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IN VITRO METABOLISM OF 17α-ETHYNYLSTEROIDS

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SUMMARY

The rates of metabolism of synthetic gestagens derived from 19-nortestosterone by rabbit liver tissue *in vitro* were compared. Over a period of 1 h norethisterone was metabolised as rapidly as 19-nortestosterone whereas D-norgestrel and lynestrenol were metabolised at a slightly lower rate. Less than 5% of L-norgestrel was metabolised. In all cases the reaction product was the tetrahydrosteroid. Lynestrenol was metabolised through norethisterone. Skeletal muscle, lung and small intestine also metabolised norethisterone and D-norgestrel but at a slower rate than liver tissue. Small amounts of norethisterone were metabolised by adipose tissue but heart and spleen were inactive. Lynestrenol and L-norgestrel were not metabolised by any of the extra-hepatic tissues studied.

INTRODUCTION

Little is known about the *in vitro* metabolism of synthetic gestagens. The available information was recently reviewed [1, 2]. No systematic study of the effect of various substituent groups on the rate of metabolism of gestagens derived from 19-nortestosterone has been carried out although Cook and Vallance[3] have studied the metabolism by rabbit liver of a series of gestagens derived from 17α-acetoxyprogesterone and an attempt was made [4] to correlate the rate of metabolism of a number of gestagens by rat liver microsomes with their biological activity. In the present investigation the rates of metabolism by rabbit tissues of three derivatives of 19-nortestosterone, norethisterone (17α-ethynyl-19-nortestosterone), norgestrel (13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one, 18-methylnorethisterone) and lynestrenol (3-deoxynorethisterone) were compared with that of 19-nortestosterone. Norgestrel exists in two stereoisomeric forms; d-norgestrel which is the biologically active isomer and l-norgestrel which is biologically inactive. Both the pure isomers and the racemic mixture were studied.

MATERIALS AND METHODS

Materials. [4-^{14}C]-Norethisterone (S.A. 11.4 mCi/mmol) was a gift from Schering A.G. Berlin, [4-^{14}C]-lynestrenol (S.A. 28.5 mCi/mmol) was a gift from Organon N.V., Oss, Holland and 4-^{14}C D, L and DL-norgestrel (S.A. 1.6 mCi/mmol) were gifts from Wyeth Laboratories, Philadelphia. [4-^{14}C]-Nortestosterone (S.A. 50 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. The labelled steroids were checked for purity by paper chromatography and radioactive scanning and were shown to be more than 98% pure. For paper chromatography, Whatman No. 42 filter paper and the solvent system light petroleum (80–100°C)–toluene–methanol–water (5:5:8:2, v/v) was used.

Preparation of tissues. Tissues were obtained from male or female New Zealand white rabbits of body wt. 2.5–4 kg. The animals were killed by cervical dislocation and cutting of the jugular vein, allowing as much blood as possible to drain from the body. The tissues were removed immediately after killing and either used at once or stored at −20°C until required. The maximum period of storage was 6–8 weeks except for tissue from the gastro-intestinal tract which was not stored for more than 2–3 days. Control experiments showed that storage of frozen tissue did not affect the enzymic activity except for the gastro-intestinal tract. All manipulations were carried out at 4°C. Each organ was washed in 0.25 M nicotinamide in 0.25 M sucrose solution and blotted dry. They were then trimmed of adherent fat and connective tissue and weighed. The entire alimentary canal was removed and small sections were washed 2–3 times by forcing ice-cold saline from a pipette through the lumen until the intestines were visibly free of excreta. The intestine was cut longitudinally and kept in ice until homogenised. Tissue from each organ was cut into small pieces, weighed and homogenised in 2 vol. ice-cold nicotinamide–sucrose solution using an Ultraturrax type TP18 tissue disintegrator at maximum speed for 3 periods of 10 s.

Incubation and extraction procedure. This was carried out as described previously [5]. In a typical incubation 50 μg unlabelled steroid and 10,000 c.p.m. labelled steroid in 0.1 ml propylene glycol were added to a tube containing 1 ml 0.04 M potassium dihydrogen phosphate pH 7.4 containing 0.0006 M NADPH, 1 ml 0.01 M potassium citrate pH 7.4, 1 ml 0.005 M manganese sulphate and 4 ml homogenate.

