylation caused by adrenalectomy was apparently not due solely to a decrease in cytochrome P-450 content nor to alterations in the type I spectral changes produced by this substrate. As shown in Table 2, adrenalectomy decreased ethylmorphine demethylation about 44 per cent, but decreased the cytochrome P-450 content only about 20 per cent. Moreover, adrenalectomy did not significantly alter the apparent affinity of microsomes for ethylmorphine as measured by the spectral binding constant, $K_{sp}$. Further, it decreased the maximal spectral change to about the same extent as it decreased microsomal cytochrome P-450 content. Also the administration of cortisone to rats did not prevent the effects of adrenalectomy on either the cytochrome P-450 content or the maximal spectral changes, even though cortisone treatment prevented the effects of adrenalectomy on ethylmorphine *N*-demethylation. Indeed when the $V_{max}$ value was related to the microsomal P-450 content, cortisone treatment increased the ethylmorphine *N*-demethylation to about 150 per cent of the control level.

The finding that adrenalectomy does not change the $K_{sp}$ value was surprising. The addition of cortisol and corticosterone to liver microsomes has been shown to evoke spectral changes having absorption maxima at 409 and 413 m$\mu$ and minima at 365 and 375 m$\mu$, respectively,[8] whereas ethylmorphine causes a difference spectrum having a maximum at 385 m$\mu$ and a minimum at 423 m$\mu$. If the corticoids and ethylmorphine were bound to the same site in liver microsomes, it might be expected that the presence of endogenous steroid would either increase the apparent $K_{sp}$ or decrease the maximal spectral change. Since neither of these parameters was altered by adrenalectomy or cortisone treatment (when the concentration of microsomal cytochrome P-450 is considered), it may be assumed that steroids were either present in very low concentrations or interacted with sites which do not bind ethylmorphine. Since cortisone reversed the effect of adrenalectomy on ethylmorphine metabolism, it seems probable that the effect of glucocorticoids is not mediated via direct interaction of the steroid molecule with cytochrome P-450, but rather through an indirect effect on the synthesis or catabolism of another component of the system.

A close correlation was found between the changes in the *N*-demethylation of

ethylmorphine and the activities of NADPH cytochrome c reductase and cytochrome P-450 reductase. The data presented in Table 2 show that adrenalectomy decreases both of these activities to about the same extent as it decreases ethylmorphine *N*-demethylation. Moreover, the administration of cortisone completely prevented the decrease in NADPH cytochrome c reductase activity caused by adrenalectomy and markedly increased the activity of cytochrome P-450 reductase. The effect on the latter becomes more impressive when the level of the cytochrome P-450 is taken into account. The cytochrome P-450 reductase activity per cytochrome P-450 was 185 per cent and ethylmorphine demethylase 156 per cent of the control levels. Clearly, of the known components of the microsomal enzyme systems, only the reductase activities were responsive to cortisone, to the degree observed in ethylmorphine metabolism. Thus, in the overall sequence of events thought to occur in drug hydroxylation, adrenal steroids seem to exert their effect at the step of reduction of cytochrome P-450.

*Acknowledgements*—We are grateful to Dr. R. Scow and Dr. F. Stefano for their helpful suggestions about the force feeding of the animals.

## REFERENCES

1. H. Remmer, *Naturwissenschaften* **45**, 522 (1958).
2. H. Remmer, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **236**, 7 (1959).
3. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 285 (1965).
4. W. Bousquet, B. Rupe and T. Miya, *J. Pharmac. exp. Ther.* **147**, 376 (1965).
5. P. L. Gigon, T. T. Gram and J. R. Gillette, *Molec. Pharmac.* **5**, 109 (1969).
6. R. Kuntzman, M. Jacobson, K. Schneidman and A. H. Conney, *J. Pharmac. exp. Ther.* **146**, 280 (1964).
7. A. H. Conney, W. Levin, M. Ikeda and R. Kuntzman, *J. biol. Chem.* **243**, 3912 (1968).
8. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
9. T. R. Tephly and G. J. Mannering, *Molec. Pharmac.* **4**, 10 (1968).
10. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 279 (1965).
11. R. Scow, *Endocrinology* **60**, 359 (1957).
12. J. B. Schenkman, J. A. Ball and R. W. Estabrook, *Biochem. Pharmac.* **16**, 1071 (1967).
13. T. Nash, *Biochem. J.* **55**, 416 (1953).
14. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
15. P. L. Gigon, T. E. Gram and J. R. Gillette, *Biochem. biophys. Res. Commun.* **310**, 558 (1968).
16. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. D. S. Davies, P. L. Gigon and J. R. Gillette, *Biochem. Pharmac.* **17**, 1865 (1968).
19. D. Bellamy, R. A. Leonard, K. Dulieu and A. Stevenson, *Gen. comp. Endocr.* **11**, 119 (1968)
20. J. R. Gillette, *Am. J. Pharm.* **141**, 64 (1969).