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IN VITRO METABOLISM OF STEROIDS BY HUMAN AND RABBIT SUBMAXILLARY GLANDS

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SUMMARY

Human and rabbit submaxillary gland homogenates were incubated *in vitro* for 2 hr with different labeled substrates under an air atmosphere with a NADPH generating system.

Both tissues converted progesterone-4-^{14}C to 20β-dihydroprogesterone to the extent of 9·7–17·3% for human and 9·5–15·7% for rabbit tissue.

When pregnenolone-4-^{14}C was incubated with rabbit submaxillary gland, 20β-dihydropregnenolone was detected with a yield of 1·7–4·4%. The labeled pregnenolone was recovered unchanged in human tissue experiments.

Both human and rabbit submaxillary glands converted androstenedione-4-^{14}C to testosterone to the extent of 19·1–31·1% and 9·9–16·5%, respectively.

No differences between sexes were observed in either human or rabbit experiments and no radioactivity was associated with the phenolic fractions.

INTRODUCTION

IN A PREVIOUS paper it was reported that rat submaxillary glands showed 17α-hydroxylase, Δ5-3β-hydroxy-dehydrogenase and C_{17-20} desmolase activities when incubated *in vitro* with labeled progesterone and/or pregnenolone [1]. The parotid glands and pancreas did not share these enzymatic activities. It was also demonstrated that the submaxillary glands of the rat had all the enzymatic machinery to perform *de novo* synthesis of cholesterol from acetate and when these glands were incubated with labeled cholesterol, a C_{20-22} desmolase activity was observed [2].

Charreau and Villee demonstrated that explants of rat submaxillary glands in organ cultures metabolized progesterone to 5α-pregnane-3,20-dione and other unidentified metabolites [3].

Early studies suggested that the salivary glands have an endocrine function [4, 5]; indeed, a purified salivary gland hormone was reported [6].

To determine if the human and rabbit submaxillary glands have some enzymatic system related to the steroid metabolism, labeled progesterone, pregnenolone and androstenedione were incubated *in vitro* with homogenates of these salivary glands.

MATERIAL AND METHODS

(a) *Tissue preparation*

Twelve New Zealand rabbits, 3 months old, were sacrificed by a blow on the head and both submaxillary glands were carefully dissected out of surrounding tissue.

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Human submaxillary specimens were surgically obtained from five patients, 3 males and 2 females, from 34 to 51 years old, with malignant carcinomas of the lips.

The tissue was weighed and homogenized with 0·1 M phosphate buffer, pH 7·4, in an all glass homogenizer. The entire procedure was performed in a cold room at 4°C. Aliquots were taken to determine the protein concentration[7].

(b) *Solvents and drugs*

All solvents were of analytical grade. Diethylether was twice redistilled through a 60 cm Vigreaux column shortly before use. Methanol was redistilled from 2,4-dinitrophenylhydrazine through a Vigreaux column. Chloroform, ligroine, acetone, hexane, cyclohexane, ethanol and ethyl acetate were twice redistilled through a Vigreaux column. Pyridine was distilled twice over KOH pellets and stored in a dark bottle in a desiccator.

(c) *Radioactive and non radioactive steroids*

Progesterone-4-^{14}C (S.A. 47 μCi/μmol), progesterone-7α-^{3}H (S.A. 14 mCi/μmol), pregnenolone-4-^{14}C (S.A. 49 μCi/μmol), pregnenolone-7α-^{3}H (S.A. 13 mCi/μmol), androstenedione-4-^{14}C (S.A. 37 μCi/μmol) and androstenedione-7α-^{3}H (S.A. 33 mCi/μmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A., and purified by paper chromatography shortly before use. Aliquots were recrystallized to constant specific activity to establish their radiochemical purity.

A Nuclear Chicago Actigraph III was utilized for radioactivity detection on chromatograms. After elution the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer using 10 ml scintillation fluid containing 4 g 2,5-diphenyloxazole and 40 mg p-bis-2-(5-phenyloxazolyl)benzene/l toluene. The efficiency for measuring ^{14}C and ^{3}H varied from 69 to 76% and 35 to 44%, respectively. When ^{3}H and ^{14}C were counted in the same sample the efficiency was 21 and 51% respectively.

Reference steroids were obtained from Steraloids, Inc., and recrystallized before use. The purity was checked by gas phase chromatography.

