Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 1181–1185. Pergamon Press. Printed in Great Britain

SUPPRESSION OF TESTICULAR ANDROGEN SYNTHESIS IN THE RAT BY ETHANE 1,2, DIMETHANESULPHONATE

DENISE E. BU'LOCK and C. MARION JACKSON

Unit of Reproductive Pharmacology, Department of Pharmacology, University of Manchester, Manchester M13 9PT, England

(*Received 2 September* 1974)

SUMMARY

Within 1 day following administration of 75 mg/kg ethane 1,2-dimethanesulphonate (EDS) to male rats and for up to 7 weeks thereafter, the ability of testis tissue in an *in vitro* system to carry out the normal reaction sequence from pregnenolone to testosterone is drastically reduced, in a manner consistent with the nature and duration of the effects of EDS on male rat fertility. The transformation of cholesterol into pregnenolone and the further metabolism of testosterone are not detectably altered.

INTRODUCTION

The action of ethane 1,2-dimethanesulphonate ($MeSO_2$-$OC_2H_4OSO_2Me$, EDS) differs from other non-steroidal antifertility chemicals in its involvement of the endocrine system [1]. Following EDS administration the antifertility effects are accompanied by temporary involution of the seminal vesicles and prostate gland, Cooper and Jackson[2]. Exogenous testosterone has a protective action on the accessory glands, maintains testis weight and delays degenerative changes in the tubules during the period of its effectiveness (Jackson *et al.*[3]). Subsequently it was shown that EDS caused marked inhibition of testosterone synthesis *in vitro* over a time range consistent with the duration of sterility, Bu'Lock and Jackson[4]. These results are amplified in the following paper.

MATERIALS AND METHODS

Radioactive steroids

[4-^{14}C]-Cholesterol (S.A. 149 μCi/mg), [4-^{14}C]-progesterone (S.A. 193 μCi/mg), (4-^{14}C)-pregnenolone (S.A. 159 μCi/mg), [4-^{14}C] 17α-hydroxyprogesterone (S.A. 192 μCi/mg) and [4-^{14}C]-testosterone (S.A. 203 μCi/mg) were purchased from the Radiochemical Centre, Amersham, Bucks., Great Britain and checked for purity by thin-layer chromatography.

Non-radioactive steroids and reagents

Cholesterol, pregnenolone, progesterone, 17α-hydroxyprogesterone, testosterone, NADP, glucose-6 phosphate and glucose-6 phosphate dehydrogenase were obtained from British Drug Houses Ltd. All solvents used were of analytical grade except cyclohexane which was M.F.C.

Preparation of tissue

Mature male rats of an American Wistar strain, average age 3–4 months, approx. 300 g, were used. EDS was administered as a single intraperitoneal injection at two dose levels (75 and 100 mg/kg body weight) in dimethylsulphoxide–water (1:3 v/v) DMSO. As a control a single i.p. injection of DMSO/water in amount equivalent to that given with EDS was administered. Unless otherwise stated the animals were killed, by a blow on the head, 7 days after treatment. The testes ($1{\cdot}75 \pm 0{\cdot}02$ g in normals, $1{\cdot}85 \pm 0{\cdot}05$ g ($P = 0{\cdot}02$) in DMSO controls, $1{\cdot}26 \pm 0{\cdot}04$ g in EDS treated) were removed and decapsulated in ice-cold Krebs–Ringer bicarbonate buffer (pH 7·4) [5] containing glucose, 2 g/l.

Incubation and extraction procedure

Following the general procedure of Bell *et al.*[6] decapsulated whole testis preparations, 1–1·5 g of tissue, were added to the radioactive precursors in 10 ml Krebs–Ringer solution and incubated at 33° in 95:5 O_2:CO_2 with continuous shaking. Samples of 1·0 ml were removed from the incubation mixture at intervals from 5 to 240 min and steroids immediately extracted from the samples with ethyl-acetate (2 × 2·0 ml). Fresh Krebs–Ringer solution (1·0 ml) was added to the incubation mixture after each withdrawal [6].

Separation and radiochromatography of products

Thin layer chromatographic systems, used on Merck Silica gel F_{254} are shown in Table 1. Progesterone, 17α-HOprogesterone, androstenedione and testosterone (10 μg of each) were added to the combined ethyl acetate extract to act as chromatographic markers. The crude extract was subjected to an initial

The following abbreviations and trivial names have been used: progesterone: 4 pregnen-3,20-dione, 17-HO progesterone: 17-hydroxy-4-pregnene-3,20dione, androstenedione: 4-androstene-3,17-dione; testosterone: 17β-hydroxy-4-androsten-3-one, pregnenolone: 3 β-hydroxy-5-pregnen-20 one: cholesterol: 5-cholestene-3B-ol: HCG: human chorionic gonadotrophin.

