Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 1455–1458. Pergamon Press. Printed in Great Britain

METABOLISM OF ESTRONE AND PROGESTERONE *IN VITRO* IN HUMAN SALIVA

TAWFIK M. A. ELATTAR

Laboratory of Hormone Research, University of Missouri School of Dentistry, Kansas City, Missouri 64108, U.S.A.

(*Received* 21 *June* 1974)

SUMMARY

[2,4,6,7-^{3}H]-estrone and [1,2,6,7-^{3}H]-progesterone were found to be metabolized by saliva of patients with chronic gingival inflammation. No metabolites were detected in saliva of subjects with clinically normal gingiva. By paper and thin-layer chromatography, isotope dilution, and the preparation of acetates and methoximes, the following metabolites were identified: estradiol-17β as the only metabolite of estrone; 5α- and 5β-pregnanediones as metabolites of progesterone.

INTRODUCTION

Rodent submandibular glands have the capacity to metabolize steroid hormones. Rat submandibular glands have been shown to convert progesterone to 5α-pregnane-3,20-dione and 4-androstene-3,17-dione [1, 2]. Mouse submandibular gland homogenates fortified with NADPH converted 4-androstene-3,17-dione predominantly to androsterone and, to a lesser extent, testosterone, 17β-hydroxy-5β-androstan-3-one and 5α-androstane-3α, 17β-diol [3].

When [4-^{14}C]-testosterone was infused into the blood supply of the submandibular gland of an adult male dog, four-fifths of the metabolite radioactivity was associated with a simple product, 4-androstene-3, 17-dione [14].

Since the saliva constitutes the external environment of the teeth and mucosa and its significance in the maintenance of health and disease of these tissues has been established, it seemed of interest to study the metabolism of representative steroid hormones, progesterone and estrone in normal human saliva and in saliva collected from patients with chronic gingival inflammation. To our knowledge, the metabolism of hormonal steroids in saliva has not been reported in the literature.

MATERIALS AND METHODS

Chemicals. [2,4,6,7-^{3}H]-estrone (114 Ci/mmol) and [1,2,6,7-^{3}H]-progesterone (80–100 Ci/mmol) were purchased from New England Nuclear Corp. and purified by chromatography on paper in solvent systems number 2 and 3. Coenzymes were products of Sigma Chemical Co. The reference steroids were purchased from Sigma Chemical Co. Reagents used were of analytical grade.

Saliva. Whole saliva samples were collected from four subjects. Two subjects with normal healthy teeth and gingiva, and two subjects with chronic gingival inflammation and advanced periodontal disease. Saliva was collected by means of spitting in sterilized 15 ml glass vials with screw caps.

Incubation. Three ml of saliva were added to 3 ml of Krebs–Ringer phosphate buffer (pH 7·4) with the following additions: NAD, 1 μmol; NADH, 1 μmol; NADP 1 μmol; glucose 6-phosphate, 12 μmol; glucose 6-phosphate dehydrogenase, 2 units; penicillin, 100 units; streptomycin, 1000 μg; and Fungizone 25 μg. Immediately prior to incubation the saliva, buffer, cofactors and antibiotics were added to 50 ml Erlenmyer flasks containing 50 μCi of [2,4,6,7-^{3}H]-estrone or 25 μCi of [1,2,6,7-^{3}H]-progesterone dissolved in 0·2 ml of propylene glycol. Control experiments were carried out by incubating radioactive substrates in buffer medium with boiled saliva and cofactors.

Extraction. The incubation media were extracted with a mixture of diethylether–chloroform (3:1, v/v). The organic extracts were evaporated to dryness at 45°C under reduced pressure.

Chromatography. The residues were subjected to paper and thin-layer chromatography. Four solvent systems were used in paper chromatography: Solvent System No. 1: Ligroin–propylene glycol [5]. Solvent System No. 2: Benzene–formamide [6]. Solvent System No. 3: Hexane–formamide [6]. Solvent System No. 4: 2,2,4-trimethylpentane–methanol–H_2O (100:91:9, by vol) [7].