(d) *Incubations*

The equivalent of 700 mg of tissue was added to each incubation flask with the appropriate substrate and a NADPH generating system (6·56 μmoles of ATP, 3·60 μmoles of NAD, 3·77 μmoles of NADP, 9·63 μmoles of glucose-6-phosphate and 50 μg of glucose-6-phosphate dehydrogenase). The final concentration of the homogenates was 30%(w/v).

The mixtures were incubated for 2 h under an air atmosphere in a Dubnoff metabolic shaker, at 37°C for human tissue and 39°C for rabbit experiments. The incubations were stopped by the addition of 5 ml of diethylether and kept at −15°C until processed. Flasks from each experiment were extracted at the start of the incubation and used as zero time controls. Heated enzymes were prepared by maintaining homogenates in a water bath at 60°C for 30 min before incubation.

(e) *Extraction*

After adding 15,000 dpm of the appropriate tritiated steroid to each incubation flask the extractions were performed as previously described[2]. The aqueous phase was extracted 3 times with 30 ml of diethylether.

The combined ether extracts were washed with 1N sodium hydroxide and with water to neutrality. The washed ether extracts were filtered through anhydrous sodium sulphate and evaporated to dryness under reduced pressure. To the dry extract 100 μg each of authentic progesterone, androstenedione and testosterone were added and the extracts were chromatographed for 6 h in system A. The recovery before the first chromatography ranged from 90 to 97%.

In the androstenedione-4-^{14}C incubations, the combined ether extracts were evaporated to dryness, resuspended with 30 ml of toluene and washed with 10 ml of 0·1 N sodium hydroxide and with water to neutrality. The combined aqueous fractions (phenolic fraction) were adjusted to pH 10·3 and extracted with diethylether according to Engel[8]. The toluene fraction (neutral fraction) was filtered through anhydrous sodium sulphate, evaporated to dryness under reduced pressure and chromatographed in system A for 6 h after adding 100 μg each of progesterone, androstenedione and testosterone. In a similar way, the ether fraction was filtered, evaporated to dryness under nitrogen and chromatographed in system C for 6 h; 100 μg each of authentic estradiol, estrone and estriol were simultaneously chromatographed on separate strips.

(f) *Chromatographic systems*

The following paper and thin layer chromatographic systems were used:

Paper chromatography

System A: hexane/formamide
System B: toluene/propylene glycol
System C: chloroform/formamide
System D; ligroine–methanol–water (5 : 4 : 1, by vol.)
System E: cyclohexane–methanol–water (10 : 10 : 1, by vol.)

Thin layer chromatography

System F: chloroform–ethanol (9 : 1, v/v)
System G: cyclohexane–ethyl acetate (1 : 1, v/v).

Paper chromatography was performed on Whatman # 1 paper previously washed with methanol and thin layer chromatography on 20 × 20 cm precoated plates (Silica Gel F 254, E. Merck AG, Darmstadt, Germany). The plates were activated at 100°–110°C for 1 h immediately before use. The reference steroids were located by ultraviolet absorption, iodine vapours or the appropriate color reaction[9].

(g) *Derivative formation*

Acetylation was performed by adding 0·2 ml of an acetic anhydride–pyridine (1 : 5, v/v) mixture to the dry extract which was left overnight in the dark. Acetate hydrolysis was performed with 0·1 N ethanolic sodium hydroxide (2). A mild oxidation was achieved by adding 0·2 ml of 0·5% chromium trioxide in 95% glacial acetic acid to the dry extract. The mixture was incubated for 2 h in the dark and the reaction was stopped with 1 ml of 20% aqueous ethanol and extracted twice with 10 ml of methylene chloride.

(h) *Identification criteria and expression of results*

The chromatographic mobility similar to that of authentic standard as free steroid and after derivative formation, and recrystallization to constant specific activity in at least four different solvents, were considered evidence of radiochemical purity.

The determination of mother liquor and crystal mass in each recrystallization was made by gas phase chromatography after having carried out the appropriate dilution. A Packard gas chromatography instrument (Model 7400) with a flame ionization detector was utilized; 1·8 m long, 0·4 cm internal diameter glass columns with either SE–30 2·5% or QF–1 1·0% on 100–120 mesh Gas Chrom Q (Applied Sciences Co.) were prepared according to Horning[10].

The results were expressed as percent conversion of the substrate and as d.p.m./mg of protein after correction of procedure losses by the recovery of the tritiated standard.

RESULTS

Human and rabbit submaxillary gland homogenates converted $4{\cdot}96 \times 10^5$ dpm of progesterone-4-^{14}C to 20β-dihydroprogesterone to the extent of 2261 ± 509 d.p.m./mg of protein (Mean $\pm$ S.D.) for human and 1269 ± 304 d.p.m./mg of protein for rabbit tissue (Tables 1 and 2).