Table 1. Chromatographic solvents

	(composition by volume)	
A.	Ethyl acetate/cyclohexane	2:1
B.	toluene/acetone	4:1
C.	cyclohexanone/cyclohexane	4:1
D.	chloroform/acetone	19:1

fractionation by running first in system A and then in the same direction in system B. The chromatograms were then scanned for radioactivity in a Packard 7201 counter (2 mm slit, 0·5 cm./min scan, ^{14}C efficiency, 30%). Areas containing activity were scraped off and eluted for further purification. The extracted mixtures from the two main zones corresponding to (a) cholesterol, progesterone and androstenedione, and (b) pregnenolone, 17α-HOprogesterone and testosterone, were each acetylated with pyridene and acetic anhydride (1:1 (v/v) 18 h), and then rechromatographed in solvent B to obtain separation of the products. Final separation of the unchanged progesterone and androstenedione from the first zone was achieved by repeated chromatography (× 3) in the same direction in solvent C. Radiochemical yields of metabolites were calculated from the integrated peak areas as plotted by the chromatogram scanner. Because of the partition of steroids between the tissue and the supernatant [7] the quoted yields for individual peaks are expressed as a percentage of the total scanned activity recovered by the ethyl acetate extraction. This was shown to be an effectively constant proportion (approx. 50%) of the total steroid in the incubation mixture.

Identification of steroids

Chromatographic criteria for identification of the steroids are summarized in Table 2 and were as follows: (i) co-chromatography of the radioactivity with visualised carrier steroids in two different chromatographic systems (B and D of Table 1); (ii) similar co-chromatographic behaviour following chemical treatments as appropriate (see Table 2) *viz*: acetylation (1:1 AC_2O/P_y), oxidation (CrO_3-P_y [8]), reduction ($NaBH_4$ in MeOH [9]). Both progesterone and testosterone were finally confirmed by recrystallization with authentic carrier steroid to specific activity constant to within ±3%, Axelrod[10] (Table 3). The radioactivity was measured in a Packard Scintillation Counter using a solution of toluene and Triton X (667:333 v/v) containing 2,5-diphenyloxazole (P.P.O) and 1,4,bis-(4 methyl-5-phenyloxazol-2-yl) benzene (DMPOPOP) 5·5 g and 0·1 g respectively/l. The counting efficiency was 67%.

Table 2. Chromatographic identification of products obtained from incubation of rat testis tissue with radioactive precursors

Product investigated and chemical reaction	Authentic Material	Chromatographic systems
[^{14}C]-Progesterone	progesterone	B, D
acetylation	progesterone	B
oxidation	progesterone	B
reduction	20β-HOpreg-4 en-3-one	B
[^{14}C]-17α-HOprogesterone	17α-HOprogesterone	B, D
acetylation	17α-HOprogesterone	B
oxidation	17α-HOprogesterone	B
[^{14}C]-Androstenedione	androstenedione	B, D
acetylation	androstenedione	B
oxidation	androstenedione	B
reduction	testosterone	B
[^{14}C]-Testosterone	testosterone	B, D
acetylation	testosterone acetate	B
oxidation	androstenedione	B
[^{14}C]-Pregnenolone	pregnenolone	B, D
acetylation	pregnenolone acetate	B
oxidation	Preg-4-ene,3,6. trione	B
[^{14}C]-Cholesterol	cholesterol	B, D
acetylation	cholesterol acetate	B

Table 3. Crystallization of radioactive products from rat testis tissue incubations to constant specific activity

Testis tissue	Substance	Specific activity of crystals (cpm/mg) 1st	2nd	3rd
Control	Progesterone	1216	1184	1124
Control	Testosterone	3065	2989	2983
EDS-treated	Progesterone	4700	4689	4642
EDS-treated	Testosterone	398	412	392

Products recovered by chromatography and mixed with 15 mg authentic carrier for recrystallizations. The solvents used for the 1st, 2nd and 3rd crystallizations were acetone-*n*-heptane, benzene-*n*-heptane and ethyl acetate-*n*-heptane respectively.

RESULTS

Controls

In a variety of experiments it was established that the course of steroid metabolism by testes from rats which had received an injection of DMSO/water was not markedly different, quantitatively or otherwise, from that in untreated controls. In what follows 'controls' are usually, but not invariably, data from DMSO-treated animals.

