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SUPPRESSION OF ADRENAL METABOLISM OF 4-^{14}C-PROGESTERONE BY TESTOSTERONE-OXIME AND 17α-METHYLTESTOSTERONE-OXIME *IN VITRO*

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Abstract—The conversion of 4-^{14}C-progesterone by rat adrenal glands to corticosteroids was studied *in vitro* following incubation with androst-4-en-17β-ol-3-one oxime (testosterone-oxime) and 17α-methyl-androst-4-en-17β-ol-3-one oxime (methyltestosterone oxime). The conversion of progesterone to 18-oxygenated steroids: aldosterone and 18-hydroxycorticosterone was decreased to more than one-half of the controls. The amount of 11-deoxycorticosterone was higher than in control experiments.

IN 1968, Rembiesa[1] observed that 17α-methyltestosterone* suppressed the formation of aldosterone and corticosterone by rat adrenal gland *in vitro*. The addition of 17α-methyltestosterone to rat adrenals also suppressed conversion of 4-^{14}C-progesterone and 4-^{14}C-11-deoxycorticosterone to the corticosteroids, included corticosterone, 18-hydroxycorticosterone, 18-hydroxy-11-deoxycorticosterone and aldosterone.[2] 18-Hydroxy-steroids were the most sensitive to the presence of the androgen. The 17α-methyltestosterone appears to act as a competitive substrate for the hydroxylating enzymes because it is itself metabolized to more polar products.

In 1967, Rembiesa *et al.*[3] described that rat adrenal glands *in vitro* and *in vivo*[1] converted 17α-methyltestosterone to 11β-hydroxy and 11-oxo-17α-methyltestosterone. The other products were also formed but in lesser amounts and a little progress was made in their identification. It is conceivable that 17α-methyltestosterone is metabolized by adrenal enzymes normally acting in the biosynthesis of corticosteroids. The rat adrenal also actively hydroxylates the C-18 methyl group to form 18-hydroxy-11-deoxycorticosterone and 18-hydroxy-corticosterone. Therefore one might guess that one or more of the unidentified adrenal metabolites of 17α-methyltestosterone would possess a hydroxyl group at C-18.

Testosterone was not as potent inhibitor as 17α-methyltestosterone.[5, 6]

Testosterone and 17α-methyltestosterone possess high androgenic activity and from this reason their future as adrenal inhibitors is questionable. We have synthetized a number of derivatives of testosterone, 17α-methyltestosterone and dehydroepiandrosterone with the purpose of reduction of androgenic activity of the mother compounds with retention of adrenal effects. In the preliminary experiments, two compounds

* Abbreviations used: testosterone = androst-4-en-17β-ol-3-one; 17α-methyltestosterone = 17α-methylandrost-4-en-17β-ol-3-one; aldosterone = 11β,21-dihydroxypregn-4-en-18-al-3,20-dione; corticosterone = 11β,21-dihydroxypregn-4-en-3,20-dione; 11-dehydrocorticosterone = 21-hydroxypregn-4-en-3,11,20-trione; 18-hydroxycorticosterone = 11β,18,21-trihydroxypregn-4-en-3,20-dione; 18-hydroxy-11-deoxycorticosterone = 18,21-dihydroxypreg-4-en-3,20-dione; progesterone = pregn-4-en-3,20-dione.

have been proved to be interesting: androst-4-en-17β-ol-3-one oxime (T oxime) and 17α-methyl-androst-4-en-17β-ol-3-one oxime (MT oxime).

MATERIALS AND METHODS

The oximes were synthetized by the method described by Brooks *et al.*[7] The purity of the compounds were examined in several chromatographic systems and by analysis of infrared spectra. *Androgenic and myotrophic properties* of these compounds were assayed in the immature castrate male rats (weight from 65 to 70 g) by daily injection for 7 days, following the procedure of Hershberger *et al.*[8] The test compounds were administered subcutaneously in an aqueous suspending medium. The aqueous suspending medium consisted of sodium chloride (0·9%), polysorbate 80 (0·4%), carboxy-methylcellulose (0·5%) and benzyl alcohol (0·9%). The animals were autopsied 24 hr after the last treatment and their androgenic and anabolic (myotrophic) activities were calculated on the basis of the weights of the seminal vesicles, ventral prostate and levator ani muscle.