When $5{\cdot}47 \times 10^5$ d.p.m. of pregnenolone-4-^{14}C were incubated with rabbit submaxillary gland, 20β-dihydropregnenolone with a yield of 341 ± 120 d.p.m./mg of protein was detected (Table 2). The labeled pregnenolone was recovered unchanged in human tissue experiments.

Both human and rabbit submaxillary glands converted $4{\cdot}18 \times 10^5$ d.p.m. of androstenedione-4-^{14}C to testosterone to the extent of 2997 ± 643 and 1127 ± 377 d.p.m./mg of protein, respectively (Tables 1 and 2).

No differences between sexes were observed in either human or rabbit experiments and no radioactivity was associated with the phenolic fractions.

Identification of compounds

The isolated radioactive steroids were purified and characterized as follows:

20β-Dihydroprogesterone. The radioactive material with an R_F(0·32) identical to authentic 20β-dihydroprogesterone in system A was eluted and aliquots were: (a) counted; (b) rechromatographed in systems B and D where a single peak of radioactivity corresponding to the mobility of authentic 20β-dihydroprogesterone was observed in each case (R_F values of 0·72 and 0·40, respectively); (c) acetylated and rechromatographed in system A, where they behaved like 20β-dihydroprogesterone acetate ($R_F = 0{\cdot}89$). After hydrolysis this material had an R_F of 0·28 in System E similar to authentic 20β-dihydroprogesterone; (d) oxidized to a radioactive material behaving like progesterone in system D ($R_F = 0{\cdot}67$); (e) recrystallized to constant specific activity as the free compound and as its acetate (Table 3).

Progesterone. The radioactive material behaving like authentic progesterone in system A ($R_F = 0{\cdot}62$) was eluted and aliquots were: (a) counted; (b) rechromatographed in systems D and E, where they behaved like authentic progesterone (R_F of 0·67 and 0·48 respectively); (c) oxidized and chromatographed in system E, having an R_F of 0·48, similar to authentic progesterone; (d) acetylated and rechromatographed in system D, in which a peak with an R_F of 0·67 similar to

Table 1. *In vitro* metabolism of $4{\cdot}96 \times 10^5$ d.m.p. (4·79 mμ moles) of progesterone-4-^{14}C (S.A. 47 μCi/μmol) and $4{\cdot}18 \times 10^5$ d.p.m. (5·13 mμmoles) of androstenedione-4-^{14}C (S.A. 37 μCi/μmol) by human submaxillary gland homogenates, incubated for 2 h at 37°C with a NADPH generating system

Patient	Age	Sex		Progesterone-4-^{14}C			Androstenedione-4-^{14}C		
				Progesterone*	20β-Dihydroprogesterone	% †	Androstenedione*	Testosterone	% †
1	38	M		13788‡	2877	17·3	9905	404	19·5
2	34	F		14809	2302	13·4	8072	2705	25·1
3	45	M		19420	2604	11·8	9141	3307	26·5
4	40	F		17202	1904	11·1	8774	3978	31·1
5	51	M		15005	1618	9·7	7517	2595	25·6
			Mean ± SD	16044 ± 2260	2261 ± 509		8681 ± 928	2997 ± 643	

*Residual substrate.
†Per cent conversion.
‡d.p.m./mg of protein.

Table 2. *In vitro* metabolism of $4{\cdot}96 \times 10^5$ d.p.m. (4·79 mμ moles) of progesterone-4-^{14}C (S.A. 47 μCi/μmol), $5{\cdot}47 \times 10^5$ d.p.m. (5·07 mμmoles) of pregnenolone-4-^{14}C (S.A. 49 μCi/μmol) and $4{\cdot}18 \times 10^5$ d.p.m. (5·13 mμmoles) of androstenedione-4-^{14}C (S.A. 37 μCi/μmol) by rabbit submaxillary gland homogenates incubated for 2 h at 39°C with a NADPH generating system

Group	No. of animals	Sex	Progesterone-4-^{14}C			Pregnenolone-4-^{14}C			Androstenedione-4-^{14}C		
			Progesterone*	20β-dihydro-progesterone	%†	Pregnenolone*	20β-dihydro-pregnenolone	%†	Androstene-dione*	Testosterone	%†
1	2	F	8617‡	1609	15·7	10942	427	3·8	8814	1478	14·4
2	2	F	6410	917	12·5	12305	218	1·7	6707	743	9·9
3	2	M	9215	1418	13·3	11774	376	3·1	7215	1178	14·0
4	2	F	7676	1264	14·1	10996	305	2·7	6147	1219	16·5
5	2	M	8419	894	9·5	11857	209	1·7	7331	819	10·0
6	2	M	9551	1517	13·7	11124	514	4·4	9176	1326	12·6
		Mean ± SD	8314 ± 1138	1269 ± 304		11499 ± 558	341 ± 120		7565 ± 1189	1127 ± 377	

*Residual substrate.
†Per cent conversion.
‡d.p.m./mg of protein.

