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STEROID BIOSYNTHESIS BY TRANSPLANTABLE GONADAL TUMORS OF RAT*

ISABEL VAN HOUTTE, R. IGLESIAS, O. IRUSTA and G. F. WASSERMANN

Laboratorio de Esteroides, Instituto de biología y Medicina Experimental, Obligado 2490-Buenos Aires, Argentina and Instituto de Medicina Experimental del Servicio Nacional de Salud, Santiago, Chile

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SUMMARY

Minces of functional transplantable tumors from ovaries and testis of AxC rats were incubated *in vitro* with several radioactive precursors.

[1-^{14}C]-sodium acetate, [1-2^{3}H]-cholesterol, [4-^{14}C]-pregnenolone, [7-^{3}H]-progesterone, [1-2^{3}H]-androstenedione and [1-2^{3}H]-testosterone were used to analyze the pathways leading to hormone production. The effect of LH and cyclic AMP on this biosynthesis was studied.

The ovarian tumor converted pregnenolone readily to progesterone; androstenedione preferentially to estrone and to some extent to 17β-estradiol, and testosterone preferentially to 17β-estradiol and to small quantities of estrone. No 17α-hydroxypregnenolone or 17α-hydroxyprogesterone was detected. The tumor did not respond to LH or 3′5′ cAMP.

The testicular tumor did not form any of the common progestins, estrogens or androgens when incubated with labelled precursors.

INTRODUCTION

DIFFERENT approaches to investigate the steroidogenic capabilities of a particular cell type have been developed, e.g. organ culture, isolated cells and tumor tissue of a specific cell class. This has been particularly useful when working with gonads, due to the remarkable complexity of these organs and the variety of their functions. Besides, some functional gonadal tumors of rat retain their endocrine activity after isologous transplantations[1]. This peculiarity has been useful to determine the biological characteristics of these tumors and to study the biosynthesis of steroid hormones[2].

In the present paper we investigate the *in vitro* transformation of labelled steroids by transplantable functional tumors of the testis (TR.MCV^{12}) and of the ovary (granulosa cell type, TOF, II^{104}) from AxC rats. Different radioactive precursors were used to analyze the pathways leading to hormone production in these tumors.

The action of LH and dibutyryl cyclic AMP on those processes was also explored.

MATERIAL AND METHODS

Six male and 5 female AxC rats were used. The females were inoculated with the ovarian granulosa cell tumor (TOF,II^{104}) and were killed after 1 month. Males were inoculated with the testicular tumor (TR.MCV^{12}) and used two months later. The tumors were dissected out and 500 mg pieces of tumoral tissue were minced. Duplicate samples were incubated for varying times in 5 ml of Krebs–Ringer

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bicarbonate buffer pH 7·4 plus 2 mg/ml of glucose, in an atmosphere of CO_2–O_2, (5:95 v/v) using a Dubnoff shaking incubator at 37°C.

The following radioactive precursors were used: [1-^{14}C]-sodium acetate (S.A.: 51 mCi/mmol) 20 μCi per sample; [1-2^{3}H]-cholesterol (S.A.: 48 Ci/mmol) 25 μCi per sample suspended in propyleneglycol; [4-^{14}C]-pregnenolone (S.A.: 45 mCi/mmol) 1 μCi per sample; [7-^{3}H]-progesterone (S.A.: 16 Ci/mmol) 3 μCi per sample; [7-^{3}H]-dehydroepiandrosterone (S.A.: 12 Ci/mmol) 0·3 μCi per sample; [1-2^{3}H]-androstenedione (S.A.: 48 Ci/mmol) 5 μCi per sample; [1-2^{3}H]-testosterone (S.A.: 45 Ci/mmol) 2·5 μCi per sample. (All radioactive substances were from New England Nuclear).

Tissue for incubation with sodium acetate or cholesterol was divided into six samples: 2 controls, 2 with 500 μg of LH per flask and 2 with dibutyryl 3′5′ cAMP (Sigma Co.) 1 mM. Times of incubation were: for acetate or cholesterol, 3 h; pregnenolone, 20, 40 or 60 min; progesterone and testosterone, 2 h; androstenedione and dehydroepiandrosterone, 1 h.

Processing of the samples

Reactions were stopped by adding 10 ml of ethyl acetate. Cold carriers were added as follows.

(a) *Incubations with androstenedione, testosterone or dehydroepiandrosterone*: androstenedione, testosterone, estrone and 17β estradiol.

