TRANSFORMATIONS OF PROGESTERONE BY SUBCELLULAR FRACTIONS OF HUMAN SKIN

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Homogenates of human skin metabolize progesterone-4-^{14}C to the same products as whole skin. Studies with subcellular fractions obtained by differential centrifugation indicate the presence in skin of a membrane-bound 5α-reductase and a soluble 20α-hydroxysteroid dehydrogenase (20α-HSD), both of which utilize TPNH as cofactor. The 20α-HSD has a pH optimum of 6.2 and a K_m of approximately 7 μM. Unlike placental preparations, the 105,000 $\times$ *g* supernatant fraction prepared from human skin has more 20α-HSD than 17β-hydroxysteroid dehydrogenase activity. This soluble skin enzyme acting on steroid hormones is distinct from previously reported particulate enzymes.

Skin has been shown to metabolize several classes of steroids [1–5]. The enzymes catalyzing the reduction of the Δ^4-bond of testosterone and progesterone [6], the Δ^4-bond and 20-ketone of cortisol, and the oxidation of the 11β-hydroxyl of cortisol (S. L. Hsia, unpublished observations) in human skin are membrane bound, as are the enzymes in the oxidation of the 17β-hydroxyl group of estradiol and testosterone in rat skin [7]. The greater portion of the estradiol-17β hydroxysteroid dehydrogenase activity in human skin preparations is membrane bound, but a small amount of activity remains in the 105,000 $\times$ *g* supernatant [8]. Voigt et al [6] and Davis [7] have suggested that a few enzymes of broad specificity may be responsible for chemical transformations of such diverse steroids as testosterone, progesterone, and estrogens.

In this study, we will describe the transformations of progesterone by subcellular fractions of human skin, confirm the finding that progesterone 5α-reductase is membrane bound, and describe a soluble 20α-hydroxysteroid dehydrogenase (20α-HSD) utilizing progesterone as substrate and TPNH as cofactor. The latter enzyme is distinct from previously reported cutaneous enzymes.

MATERIALS AND METHODS

Chemicals

Radioactive steroids were purchased from New England Nuclear Company. Specific activities were approximately 20 mCi/mmole for ^{14}C-containing compounds and 33.5 Ci/mmole for tritiated compounds. Solutions of the radioactive compounds were evaporated under nitrogen and the residue dissolved in methanol. After examination for chromatographic purity in system PG (see below), stock solutions containing approximately 10^6 dpm (8.6 nmoles) of progesterone-4-^{14}C in each 0.1 ml were stored at −10°C. Reference steroids and coenzymes were purchased from Southwestern Biochemicals, Mann Research Laboratories, and Sigma Chemical Company. 20α-Hydroxypregn-4-en-3-one (20α-DHP) was a gift from the Upjohn Company.

Preparation of Tissue Homogenates

Specimens of human foreskin were collected at circumcision and frozen at −60°C until the day of the experiment. In some experiments, freshly obtained tissue was used, but no significant differences from preparations utilizing frozen tissues were noted. The tissue was weighed and minced with scissors. The minced tissue was added to enough buffer (0.1 M sodium phosphate, pH 7.4, containing 0.25 M sucrose) to give a 10% (w/v) homogenate. The skin, while chilled in an ice bath, was homogenized for 1 min with a Polytron homogenizer (Kinematica GMBH, Lucern, Switzerland) using a setting of 5, and then with a motor-driven ground-glass homogenizer (12 strokes) as described by Voigt et al [6].

The homogenate was centrifuged at 100 $\times$ *g* for 30 min to remove debris and the supernatant (designated "whole homogenate") fraction was further centrifuged at 10,000 $\times$ *g* for 30 min. The 10,000 $\times$ *g* pellet was washed once in buffer and resuspended in a volume of buffer equal to that of the 10,000 $\times$ *g* supernatant. The 10,000 $\times$ *g* supernatant was centrifuged at 105,000 $\times$ *g* for 1 hr and the 105,000 $\times$ *g* pellet washed with buffer. All centrifugations were at 4°C.

