Metabolism of Progesterone-4-^{14}C *in Vitro* in Human Skin and Vaginal Mucosa*

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ABSTRACT: Progesterone-4-^{14}C was found to be actively metabolized by minces of human skin and vaginal mucosa. By paper and thin-layer chromatography, isotopic dilution, and the preparation of chromic acid oxidation products, acetates and methoximes, the following metabolites were identified: 5α-pregnane-3,20-dione, 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, 5α-pregnane-3β,20α-diol, Δ^4-pregnen-20α-ol-3-one, and 5α-pregnan-3α-20α-diol. The major metabolic pathways of progesterone in human skin and vaginal mucosa involve reduction of the 20-one to 20α-ol, reduction of the 3-one to 3α-ol and 3β-ol, and the saturation of the 4–5 double bond to form 5α-pregnanes. No 5β metabolites could be detected.

Human skin has now been shown to metabolize several steroid hormones. The interconversion of testosterone and androstenedione (Gomez and Hsia, 1968), estradiol and estrone (Frost *et al.*, 1966; Weinstein *et al.*, 1968), cortisol and cortisone (Hsia and Hao, 1966, 1967), and the reduction of the 20-one of cortisol and cortisone to 20α- and 20β-ols have been demonstrated to occur in skin from various anatomic sites. The conjugation *in vitro* of dehydroepiandrosterone to form its sulfate by human skin has also been demonstrated (Gallegos and Berliner, 1967; Faredin *et al.*, 1968). Whereas testosterone, a C-19 steroid with only two oxygen groups, is reduced in ring A to form several 5α-androstane derivatives in skin from various sites, the reduction of ring A of cortisol, a polyhydroxylated C-21 steroid, to form allodihydrocortisol occurred only in foreskin. From both compounds, only 5α metabolites were found in contrast to the systemic formation and urinary excretion of both 5α and 5β products (Fukushima *et al.*, 1960). Because of the differences of metabolism of cortisol in skin from various anatomic sites and the formation of only 5α reduction products from both testosterone and cortisol, it was of interest to determine the nature of the metabolism in human skin of progesterone,[1] a C-21 steroid like cortisol, but having only two oxygen groups, like testosterone.

Experimental Section

Chemicals. Progesterone-4-^{14}C (specific activity, 57.3 mCi/mmole) was purchased from New England Nuclear Corp. and its radiopurity verified with paper chromatography in systems PG and Bush A, described below. A stock solution was prepared to contain 10^6 dpm/0.1 ml of methanol and stored at −10°. Coenzymes were purchased from Sigma Chemical Co., methoxyamine hydrochloride from Eastman Organic Chemicals, and reference steroids from Mann Research Laboratories, Ikapharm (Ramat-Gan, Israel), and Steraloids, Inc. Δ^4-Pregnen-20α-ol-3-one was generously supplied by the Upjohn Co.

Specimens. Specimens of neonatal foreskin were obtained at circumcision; specimens of abdominal skin and vaginal mucosa, from adults during surgery. After removal of subcutaneous fat, skin samples were minced with fine scissors, kept on ice in cotton gauze moistened with a normal saline solution, and used within 1 hr.

Incubation. The medium consisted of 5 ml of Krebs–Ringer phosphate buffer (pH 7.4) containing NAD, 3.3 μmoles; NADH, 3.3 μmoles; NADP, 3.3 μmoles; glucose 6-phosphate dehydrogenase, 2 Kornberg units; glucose 6-phosphate, 12 μmoles; penicillin G, 500 units; streptomycin, 500 μg; and gentamycin sulfate, 200 μg. The minced tissue (200–300 mg) and progesterone-4-^{14}C (1.0×10^6 dpm in 0.1 ml of methanol) were added, and incubation was carried out for 4–5 hr at 37° in a Dubnoff shaking incubator.

Extraction. After incubations were terminated by the addition of 15 ml of methanol–dichloromethane (2:1), 100 μg each of carrier steroids (varied depending upon experiment) were added to each flask. Mixtures were then kept in a freezer at −10° overnight. Steroids were extracted and prepared for chromatography as previously described (Gomez and Hsia, 1968). The recovery of ^{14}C was greater than 93%.