The tubes were shaken in air at 37°C for varying periods of time. All incubations were carried out in

duplicate with suitable controls and for each set of incubations the rate of metabolism of the standard steroid 19-nortestosterone was studied under similar conditions. At the end of the incubation period 30 ml ice-cold acetone was added to each tube to terminate the reaction. The tubes were stoppered, shaken and the mixture centrifuged for 10 min. at 500 *g* at 4°C. The supernatant was removed and the remaining tissue pellet resuspended in a further 30 ml ice-cold acetone. After centrifugation the supernatants were combined and evaporated to a small volume (about 5 ml) and diluted with 25 ml water. The aqueous phase was extracted with 60 ml redistilled chloroform, the chloroform phase was dried with anhydrous sodium sulphate and evaporated to dryness *in vacuo*.

For extracts of incubations with norgestrel, norethisterone or 19-nortestosterone the extract obtained from evaporation of the chloroform was submitted to a hexane–methanol partition [6], the aqueous methanol phase was separated and evaporated to dryness. The residue was dissolved in ethanol and samples taken for estimation of radioactivity and for gas liquid chromatography. Since lynestrenol remained in the hexane phase of the hexane–methanol partition, the residues obtained from the chloroform extracts of incubations with lynestrenol were dissolved in 5 ml redistilled toluene and chromatographed on 1 cm diameter columns containing 3 g alumina (neutral, water content 2%, M. Woelm, Germany). Lynestrenol and its metabolites were eluted from the column with 40 ml 1% ethanol in toluene. The solvent was evaporated at 40°C in a rotary evaporator and the residue treated as described above. The mean recovery of lynestrenol and norethisterone through this procedure for four estimates of each steroid were 95.1% ± 1.2% and 92.5 ± 1.6% respectively.

Chromatography. Ascending unidimensional thin layer chromatography was performed using 0.25 mm plates (Merck, Darmstadt) of silica gel G or H and the solvent system cyclohexane–ethyl acetate (1:1, V/V) or alumina type T and the solvent system toluene–acetone (4:1, V/V).

For gas liquid chromatography a Pye 104 instrument with flame-ionization detector was used. Columns (5' glass) were packed with 3% SE 30 on Gaschrome Q (80–100 mesh). The column temperature was 220°C and the nitrogen flow-rate 50 ml per min. Trimethylsilyl ether derivatives of the steroids were prepared according to Chambaz and Horning[7] except that light petroleum (boiling range 80–100°C) was used as solvent. Cholestanone was used as an internal standard for gas liquid chromatography.

RESULTS

Metabolism by hepatic tissue

The rate of metabolism of 19-nortestosterone and its derivatives by female rabbit liver preparations using a tissue:steroid ratio of 40,000:1 is shown in Table 1 and some of the mean values are shown in Fig. 1. 19-nortestosterone, which was used as the standard compound, was metabolised quickly, more than half of the steroid being metabolised within 30 min. of incubation and no unmetabolised steroid was recovered after 5 h incubation. During the first 30 min. of incubation norethisterone and lynestrenol were metabolised as rapidly as 19-nortestosterone. D-Norgestrel was metabolised at a slightly slower rate whereas almost no metabolism of L-norgestrel occurred. With longer incubation times, up to 5 h, there was a decrease in the rate of metabolism. During this period 19-nortestosterone and norethisterone continued to be metabolised at the same rate until all the substrate had disappeared. Lynestrenol and D-norgestrel were metabolised more slowly and for the former the conversion to norethisterone appeared to be the limiting factor. L-Norgestrel was resistant to reduction and even after 5 h incubation 90% of the steroid could be recovered unchanged. The metabolism of racemic DL-norgestrel was intermediate between that of the two stereoisomers.

As shown in Table 1 the main route of metabolism was reduction of Ring A to form the tetrahydro steroids and the amount of incubated steroid metabolised could be quantitatively accounted for as the tetrahydro-compound. Lynestrenol was first metabolised to norethisterone which was then rapidly reduced to tetrahydronorethisterone. The tetrahydro steroids were characterised by their gas-liquid and thin-layer chromatographic behaviour but their configuration was not rigorously identified. For both norethisterone and norgestrel the metabolite appeared to have the 3α, 5β configuration. This was examined further in the case of norgestrel by incubating possible intermediates under the same conditions (Table 2). Whereas 5β-dihydronorgestrel was readily converted to tetrahydronorgestrel the rate of conversion of the 5α-dihydro compound was very slow. Conversion of

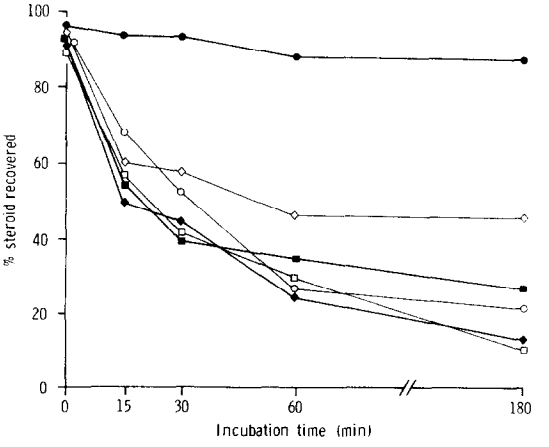

Fig. 1. Rate of metabolism of 19-norsteroids by rabbit liver tissue. 19-nortesterone, ◆——◆; norethisterone, □——□; d-norgestrel, ○——○; lynestrenol, ■——■; dl-norgestrel, ◇——◇; l-norgestrel, ●——●. Values are means from Table 1.

