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BIOSYNTHESIS OF CORTICOSTERONE AND DEOXYCORTICOSTERONE IN SPRAGUE–DAWLEY MALE AND FEMALE RATS AFTER ADMINISTRATION OF 7,12-DIMETHYLBENZANTHRACENE*

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SUMMARY

7,12-Dimethylbenzanthracene was administered to 50-day old male and female Sprague–Dawley rats. Some aspects of adrenal metabolism in the adrenal cortex were studied 15 days after treatment: *in vitro* corticosterone biosynthesis, mitochondrial 11β-hydroxylase activity and cytochrome P-450 content. Plasma corticosterone levels were also measured. Untreated and treated animals gave identical results. Thus, the rat adrenal cortex does not seem to play any role in the induction of DMBA‡ mammary tumours by secretion of corticosteroids.

INTRODUCTION

The possibility of obtaining objective remission up to several months has led to the widespread use of adrenalectomy in the treatment of disseminated breast carcinoma [1, 2]. The mechanism by which this ablation induces remission is not yet clearly understood. It is probable that this operation suppresses a source of estrogens other than the one affected by castration, although the plasma estrogen levels in menopausal women are extremely low (1–2 ng/100 ml). Bulbrook[3] has reported on the possible significance of the secretion of corticosteroids by the adrenal glands.

In 1961, Huggins[4] proposed an experimental model using 7,12-dimethylbenzanthracene (DMBA) as a carcinogen. The administration of appropriate doses of DMBA to mature Sprague–Dawley rats produced a necrosis of the adrenal cortex, followed either by the death of the animal or regeneration of the adrenal tissue. In the latter case, the animals show cancer formation in the mammary glands after a minimum lag period of 50 days.

More recently, Jull[5] showed that adrenal glands removed 48 h after injection of DMBA and incubated in the presence of [^{14}C]-labelled cholesterol produce less corticosterone than do normal adrenal glands.

In a preliminary study [6], we showed that, when the adrenals were removed 15 days after treatment, i.e. after tissue regeneration, there was an increase in the transformation of progesterone into corticosterone.

The aim of the present paper is to report on our work on the biosynthesis of corticosteroids in Sprague–Dawley male and female rats treated with DMBA.

MATERIALS AND METHODS

Sprague–Dawley male and female rats (Charles Rivers or Ifa–Credo), aged 45 days, were housed 3 per cage at a room temperature of 22 ± 1°, with a light–dark cycle. They were maintained on a standard rat pellet diet and tap water *ad libitum*.

All unlabelled steroids were purchased from Sigma (St. Louis, Missouri, U.S.A.) and the cofactors from Boehringer Co (Mannheim, Germany). The radioactive substrates were obtained from the Radiochemical Centre, (Amersham, England); purity was verified by t.l.c. and reverse isotopic dilution. The solvents were analytical grade from Merck (Germany) unless otherwise indicated.

Rats were acclimatized to laboratory conditions for 5 days after which, a single dose of 20 mg DMBA dissolved in 1 ml olive oil was administered by gastric intubation to each animal. They were given isotonic saline to compensate mineral losses due to adrenal necrosis during the first few days (4–7 days).

The animals were sacrificed by decapitation 15 days after treatment. Arterial and venous blood were collected from jugular and carotid vessels in heparinized tubes, centrifuged immediately and kept at −20°.

In certain experiments, the animals were anaesthetized with Nembutal (8.5 mg/200 g body wt); some

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‡ The abbreviations used are: DMBA: 7,12-dimethylbenzanthracene; DOC: deoxycorticosterone; B: corticosterone; P: progesterone

were decapitated immediately, while others were killed 50 min after injection to minimize the stress induced by anaesthesia

Incubation of the adrenal specimens

The adrenals were removed, cleared of adherent fat and divided up into 4 parts. They were incubated for 2 h at 37° in a Dubnoff incubator under a mixture of oxygen and carbon dioxide (95/5) in the presence of 13.5 μCi of [7α-^{3}H] progesterone (S.A. = 14.6 Ci/mmol) and a NADPH generating system (NADP: 5 μM; glucose-6-phosphate; 5 μM; glucose-6-phosphate dehydrogenase: 5 I.U.) dissolved in 2 ml of Krebs–Ringer bicarbonate buffer at pH 7.4

The metabolites were purified successively by paper and thin layer chromatography:

Corticosterone. Paper chromatography in IBMW (isooctane–benzene–methanol–water: 26:13:16:4 by vol.) and BL_1 (petroleum ether–benzene–methanol–water: 30:70:50:50 by vol.) systems.