Chromatography. The following chromatographic systems were used: (A) paper—system PG, ligroin–propylene glycol (Savard, 1953); system PE, ligroin–2-phenoxyethanol (Savard, 1954); and Bush system A, heptane–methanol–water (5:4:1) (Bush, 1952); and (B) thin layer (40 × 5 cm plates)—system TLC-CM-2, chloroform–methanol (98:2); and system TLC-CM-4, chloroform–methanol (96:4).

Reference compounds with an α,β-unsaturated ketone

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[1] The following trivial name is used: progesterone, Δ^4-pregnene-3,20-dione.

structure were visualized under short-wave ultraviolet light and diketones were stained with 2,4-dinitrophenylhydrazine in HCl (Axelrod, 1953). Diols were visualized either by exposure to iodine vapor or by the application of fuming H_2SO_4. Keto steroids were detected by a modified Zimmerman reaction using 2.5 N KOH in 90% aqueous methanol and 2% *m*-dinitrobenzene in ethanol and heating to 100–120°.

Isotopic Dilutions. Radioactive metabolites were located, eluted, diluted with carrier steroids, and recrystallized to constant specific activity according to established procedure (Hsia and Hao, 1966). The amount of radioactivity represented by each peak was expressed as a per cent of total radioactivity on the chromatogram (Figure 1).

Preparation of Derivatives. ACETATES. These were prepared by dissolving the steroids in a few drops of pyridine, adding an equal volume of acetic anhydride, and letting the mixture react overnight in a closed vessel at room temperature. Excess acetic anhydride was destroyed by adding ice water. The resulting precipitate was collected by filtration, washed with water, and then crystallized from suitable solvents. Steroids acetylated in microgram quantities were extracted by partitioning between water and dichloromethane.

O-METHOXIMES. Steroids were treated with methoxyamine hydrochloride as described by Fales and Luukkainen (1965) and the products extracted or precipitated with water as described for acetates. When milligram quantities of the steroid ketones were reacted to form methoximes, the completeness of the reaction was checked by gas chromatography (0.75% SE-30 column at 230°) and by demonstrating the disappearance of the absorption bands for ketone groups (at 5.88 μ) with infrared spectroscopy.

CHROMIC ACID OXIDATION. Secondary hydroxyl groups of steroids were oxidized to ketone groups with chromic acid as described by Lieberman *et al.* (1953).

Results

Incubation of Progesterone-4-^{14}C. Incubation of progesterone-4-^{14}C with specimens of human foreskin, abdominal skin, and vaginal mucosa resulted in the production of metabolites with similar chromatographic mobilities. A scan of a chromatogram from a typical experiment using foreskin (Figure 1) shows the presence of six distinct radioactive areas (peaks I–VI). Peak II corresponds to the location of the progesterone and peak IV to the location of the possible reduction product, Δ^4-pregnen-20α-ol-3-one. No radioactive peak with the mobility of Δ^4-pregnen-20β-ol-3-one was found. The chromatogram from a control incubation containing cofactors but no skin shows a single large radioactive peak, corresponding in mobility to that of the substrate, and a small amount of radioactivity at the origin which may represent a decomposition product since it was not present prior to incubation.

Identification of Metabolites. Reverse isotopic dilution, crystallization to constant specific activity, and preparation of derivatives were performed with metabolites from experiments with foreskin.

Identification of 5α-Pregnane-3,20-dione (Peak I). Peak I was less polar than progesterone, and had the mobility of 5α- and 5β-pregnane-3,20-diones in systems