Incubation and extraction. Male Wistar rats were used in these experiments. The rats were killed by decapitation and their adrenal glands were removed, quartered and placed in 25 ml Erlenmeyer flasks. Each flask contained 3 ml of cold incubation medium and 2 adrenal glands. The weight of adrenals was 36·5 ± 2·5 mg/flask. The conditions for incubation were as described previously.[2,3] In summary, Krebs–Ringer bicarbonate medium containing 200 mg of glucose/100 ml was used. The incubations were carried out under a gas phase mixture of 5% CO_2–95% O_2 in a Dubnoff-like incubator at 38°. The compounds dissolved in 50 μl of ethanol–propylene glycol (1:1) were added to both the preincubation and incubation media (final concentration 1×10^{-4} M). After 30 min of preincubation the medium was replaced and 3 hr incubation was carried out. After preincubation, 0·1 μc (1·45 μg) of 4-^{14}C-progesterone (Radiochemical Centre, Amersham, England) was added. At the end of incubation mixtures were rapidly frozen. The adrenal tissue and media were extracted twice with equal volumes of methylene chloride and the extracts were then evaporated in a stream of nitrogen.

Chromatography. The compounds to be separated or identified by paper chromatography were spotted on paper strips 2 cm wide and 45 cm long, cut from Whatman No. 1 chromatographic paper. Steroid mixtures extracted from the incubation medium were fractionated by thin layer chromatography on silica gel G.

Chromatographic systems used in this study are listed in Table 2.

Radioactivity measurements. Radioactive substances were detected with the aid of radiochromatogram scanner.[9] The radioactivity was determined in a liquid scintillation counter U.S.B.—2 (BUTJ—Poland) at 65 per cent efficiency for ^{14}C. The scintillation solution was prepared by adding 3·0 g of PPO and 0·30 g of POPOP (Nuclear Enterprises, Ltd, Scotland) to 1 l. of toluene containing 1% of methanol.

U.v. absorption. Substances on the paper chromatograms which absorbed short-wave u.v. light were detected by a contact photography on a photocopy paper with u.v. light.

RESULTS AND DISCUSSION

Table 1 summarizes androgenic and myotrophic properties of tested compounds. It can be seen that substitution at C-3-oxo-group of testosterone and 17α-methyltesto-

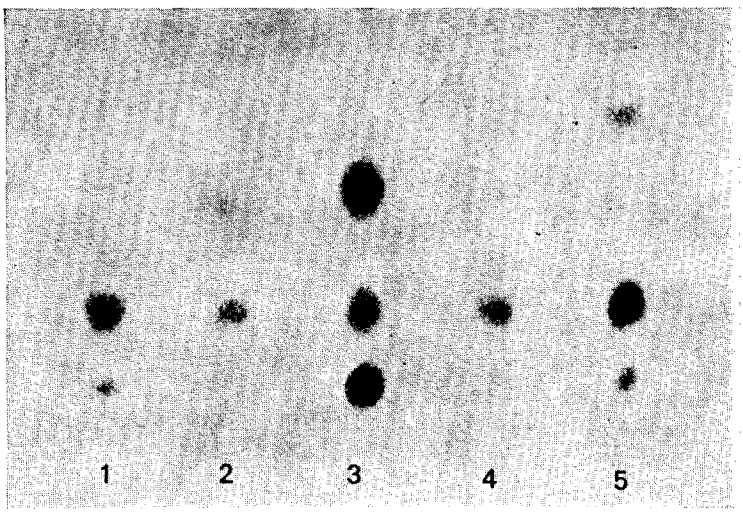

FIG. 1. Typical example of the thin-layer chromatogram (developed in chloroform–ethanol 90:10) of a methylene chloride extract of the medium in which rat adrenal tissue (36 mg/flask) was incubated:

1. Control flask.
2. With testosterone-oxime (10^{-4} M).
3. Standards (from the bottom): aldosterone, corticosterone and 11-dehydrocorticosterone.
4. With 17α-methyltestosterone-oxime (10^{-4} M).
5. Control flask.

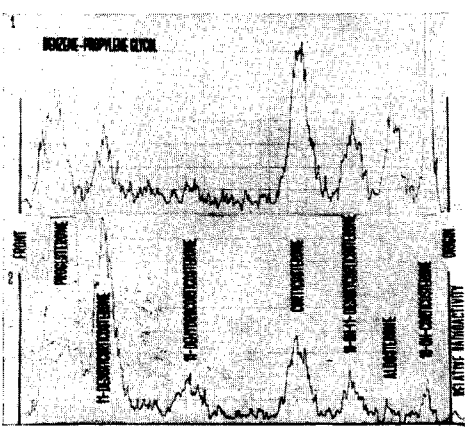

FIG. 2. Typical example of the radioactive scan of paper chromatogram (developed in benzene–propylene glycol system) of a methylene chloride extract of the medium in which rat adrenal tissue was incubated with approximately 1·45 μg (0·1 μc) of 4-^{14}C-progesterone. Control scan No. 1. With testosterone-oxime (10^{-4} M) No. 2.

sterone by C=N—OH group brings about a marked reduction in androgenic activity and in lesser degree of anabolic effect.