Enzymatic Assay of Subcellular Fractions

A variable volume of the subcellular fraction to be assayed was mixed with 0.1 M phosphate buffer (pH 7.4) to give a total incubation volume of 5 ml. In experiments utilizing cofactors, 3.6 μmoles each of TPN, DPN, DPNH, and/or TPNH were added. In some experiments a TPNH generating system consisting of TPN (3.3 μmoles), glucose-6-phosphate (12 μmoles), and glucose-6-phosphate dehydrogenase (2 units) was used. Reaction was begun by the addition of 0.1 ml (8.6 μmoles) of

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progesterone-4-^{14}C stock solution. After incubation for 3 hr at 37°C, the reaction was stopped and the steroids extracted as previously described [2]. Metabolites formed were separated by chromatography in system PG and quantitated by scanning.

20α-HSD Assay

Because quantitative recovery of radioactive steroids by exhaustive extraction with organic solvents is tedious, the single extraction method described by Davis et al [7] was employed. The incubation was carried out in a 50-ml screw-cap centrifuge tube in which 0.1 ml of a methanolic solution containing 8.6 nmoles of progesterone-4-^{14}C had been evaporated. One milliliter of enzyme solution and 0.2 ml of buffer containing 3 μmoles of TPNH were added and the mixture incubated at 37°C for 1 hr. The incubation was terminated by the addition of 100 μg each of carrier progesterone and 20α-DHP and 6 ml dichloromethane. The tube was tightly capped, shaken vigorously, and the solvents allowed to separate into two phases. Approximately 5 ml of the lower (dichloromethane) phase was transferred by pipette to a conical-bottom tube and evaporated to dryness under a nitrogen jet. The residue was dissolved in 0.2 ml of methanol and chromatographed in system PG. The amount of product produced was calculated from the scanning of the chromatogram by use of a correction factor for the differential extraction of substrate and product. The correction factor was determined as follows: a mixture of progesterone-4-^{14}C and 20α-DHP-1-^{3}H was prepared which gave an isotope ratio (dpm^{3}H/dpm^{14}C) of 7.95. An aliquot was evaporated in 6 centrifuge tubes and 1 ml of methanolic carrier solution and 6 ml of dichloromethane added in rapid succession. The ^{3}H and ^{14}C in the extracts were determined and recovery of substrate and product calculated. The mean recovery for ^{14}C was 55.5% with a standard deviation of 5.0% and for ^{3}H was 76.3% with a standard deviation of 5.0%. From these values, a correction factor of 0.7 was calculated which then was used to correct for the preferential extraction of product. In experiments measuring the conversion of estrone-4-^{14}C to estradiol-^{14}C, a different factor (0.9) determined in a similar manner was used.

Time studies utilizing the single extraction method indicated that conversion of progesterone to 20α-DHP was linear for 3 hr.

Chromatography

Residues were subjected to thin-layer chromatography or paper chromatography in the following systems:

System PG was the ligroin–propylene glycol system described by Savard [9] and was used for progesterone and its metabolites.

System BHMW was the benzene:heptane:methanol:water (4:6:7:3) system described by Bush [10] and was used to measure the conversion of estrone to estradiol-17β as previously described [3].

After development, paper strips were either dried for 50 min under vacuum at 50°C or allowed to dry in the air for 24 hr. The distribution of radioactivity was monitored by a Vanguard Autoscanner 880 with automatic data system. The amount of radioactivity represented by each peak was calculated as a percentage of the total radioactivity in the chromatogram. Reference compounds with an α, β-unsaturated ketone structure were detected by examining the strip under a short-wave ultraviolet lamp.

System TLC-CM-4 was a thin-layer chromatography system using chloroform:methanol (96:4) as the solvent. The chloroform was stabilized by 0.75% ethanol immediately after distillation. Plates were allowed to develop until the solvent front had ascended 35 cm, which usually required 3 hr. Steroids on thin-layer chromatography plates were visualized by exposure to iodine vapor. Thin-layer chromatography was performed on 40 × 5 cm glass plates coated with silica gel H (thickness 3/8 mm). Plates were air dried for 30 min, activated at 100°C for 30 min, and stored in a desiccator until used.