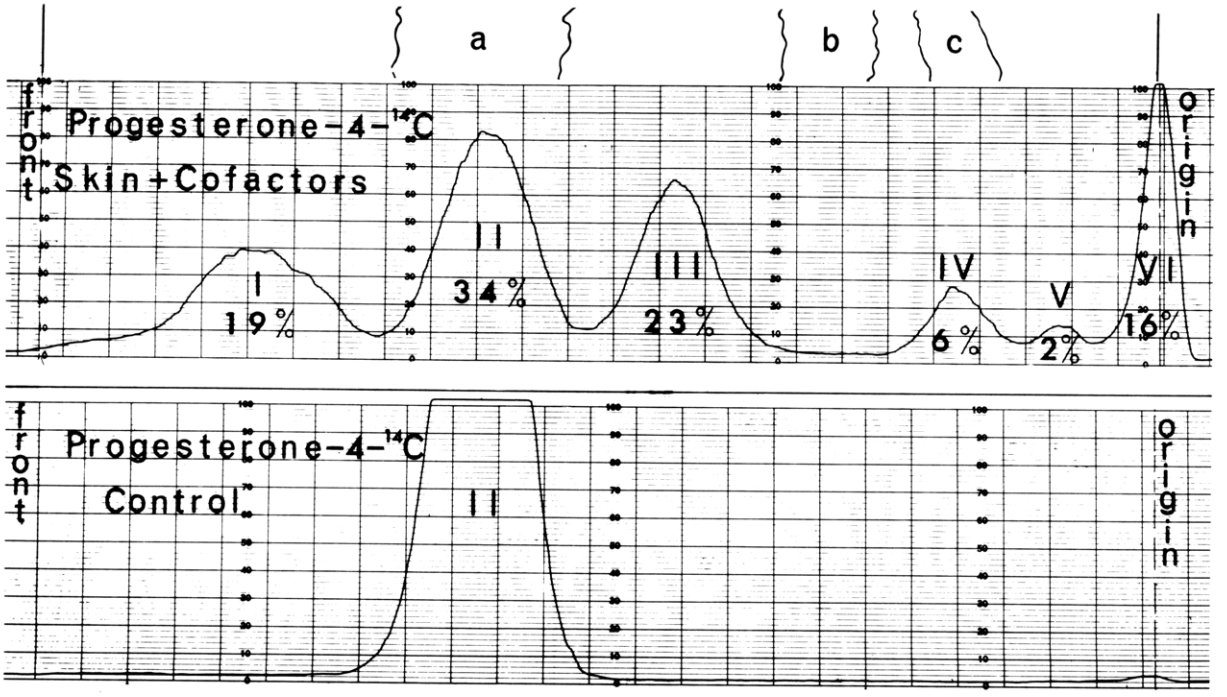


FIGURE 1: Scans of chromatograms obtained after incubations of progesterone-4-^{14}C with foreskin (above) and without skin (below). The outlined regions of the chromatogram above the upper scan indicate the locations of carrier progesterone (a), Δ^4-pregnen-3-one-20β-ol (b), and Δ^4-pregnen-3-one-20α-ol (c).

PE, PG, and Bush A. Treatment with acetic anhydride in pyridine did not change its mobility, indicating the absence of readily acetylable hydroxyl groups.

The metabolite in peak I from an experiment in which carrier 5β-pregnane-3,20-dione was added after incubation was eluted and subjected to reverse isotopic dilution with 5β-pregnane-3,20-dione (24.6 mg). The specific activity decreased successively after each crystallization so that 5β-pregnane-3,20-dione-^{14}C, if present, could not account for more than 2% of the ^{14}C in this peak. On the other hand, a similar experiment using 5α-pregnane-3,20-dione (26.5 mg) as carrier for isotopic dilution resulted in a constant specific activity near the calculated value (Table II). Preparation of the dimethoxime and further crystallization did not change the specific activity. The data indicated that 99% of the radioactivity in peak I could be in 5α-pregnane-3,20-dione.

Identification of Unmetabolized Progesterone and 5α-Pregnan-3α-ol-20-one (peak II). Material from this peak had the same mobility as progesterone in systems PG and Bush A but when subjected to reverse isotopic dilution with progesterone (25.0 mg), the specific activity dropped to 79% of the calculated value (Table I). The presence of another compound with the same mobility was confirmed by chromic acid oxidation. A portion of the radioactivity was converted into a substance with the mobility of 5α-pregnane-3,20-dione. This oxidation product was examined by isotopic dilution with 5α-pregnane-3,20-dione and a constant specific activity of 97% of the calculated value was obtained.

The metabolite in peak II was separated from progesterone-4-^{14}C by chromatography in system TLC-CM-2. In addition to an area corresponding to carrier progesterone (IIa), a more slowly moving radioactive peak (IIb) was detected. Following chromatographic comparisons with likely metabolites, peak IIb was examined by isotopic dilutions with 5α-pregnan-3α-ol-20-one (15.3 mg). Constant specific activity was obtained after crystallizations of the free compound and the acetate (Table II).

Identification of 5α-Pregnan-3β-ol-20-one (peak III). The radioactive material eluted from peak III was oxidized with chromic acid. The oxidation product had the mobility of 5α-pregnane-3,20-dione, and its identity was confirmed by isotopic dilution. Peak III was rechromatographed in system TLC-CM-2 to give a single peak with the R_F of 5α-pregnan-3β-ol-20-one, and reverse isotopic dilution with 28.5 mg of the carrier resulted in a constant specific activity at essentially the calculated value (Table II).