In the preliminary experiments the lipid material produced by the rat adrenal glands *in vitro* with and without tested compounds has been separated by TLC. The chromatograms were developed in chloroform–ethanol (90:10) system and sprayed

TABLE 1. ANDROGENIC AND MYOGENIC ACTIVITY OF TESTOSTERONE-OXIME AND 17α-METHYLTESTOSTERONE-OXIME

Compounds	Relative potencies			Myogenic and androgenic ratio	
	Seminal vesicles (S.V.)	Ventral prostate (V.P.)	Levator ani (L.A.)	L.A.:S.V.	L.A.:V.P.
Testosterone	1·00	1·00	1·00	1·00	1·00
Testosterone-oxime	0·21	0·32	0·73	3·47	2·30
17α-Methyltestosterone	1·00	1·00	1·00	1·00	1·00
17α-Methyltestosterone-oxime	0·39	0·47	0·66	1·74	1·45

All compounds were injected daily for 7 days in dose 0·6 mg/rat.

with solution of blue tetrazolium. Using the reference standards as a guide it was apparent that the control sample of lipid material produced by the rat adrenals contained considerable quantities of aldosterone and corticosterone. Of all the tested compounds used as additions to the medium, only T-oxime and MT-oxime caused the marked lowering of these steroids in lipid material produced by rat adrenal glands during incubation (Fig. 1).

As further confirmation of the activity of T-oxime and MT-oxime and also in order to obtain some preliminary information on mechanisms, studies with 4-^{14}C-progesterone were performed.

The results reported here are based on the identification of radioactive transformation products of 4-^{14}C-progesterone. After incubation each extract was at first chromatographed in the Zaffaroni benzene–propylene glycol system in the presence of 50 μg of unlabeled standards. The chromatograms were scanned, and after elution the amount of material in each radioactive peak was rechromatographed once more in order to purify the corresponding compounds.

For identification of steroids, the material in the radioactive peaks was subjected to acetylation, oxidation, reduction and saponification, as explained in the previous publications.[1, 2, 10] Steps used to identify radioactive compounds are summarized in Table 2.

Following the extraction, the crude residue was chromatographed in benzene–propylene glycol system which separated radioactive products. Each from seven zones (Fig. 2) was eluted and an aliquot was counted for radioactivity. MT-oxime and T-oxime added to a quartered rat adrenal preparation caused decrease in the *in vitro* conversion of 4-^{14}C-progesterone to 18-oxygenated steroids: 18-hydroxycorticosterone, aldosterone and 18-hydroxy-11-deoxycorticosterone. In the presence of a tracer dose of progesterone (1·45 μg), the formation of these steroids was decreased to more than 50 per cent of control values. In these experiments it was demonstrated that MT-oxime and T-oxime also suppressed the conversion of progesterone to corticosterone.

TABLE 2. PROCEDURES USED TO IDENTIFY RADIOACTIVE COMPOUNDS ISOLATED FROM INCUBATION OF 4-^{14}C-PROGESTERONE

^{14}C Peak in position of:	^{14}C Peak in position of:	^{14}C Peak in position of:	^{14}C Peak in position of:	^{14}C Peak in position of:	^{14}C Peak in position of:
18-OH-Corticosterone ↓ (a, b, c) oxidation ↓ (HJO_4) 11β,18-Dihydroxy-3-keto-4-etienic acid (c, d) ↓ reduction ↓ ($NaBH_4$) 11β,3β-18-Trihydroxy-4-etienic acid (b) ↓ oxidation ↓ (CrO_3) 3,11-Diketo-4-etienic acid (d, e)	Aldosterone (a, b) ↓ 21-acetylation Aldosterone-21-monoacetate (c, d) Aldosterone (a, b) ↓ oxidation ↓ (HJO_4) 3-Keto-11β-18-epoxy-4-etienic acid (d, c) ↓ reduction ↓ ($NaBH_4$) 11β,3β-18-Trihydroxy-4-etienic acid (b) ↓ oxidation ↓ (CrO_3) 3,11-Diketo-4-etienic acid (d, e)	18-OH-Deoxycorticosterone (c) ↓ oxidation ↓ ($NaBiO_3$) —18-OH-3-Keto-4-etienic acid 20→18 lactone (c)	Corticosterone (a, c) ↓ acetylation Corticosterone acetate (d, e) ↓ oxidation ↓ (CrO_3) 11-Dehydrocorticosterone acetate (d, e) ↓ saponification 11-Dehydrocorticosterone (c, d) ↓ oxidation ↓ (CrO_3) Adrenosterone (d, e)	11-Dehydrocorticosterone (c) ↓ acetylation Dehydrocorticosterone acetate (d) ↓ oxidation ↓ (CrO_3) 11-Dehydrocorticosterone acetate (d) ↓ saponification 11-Dehydrocorticosterone (d)	11-Deoxycorticosterone ↓ (c, d) acetylation 11-Deoxycorticosterone acetate (d) ↓ oxidation ↓ (CrO_3) 11-Deoxycorticosterone acetate (d) ↓ saponification 11-Deoxycorticosterone (c)