Thin-layer chromatography was performed on 20 × 5 cm. glass plates coated with Merck silica gel GF-254 (thickness 3/8 mm). Two solvent systems were used for thin-layer chromatography: Solvent system No. 5: Chloroform–ether (9:1, v/v). Solvent system No. 6: Benzene–ethyl acetate (185:15, v/v).

After development, the distribution of radioactivity on paper and thin-layer chromatograms were monitored by a Packard Radiochromatogram Scanner 7201.

Reference compounds of estrogens were detected by treatment of paper strips with 1% ferric chloride and 1% potassium ferricyanide [8]. Estrogens on thin layer chromatograms were detected by subjecting the chromatograms to iodine vapor. Progesterone was detected by examining paper and thin layer chromatograms under a mineral light UVS 12 lamp. The diketones were stained with 2,4-dinitrophenylhydrazine in HCl [9].

Isotopic dilutions. In addition to comparison of chromatographic behaviors, identification of metabolites was established by dilution with carrier steroids and crystallization to constant specific activity [10].

Measurement of radioactivity. Tritium was determined in a Packard Tri-Carb liquid scintillation spectrometer Model 3320. Samples were counted in toluene containing 5 g PPO and 0·3 g dimethyl-POPOP/l. The efficiency for counting ^3H in the toluene scintillator was 45%.

Preparations of derivatives. The acetates were prepared by reaction of steroids with a mixture of acetic anhydride–pyridine (1:1 v/v) at room temperature overnight and then recrystallization from suitable solvents. The O-methoximes were prepared by reaction of steroids with methoxyamine hydrochloride as described [11], and then recrystallized from suitable solvents.

RESULTS

Incubation with [2,4,6,7-^3H]-estrone. Incubation of [2,4,6,7-^{3}H]-estrone with saliva of a clinically healthy subject did not result in any transformation products. On the other hand estradiol-17β was the only conversion product of [2,4,6,7-^{3}H]-estrone in saliva of a patient with chronic gingival inflammation Table 1.

Identification of unmetabolized [2,4,6,7-^3H]-estrone. The radioactive extracts of incubation media of [2,4,6,7-^{3}H]-estrone with normal and inflamed saliva were chomatographed on two strips of Whatman filter paper No. 1 in solvent system No. 2 with reference standards of estrone, estradiol-17β, and estriol, on a separate paper strip, for two and a half hours. A major radioactive peak corresponding to a standard of estradiol-17β (R_F 0·3) was detected only on the radiochromatogram of the extract of saliva collected from a patient with chronic gingival inflammation. Traces of radioactivity were detected on the origin of each radiochromatogram and corresponded to a reference standard of estriol. The radioactive peaks corresponding to estrone reference standard were eluted, acetylated, and the acetates were chromatographed on silica gel thin layer in solvent system No. 6 for 45 min. One single peak of radioactivity which corresponded to a reference standard of estrone acetate (R_F 0·3) was found on each chromatogram. The radioactive peaks were eluted with methanol and aliquots were examined by reverse isotope dilution with estrone acetate.

Table 1. *In vitro* conversion of [2,4,6,7-^{3}H]-estrone in saliva of a clinically healthy subject and a subject with chronic gingival inflammation

Experimental step	Normal saliva	Inflamed saliva
Saliva volume (ml)	3	3
Radioactivity incubated (μCi)	50	50
Radioactivity recovered (μCi)	48·9	47·3
Percentage of recovery	97	94
Radioactivity corresponding to estrone standard (μCi)	47·9	44
Radioactivity corresponding to estradiol-17β standard (μCi)	none	1·82
Radioactivity corresponding to estriol standard (μCi), and which remains in first chromatography in solvent system No. 2.	0·1	0·5

Table 2. Radiochemical purity of estradiol-17β in saliva of a subject with chronic gingival inflammation

Solvents	Crystallization number	Specific Activity (d.p.m./μmol) Crystals	Mother Liquor
Acetone–pentane	1	2814	2884
Acetone–hexane	2	2729	2751
Acetone–water	3	2748	2741
Methanol–water	4	2796	2734
Dichloromethane–hexane	5	2774	2679
Ethanol-pet. ether	6	2808	2718

Estradiol-17β-diacetate (25 mg) was added to an aliquot containing 353,550 d.p.m. of ^{3}H from the area of silica gel thin layer radiochromatogram, corresponding to estradiol-17β-diacetate. The calculated specific activity was 2901 d.p.m./μmol. The data indicate that 95% of the ^{3}H examined could be in estradiol-17β-diacetate.