Identification of Δ^4-Pregnen-20α-ol-3-one and 5α-Pregnane-3α,20α-diol (peak IV). The radioactive material in peak IV had the mobility of Δ^4-pregnen-20α-ol-3-one in systems PG and Bush A. Isotopic dilution with that compound, however, yielded a final specific activity of only 84% of the calculated value (Table I). Oxidation of the material in peak IV with chromic acid overnight converted the carrier Δ^4-pregnen-20α-ol-3-one into an ultraviolet light absorbing material with the mobility of progesterone, but only a portion of the radioactivity moved with it, while the remainder moved with 5α-pregnane-3,20-dione. Oxidation for 1 hr led to products with mobilities of peaks I and II, and an additional compound whose chromatographic mobility was between peaks II and IV. When further oxidized, this intermediate was converted entirely into peak I. These data suggested the presence of a saturated diol in peak IV in addition to the 20α-reduction product of progesterone. This was further demonstrated by treating the radioactive materials in peak IV and carrier Δ^4-pregnen-20α-ol-3-one with methoxyamine hydrochloride. The carrier and a portion of the radioactive material were converted into a nonpolar ultraviolet light absorbing compound which was presumed to be the 3-methoxime. A portion of the radioactivity retained the initial mobility. Since this component in peak IV did not form a methoxime, it could be a diol. Its 5α structure was substantiated with conversion by chromic acid oxidation to 5α-pregnane-3,20-dione which was identified by isotopic dilution. In a subsequent experiment, the two components of peak IV were resolved by chromatography in system TLC-CM-4 or by overrunning the chromatograms in system PG; the ketol (peak IVa) migrated ahead of the diol (peak IVb). The radioactive material in peak IVb had the mobility of 5α-pregnane-3α,20α-diol and isotopic dilution with 15.9 mg of carrier indicated that 95% of the radioactivity could be in this compound (Table II). The amount of diol in peak IV was found to vary with time of incubation; little was present at first, but it formed the major part of peak IV after 3–4 hr.

Identification of 5α-Pregnane-3β,20α-diol (Peak V).

TABLE I: Radiochemical Purity of Δ^4-Pregnene Derivatives.

Solvents	No. of Crystn	Sp Act. (dpm/μmole)	% of Calcd Sp Act.
Progesterone (peak II)			
Acetone–hexane	4	395	
Cl_2CH_2	4	364	
Acetone–hexane	8	389	79
Δ^4-Pregnen-20α-ol-3-one (peak IV)			
Cl_2CH_2–hexane	8	350	
Acetone–hexane	2	337	
Acetate			
Methanol–water	6	293	

TABLE II: Radiochemical Purity of 5α-Pregnane Derivatives.

Solvents	No. of Crystn	Sp Act. (dpm/μmole)	% of Calcd Sp Act.
5α-Pregnane-3,20-dione (peak I)			
Acetone–hexane	3	435	
Cl_2CH_2–hexane	6	476	
Ethyl acetate–hexane	3	477	
Dimethoxime			
Cl_2CH_2–hexane	4	430	
Methanol–hexane	3	471	99
5α-Pregnan-3α-ol-20-one (peak II)			
Cl_2CH_2–hexane	8	382	
Acetate			
Methanol–water	4	374	97
5α-Pregnan-3β-ol-20-one (peak III)			
Cl_2CH_2	4	367	
Acetone–hexane	2	368	
Acetate			
Cl_2CH_2–hexane	2	340	
Methanol–water	4	367	99
5α-Pregnane-3α,20α-diol (peak IV)			
Ethanol–benzene	6	403	
Diacetate			
Methanol–water	4	391	95
5α-Pregnane-3β,20α-diol (peak V)			
Cl_2CH_2–hexane	4	272	
Acetone–hexane	6	283	
Diacetate			
Methanol–water	4	309	99

After oxidation with chromic acid, peak V yielded a single product which was identified by chromatographic mobility and isotopic dilution as 5α-pregnane-3,20-dione. The major metabolite in peak V was identified by isotopic dilution with 21.4 mg of 5α-pregnane-3β,20α-diol (Table II).

Peak VI. This peak appears to contain multiple polar compounds whose identification is now in progress.

Control of Microbial Growth. Samples of selected incubation media were cultured for fungi and bacteria; these yielded no growth either aerobically or anaerobically.