a = Bush B_5, b = chloroform-formamide, c = benzene-propylene glycol, d = cyclohexane-benzene-propylene glycol, e = cyclohexane-propylene glycol.

TABLE 3. EFFECT OF 17α-METHYLTESTOSTERONE-OXIME AND TESTOSTERONE-OXIME ON THE METABOLISM OF 4-^{14}C-PROGESTERONE BY THE RAT ADRENAL GLANDS *IN VITRO*

		cpm × 10^3 per zone							
	Adrenal tissue mg/flask	Unconverted progesterone	18-OH-B	Aldosterone	18-OH-DOC	B	11-Dehydro-B	DOC	Total radio-activity recover from all zones†
Controls	35·0 ± 1·4*	28 ± 2·1	14 ± 1·1	9 ± 0·9	9 ± 0·2	23 ± 1·2	6 ± 0·3	12 ± 0·9	101
17α-Methyltestosterone-oxime (10^{-4})	36·0 ± 2·3	54 ± 7·1	2 ± 0·3	2 ± 0·0	6 ± 0·8	14 ± 0·6	7 ± 0·3	19 ± 1·2	104
Controls	38·7 ± 1·5	18 ± 2·7	12 ± 0·5	7 ± 0·8	7 ± 0·9	18 ± 2·6	7 ± 2·0	13 ± 1·7	82
Testosterone oxime (10^{-4})	36·6 ± 2·0	33 ± 3·6	4 ± 0·9	4 ± 1·4	5 ± 0·8	10 ± 3·2	7 ± 0·3	23 ± 1·7	86

* = Average of 5 experiments ± S.E.

† = The sum of all cpm eluted in the 7 fractions. The recovery from benzene–propylene glycol chromatography was 89 ± 5 per cent of the radioactivity applied before chromatography.

18-OH-B = 18-hydroxycorticosterone; 18-OH-DOC = 18-hydroxy-11-deoxycorticosterone; B = corticosterone; DOC = 11-deoxycorticosterone.

Radioactivity of fractions obtained following benzene–propylene glycol chromatography.

The amount of 11-deoxycorticosterone was higher than in control experiments. The amount of 11-dehydrocorticosterone was also higher but not significantly altered. The results are presented in Table 3 and Fig. 3.

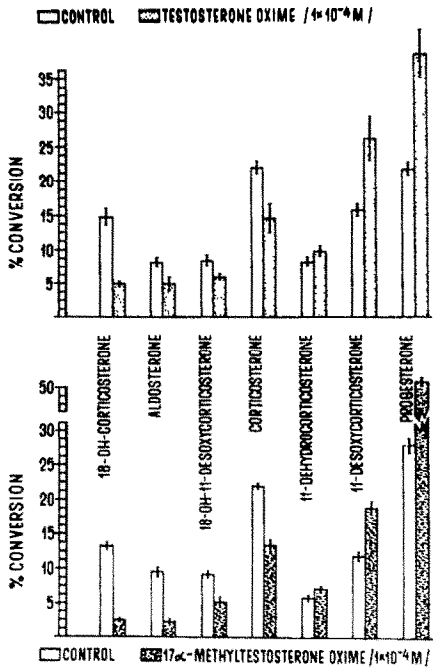

FIG. 3. Effect of the 17α-methyltestosterone-oxime and testosterone-oxime on the percentage distribution of radioactivity after incubation of rat adrenal tissue with 4-^{14}C-progesterone.

It appears that oxo group in position C-3 is necessary for androgenic effect of 17α-methyltestosterone and testosterone but not for inhibition of 18-hydroxylase and 11β-hydroxylase in adrenal gland. Substitution of C-3-oxo by C=N—OH group retained adrenal effects of androgens.

The mechanism of action of 17α-methyltestosterone, testosterone and their oximes in the inhibition of steroid hydroxylation is unknown. Although the mode of inhibition by androgens and other similar inhibitors is not known, their participation in the hydroxylation reaction as preferential substrates for these enzymes should be considered. The possibility of hydroxylation of these blocking agents as a competing reaction with steroid hydroxylation is presently under examination.

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