Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 135–137. Pergamon Press. Printed in Great Britain

# THE PRESENCE OF 6β-STEROID HYDROXYLASE IN HUMAN CORNEA

*Alfredo J. Gallegos, †Primo Delgado Partida and ‡Pedro Garzón

*División de Biología de la Reproducción, Unidad de Investigación Científica del Centro Médico Nacional—IMSS, Apartado Postal 66-737, México 12, D.F. †Departamento de Oftalmología, Centro Médico La Raza—IMSS, México, D.F. ‡Sección de Biología Molecular, División de Biología de la Reproducción, Unidad de Investigación Científica del Centro Médico Nacional—IMSS, México, D.F., México

(*Received* 17 *April* 1975)

SUMMARY

Human cornea were excised at the time of surgical procedure from patients undergoing radical surgery. Three separate incubations were performed in Eagle's minimum essential medium with [4-$^{14}$C]-progesterone as precursor for five days. From the initial radioactivity, 6·9% was biotransformed to 6β-hydroxy-4-pregnene-3,20-dione. This steroid was identified by paper chromatography, derivative formation, and recrystallizations to constant S.A.

## INTRODUCTION

The use of topically applied natural and synthetic anti-inflammatory steroids for treatment of several ocular conditions [1–5], and our initial studies of steroid metabolism by corneal tissues [6], prompted us to explore the existence of a hydroxylating mechanism which could turn hydrophobic steroid hormones such as progesterone into more polar compounds (hydrophilic), and thus partially explain the transport and distribution of relatively water insoluble compounds such as steroid molecules into the aqueous physiological media.

Hydroxylation at the C-6 position of the steroid molecule has been shown to occur in different tissues such as placenta [7, 8], liver [9], kidney [10], adrenal slices [11], neoplastic testicular tissue [12], and skin [13]. Also the 6β-hydroxy steroid hormone derivatives have been isolated from human urine [14–16].

Our present results demonstrate the formation of 6β-hydroxy-4-pregnene-3,20-dione by human cornea incubations utilizing progesterone as precursor.

## EXPERIMENTAL PROCEDURE

Three human cornea were taken at the time of ocular surgery. After rinsing with warm saline solution, they were placed separately into culture media flasks containing Eagle's growth minimum essential medium [17] supplemented with penicillin 100 U/ml, streptomycin sulfate 100 μg/ml, 10 mmol glutamine, and [4-$^{14}$C]-progesterone (S.A. 52·8 mCi/mmol) in a final concentration of 13·3 nmoles/incubation flask. The radioactive precursor was solubilized in the growth media by the use of 2 μl/ml of propylene glycol [19].

In addition to the corneal samples, suitable controls both for sterilization and serum media effects on the progesterone molecule were incubated at 37°C for a 5-day period in an atmosphere of 95% air-5% $CO_2$. Metabolic processes were stopped by the addition of 3 vol. of warm acetone (40°C). The extraction procedure as well as the criteria for steroid metabolites identification have been described elsewhere [13, 18].

[4-$^{14}$C]-Progesterone was obtained from New England Nuclear (S.A. 52·8 mCi/mmol) and purified by descendent paper chromatography in the hexane–formamide system [18]. 6β-Hydroxy-4-pregnene-3,20-dione, was a gift from Upjohn Co., Kalamazoo, Mich. Purification was achieved by repeated crystallizations in 70% methanol. A solution was prepared from which 50 μg was used as radioinert carrier both in side by side and in mixed chromatograms. Also 20 mg were used to dilute the radioactive compounds before crystallization to constant S.A.

Radioactivity was detected in the chromatograms with an Actigraph III (Nuclear Chicago). Quantification of radioactivity was assessed by a Mark II liquid scintillation spectrometer.

## RESULTS

No fungal or bacterial contamination was detected. Also, controls without tissue revealed two radioactive areas after chromatography in hexane–formamide system, one at the origin and the other migrating with pure carrier progesterone (50 μg) as revealed by its absorbance under U.V. light. The first accounted for 8% and the second 92% of the total recovered radioactivity.

The corneal organic extracts revealed [6] five separate radioactive areas when chromatographed in hexane–formamide system. Four of them were identi-

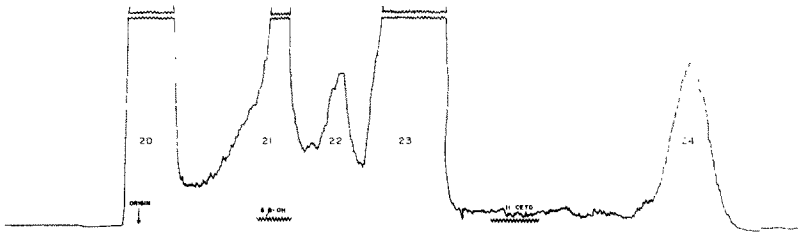


Fig. 1. Represents a paper radiochromatogram developed in benzene formamide system after pooling the most polar fractions obtained from the three human corneas. Fractions I, III and IV are in the process of final identification; II and V correspond respectively to 6β-hydroxy-pregnene-3,20-dione and 4-pregnene-3,20-dione.

fied previously, the exception being those compounds remaining at the origin of the chromatograms:

I Origin (propositus)
II 20α-hydroxy-4-pregnene-3,20-dione
III 20β-hydroxy-4-pregnene-3,20-dione
IV 4-pregnene-3,20-dione
V 5α-Pregnan-3,20-dione.