(b) *Incubations with progesterone*: as for a) but in addition: progesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 20β-dihydroprogesterone.

(c) *Incubations with acetate, cholesterol or pregnenolone*: as for (b) but in addition: pregnenolone, 17α-hydroxypregnenolone.

After homogenization the samples were extracted with ethyl acetate (1·5 vol. × 4). The extracts were evaporated and the phenolic steroids separated from neutral steroids following the method of Engel *et al.*[3]. The estrogens were then run on thin layer chromatograms (t.l.c.) in benzene–methanol (95:5 v/v). The areas corresponding to estrone and 17β-estradiol were eluted, rechromatographed on t.l.c. in chloroform–ether (4:1 v/v).

Neutral fractions were purified by paper chromatography (PC) using the methylcyclohexane–propyleneglycol system for 9·5 h. When cholesterol or pregnenolone was the substrate, the following radioactive zones were investigated.

(a) Testosterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone. These steroids were eluted and rechromatographed on PC using toluene–propyleneglycol as a solvent system. Testosterone and 17α-hydroxyprogesterone were then eluted, acetylated[4] and rechromatographed in t.l.c. in chloroform–ether (4:1 v/v). The 17α-hydroxypregnenolone area was eluted, and directly purified by recrystallization.

(b) 20α-dihydroprogesterone, 20β-dihydroprogesterone and dehydroepiandrosterone were eluted and acetylated.

(c) The androstenedione and pregnenolone area was eluted, acetylated and rechromatographed on t.l.c. in benzene–ethyl acetate (5:1 v/v).

(d) The progesterone area was eluted and reduced to 20β-dihydroprogesterone (5), chromatographed on t.l.c. in benzene:ethyl acetate (4:1 v/v). The U.V. absorbing area was acetylated and rechromatographed in benzene–ethyl acetate (5:1 v/v).

When other substrates were used, a similar technique was utilized for the isolation of the metabolites produced.

Recoveries of the U.V. absorbing steroids were quantitated by U.V. absorption in a Beckman DB-G spectrometer using Allen's correction [6].

Radioactivity was measured using a packard Tri Carb liquid scintillation spectrometer model 3320. The chromatograms were scanned in a Packard radiochromatogram scanner model 7201.

The criteria for final identification of the compounds formed was based on the constant specific activity of crystals obtained in the course of repeated crystallizations [7].

RESULTS

Ovarian tumor TOF, II[104]

Incubations with ^{14}C *acetate*: In these experiments substances in the area of progesterone in the first p.c. were carefully purified. At the end of this procedure the following amounts of radioactivity associated with the carrier were detected (Mean ± S.D.). Controls: 4375 ± 1835, with LH: 5010 ± 3400, with 3'5' cAMP: 6510 ± 4060 d.p.m.

No radioactivity associated with the other carriers at the end of the purification procedure was found.

Incubations with [^3H]-*cholesterol.* With this substrate no radioactive peaks behaving like the carriers were seen after purification. No variations in the pattern of radioactivity were observed in samples incubated with LH or 3'5' cAMP as compared with controls.

Incubations with [^{14}C]-*pregnenolone.* Samples of the tumor were incubated for 20, 40 and 60 min. The pattern of biosynthesis was essentially similar in all intervals studied.

Regardless of the time of incubation small amounts of radioactivity remained associated with the precursor at the end of the purification procedure.

The cold carriers 17α-hydroxyprogesterone and 17α-hydroxypregnenolone were devoid of radioactivity after the purification. A significant amount of radioactivity remained associated with the 20β-dihydroprogesterone carrier after chromatography and acetylation. The radioactivity associated with carrier progesterone was recrystallized to constant specific activity (Table 1) and found to be authentic progesterone in a yield of 5% (Table 1).

Incubation with [^3H]-*progesterone.* The tissue was incubated for 2 h with [^3H]-progesterone, the radioactivity that remained with the substrate had then almost disappeared. No radioactivity was associated with the carriers added, but a small amount was associated with 20β-dihydroprogesterone. In this incubation, 17α-hydroxy-progesterone was carefully looked for but no trace of radioactivity was associated with this steroid.

Incubation with androstenedione. The samples incubated with androstenedione produced estrone as the main compound and some 17β-estradiol. Little radioactivity was associated with carrier testosterone. Estrone and 17β-estradiol were recrystallized to constant specific activity (see Table 1).