Deoxycorticosterone. Paper chromatography in IBMW. Bu-Fw (butylacetate–formamide–water· 20:1:1 by vol.) and Bush A (petroleum ether–methanol–water: 50:40:10 by vol.) systems. After acetylation, the deoxycorticosterone acetate was purified by t.l.c. in B–AE (benzene–ethyl acetate. 8:3 v/v) system.

Progesterone. Paper chromatography in IBMW, L–PG (ligroin–propylene glycol: 50:50 v/v) and Bush A systems.

The metabolites were then recrystallized until the $^3H/^{14}C$ ratio remained constant (Table 1). They were examined for the presence of oestrogen using the procedure described by Cédard[7].

Estimation of 11β-hydroxylase in the mitochondria

Decapitation was performed without anaesthesia; the adrenals were removed and weighed. They were then homogenized in a sucrose 0.25 M–EDTA 0.001 M buffer, so that the final concentration of the homogenate was 35 mg of tissue per ml. The mitochondria were isolated in the usual way, centrifugation at 900 *g* for 10 min then 10,000 *g* for 10 min The sediment was placed in suspension in EDTA sucrose buffer and centrifuged at 10,000 *g* for 10 min, then resuspended in 2 ml of the same buffer solution A 50 μl aliquot of this suspension was then removed and protein measured by Lowry's procedure [8].

11β-hydroxylase activity was measured in 0.5 ml of this suspension to which was added 4.5 ml of T E A buffer (0.25 M sucrose, 20 mM KCl, 10 mM potassium phosphate buffer pH 7.4, 15 mM triethanolamine chloride buffer pH 7.4, and 5 mM $MgCl_2$) at pH 7.4 prepared using Cammer and Estabrook's technique [9]. Deoxycorticosterone (250 μg) was added dissolved in 10 μl of ethanol.

The solution was then preincubated for 10 min at 37° in air in a Dubnoff incubator; a 0.5 ml sample was removed and poured into a tube containing 0.5 ml of $HgCl_2$ (5 mg/ml)

At zero time, 30 μl of 2 M isocitrate, i.e. 6 μmol, were added to the incubation beaker and incubation continued. After 2, 4 and 6 min respectively, 0.5 ml aliquots were removed and poured into tubes containing 0.5 ml of $HgCl_2$. Ten ml of dichloromethane were added to each tube and extraction was carried out after stirring.

After centrifugation, the upper part of the solution was aspirated and 5 ml were then removed from the lower part and added to 4 ml of an ethanol–sulphuric acid mixture (35:65 v/v) This solution was shaken and left at room temperature for 1 h. It was then centrifuged and the upper layer discarded.

Each determination of corticosterone was carried out in duplicate, with subtraction of the blank dichloromethane), using a Farrand spectrofluorimeter (stimulation: 475 nm, emission. 525 nm).

Determination of plasma corticosterone

Plasma corticosterone was determined in the same way, using 1.0 ml of a mixture of venous and arterial plasma.

Table 1. Crystallizations to constant specific activity of corticosterone, deoxycorticosterone and progesterone (one experiment)

Steroid isolated	Recrystallized as free or derivative	Crystallization number	S A d.p.m. ^3H/mg	$^3H/^{14}C$
Corticosterone	free	1	49 135	15.60
		2	53 379	17.00
		ML 2	52 518	17 10
Deoxycorticosterone	acetate	1	2 081	0 96
		2	2 106	0 97
		ML 2	2 110	1 02
Progesterone	free	1	16 428	46 50
		2	15 997	46 30
		ML 2	17 014	46 90

ML· Mother liquor. DMBA treated female rats were killed 50 min after anaesthesia. adrenals were incubated with 13.5 μCi of [7α-^{3}H]progesterone.