The most polar fraction (I) from the three corneal samples averaged 41·59 of the original incubated radioactive progesterone. Once they were pooled ($1{\cdot}94 \times 10^6$ d.p.m.), another chromatogram was performed in benzene–formamide system.
Five radioactive areas were detected (see Fig. 1):

I Origin
II 6β-hydroxy-4-pregnene-3,20-dione
III Unknown
IV Unknown
V 4-pregnene-3,20-dione.

Peaks I, III, and IV represented 28·5% of the initial radioactivity and are in the final process of identification. Peak II accounted for 6·91% of the original radioactivity and migrated with an $R_F$ of 0·34 in the hexane–benzene (1:1 v/v) system. Upon oxidation with 0·5% $CrO_3$ in 90% acetic acid at room temperature (20°C) 4-pregnene-3,6,20-trione was obtained and had an $R_F$ of 0·6 in benzene–formamide system with mobility identical to 50 μg of the pure reference compound. The acetylated derivative had an $R_F$ of 0·65 as the reference compound in the hexane–formamide system.

Table 1. Recrystallizations of 6β-hydroxy-4-pregnene-3,20-dione to constant specific activities

| | Values presented in d.p.m./mg Recrystallizations | | | | |
|---|---|---|---|---|---|
| | First | Second | Third | Fourth | Fifth |
| | 431 (A) | 368 (B) | 250 (C) | 240 (C) | 246 (C) |
| Mother liquors d.p.m. | 3664 | 1540 | 770 | 736 | 742 |

Solvents used to recrystallize are indicated in parentheses: (A) 70% Methanol (B) 100% Hexane (C) Hexane–Benzene (1:1).

Four recrystallizations to constant S.A. were performed with pure 6β-hydroxy-4-pregnene-3,20-dione in different solvents. Results are depicted in Table 1.

Peak V accounted for 7·4% of the initial radioactivity after paper chromatography in benzene–formamide system. It had mobility identical to the radioinert 4-pregnene-3,20-dione in both hexane–benzene (1:1 v/v) and in benzene–formamide systems. This compound was not acetylable using acetic anhydride–pyridine reaction.

## DISCUSSION AND CONCLUSIONS

Previous studies of the biotransformation of progesterone by primary explants [13], tissue culture cells [13], minced organ culture *in vitro*, neoplastic tissues [12], and adrenal slices [11] have shown the presence of hydroxylating mechanisms at the C-6 position of the progesterone molecule. Other experiments performed *in vitro* and *in vivo* [10, 14–16] using cortisol as precursor also indicate the presence of this hydroxylating system which has been labelled as an important metabolic process.

The results presented here indicate the formation of 6β-hydroxy-4-pregnene-3,20-dione from progesterone after a 5-day *in vitro* incubation period with human corneal tissue. Other metabolic products have not been fully identified; they represent 28·5% of the initial recovered radioactivity.

Further experiments now in progress using anti-inflammatory steroids incubated with human corneal and other tissues might indicate the importance of this hydroxylating mechanism in the formation of partially water soluble compounds from practically water insoluble steroids thus partially explaining some of the diffusion and transport of this type of compounds in the human eye.

## REFERENCES

1. Yotsuyanagi T. and Higuchi W. I.: *J. Pharm. Pharmacol.* **24** (1972) 934–941.
2. Cleary G. W. and Gatz J. L.: *Colloid Interface Sci.* **45** (1973) 507–511.
3. Toczolowski J.: *Polish Med. J.* **X** No. 3 (1971) 757–763.
4. Sanchez J. and Polak F. M.: *Invest. Ophthal.* **13** (1974) 17–22.

5. Cox W. V., Kupferman A. and Leibowitz H. M.: *Archs Ophthal.* **88** (1972) 308–313.
6. Garzón P., Delgado-Partida P. and Gallegos A. J.: *J. steroid Biochem.* (in press).
7. Berliner D. L. and Shalhanik H. A.: *J. clin. Endocr. Metab.* (1956) 903–904.
8. Hagopian M., Pincus G., Carlo J. and Romanoff E. B.: *Endocrinology* **58** (1956) 387–388.
9. Cohn G. L., Upton U. and Bondy P. K.: *J. clin. Endocr. Metab.* **21** (1961) 1328–1330.
10. Lipman M. M., Katz F. H. and Jailer J. W.: *J. clin. Endocr. Metab.* **22** (1962) 268–272.
11. Touchtone J. C., Kasparow M. and Rosenthal: *Fedn Proc.* **18** (1959) 340.
12. Domínguez O. V.: *J. clin. Endocr. Metab.* **21** (1961) 663–674.
13. Garzón P. and Berliner D. L.: In *Advances in Endocrinology.* Proc. of the VII Pan-American Congress of Endocrinology (Edited by E. Mattar). Excerpta Medica (1970) pp. 153–159.
14. Ulstrom R. A., Colle E., Burley and Gunville R.: *J. clin. Endocr. Metab.* **20** (1960) 1080–1094.
15. Frantz A. G., Katz F. H. and Jailer J. W.: *J. clin. Endocr. Metab.* **21** (1961) 1290–1303.
16. Katz F. H., Lipman M. M., Frantz A. G. and Jailer J. W.: *J. clin. Endocr. Metab.* **22** (1962) 71–77.
17. Eagle H.: *Science* **130** (1959) 432–437.
18. Zaffaroni A.: *Recent. Prog. Horm. Res.* **8** (1950) 51–86.
19. Berliner D. L. and Ruhman A. G.: *J. Invest. Derm.* **49** (1967) 117–122.