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PROGESTERONE METABOLISM BY HUMAN CORNEA

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SUMMARY

Human cornea excised from patients with wounded eyes were incubated *in vitro* for a 5-day period in the presence of [4-^{14}C]-progesterone. The following C_{21} steroid metabolites were identified by paper chromatography, derivative formation, and crystallizations to specific activities: 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, and 5α-pregnan-3,20-dione.

INTRODUCTION

Topically administered corticosteroids are widely used in the treatment of ocular inflammatory disease [1–5]. Although there are experimental data indicating optimal steroid concentrations and frequencies of administrations on corneal wounds, nevertheless [6–8] cataracts and glaucoma are the main ophthalmological complications that develop after the prolonged use of corticosteroids [2, 9].

Histological and histochemical data obtained after long-term topical application of corticosteroids reveal changes in epithelia continuity, cell proliferation and mucopolysaccharides content in corneal stroma [6, 7, 10]. However, no data are available at the present time in relation to steroid biosynthesis and biotransformation in this tissue.

Coenzymes, necessary for steroid oxidation-reduction reactions (NADP and NADPH), are formed in corneal tissues via the pentose phosphate pathway [11–15]. Therefore, in theory, ketonic groups present at the C-3 and C-20 positions of [4-^{14}C]-progesterone and double bonds in position 3–4 could be reduced, if the proper enzymes were present in the tissue.

EXPERIMENTAL PROCEDURE

The corneal samples excised from patients who underwent radical surgery of wounded eyes were taken at the time of surgical procedure.

After immediate rinsing with warm saline solution, each specimen was immersed in warm Eagle's minimum essential medium supplemented with penicillin 100 U/ml, 10 mM glutamine, streptomycin sulfate 100 μg ml [16], and 0.7 μCi of [4-^{14}C]-progesterone (equivalent to 13.3 nmol) as substrate.

[4-^{14}C]-Progesterone (S.A. 52.8 mCi/mmol) was purified by paper chromatography [17] and then dissolved in Eagle's growth media containing 2 μl per ml of propylene glycol per ml of Eagle's growth media [18].

Separate corneal samples and suitable controls for both sterilization and serum media effects on the progesterone were performed by allowing them to grow 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 acetone was then removed by rotatory evaporation at 50 C. The remaining aq. phase of each sample was then extracted 4 times with 2 vol. of chloroform. The chloroform extracts were evaporated *in vacuo* and the residue was resuspended in chloroform–methanol (1:1 v/v). Radioactive contents of chloroform–methanol sample extracts were estimated. The following purified steroids (50 μg of each) were added as indicators and then run separately in parallel chromatograms: 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, and 4-pregnen-3,20-dione in Zaffaroni's hexane formamide system [17]. In order to distinguish between different fractions, the chromatograms were scanned with an Actigraph III (Nuclear Chicago).

The radioactive zones were eluted with warm methanol and counted in a liquid scintillation spectrometer (Nuclear Chicago Mod. Mark II). Visualization of carrier steroids on paper chromatograms was made from their absorbance under U.V. lamp (340 nm) and with Zimmerman color reaction for pregnanediones.

The following criteria were used to identify the [4-^{14}C]-progesterone metabolites:

(a) Derivative formation by acetylation with acetic anhydride and pyridine and oxidation by CrO_3 [19].

(b) Chromatography in different solvent systems with 50 μg of the pure and appropriate carriers [17, 20].

(c) Recrystallizations to constant S.A. with 50 or 20 mg of pure reference compounds [17, 20].

RESULTS

Recovery of the different fractions after paper chromatography in hexane–formamide system is described in Table 1.

Figure 1 represents the typical radioactive peaks obtained after hexane–formamide paper chromatography of the three processed human corneal tissues. Five areas were eluted, corresponding to:

(I) Unidentified polar steroid metabolites (origin).
(II) 20α-hydroxy-4-pregnen-3-one.
(III) 20β-hydroxy-4-pregnen-3-one.
(IV) 4-pregnen-3,20-dione.
(V) 5α-pregnan-3,20-dione.

The most polar fraction (I) represented an average of 46.9% of the recovered radioactivity. Further work is being carried out to identify these polar metabolites.

Fraction II represented an average of 12.42% of the recovered radioactivity and was identified as 20α-hydroxy-4-pregnen-3-one. This compound migrated slightly from the origin in hexane-formamide and was resolved by hexane overflow for 6 h. It ran identically with the purified reference compound, both in hexane (Fig. 2), and hexane–benzene systems. Oxidation with CrO_3 converted it to progesterone. Acetylation yielded a derivative which ran identically with the acetate of the reference compound. Crystallizations to constant S.A. were performed in 70% methanol, hexane–benzene (1:1 v/v), and hexane (Table 2).

Table 1. Distribution of radioactivity in five different areas after hexane formamide to the front in paper chromatography (values represented in d.p.m.)