Table 1. Rate of metabolism of 19-nortestosterone and its derivatives by liver tissue from female rabbits (values are mean + S.D. for % of steroid recovered after incubation. Figures in parentheses denote number of incubations. TH denotes tetrahydrosteroid)

	% Steroid recovered											
Steroid incubated	19-nortestosterone	Norethisterone (Net)		DL-Norgestrel (NG)		D-Norgestrel		L-Norgestrel		Lynestrenol (Lyn)		
Steroid recovered	19-nortestosterone	Net	THNET	Ng	THNg	Ng	THNg	Ng	THNg	Lyn	Net	THNET
Time of incubation (min)												
0	92.0 ± 5.5 (9)	90.6 ± 10.4 (11)	0 (11)	92.5 ± 7.3 (8)	0 (8)	94.7 ± 2.5 (4)	0 (4)	94.1 ± 1.8 (4)	0 (4)	63.04 ± 4.1 (3)	0 (3)	0 (3)
10	43.7 ± 28.0 (3)	67.9 ± 9.1 (6)	22.9 ± 7.7 (6)	62.8 ± 8.3 (8)	26.4 ± 6.1 (8)	63.0 ± 10.3 (4)	29.3 ± 9.1 (4)	92.6 ± 2.0 (4)	1.0 ± 0.5 (4)	63.0 ± 4.1 (3)	18.0 ± 1.4 (3)	14.2 ± 0.7 (3)
15	50.1 ± 12.3 (4)	56.9 ± 19.7 (3)	19.1 ± 1.0 (3)	60.5 ± 13.0 (6)	27.5 ± 6.7 (6)	68.0 ± 7.2 (4)	23.4 ± 5.2 (4)	93.1 ± 1.4 (4)	0.5 ± 0.4 (4)	55.9 ± 2.3 (4)	24.0 ± 3.6 (4)	16.6 ± 2.5 (4)
20	53.6 ± 5.3 (3)	62.6 ± 1.3 (3)	17.3 ± 1.3 (3)	49.2 ± 10.2 (5)	37.9 ± 4.4 (5)	45.5 ± 3.9 (4)	47.7 ± 6.6 (4)	91.4 ± 2.7 (4)	1.9 ± 0.8 (4)	52.9 ± 1.7 (3)	26.3 ± 0.9 (3)	18.2 ± 2.4 (3)
30	45.1 ± 11.3 (3)	42.4 ± 15.5 (8)	47.1 ± 14.5 (8)	58.5 ± 19.7 (8)	30.1 ± 16.8 (8)	52.4 ± 18.3 (4)	38.5 ± 19.3 (4)	92.3 ± 2.3 (4)	1.3 ± 0.9 (4)	40.2 ± 1.6 (4)	33.0 ± 1.8 (6)	22.9 ± 2.2 (6)
60	24.7 ± 17.0 (4)	29.4 ± 21.1 (4)	60.7 ± 20.7 (4)	45.9 ± 13.8 (8)	38.4 ± 11.0 (8)	27.0 ± 9.0 (4)	60.5 ± 6.6 (4)	88.6 ± 3.9 (4)	3.9 ± 1.5 (4)	35.6 ± 6.1 (5)	27.8 ± 1.8 (5)	15.1 ± 2.2 (5)
180	13.0 ± 12.0 (3)	9.9 ± 0 (4)	85.8 ± 9.9 (4)	44.2 ± 18.1 (8)	42.8 ± 19.2 (8)	22.3 ± 8.1 (4)	66.4 ± 5.9 (4)	89.0 ± 3.4 (4)	3.6 ± 1.3 (4)	27.5 ± 2.9 (4)	35.8 ± 2.7 (4)	25.0 ± 4.4 (4)
300	0 (3)	0 (2)	85.5 ± 1.4 (3)	43.4 ± 13.0 (8)	47.1 ± 12.6 (8)	11.6 ± 5.0 (4)	78.3 ± 9.9 (4)	88.8 ± 3.0 (4)	4.2 ± 1.2 (4)	20.0 ± 9.0 (4)	39.7 ± 3.8 (4)	38.0 ± 1.3 (4)

Table 2. Incubation of D-norgestrel and possible intermediates in the production of tetrahydronorgestrel by rabbit tissue (Values are mean ± S.D. for 3 incubations; incubation time 10 min.)