Table 3. Recrystallization to constant specific activity of the radioactive material behaving like 20β-dihydroprogesterone and progesterone obtained from *in vitro* incubations of progesterone-4-^{14}C with human and rabbit submaxillary glands

	Specific activity (d.p.m./mg)*			
	Human experiments		Rabbit experiments	
	Mother liquor	Crystals	Mother liquor	Crystals
20β-Dihydroprogesterone				
Aqueous acetone	2357	2245	1714	1847
Aqueous methanol	2462	2302	1605	1772
Chloroform–ethanol (5 : 1) (acetylation)	2123	2399	1802	1694
Aqueous methanol	2215	2074	1724	1832
Progesterone				
Aqueous methanol	3627	3581	2623	2570
Cyclohexane–diethylether	3472	3427	2772	2666
Hexane–ethanol (3 : 1)	3519	3619	2549	2792
Aqueous acetone	3507	3595	2671	2594

* 10 mg of the authentic steroid was added before recrystallization.

authentic progesterone was observed; (e) recrystallized to constant specific activity (Table 3).

20β-Dihydropregnenolone. The radioactive material with an R_F of 0·13 in system A, obtained from pregnenolone incubations with rabbit tissue, was eluted and aliquots were: (a) counted; (b) rechromatographed in systems F and G where they behaved like authentic 20β-dihydropregnenolone (R_F values of 0·51 and 0·32, respectively); (c) acetylated to a material behaving like 20β-dihydropregnenolone acetate in system A (R_F 0·89). After hydrolysis a radioactive material with an R_F similar to 20β-dihydropregnenolone in system F was observed; (d) recrystallized to constant specific activity as the free compound and as its acetate (Table 4).

Pregnenolone. The radioactive material behaving like authentic pregnenolone in system A (R_f: 0·50) was eluted and aliquots were: (a) counted, (b) rechromatographed in systems B and D where a peak was observed, corresponding to the mobility of authentic pregnenolone (R_F 0·76 and 0·53, respectively); (c) acetylated to a material behaving like pregnenolone acetate, in system A (R_F = 0·95). After hydrolysis, this material had a mobility similar to pregnenolone in system E ($R_F = 0{\cdot}31$); (d) recrystallized to constant specific activity as the free compound and as its acetate (Table 4).

Testosterone. The radioactive material obtained from androstenedione incubations with identical mobility to authentic testosterone in system A after 10 h was eluted and aliquots were: (a) counted; (b) rechromatographed in systems B and E where they behaved like testosterone (R_F values of 0·52 and 0·15); (c) oxidized to androstenedione, as revealed by chromatography in system D (R_F = 0·30); (d) acetylated to testosterone acetate, as revealed by chromatography in system A ($R_F = 0{\cdot}84$). After hydrolysis this material behaved like testosterone in

Table 4. Recrystallization to constant specific activity of the radioactive material behaving like 20β-dihydropregnenolone and pregnenolone obtained from *in vitro* incubations of pregnenolone-4-^{14}C with human and rabbit submaxillary glands

	Specific activity (d.p.m./mg)*			
	Human experiments		Rabbit experiments	
	Mother liquor	Crystals	Mother liquor	Crystals
20β-Dihydropregnenolone†				
Petroleum ether	—†	—	1317	1462
Aqueous ethanol	—	—	1214	1229
Cyclohexane–methanol (acetylation)	—	—	1425	1410
Aqueous acetone	—	—	1345	1307
Pregnenolone				
Aqueous methanol	2915	3032	2528	2642
Aqueous acetone	2801	2921	2649	2477
Aqueous ethanol (acetylation)	2972	3018	2577	2525
Aqueous methanol	2894	3057	2694	2489

*10 mg of the authentic steroid was added before recrystallization.
†Not detected in human tissue experiments.

system E ($R_F = 0{\cdot}15$); (e) recrystallized to constant specific activity as the free compound and as its acetate (Table 5).