Sample	Recovered radioactivity (d.p.m.)	I	II	III	IV	V
1 %	1,828,570	1,252,570 (68.5)	157,940 (8.64)	32,560 (1.78)	203,660 (11.13)	120,000 (6.56)
2 %	1,218,800	527,600 (43.29)	230,850 (18.94)	17,530 (1.44)	285,000 (23.38)	120,490 (9.89)
3 %	1,084,296	158,840 (14.65)	124,360 (11.47)	95,387 (8.8)	306,054 (28.23)	118,860 (10.96)

Fraction III represented 3.5% of the recovered radioactivity. It was identified as 20β-hydroxy-4-pregnen-3-one. This compound migrated midway between the origin and the progesterone area in the hexane-formamide system (Fig. 1.). It ran identically to the reference compound in both hexane and hexane-benzene systems. Progesterone was formed upon oxidation with CrO_3. The acetylated compound migrated to the front of the hexane chromatographic system. Crystallizations were performed in 70% methanol, hexane–benzene (1:1 v/v), and hexane (Table 2).

Fraction IV represented 19.23% of the recovered radioactivity. It was identified as 4-pregnen-3,20-dione (Fig. 1.). In hexane as well as heptane–formamide systems the radioactive compound migrated at the same rate as the reference compound. This compound could not be acetylated.

Fraction V represented 8.7% of the total recovered radioactivity. By dripping in hexane for 45 min., 5α-pregnan-3,20-dione was distinguished from progesterone (peak IV). The radioactive peak was found to correspond with the carrier and would not acetylate. It also resisted to oxidation with CrO_3. Crystallizations were performed in pure heptane (Table 2).

Control incubation demonstrated, first, that the progesterone was not transformed by enzymes which could be present in calf serum of the culture media and, secondly, that no bacteria or fungi were present throughout the experiment.

DISCUSSION AND CONCLUSIONS

The presence of 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, and 5α-pregnan-3,20-dione obtained after human corneal incubations with [4-^{14}C]-progesterone during a 5-day period demonstrates the existence of NADPH and dependent enzyme systems, similar to those previously reported to occur in other non-endocrine and/or target tissues [21–23].

Since radioactivity remaining at the origin of the hexane–formamide paper chromatography accounted

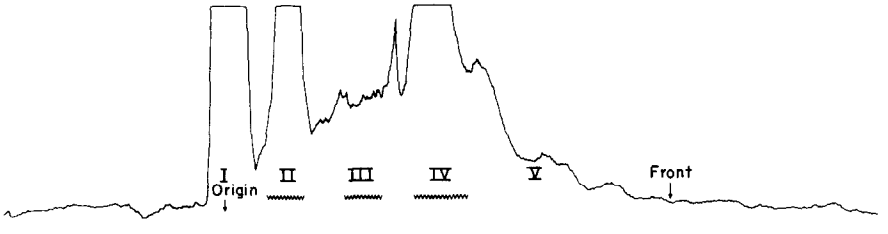

Fig. 1. Human cornea incubated for a 5-day period with [4-^{14}C]-progesterone. After hexane–formamide paper chromatography five radioactive areas were identified: (I) Unknown polar compounds, (II) 20α-hydroxy-4-pregnen-3-one, (III) 20β-hydroxy-4-pregnen-3-one, (IV) 4-pregnen-3,20-dione, (V) 5α-pregnan-3,20-dione.

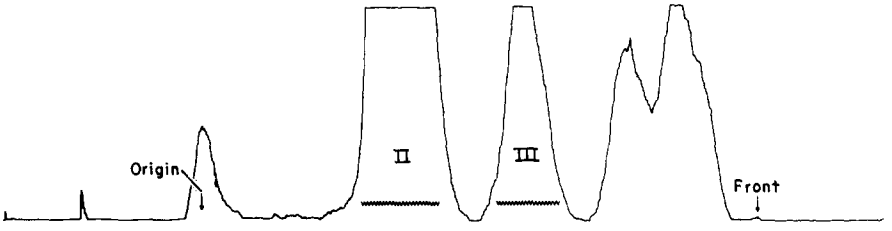

Fig. 2. Radioactive zones II and III resolved in hexane–benzene formamide system with identical mobility as: (1) 20α-hydroxy-4-pregnen-3-one. (2) 20β-hydroxy-4-pregnen-3-one.

Table 2. Identification of [4-^{14}C]-progesterone metabolites by crystallizations to constant S.A. (values are represented in d.p.m./mg)

Crystallizations	20α-hydroxy-4-pregnen-3-one (a)	20β-hydroxy-4-pregnen-3-one (b)	5α-pregnan-3,20-dione (c)
First	528	62	2041
Second	516	73	1747
Third	531	59	1672
Fourth	523	68	1840

Solvents:

(a) 70% methanol, hexane–benzene (50:50 v/v), 100% hexane.

(b) 70% methanol, hexane-benzene (1:1 v/v), 100% hexane.

(c) Heptane, heptane, heptane, heptane.

for 46.9% of the recovered radioactivity, it is expected that more polar steroid derivatives will be found.

Although the pentose phosphate pathway can account for up to 70% of the glucose utilization, only pyruvate → lactate, oxo-glutarate → isocitrate reactions and possible fatty acid synthesis have been recognized as NADPH dependent enzyme mediated reactions [12–14]. Since most of the steroid enzymatic reactions involve NADH and NADPH, it would be expected that biotransformation of corticosteroids by corneal layers might be an important area to be explored in an attempt to explain steroid induced cataracts and glaucoma, and cell proliferation and/or inhibition.

Also, further experiments using transcription and translation inhibitors as well as corneal tissues exposed for short periods of time to steroid hormone precursors will allow us to know if the described enzyme mediated reactions are due to naturally occurring or adaptive enzymes.

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