Analysis of Kinetic Data

The kinetic data were analyzed on an IBM 360/65 computer with the aid of the Hyperb program of Hanson et al [11] based on the method of Bliss and James [12].

RESULTS

Incubations with subcellular fractions. Incubation of progesterone with 2 ml (equivalent to 200 mg of skin) of "whole homogenate" and all four cofactors (see *Materials and Methods*) resulted in chromatograms showing 6 areas of radioactivity in a pattern identical to that obtained previously by incubation with whole skin [4]. The mobility of Peaks I to V in system PG corresponded to that of the metabolites previously identified:

Peak I: 5α-pregnane-3,17-dione
Peak II: progesterone and 3α-hydroxy-5α-pregnan-20-one
Peak III: 3β-hydroxy-5α-pregnan-20-one
Peak IV: 20α-hydroxypregn-4-en-3-one (20α-DHP) and 5α-pregnane-3α,20α-diol
Peak V: 5α-pregnane-3β,20α-diol

The 6th area of radioactivity remained at the origin and was not previously identified.

Incubation of progesterone with 2 ml of the microsomal fraction (100,000 × *g* pellet) and cofactors resulted in a chromatogram lacking peaks IV and V, indicating the absence of metabolites with side chains reduced at C-20. The formations of peaks I and III indicate the reduction of the Δ^4-double bond of progesterone, resulting in the formation of products previously identified as 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one.

In contrast, incubation with the soluble (100,000 × *g* supernatant) fraction and the same cofactors produced a large peak IV, while radioactivity in peaks I and III was minimal. Since incubations with whole skin produce a peak IV consisting of two metabolites, 20α-hydroxypregn-4-en-3-one (20α-DHP) and 5α-pregnane-3α,20α-diol, the peak IV produced by the soluble fraction was eluted from the chromatogram, mixed with progesterone, 20α-DHP and 5α-pregnane-3α,20α-diol standards, and chromatographed in system TLC-CM-4. Virtually all of the radioactivity in this chromatogram had the mobility of 20α-DHP and no discernible peak corresponded to the position of the 5α-pregnane-3α,20α-diol.

These data indicate that the 5α-reductase for progesterone is predominantly particulate while the 20α-HSD is a soluble enzyme. The localization of the 3α or 3β-hydroxysteroid dehydrogenases must await studies utilizing substrates without

the Δ^4-double bond, because the reduction of the ketone at position 3 is markedly retarded by α, β-unsaturation at C_4. Thus, the absence of reduction of the 3-ketone by the soluble fraction may indicate a lack of reduction of the Δ^4-double bond rather than the absence of soluble 3α- or 3β-hydroxysteroid dehydrogenases in this fraction.

Cofactor specificity of 5α-reductase. An aliquot of the microsomal fraction was incubated with progesterone-4-^{14}C with and without the addition of TPNH or DPNH. In the absence of TPNH, no 5α-metabolites were formed, while with TPNH present 551 pmoles were produced. These results indicate that the reduction of the double bond of progesterone, like that of testosterone [6], required TPNH and that DPNH does not function as a cofactor.

Cofactor specificity of 20α-HSD. Incubation of progesterone-4-^{14}C with the soluble fraction in the absence of added cofactors resulted in the formation of 160 pmoles of 20α-DHP. Addition of DPNH resulted in a slight stimulation, yielding 360 pmoles, while the addition of TPNH markedly stimulated the reaction, yielding 2,710 pmoles. These data indicate that cutaneous 20α-HSD utilizes TPNH as the preferred cofactor.

Stability of cutaneous 20α-HSD. To examine the stability of 20α-HSD prior to further characterization, a single batch of the 105,000 × g supernatant was assayed immediately after preparation, after storage for 4 days at 4°C, and for 1 month at −60°C. The results indicated that the activity was stable on freezing and at refrigerator temperature for the periods tested.