Identification of metabolites of [2,4,6,7-^3H]-estrone in inflamed saliva. The radioactive material from the area corresponding to a reference standard of estradiol-17β in the first chromatogram was eluted, acetylated and chromatographed on silica gel thin layer in system No. 6 for 45 min. A single peak of radioactivity corresponding to a reference standard of estradiol-17β-diacetate (R_F 0·5) was detected. The radioactivity was eluted and final identification was accomplished by means of reverse isotope dilution technique as shown in Table 2.

The diffused pattern of radioactivity remaining on the origin and which constituted 1·5% of the chromatographed radioactivity did not warrant its further identification.

Incubation with [1,2,6,7-^3H]-progesterone. [1,2,6,7-^{3}H]-progesterone was incubated with saliva of a clinically healthy subject and a patient with chronic gingival inflammation (Table 3). The radioactive extracts of incubation media were chromatographed on two strips of Whatman filter paper No. 1 in solvent system No. 3 for a period of 3 h. Reference standards of progesterone and 5α-pregnanedione were chromatographed simultaneously on a separate paper strip. The radioactive extracts of incubation media

Table 3. *In vitro* conversion of [2,4,6,7-^{3}H]-progesterone in saliva of a clinically healthy subject and a subject with chronic gingival inflammation

Experimental step	Normal saliva	Inflamed saliva
Saliva vol.	3 ml	3 ml
Radioactivity incubated (μCi)	25	25
Radioactivity recovered (μCi)	23·9	22·4
Percentage recovery	95	90
Radioactivity corresponding to unconverted progesterone (μCi)	22·4	19·6
Radioactivity corresponding to 5α-pregnanedione (μCi)	no conversion	0·27
Radioactivity corresponding 5β-pregnanedione (μCi)	no conversion	1·77
Ratio of 5α-pregnanedione to total radioactivity chromatographed		0·012
Ratio of 5β-pregnanedione to total radioactivity chromatographed		0·079

of normal saliva showed only one peak of radioactivity which corresponded to a reference standard of progesterone (R_F 0·5). The radioactive extracts of incubation media with inflamed saliva showed, in addition to a progesterone peak, a second peak which corresponded to a reference standard of 5α-pregnanedione (R_F 0·7). The amounts of radioactivity incorporated into the radioactive peaks of progesterone and its metabolites in paper chromatograms, are shown in Table 3.

Identification of unmetabolized [1,2,6,7-^{3}H]-progesterone in normal and inflamed saliva. The radioactive peaks, on the two chromatograms, which corresponded to a reference standard of progesterone were eluted with methanol and the eluates were chromatographed on paper in solvent system No. 4 for 3 h. One radioactive peak corresponding to a standard of progesterone (R_F 0·3) was detected. The radioactivity, corresponding to progesterone, was eluted, acetylated and rechromatographed on silica gel thin layer in solvent system No. 5 for 45 min. One peak of radioactivity corresponding to a reference standard of progesterone (R_F 0·5) was found. The final identity of progesterone was established by means of reverse isotope dilution technique.