In control incubations, the pattern of major metabolites produced from labelled substrates was consistent with the well-established sequence for the rat [11, 12], *i.e.*

cholesterol → pregnenolone → progesterone

→ 17α-hydroxyprogesterone → androstenedione

⇄ testosterone → more polar metabolites.

Although under the incubation conditions used, the transformation of cholesterol was relatively slow, during a 4 h incubation accumulations of progesterone and testosterone were seen. Testosterone itself underwent a rather slow conversion to more polar products and some back reaction to androstenedione.

The effect of EDS treatment

Direct addition of EDS to the incubation mixture in amounts of up to 90 μg/g tissue had no effect over a 2 h period. In testes from EDS-treated animals the ability to transform pregnenolone, progesterone and 17α-hydroxyprogesterone into testosterone was dramatically reduced but the initial conversion of cholesterol and the further metabolism of testosterone were little, if at all, affected. This is shown by the data

Table 4. Products from incubations of various substrates with rat testis tissue

Substrate	Tissue	$T_{\frac{1}{2}}$ (h)	Recovered activity (%) after 1 h in: Substrate	Major product(s)	Minor products
Cholesterol	Control	2·75	66 (75–59)	pregnenolone + further products 25 (34–20)	see Table 3
	treated	2·75	64 (77–59)	pregnenolone + further products 26 (36–16)	see Table 3
Pregnenolone	Control	0·2	6 (10–4)	testosterone + polar products 72 (78–65)	progesterone, 17α-HO-progesterone,
	treated	0·8	47 (51–44)	testosterone + polar products 27 (29–25)	androstenedione
Progesterone	Control	0·1	8 (6–15)	testosterone + polar products 71 (73–66)	17α-HOprogesterone
	treated	2·5	63 (61–67)	testosterone + polar products 13 (18–6)	androstenedione
17α-HO-progesterone	Control	0·3	13 (11–15)	testosterone + polar products 71 (73–66)	androstenedione
	treated	1·2	53 (51–55)	testosterone + polar products 39 (34–42)	
Testosterone	Control	2·5	68 (75–64)	Unidentified polar products 8 (14–5)	androstenedione
	treated	2·5	65 (74–58)	Unidentified polar products 9 (13–6)	

(4-^{14}C) Pregnenolone (0·1 μC; 51 mCi/m.mol); progesterone (0·1 μC; 61 mCi/m.mol); 17-hydroxyprogesterone (0·1 μC; 61 mCi/m.mol); testosterone (0·1 μC; 59 mCi/m.mol) and (4-^{14}C) cholesterol (0·2 μC; 58 mCi/m.mol) were incubated with one whole decapsulated testis in Krebs-Ringer buffer solution with glucose at pH 7·4, in 95% oxygen at 33°C. The incubations were carried out for 1 h. Each result is the mean of at least 4 separate incubations and the range is shown in brackets. Controls are from DMSO-treated animals. Recovered activity is given as a percentage of that recoverable from a sample taken at zero time. Half-life data from similar measurements at from 5′ to 2 h (for cholesterol, 4 h).

Table 5. Yield of metabolites formed from cholesterol by control rat testis tissue and tissue from rats treated with EDS (100 mg/kg i.p.) 7 days previously

	Recovered activity* (%) Cholesterol	Pregnenolone	Progesterone	Testosterone	Polar products
EDS + DMSO	38 (45–30)	22 (25–20)	12 (17–9)	5 (8–3)	10 (15–5)
DMSO	38 (46–28)	8 (11–5)	21 (28–16)	15 (18–11)	8 (12–7)

(4-^{14}C) Cholesterol (0·2 μC; 58 mCi/m.mol) incubated with a single decapsulated whole testis in Krebs-Ringer Buffer + glucose (pH 7·4) in 95% O_2 at 33°C for 4 h.

Recovered activity as in Table 2.

* The mean of 4 separate incubations for tissue from EDS-treated rats and 3 for control (DMSO) rats, with the range shown in brackets.

collected in Table 4. By observing the decay of the amounts of labelled substrate recovered during 2 to 4 h incubations the half reaction time, $T_{\frac{1}{2}}$, can be estimated; the results show that in testes from treated animals the initial transformations of pregnenolone, progesterone and 17α-hydroxyprogesterone were respectively approx. 0·25, 0·04 and 0·25 as rapid as in the controls while the rate of further transformation of testosterone remained unaffected. Consistent with these data the accumulations of testosterone after 1 h incubations of testes from treated animals with pregnenolone, progesterone or 17α-hydroxyprogesterone were very much less than the controls (Table 4).