Androstenedione. The material behaving like androstenedione in system A after 10 h was eluted and aliquots were: (a) counted; (b) rechromatographed in systems B and D where they behaved like authentic androstenedione (R_F values of 0·92 and 0·30, respectively); (c) oxidized and rechromatographed in system D, where they behaved like androstenedione; (d) acetylated and rechromatographed in system A, where they behaved like authentic androstenedione; (e) recrystallized to constant specific activity (Table 5).

DISCUSSION

In the present paper it has been demonstrated that rabbit and human submaxillary glands *in vitro* have 17β (C_{19} steroid) and 20β steroid reductase activities.

When rabbit submaxillary gland homogenates were incubated with labeled progesterone or pregnenolone the 20β-dihydrogenated derivative was formed (Table 2). The percent conversion varied from 9·5 to 15·7% for 20β-dihydroprogesterone and from 1·7 to 4·4% for 20β-dihydropregnenolone, respectively.

In a similar way, human submaxillary gland homogenates converted labeled progesterone to 20β-dihydroprogesterone with a percent conversion of 9·7 to 17·3% (Table 1). However, when pregnenolone-4-^{14}C was used as substrate no radioactive material behaving like 20β-dihydropregnenolone was obtained and the substrate was recovered unchanged.

Both human and rabbit submaxillary gland homogenates, when incubated *in*

Table 5. Recrystallization to constant specific activity of the radioactive material behaving like testosterone and androstenedione obtained from *in vitro* incubations of androstenedione-4-^{14}C with human and rabbit submaxillary glands

	Specific activity (d.p.m./mg)*			
	Human experiments		Rabbit experiments	
	Mother liquor	Crystals	Mother liquor	Crystals
Testosterone				
Aqueous ethanol	3429	3225	2774	2635
Cyclohexane–acetone	3274	3412	2629	2742
Aqueous methanol (acetylation)	3421	3332	2812	2622
Aqueous acetone	3372	3505	2759	2718
Androstenedione				
Aqueous acetone	3828	3805	2423	2402
Aqueous ethanol	3945	3826	2617	2414
Cyclohexane–acetone	3779	3940	2572	2613
Aqueous methanol	3837	3921	2518	2578

*10 mg of the authentic steroid was added before recrystallization.

vitro with androstenedione-4-^{14}C, biosynthesized a material behaving like testosterone. The percent conversion varied from 19·5 to 31·1% for human (Table 1) and from 9·9 to 16·5% for rabbit (Table 2) submaxillary glands.

No differences between sexes were observed either in human or rabbit experiments.

There are striking differences between human and rabbit enzymatic activity and that observed when rat submaxillary glands were incubated.

The rat tissue converted labeled progesterone to 17α-hydroxyprogesterone and androstenedione in the female and to androstenedione in the male[1]. When male rat submaxillary homogenates were incubated with 17α-hydroxyprogesterone-4-^{14}C a radioactive material behaving like androstenedione was obtained [1]. Male and female tissue converted pregnenolone to progesterone and dehydroepiandrosterone.

Charreau and Villee observed that the submaxillary gland of the rat in organ culture took up progesterone and converted it to 5α-pregnane-3,20-dione and to other unidentified compounds more polar than progesterone[3].

Recently it has been demonstrated that the rat submaxillary gland has all the necessary enzymes to perform *de novo* synthesis of cholesterol from acetate and when cholesterol-4-^{14}C was used as substrate, pregnenolone and dehydroepiandrosterone were obtained[2].

Furthermore, the submaxillary glands of the rat have sexual differences in structure and exocrine production[11, 12], these properties not being shared by human or rabbit species.

The fact that only 20β-reductase activity was found when progesterone was incubated with rabbit submaxillary glands is in contrast to the presence of en-

zymes capable of 20α- and 20β-reduction, 3β-reduction, 5α-reduction and 6β-hydroxylation reported for rabbit kidney homogenates[13]. Incidentally no 20β-reductase activity was detected in the liver or mammary gland[13].

No 20α-reductase activity was observed in human and rabbit submaxillary glands, an enzyme which, on the other hand, has been found to be active in several other tissues[14].

Human and rabbit submaxillary glands have a nearly similar pattern of metabolism when incubated with labeled progesterone, pregnenolone or androstenedione. The only exception was the 20β-reduction of pregnenolone by the rabbit tissue, not shared by the human one. There are also similarities in the excretory products of progesterone isolated from human and rabbit urine[15]. In this sense, the rabbit could serve as an experimental model to determine the *in vivo* role of the human submaxillary gland in the metabolism of steroids.

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