Table 2. Conversion of progesterone into deoxycorticosterone and corticosterone by the adrenal gland of female rats

	Experiment No. 1		Experiment No. 2	
	Control $n = 10$	Treated $n = 12$	Control $n = 10$	Treated $n = 13$
Fresh weight tissue in mg	302	324	298	340
P metabolized in %	85.3	88.7	83.7	90.2
Corticosterone produced B in %	45.0	49.1	37.6	39.4
Deoxycorticosterone produced DOC in %	1.1	0.57	0.57	0.34
DOC/B	0.02	0.01	0.01	0.01

n = Number of animals used for one experiment. Experiment 1 (time 0): animals decapitated immediately after anaesthesia. Experiment 2 (time 50): animals decapitated 50 min after anaesthesia. Adrenals were incubated with 13.5 μCi of [7α-^{3}H]-progesterone, at 37° for 2 h under a stream of O_2–CO_2 (95:5). The metabolites were purified by repetitive chromatography, then recrystallized until the $^3H/^{14}C$ ratio remained constant.

Determination of cytochrome P-450

Mitochondrial suspension (0.7 ml) was made up to 9 ml using T.E.A. buffer at pH 6.3. Cytochrome P-450 was measured with an Aminco–Chance differential spectrophotometer. Type II cytochrome was measured between 420 and 390 nm, Type I between 450 and 490 nm, using the procedure described by Colby and Brownie[10]. These same experiments were performed on a group of control animals.

RESULTS

Incubation of adrenal glands in the presence of progesterone

There is no difference between the treated and control groups of female animals (Table 2). The DOC/B ratios at time zero and at time 50 min were identical. The production of corticosterone from progesterone did not differ between animals treated with DMBA and control animals, but increased production was found immediately after anaesthesia.

In Table 3, we summarize the results of 3 series of experiments carried out on male rats. The production of corticosterone is identical in normal and treated animals. It was, however, higher in experiment No. 1 than in experiments No. 2 and No. 3.

It must be emphasized that approximately 90% of the progesterone substrate was metabolized (Tables 2 and 3).

Levels of plasma corticosterone

In female rats, plasma corticosterone was significantly higher immediately after anaesthesia than 50 min later ($P = 0.02$) (Table 4). Corticosterone levels were roughly identical in animals treated with DMBA and in control animals ($P < 0.10$).

In male rats, as shown in Table 5, there was practically no significant difference between the various groups of animals, whether corticosterone was determined immediately after injection of Nembutal or 50 min after this injection. The results were roughly identical in the control animals ($P < 0.10$).

Measurement of the mitochondrial 11β-hydroxylase activity

Whatever the age of the animals at the time of sacrifice after administration of DMBA (15, 30, 60

Table 3. Conversion of progesterone into deoxycorticosterone and corticosterone by the adrenal glands of male rats

	Experiment No. 1		Experiment No. 2		Experiment No. 3	
	Control $n = 13$	Treated $n = 13$	Control $n = 7$	Treated $n = 8$	Control $n = 7$	Treated $n = 9$
Fresh weight tissue in mg	340	340	159	167	203	215
P metabolized in %	97.7	98.2	90.0	92.3	90.1	92.9
B produced in %	38.4	39.4	27.7	25 1	27.7	21.9
DOC produced in %	0.7	0.6	2.1	2.3	2.3	2.3
DOC/B	0.02	0.01	0.07	0.07	0.08	0.10

n = Number of animals used for one experiment. Experiments 1 and 2 (Time 0): animals decapitated immediately after anaesthesia. Experiment 3 (Time 50): animals decapitated 50 min after anaesthesia. Adrenals were incubated for 2 h at 37° with 13.5 μCi of [7α-^{3}H]-progesterone under a stream of O_2–CO_2 (95:5). The metabolites were purified by repetitive chromatography then recrystallized until the $^3H/^{14}C$ ratio remained constant.

Table 4. Levels of plasma corticosterone in untreated and treated female rats

Animals	Immediately after anaesthesia (Experiment 1)	50 min after anaesthesia (Experiment 2)	P values (Exp. 1 v Exp 2)
Control	19.27* ± 3.81 (n = 12)	6 60 ± 1.93 (n = 10)	0.02 (S)
Treated	17.72 ± 3 00 (n = 12)	7 73 ± 1.21 (n = 10)	0.01 (S)
P values (control v. treated)	<0 10 (NS)	<0.10 (NS)	

* In μg/100 ml of plasma, mean with S.E.M. P values: from Student's "t" Test. NS. not significant. S: significative. The levels of plasma corticosterone were determined in 1.0 ml plasma by fluorimetry.

days), the production of mitochondrial corticosterone was roughly the same in treated animals as in controls (Table 6). In the series where Ifa Credo rats were used, we found a difference between the levels of corticosterone production in treated animals (0.16) and control animals (0.22). In males (Table 7), we found a difference in corticosterone production when expressed in nM/mg protein/min. However, this difference does not appear when the production is expressed in nM/mg of adrenal gland/min.