The results of the incubations with labelled cholesterol support the same conclusions in a somewhat different manner. As noted, the rate at which the substrate is transformed is unaffected (Table 5), but the pattern of metabolites produced is markedly changed. For example, after 4 h the principal metabolites seen in controls were progesterone and testosterone (Table 5), whereas in the incubations with testes from EDS-treated animals accumulations of steroids occurred earlier in the sequence, *viz.* pregnenolone and progesterone. The yield of "polar products" from the incubation with testis tissue from EDS-treated rats is somewhat higher than might be expected if these are exclusively formed *via* testosterone, but these materials, even in control incubations, are a complex mixture which has not been resolved and which may well include various different diols and certain types of moderately polar conjugates, formed from any of the labelled steroids present in the incubations.

The addition of excess NADPH, generated *in situ* by including NADP, glucose-6 phosphate and glucose-6 phosphate dehydrogenase in the incubations, as co-factor somewhat increases the transformation of progesterone into testosterone by tissue from EDS-treated animals (Table 6), but the difference from controls (in which NADPH has no effect) remains most marked.

Table 6. Metabolism of progesterone in the presence of NADPH by testis tissue from control and EDS-treated rats

	Recovered Activity* (%) Progesterone	Testosterone
Control	1·6–1·4	47–51
Control + NADPH	1·9–1·6	49–51
EDS	55–54	17–14
EDS + NADPH	37–34	34–31

(4-^{14}C)progesterone (0·1 μC; 61 mCi/m.mol) incubated with 500 mg decapsulated testis tissue in 5 ml Krebs-Ringer Buffer solution or 2·5 ml Krebs-Ringer buffer, pH 7·4 + 1 ml NADPH solution (5 mg) 1·5 ml glucose-6-phosphate solution (30 mg) and 0·15 ml glucose-6-phosphate dehydrogenase (20 E.U.) in 95% O_2 at 33°C.

* Results for two incubations.

Table 7. Yield of testosterone (+ more polar metabolites) from 1 h incubations of (4-^{14}C)progesterone with testis tissue during 9 weeks following EDS administration. (Number of animals in parentheses)

Days after EDS	% Recovered activity Dose of EDS administered 75 mg/kg	100 mg/kg	Controls
1	52 (4)	73 (2)	80–60 (6)
2	46 (4)	48 (2)	80–60 (4)
3	28 (4)	—	80–60 (4)
7*	13 (7)	13 (17)	78–64 (30)
14	25 (3)	18 (4)	67–74 (4)
21	—	21 (4)	65–80 (4)
28	48 (2)	—	62–70 (2)
35	—	43 (2)	62–72 (2)
49	56 (2)	—	54–78 (2)
63	—	74 (2)	72–85 (2)

Results for control (DMSO) animals show the range; those for treated animals are expressed as mean values.
* Recovered activity 7 days after EDS 13 ± 2·0; 12·5 ± 1·1 (75 & 100 mg/kg respectively). cf. DMSO controls, 70·6 ± 1·8 ($P = 0$).

Duration of the EDS effect

The magnitude of the effect of EDS treatment on the ability of the testis to carry out androgen metabolism is most significantly measured in terms of its effect on testosterone production. As shown in Table 7, the suppressive effect of a single dose of EDS at either 75 or 100 mg/kg body weight on the conversion of added progesterone into testosterone and its more polar metabolites is apparent 1 or 2 days after injection and reaches a maximum after 7 days. Following the lower dose, recovery is marked by 21 days and almost complete after 7 weeks. At the higher dose level, the period of substantial inhibition is somewhat longer and recovery is complete by 9 weeks.

DISCUSSION

The antispermatogenic activity of EDS in the rat [1] involves both pre- and post-meitoic phases; a single dose at 75 mg/kg causes infertility through weeks 2 to 8 after administration (at 100 mg/kg, 2–12 weeks). The same dosage produced a marked reduction of testis weight for approximately the same period with a temporary involution of the prostate and seminal vesicles [2]. From the overall pattern produced by EDS it was suggested [13] that at least part of its action was to inhibit testicular androgen synthesis; consistent with this view, administration of testosterone propionate (TP) will maintain the accessory glands and partially antagonize the antispermatogenic effect [3].

While the above biological effects take some days to develop, a decline in the capacity of the treated testis to form testosterone (as assessed by the *in vitro* technique) can be recognised after 24 h, becomes maximal after 7 days, and is no longer significant between 7–9 weeks. It is established that injected EDS quickly passes the blood-testis barrier and apparently has free access to the seminiferous epithelium and interstitium [14].