Regional Differences in Progesterone Metabolism (Figure 2). Relatively more progesterone-4-^{14}C was reduced to 5α compounds in experiments with foreskin (52%) and vaginal mucosa (63%) than in experiments with abdominal skin (27%).

Discussion

These data reveal the presence of possibly four enzyme systems in human skin which can act upon progesterone: a 20α-ol-dehydrogenase, 3α-ol- and 3β-ol-dehydrogenases, and a 5α-reductase. Whether or not these enzymes in skin are identical with those which act upon cortisone, hydrocortisone, and testosterone is not yet known. Hsia and Hao (1966) found 5α-reductase activity for cortisol in human foreskin, but not in abdominal skin. Gomez and Hsia (1968) found 5α-reductase activity for testosterone in both foreskin and abdominal skin. In the present experiments, 5α-reductase activity for progesterone was present in foreskin, vaginal mucosa, and abdominal skin, but such activity was minimal in abdominal skin (Figure 2). There is apparently heterogeneity of 5α-reductases for these compounds in skin. The data of McGuire and Tomkins (1960) suggest the presence in rat liver of at least five separate microsomal 5α-reductases specific for different Δ^4-3-keto steroids. Moreover, separate 5β-reductases were found in the soluble fraction of rat liver (Tomkins, 1957).

The chief urinary metabolites of progesterone in women are 5β-pregnane-3α,20α-diol and 5β-pregnan-3α-ol-20-one (Loraine and Bell, 1966) but it is of interest that excess thyroid hormone appears to favor the formation of 5α isomers (Bradlow *et al.*, 1966). After incubation of progesterone with human proliferative endometrium *in vitro*, Bryson and Sweat (1967) found that 20% of the product was 5α-pregnane-3,20-dione and only 3% 5β-pregnane-3,20-dione. In our present experiments with skin, only 5α metabolites were found. In human liver, Atherden (1959) found

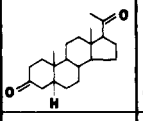
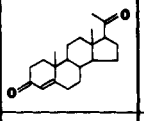
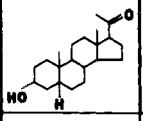
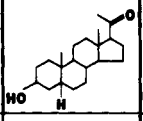
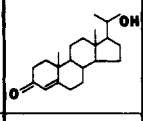
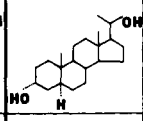
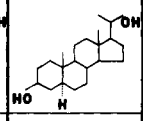
PEAK / TISSUE	I	IIa	IIb	III	IVa	IVb	V	VI
								
FORESKIN	19	28	7.4	22	4.8	0.9	2.6	16
VAGINAL MUCOSA	21	20	3.5	31	4.9	1.4	5.6	13
ABDOMINAL SKIN	4.3	49	1.6	16	14	4.5	0.5	9.7

FIGURE 2: Comparison of the relative amounts of metabolites formed by tissue from different body regions. Except for 5α-pregnane-3α,20α-diol, relatively less 5α metabolites are formed in abdominal skin (27%) than in foreskin (52%) or vaginal mucosa (63%). Similar amounts of various metabolites were obtained with abdominal skin from men and women. Values are averages of two experiments with each tissue. Peak VI contains metabolites not yet identified. Values given in per cent of total radioactivity.

both 5α and 5β metabolites of progesterone, with more of the 5β compounds being formed. In rat liver, on the other hand, only 5α metabolites have been detected (Shirley and Cooke, 1968). It appears, then, that there are organ to organ variations within a species with regard to relative amounts of 5α and 5β reductase activities for progesterone.

The significance of this system to metabolize progesterone in human skin is not apparent at present, but since skin is a relatively large organ its contribution to the metabolism of steroid hormones merits attention. After perfusion of human fetuses with progesterone-4-^{14}C, several radioactive metabolites were identified in various tissues (Solomon *et al.*, 1967). Human fetal blood has been shown to form Δ^4-pregnen-3-one-20α-ol from progesterone (Nancarrow and Seamark, 1968). Since the activity of the 20α-hydroxysteroid dehydrogenase which forms this compound declined rapidly during the first 60 days postpartum, it was suggested that metabolism of progesterone to a less biologically active compound fulfills a protective role in regulating the level of progesterone reaching the fetus. Although the potential for skin to metabolize progesterone persists into adulthood it may also serve to protect the fetus *in utero* against high levels of progesterone in amniotic fluid.

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