Table 4. Radiochemical purity of metabolites of progesterone in inflamed human saliva

Solvents	No. of Crystns	S.A. (d.p.m./μmol)	Calculated S.A. %
5α-Pregnanedione Acetone–pentane	3	7331	94
Dimethoxime Cl_2CH_2–hexane	3	5680	89
5β-Pregnanedione Acetone–hexane	3	45646	87
Dimethoxime Cl_2CH_2–hexane	3	35009	96

The identification of metabolites of [1,2,6,7-^{3}H]-progesterone in inflamed saliva. The radioactive peak corresponding to a reference standard of 5α-pregnanedione (R_F 0·7), on the initial paper chromatogram, and which contained 9% of the chromatographed radioactivity, was eluted and rechromatographed on paper in system No. 1 for a period of 2·5 h. Two radioactive peaks corresponding to reference standards of 5α-pregnanedione (R_F 0·6) and 5β-pregnanedione (R_F 0·9) were detected. The radioactive peaks were eluted and further identification was done by reverse isotope dilution technique of the free compounds and their dimethoximes as shown in Table 4.

DISCUSSION

The *in vitro* metabolism of [2,4,6,7-^{3}H]-estrone and [1,2,6,7-^{3}H]-progesterone in human saliva collected from patients with chronic gingival inflammation was mainly reductive. In presence of pyridine nucleotide cofactors, the major metabolites detected were estradiol-17β, and 5α- and 5β-pregnanediones. The conversion demonstrates the activity of 17β-hydroxysteroid dehydrogenase, 5α- and 5β-reductases.

The main source of metabolic activity in saliva is associated with the oral leukocyte protoplasm [12], the epithelial cell fraction [13] or due to the bacterial content [14]. Leukocytes regularly found in human saliva generally increase in number during development of gingivitis [15].

The failure to detect, in current experiments, any conversion products of [2,4,6,7-^{3}H]-estrone and [1,2,6,7-^{3}H]-progesterone in saliva of subjects with healthy gingiva might indicate, indirectly, the association of the steroid enzymes, 17β-hydroxysteroid dehyphogenase, 5α- and 5β-reductases with the oral leukocytes.

Further research on the efficiency of oral leukocytes and epithelial cells to metabolize steroid hormones is needed to explain the source for steroid metabolism in saliva. The possibility of bacterial action on steroid hormones metabolism is remote since antibiotics, penicillin, streptomycin, and fungizone were used in saliva incubations.

Acknowledgements—The author is grateful to Mr. Anil Patel for his expert technical assistance. This work was supported by PHS Research Grant DE-03031-03 from the National Institute of Dental Research.

REFERENCES

1. Rosner J. M., Camara S. A., Cardinali D. D. and Dieguez M. E.: *Acta physiol. latino am.* **15** (1965) 221–223.
2. Rosner J. M., Mocome J. C. and Cardinali D. P.: *Endocrinology* **85** (1969) 1000–1003.
3. Coffey J. C.: *Steroids* **22** (1973) 247–257.
4. Winer A. L., Ofner P. and Sweeney E. A.: *Endocrinology* **87** (1970) 406–409.
5. Savard K.: *J. biol. Chem.* **202** (1953) 457–477.
6. Zaffaroni A. and Burton R. B.: *J. biol. Chem.* **193** (1951) 749–767.

7. Bush I. E.: *Biochem. J.* **50** (1951) 370–378.
8. Burton R. B., Zaffaroni A. and Keutmann E. H.: *J. biol. Chem.* **188** (1951) 763–771.
9. Axelrod L. R.: *J. biol. Chem.* **205** (1953) 173–184.
10. Axelrod L. R., Matthijssen C., Goldzieher J. W. and Pulliam J. E.: *Acta endocr. Copenh.* suppl. 99 **49** (1965) 7–77.
11. Fales H. M. and Luukkainen T.: *Analyt. Chem.* **37** (1965) 955–957.
12. Eichel B. and Lisanti V. F.: *Archs oral Biol.* **9** (1964) 299–314.
13. Tonzetich J. and Friedman S. D.: *Ann. N. Y. Acad. Sci.* **131**, Part 2 (1965) 815–829.
14. Tatevossian A. and Jenkins G. N.: *Archs oral Biol.* **14** (1969) 1121–1123.
15. Astrup T., Henrichsen J. and Kwaan H. C.: *Blood* **29** (1967) 134–138.