Clearly impaired production of testicular testosterone goes far towards explaining the antispermatogenic effects of EDS. From the data of Table 7 it is apparent that the levels of androgen available to the developing sperm will be restricted for up to 7 weeks after administration, particularly so in the first 2–3 weeks. Administration of testosterone (from 3 days prior to EDS treatment to 6 days after treatment, at 3 mg/day) protects spermatogenic cells which are in their post-meiotic phases during the first 4 weeks of EDS-induced infertility, but not those in meiosis or pre-meiotic stages of development; the supply of exogenous testosterone is sufficient to maintain testis weight until week 3 [3].

Testis tissue from EDS-treated and EDS + TP treated animals (weeks 1–3) show no difference in their capacity to form testosterone from progesterone [15]; although the capacity of testis tissue from TP-treated rats to form testosterone is considerably reduced (cf. Lacy.[16]) it is significantly higher than in EDS-treated and EDS + TP-treated animals. Administration of human chorionic gonadotrophin (HCG) (from 3 days before injection to 6 days after, 100 i.u./day) would be expected to stimulate testicular steroidogenesis. In fact, although histologically spermatogenesis then appears normal, fertility data [3] suggest that endogenous steroidogenesis in the EDS-treated animal is insufficient to provide the adequate hormonal support for post-meiotic cells. We were unable to detect any difference in the capacity to form testosterone from progesterone in the testes from EDS-treated and EDS + HCG-treated animals during weeks 1–3 (15*cf. 3).

Since EDS treatment affects several reactions in steroidogenesis rather than any one enzyme, and since it has no short-term effect in the *in vitro* system, the possibility of an indirect mechanism of action has not been discounted. Since EDS does not produce any alteration in the oestrous cycle or fertility of the female rat [17], a direct effect on the hypothalamic pituitary axis in the male rat seems unlikely.

The results summarized in Tables 4 and 5 show that whether the steroid substrates are exogenous or are generated *in situ* from exogenous cholesterol, the reactions affected are specifically those carried out by enzymes in the smooth endoplasmic reticulum of the Leydig cells of the interstitium [12, 18]. Directly or indirectly, the Leydig cells seem to be the focal point of the effects produced by EDS.

Acknowledgements—This work was supported by grants from the Ford Foundation and the Medical Research Council.

The authors are grateful to Dr. Harold Jackson for guidance during the course of the project and to Professor L. G. Lajtha for various facilities at The Paterson Laboratories, Christie Hospital, Manchester.

REFERENCES

1. Jackson H.: *Br. med. Bull.* **20** (1964) 107–114.
2. Cooper E. R. A. and Jackson, H.: *J. reprod. Fert.* **23** (1970) 103–108.

3. Jackson H., Jackson C. M. and Jones P.: *J. reprod. Fert.* **34** (1973) 133–135.
4. Bu'Lock D. E. and Jackson C. M.: *Gynecol. Invest.* **2** (1971/72) 305–308.
5. Krebs H. A. and Hensleit K.: *Hoppe-Seyler Z. physiol. Chem.* **210** (1932) 33–66.
6. Bell J. G. B., Vinson, G. P., Hopkin D. J. and Lacy D.: *Biochim. biophys. Acta* **164** (1968) 412–420.
7. Vinson G. P.: *J. Endocr.* **34** (1966) 355–363.
8. Menini E. and Norymberski J. K.: *Biochem. J.* **84** (1962) 195–201.
9. Moshang T., Rudd B. T., Eberlein W. R. and Bongiovanni A.: *Steroids* **15** (1970) 195–207.
10. Axelrod L. R., Matthijssen C., Goldzieher J. W. and Pulliam J. E.: *Acta endocr., Copenh.* **49** Suppl. 99 (1965) 1–77.
11. Eik-Nes K. B.: *The Androgens of the Testis* (Edited by K. B. Eik-Nes). Marcel Dekker, New York (1970) pp. 1–34.
12. Hall P. F.: in *The Testis* (Edited by Johnson W. D., Gomes W. R. and Vandenmark N. L.). Academic Press, New York, Vol. 2 (1970) pp. 1–71.
13. Jackson H.: *Nobel Symposium* 15, *Control of Human Fertility* Almqvist & Wiskell, Stockholm (1971) pp. 119–136.
14. Waites G. M. H., Jones A. R. and Main S. J.: *J. reprod. fert.* **31** (1972) 508–509.
15. Bu'Lock D. E. and Jackson C. M.: (unpublished data).
16. Lacy D., Fyson P., Collins P., Tsang W. N. and Pettit J. J.: *Adv. Biosci.* **10** (1973) 27–62.
17. Jackson C. M. and Jackson H.: (unpublished data).
18. Tamaoki B.: *J. steroid Biochem.* **4** (1973) 89–118.