Time course of side chain reduction. The single extraction method was used to further study 20α-HSD. Figure 1 shows the time course of the reduction of the 20-ketone of progesterone using this method. At 37°C the reaction was linear for at least 3 hr. For convenience, an incubation time of 1 hr was subsequently used.

pH Optimum of 20α-HSD. To determine the optimum pH for this reaction, supernatant (pH 7.4) was dialyzed for 9 hr at 4°C against 0.1 M phosphate buffer (pH 7.0). Aliquots of the supernatant were then adjusted to various pH values by addition of small volumes of HCl or NaOH and the activity assayed. The maximum production of 20α-DHP occurred at pH 6.0. Since phosphate is a poor buffer in this pH range, the dialysis was repeated using buffer containing 0.1 M phosphate and 0.05 M citrate and having a pH of 6.0. After dialyzing and adjusting to the desired pH value, the activity was assayed. The results are summarized in Figure 2. Activity was greatest at pH 6.2 and declined rapidly at more acidic or basic pH's. Between pH 7.5 and 8.5 the activity remained constant.

K_m of 20α-HSD for progesterone. The effect of varying substrate concentration in the reaction mixture was examined. The results (Fig. 3a) indicated a K_m of approximately 7 μ M.

Linearity of assay for 20α-HSD. The variation of activity with different volumes of supernatant (varying amounts of enzyme) was examined (Fig. 3b). The reaction rates were found to be linear with a protein concentration up to 3.5 mg per incubation mixture (equivalent to 1 ml of undiluted supernatant per incubation mixture).

Assay of 20α-HSD and 17β-HSD in 105,000 × g supernatant. Davis [8] found that a minor portion

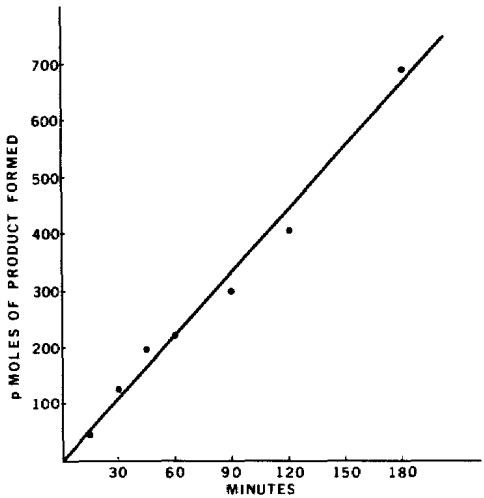

FIG. 1. Time course of the 20α-hydrosteroid dehydrogenase reaction using the single extraction method.

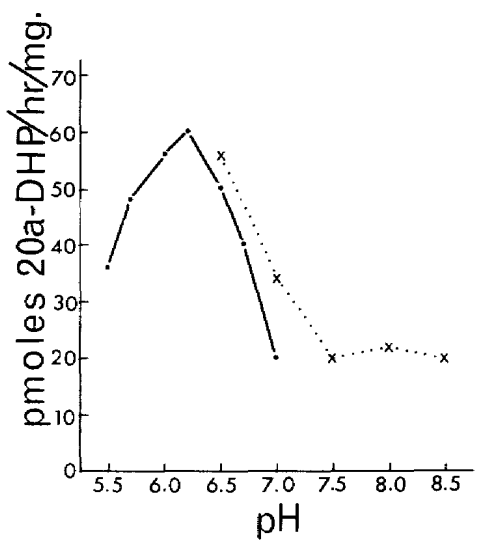

FIG. 2. The pH dependence of the 20α-hydrosteroid dehydrogenase reaction. Abscissa, pH; ordinate, pmoles of 20α-hydroxypregn-4-en-3-one (20α-DHP) formed per mg protein in 1 hr. Buffers: 0.1 M phosphate buffer (×···×···×), 0.1 M phosphate and 0.05 M citrate buffer (·-·-·-·).