Steroid incubated	% Conversion to tetrahydronorgestrel
D-Norgestrel	48.1 ± 8.4
5α-Dihydronorgestrel (13β-ethyl-17α-ethynyl-5α-gonan-3-on-17β-ol)	4.1 ± 1.2
5β-Dihydronorgestrel (13β-ethyl-17α-ethynyl-5β-gonan-3-on-17β-ol)	80.1 ± 4.1
3α-Dihydronorgestrel (13β-ethyl-17α-ethynyl-gon-4-ene-3α, 17β-diol)	2.1 ± 1.5
3β-Dihydronorgestrel (13β-ethyl-17α-ethynyl-gon-4-ene-3β, 17β-diol)	3.1 ± 2.0

the 3α- and 3β-hydroxy-4-ene steroids was also of a low order suggesting that the major pathway for the metabolism of norgestrel was via 5β-dihydronorgestrel.

Metabolism by extra-hepatic tissues

The incubation conditions used to study metabolism of the steroids by liver tissue were also used to compare the extent of metabolism when incubated for 120 min. with other tissues. Organs from a single animal were used simultaneously.

There were no significant differences between the rate of metabolism of the various steroids by tissues from male animals and those from female ones. The combined results are shown in Table 3. The absence of a sex difference in metabolism is in agreement with the findings of Taylor[8] from studies of the metabolism of progesterone by rabbit liver homogenates. Kidney, spleen and heart were unable to metabolise any of the gestagens. Neither L-norgestrel nor lynestrenol were metabolised by the non-hepatic tissues. Adipose tissue and kidney were not able to metabolise norgestrel although small amounts of metabolism, 10.5% and 2.2% respectively occurred with norethisterone. Both norethisterone and 19-nortestosterone underwent considerable metabolism on incubation with skeletal muscle, lung and small intestine and although D- and DL-norgestrel were metabolised by these tissues it was to a much lesser extent than in the case of the other two compounds. When metabolism occurred the products, as with liver tissue, were the tetrahydro compounds.

DISCUSSION

One of the main metabolic pathways for the inactivation of the naturally-occurring steroid hormones as well as most synthetic steroids is reduction of ring A to the tetrahydro compounds and this step may be the rate limiting one [9, 10]. Under our incubation conditions ring A reduction appeared to be the major metabolic reaction occurring since disappearance of the substrate, with the exception of lynestrenol, was quantitatively accounted for by the tetrahydro metabolites.

In the present investigations when the 17α-ethynyl group was introduced into 19-nortestosterone, the rate of metabolism of the resulting compound, norethisterone was not significantly different from that of 19-nortestosterone. However, when the angular methyl group at C-13 was substituted by an ethyl group as in norgestrel, there was a slight decrease in the rate of metabolism of the D-isomer and a very marked decrease for the L-isomer. Using rat liver preparations, Gerhards *et al.*[11] showed that norgestrel was less rapidly reduced in ring A than norethisterone. The conversion of the 3-deoxysteroid lynestrenol to norethisterone was a rapid reaction initially but, on longer incubation, the rate-limiting step in the metabolism of lynestrenol appeared to be its conversion to norethisterone. Dericks-Tan *et al.*[4] found that 3-oxo group was necessary for the hydrogenation of the double bond at C-4. The *in vitro* conversion of lynestrenol to norethisterone by rabbit liver preparations has been described previously by Mazaheri *et al.*[12] and this conversion has now also been demonstrated *in vivo* in humans [13, 14]. The liver would seem to be the major organ involved in this transformation since *in vitro* other tissues appear inactive (see Table 3).

These *in vitro* findings agree with results of *in vivo* metabolic studies where it has been shown that, for both norgestrel and norethisterone, ring A-reduced metabolites are the predominant products in urine [2]. 3α, 5β-Tetrahydronorgestrel is quantitatively the most important metabolite of DL- and D-norgestrel in humans [15, 16]. It is also of interest that in the present study L-norgestrel was metabolised in ring A only very slowly. *In vivo* metabolic studies have also shown the L-isomer to be less readily reduced in ring A than the D-isomer but to be more readily metabolised for hydroxylation reactions [16].