Measurement of cytochrome P-450

There was no difference in cytochrome P-450 levels between treated aand control animals (Table 8). This was in agreement with the above results.

Determination of plasma corticosterone

Plasma corticosterone levels were determined several times. We wanted to see whether these levels changed in relation to the interval after administration of DMBA. As shown in Table 9, there was no significant difference between determinations carried out 15 days, one month or two months after administration of DMBA ($P < 0.10$). Furthermore, we found identical values in treated and control animals ($P < 0.10$). One determination was carried out under special conditions (animals kept in different soundproof rooms) in Dr. Brownie's laboratory in the U.S.A. and it was noted that the levels of plasma corticosterone were particularly low (around 5 μg). The significance of this result will be discussed below

Table 5 Levels of plasma corticosterone in control and treated male rats

Animals	Immediately after anaesthesia (Experiment 2)	50 min after anaesthesia (Experiment 3)	P values (Exp. 2 v Exp 3)
Control	10.30* ± 2.31 (n = 9)	12.81 ± 0.98 (n = 6)	<0.10 (NS)
Treated	10.83 ± 2.90 (n = 5)	9.29 ± 1.85 (n = 5)	<0 10 (NS)
P values (control v. treated)	<0.10 (NS)	<0.10 (NS)	

* In μg/100 ml of plasma, mean with S.E M. P values: from Student's "t" Test The levels of plasma corticosterone were determined in 1.0 ml plasma by fluorimetry

Table 6. Mitochondrial production of corticosterone in control and treated female rats

Time after DMBA	n	nM/mg adrenal/min Control	nM/mg adrenal/min Treated	nM/mg protein/min Control	nM/mg protein/min Treated
15	11	0.18	0 15	13 32	11 21
	12	0.29*	0 26	10 79	14.94
30	12	0.22	0 21	15.86	16.03
60	12	0 15	0 13	13.30	12 60
15	12	0 22†	0 16	15.75	12 70

n: Number of animals used in each experiment. * Experiment conducted in Doctor Brownie's laboratory (U S A.) † The only experiment realized with Ifa–Credo rats After decapitation, the adrenals were removed and homogenized in a sucrose–E.D T A. buffer The mitochondria were isolated and 11β-hydroxylase activity measured by incubating them with deoxycorticosterone.

Table 7. Mitochondrial production of corticosterone in control and treated male rats (one experiment)

	Control $n = 10$	Treated $n = 10$
nM/mg prot./min	16.6	27.4
nM/mg adrenal/min	0.229	0.265

11β-hydroxylase activity was measured by incubating mitochondria with deoxycorticosterone and corticosterone was then determined by spectrofluorimetry at 525 nm.

Table 8. Determination of mitochondrial cytochrome P-450 in 65 days old rat

	Control $n = 18$	Treated $n = 18$
Type II in nmol P-450	0.00675	0.0102
Type I in nmol P-450	0.0434	0.0407
P-450 total	24.414*	13.896
P-450 by rat	2.219	1.263
P-450 by mg adrenal	0.0372	0.0281
P-450 by mg protein	1.398	1.155

* Results expressed in nmol (single experiment). 0.7 ml of mitochondrial suspension was made up to 9 ml using T.E.A. buffer. Cytochrome P-450 was measured with an Aminco–Chance differential spectrofluorimeter: type II between 420 and 390 nm, type I 450 and 490 nm.

In male animals, the levels of plasma corticosterone were about 8 μg, in control or treated animals.

DISCUSSION

The study of metabolic changes induced by the administration of DMBA to Sprague–Dawley male and female rats under the conditions described previously led to the following conclusions:

Female animals. We did not find the increase in corticosterone biosynthesis from progesterone described by Joubert and Drosdowsky[6]. In fact, in experiment 1 when animals were decapitated immediately after anaesthesia, the percentage of corticosterone synthesized was 45–49% whereas it was only 38% in experiment 2.

The ratio DOC/B lay between 0.02 and 0.01, whereas Joubert and Drosdowsky reported it as being between 0.3 and 0.03.