Incubation with testosterone. The tissue incubated with testosterone converted this steroid principally to 17β-estradiol, this compound was identified by recrystallizations and the yield was 15% of the precursor (Table 1). Small amounts of

Table 1. Steroids formed in incubations with the ovarian granulosa cell tumor[a]

Precursor	Product	Crystals S.A. in d.p.m./mg[b]	Conversion in % of substrate[c]	Time of incubation
[4-^{14}C]-Pregnenolone (1 μCi)	Progesterone	2324 2324 2263 2157	6%	1 h
[1-2^{3}H]-Androstenedione (5 μCi)	Estrone	2460 2440 2300 2420	2%	1 h
	17β-estradiol	42 32 30 28	0·03%	1 h
[1-2^{3}H]-Testosterone (2·5 μCi)	Estrone	930 906 990 982	1%	2 h
	17β-estradiol	13946 13139 13959 13411	15%	2 h

[a]Conditions of incubation as reported in Material and Methods.
[b]Crystallizations were done using 30 mg of authentic steroid.
[c]Calculations were made taking into consideration the amount of radioactivity when the constant specific activity was reached and correction to 100% of recovery.

estrone were also found. The amount of radioactivity associated with androstenedione carrier was too low to allow further attempts at identification.

Testicular tumor TR.MCV[12]

Incubation with [^{14}C]-*acetate or* [^{3}H]-*cholesterol.* In these experiments no radioactivity was associated with added carriers after purification.

Incubation with [^{14}C]-*pregnenolone or* [^{3}H]-*progesterone.* The substrates were completely converted to radioactive substances which could not be identified as any of the added carriers.

Incubation with [^{14}C]-*dehydroepiandrosterone or* [^{3}H]-*androstenedione.* Using [^{3}H]-androstenedione as a substrate neither 17β-estradiol nor estrone were found. With both substrates, radioactivity was associated with testosterone after chromatography and acetylation but in such small amounts as to preclude recrystallization.

DISCUSSION

The functional ovarian tumor TOF, II[104] has been reported to produce estrogens and the factors influencing its growth have been extensively investigated [8]. This tumor, when incubated with radioactive precursors, shows

active biosynthesis. From our results the following conclusions can be drawn:

Pregnenolone was actively converted to progesterone. Incubation with acetate as substrate produced trace amounts of progesterone. This conversion was not modified by LH or dibutyryl cyclic AMP.

Testosterone was actively transformed to 17β-estradiol and androstenedione converted preferentially to estrone.

Cholesterol was converted to compounds that could not be identified.

Progesterone used as substrate was totally converted to unidentified radioactive compounds and probably to small amounts of 20β-dihydroprogesterone.

In no case could 17 hydroxylation of pregnenolone or progesterone be demonstrated (Table 2).

Table 2. General scheme of the results found in the different incubations with ovarian tumor tissue

Substrate added	Products		
	Demonstrated[a]	Probable[b]	Absent[c]
Acetate	—	Progesterone	
Cholesterol	—	—	
Pregnenolone	Progesterone	20β-dihydroprogesterone	17α-hydroxyprogesterone 17α-hydroxypregnenolone
Progesterone	—	20β-dihydroprogesterone	17α-hydroxyprogesterone
Androstenedione	Estrone 17β-estradiol	Testosterone	—
Testosterone	17β-estradiol Estrone	Androstenedione	—

[a]Identified by means of crystallization to constant specific activity.
[b]Results based on evidence from chromatographic behaviour and derivative formation.
[c]Steroids not found after careful and extensive investigation.

This tumor is thus apparently able to transform the acetate to progestins and the C^{19} steroids to estrogens but lacks the capacity to transform the C^{21} steroids used to androgens.

The testicular tumor showed an androgenic and estrogenic activity in biological tests in inoculated animals [9]. This transplantable tumor failed to convert the substrates used to any of the steroids investigated (progestins, estrogens and androgens).

Pasqualini *et al.* [2] demonstrated a decrease in testosterone biosynthesis *in vitro* between the fourth and sixth transplants of a transplantable rat testicular tumor. Perhaps a similar diminution of steroidogenesis *in vitro* by this tumoral tissue has occurred.

It is also possible that the biological activities of these tumors may be mediated by a substance(s) which does not have the common estrogenic or androgenic structures [2].

The possibility that this tumor synthesizes some corticoid molecules as was described for testicular tumors of mice [10] was not investigated in the present paper.

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