It has been recognised for some time that extrahepatic tissues can metabolise steroids [17]. The kidney has been shown to metabolise steroid hormones in various animal species [18] and ring A reduction of progesterone was shown with rabbit kidney tissue [19]. In contrast, in the present study 19-nortestosterone and norethisterone were only slowly metabolised by kidney tissue and the other gestagens not at all. Our study demonstrated that skeletal muscle was able to metabolise the 19-nor steroids with the exception of L-norgestrel and lynestrenol; for the latter steroid presumably hydroxylation at C-3 could not be performed. Thomas and Dorfman[20] identified 5α-androstane-3, 17-dione, 3β-hydroxy-5α-androstan-17-one and testosterone from the incubation of ^{14}C androstenedione with rabbit skeletal tissue.

The marked metabolism of the steroids by rabbit lung tissue was surprising, although during the time that these studies were being carried out the metabolism of testosterone to androstenedione, 5α-androstanolone and dihydrotestosterone by rabbit lung tissue was described [21]. Similar products were

Table 3. Rate of metabolism of 19-nortestosterone and its derivatives by rabbit tissues (Values are mean ± S.D. for % of steroid recovered after incubation for 120 min. with tissues from 3 female and 3 male animals. TH denotes tetrahydrosteroid).

	% Steroid recovered											
Steroid incubated	19-nortestosterone	Norethisterone (Net)		DL-Norgestrel (Ng)		D-Norgestrel		L-Norgestrel		Lynestrenol (Lyn)		
Steroid recovered	19-nortestosterone	Net	THNET	Ng	THNg	Ng	THNg	Ng	THNg	Lyn	Net	THNET
Tissue												
Liver	19.6 ± 2.0	34.2 ± 7.6	64.9 ± 6.7	47.2 ± 3.6	48.8 ± 5.3	42.0 ± 3.5	52.9 ± 3.6	89.5 ± 1.4	2.5 ± 2.1	36.4 ± 7.1	20.1 ± 8.3	35.0 ± 3.8
Kidney	79.1 ± 5.2	89.0 ± 4.3	2.2 ± 1.3	82.6 ± 6.9	0	81.9 ± 5.6	0	90.7 ± 4.3	0	85.0 ± 3.4	0	0
Skeletal muscle	60.2 ± 2.8	66.6 ± 5.2	29.0 ± 3.2	88.5 ± 3.6	9.2 ± 2.3	77.5 ± 5.0	16.3 ± 2.9	91.7 ± 4.3	0	88.7 ± 6.2	0	0
Lung	39.0 ± 1.9	42.7 ± 3.9	44.9 ± 6.6	87.2 ± 4.5	8.9 ± 1.9	83.1 ± 5.2	13.4 ± 2.3	91.0 ± 3.7	0	94.5 ± 2.1	0	0
Spleen	90.2 ± 2.5	88.2 ± 3.2	0	87.0 ± 3.8	0	87.0 ± 4.7	0	90.0 ± 5.7	0	69.7 ± 30.7	0	0
Small intestine	43.8 ± 2.0	60.7 ± 9.0	32.6 ± 3.5	88.0 ± 1.6	7.7 ± 2.2	82.9 ± 3.1	13.0 ± 1.8	92.2 ± 5.3	0	93.8 ± 2.5	0	0
Adipose	81.5 ± 8.9	84.0 ± 2.8	10.7 ± 1.5	81.7 ± 5.0	0	83.6 ± 7.1	0	89.6 ± 2.8	0	86.3 ± 8.0	0	0
Heart	81.5 ± 4.3	82.2 ± 6.0	0	83.8 ± 10.2	0	80.3 ± 10.4	0	90.4 ± 2.4	0	86.0 ± 2.4	0	0

obtained from rat and dog lung tissue [22, 23] and more recently the metabolism of DHA and androstenedione by adult human lung was reported [24]. In our investigations no metabolism of the steroids occurred on incubation with spleen tissue and, although the metabolism of androgens or gestagens has not previously been investigated with this tissue, the spleen does not metabolise cortisone under conditions where this compound is metabolised by liver and kidney tissue [25]. Intestinal tissue can metabolise ring A of steroids [26, 27]. In our experiments both 19-nortestosterone and norethisterone were rapidly metabolised by intestinal tissue although DL- and D-norgestrel were metabolised comparatively slowly. Adipose tissue was only able to metabolise 19-nortestosterone and norethisterone to a small extent. Incubation with heart tissue produced no change and this tissue was unable to metabolise cortisone [25].

These studies suggest that, although the liver is the major tissue involved in the metabolism of the synthetic gestagens, the lung, skeletal muscle and intestinal tissue may make an important contribution.

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