Furthermore, there did not appear to be an increased 11β-hydroxylase activity when measured by incubating mitochondria in the presence of deoxycorticosterone. This was also the case with cytochrome P-450 levels which remained unchanged as did those of corticosterone in the blood. Although the levels of plasma corticosterone did not differ significantly between treated and control animals, we did observe some dispersion with extreme values between 5 and 30 μg. On the other hand, in female rats from Ifa–Credo Co. where cancer formation was about 100%—whereas it was only 70% in Charles Rivers rats—the production of mitochondrial corticosterone in treated animals (0.16 nM/g of adrenal gland/min) and in controls (0.22) was different. However, the latter results were obtained in only one series of animals.

Male rat. No change was observed in treated animals when compared with controls as regards: biosynthesis of corticosterone from progesterone, mitochondrial 11β-hydroxylase activity and plasma corticosterone levels.

One should, however, note that, in experiment 1 carried out by F.J., the ratio DOC/B was 0.02–0.01 owing to lesser production of deoxycorticosterone, compared with experiments 2 and 3 where ratio DOC/B was about 0.07.

Corticosterone levels in the plasma (about 8 μg/100 ml) were similar to those obtained by Kitay[11]: 9.2 μg/100 ml.

The increase in 11β-hydroxylase activity which we first noted was probably due to anaesthetic stress. In our preliminary studies, we noticed an increase in plasma corticosterone levels following anaesthesia. Dallmann and Jones[12], and Cook *et al.*[13] also

Table 9. Plasma corticosterone levels (in rats used for determination of corticosterone production in mitochondria)

Strain	Time after DMBA in days	Sex	Control	Treated	P values control/treated
Charles Rivers	60	F	13.25 ± 4.29* $n = 12$	16.33 ± 4.40 $n = 12$	<0.10 (NS)
Charles Rivers	30	F	19.37 ± 3.94 $n = 8$	17.50 ± 3.69 $n = 10$	<0.10 (NS)
Charles Rivers	15	F	13.80 ± 2.78 $n = 9$	13.10 ± 3.11 $n = 11$	<0.10 (NS)
Ifa–Credo	15	F	13.40 ± 6.23 $n = 12$	16.91 ± 5.66 $n = 12$	<0.10 (NS)
Holtzmann†	15	F	5.83 ± 1.77 $n = 12$	4.47 ± 0.91 $n = 12$	<0.10 (NS)
Charles Rivers	15	M	7.83 ± 1.18	8.53 ± 0.85	<0.10 (NS)

* In μg/100 ml of plasma, mean with S.E.M. † Experiment conducted in Doctor Brownie's laboratory (U.S.A.). P values: (between rats at different time after DMBA treatment). 60/30: NS, 60/15: NS, 30/15: NS.

showed that females were much more sensitive to stress than males, and that corticosterone levels increased tenfold 15 min after Nembutal or ether anaesthesia and did not return to normal until 40 min later. Thus, we compared corticosterone levels in animals decapitated immediately after anaesthesia and those sacrificed 50 min later. Our results confirm those reported by the above authors and the values in Table 9 show that animals decapitated without anaesthesia, or other special precautions, have high plasma corticosterone levels (15 μg/100 ml of plasma) compared with those obtained in animals sacrificed under minimum stress (5 μg/100 ml).

If the adrenal gland of DMBA treated rats does play a role either in the induction or the maintenance of breast carcinoma, it does not seem to operate through its corticosteroid secretion, that is to say that DMBA treatment does not influence corticosteroid secretion. On the other hand, the role of the ovaries is quite definite (Sinha[14], Jensen[15]) and it is known, that in animals castrated 30 days before administration of DMBA, tumor production does not occur, in relation with either a lack or an insufficient quantity of oestrogens.

Furthermore, Meites[16] showed that the pituitary also plays an important role by its secretion of prolactin and somatotrophin. Vignon and Rochefort[17] have recently emphasized the role of prolactin, showing that this hormone induces a restoration then an increase in oestradiol sites in castrated female rats treated with DMBA. Oophorectomy reduces carcinogenesis and combined with adrenalectomy or hypophysectomy, completely inhibits the process.

Oestrogens and prolactin thus appear to be the only hormones necessary for the first stages of tumor development in the mammary gland of the rat.

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