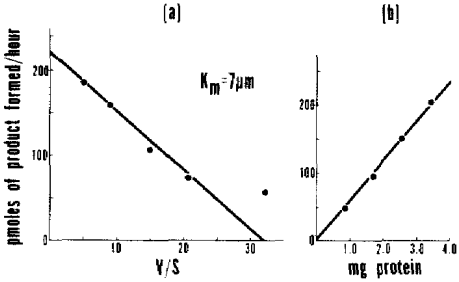

FIG. 3. *a:* Kinetic plot of the 20α-hydrosteroid dehydrogenase reaction by the method of Eadie. *b:* Dependence of product (20α-DHP) formation on amount of enzyme preparation. In both experiments the 105,000 × *g* supernatant was adjusted to pH 6.2 by dialysis against PO_4-citrate buffer of the same pH and the single extraction method used to measure enzyme activity

TABLE. *17 β-HSD and 20α-HSD activity in skin supernatant*[a]

Substrate	Enzyme	Cofactor	Product (pmoles)	
			Measured	Corrected[b]
Estrone	1 ml	TPNH	116	85
Estrone	1 ml	DPNH	154	123
Estrone	1 ml	—	93	62
Estrone	—	—	31	—
Progesterone	1 ml	TPNH	379	379
Progesterone	1 ml	DPNH	48	48
Progesterone	1 ml	—	6	6
Progesterone	—	—	0	—

[a]Assay system was the single extraction method as described in *Materials and Methods*.

[b]Corrected for spontaneous decomposition of substrate.

of human cutaneous 17β-HSD remained in the 105,000 × *g* supernatant. In view of the report by Purdy et al [13] that 20α-HSD activity of human placenta is a minor activity of a soluble 17β-HSD catalyzing the reduction of estrone to form estradiol-17β, the 105,000 × *g* supernatant was simultaneously assayed for 20α-HSD and 17β-HSD activities. The results are shown in the Table. With estrone as substrate, a net production of 62 pmoles of estradiol-17β was found in the absence of cofactors, 85 pmoles with TPNH added, and 123 with DPNH added. The level of estrone decomposition found is similar to that found in Davis [8]. With progesterone as substrate only 6 pmoles of 20α-DHP were produced in the absence of cofactor, 48 pmoles with DPNH added, and 379 pmoles with TPNH added.

DISCUSSION

The 5α-reductase from human skin which converts testosterone to dihydrotestosterone is a membrane-bound, TPNH-lined enzyme. Cross-inhibition studies suggest that the same enzyme may also reduce the Δ^4-double bond of progesterone [6]. The present data confirm that the progesterone reductase activity is predominantly membrane bound and, as with testosterone as substrate, is TPNH dependent. Little is known of the 3α-HSD and 3β-HSD of human skin, but Davis [8] has shown that the cutaneous 17β-HSD is also predominantly membrane bound and utilizes DPNH in preference to TPNH. Since virtually all the 20α-HSD activity in skin is in the soluble fraction, it appears that this activity is catalyzed by a different enzyme from that responsible for cutaneous 17β-HSD activity. An additional difference is cofactory specificity: the 20α-HSD utilizes TPNH in preference to DPNH. These data indicate that cutaneous 20α-HSD is distinct from the microsomal enzymes of broad specificity described by Voigt et al [6] and Davis [8].

Purdy et al [13] have investigated the 20α-HSD activity of human placenta and reported this activity to be a minor activity of the placental 17β-HSD which utilizes estradiol-17β or estrone as substrates. Estrogens appear to be the preferred substrates of the placental preparations, since the K_m for estrone was 5.6 μM while that for progesterone was 180 μM. The apparent K_m for progesterone of the cutaneous 20α-HSD described in the present study is about 1/25 that reported by Purdy et al [13] for the placental enzyme. Likewise, in placental preparations, the maximum reaction velocity with estrone as substrate is approximately 46 times greater than with progesterone [13]. The low 17β-HSD activity in skin supernatants appears to be less than that of 20α-HSD, indicating that in skin the reduction of the progesterone side chain is unlikely to be due to the placental enzymes.

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