ISOLATION OF 3β,20α-HYDROXYSTEROID OXIDOREDUCTASE FROM SHEEP FETAL BLOOD

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ABSTRACT

3β,20α-Hydroxysteroid oxidoreductase has been isolated from ovine fetal blood by a 2,370-fold purification scheme of ammonium sulfate fractionation, calcium phosphate gel adsorption, affinity chromatography, and fast performance liquid chromatography. A new high performance liquid chromatography-based assay for measuring 20α-reductase activity is described. The enzyme is a monomer with a molecular weight of 35,000 and uses NADPH as a cofactor for reductase activity. It reduces progesterone to 4-pregnen-20α-ol-3-one or 5α-dihydrotestosterone to 5α-androstan-3β,17β-diol with kinetic characteristics of Km = 30.8 μM and Vmax = 0.7 nmol min^{-1} (nmol of enzyme)$^{-1}$ or Km = 74 μM and Vmax = 1.3 nmol min^{-1} (nmol of enzyme)$^{-1}$, respectively. 5α-Dihydrotestosterone competitively inhibits 20α-reductase activity with a Ki value of 102 μM.

INTRODUCTION

Bovine and ovine fetal blood rapidly convert progesterone to 4–pregnen-20α-ol-3-one (1). Indeed, 20α-reductase activity has been found in fetal erythrocytes from a number of species in the family

Bovidae (2,3). Similarly, 5α-dihydrotestosterone is metabolized to 5α-androstan-3β,17β-diol in bovine and ovine blood, metabolic activity earlier believed due to a separate C_{19}-specific 3β-hydroxysteroid oxidoreductase. Isolation of the enzyme from bovine fetal blood permitted us to demonstrate that 3β-reductase and 20α-reductase activity are both produced by a *single* 3β,20α-hydroxysteroid (3β,20α-HSD) oxidoreductase enzyme (3). Moreover, both 3β- and 20α-reductase activities were associated with the *same* catalytically active site (4).

Remarkably, 3β,20α-HSD enzyme activity practically disappears from the blood of the newborn shortly after parturition (5). To discover the developmental function of the enzyme in fetal blood, it is important to isolate and characterize the 3β,20α-HSD enzymic protein so that antibodies can be produced from it for accurately measuring its evolution in the developing embryo and fetus. The present report describes the first isolation and characterization of 3β,20α-HSD from ovine fetal erythrocytes and its comparison with the analogous bovine enzyme which has been previously reported (3,4).

MATERIALS AND METHODS

Reagents

Progesterone, 4-pregnen-20α-ol-3-one, 5α-dihydrotestosterone, and 5α-androstan-3β,17β-diol were purchased from Steraloids, Inc., Wilton, NH. Radioisotopes [4-^{14}C]progesterone (50 mCi/mmol) and 5α-[4–^{14}C]androstan-17β-ol-3-one (58 mCi/mmol) were purchased

from New England Nuclear, Boston, MA, and chromatographically purified prior to use with silica gel G plates from Eastman Kodak Co., Rochester, NY (#13181), developed with benzene/ethyl acetate (3:1). Cibacron Blue Agarose, NADPH, calcium chloride, sodium phosphate (tribasic), Sephadex gels, and inorganic chemicals were from Sigma Chemical Co. of St. Louis, MO. Ammonium sulfate was from Schwarz-Mann Co., Spring Valley, NY. Organic solvents were from Fisher Scientific Co., St. Louis, MO.

Erythrocytes

Ovine fetal blood was collected by heart puncture or from the jugular vein of the fetuses through hysterotomy of late-term (>100 days) pregnant ewes (under general anesthesia). The erythrocytes were washed twice by mixing the blood cells with two volumes of cold 0.9% NaCl solution per volume of erythrocytes, followed by centrifugation at 1,500 rpm for 15 min, and then stored at –20°C until further purification. The thawed cells were mixed with 1 vol 10 m**M** phosphate buffer, centrifuged at 15,000 rpm for 30 min, and the supernatant was subjected to ammonium sulfate fractionation (30% to 60% $(NH_4)_2SO_4$ saturation), as previously described (3,4). The collected fraction, which contained most of the enzyme (and large quantities of hemoglobin), was dialyzed against distilled water at room temperature. The retentate was lyophilized and then stored at 4°C. Crude, lyophilized enzyme, shipped by airfreight between Sydney, Australia and St. Louis, MO, did not measurably lose *any* activity. Upon receipt, the preparation was stored at -20°C.

Methods of enzyme assays

1. Radioisotope method. 20α-Reductase activity (progesterone to 4–pregnen-20α-ol-3-one) is assayed in a 10 mm x 75 mm test tube containing 1.0 mL solutions of 0.1 **M** potassium phosphate buffer, pH 6.0, containing 1.0 m**M** NADPH, 0.1 μmol of progesterone (100 μ**M**) containing 100,000 cpm of [4-^{14}C]progesterone. The assay mixture is incubated for 30 min at 37°C in a Temp-Blok (model H 2025-1) block heater from Cole-Parmer Co., Chicago, IL. The steroids are extracted with 1.5 mL of diethyl ether/ethyl acetate (1:1), the organic extract is transferred to a test tube and concentrated to dryness at 37°C (block heater) in a stream of dry air. The residue is

dissolved in 40 μL of diethyl ether/ethyl acetate (1:1) and applied to a 20 cm x 20 cm thin-layer chromatography (TLC) sheet of silica gel G containing a fluorescent indictor (#13181, Eastman Kodak Co.). The chromatograms are developed with benzene/ethyl acetate (3:1), dried, and then visualized under ultraviolet light (254 nm) to locate progesterone and 4-pregnen-20α-ol-3-one. The spots containing each of the steroids are cut out from the TLC sheet and then transferred to 10-mL vials, each containing 5.0 mL of ACS brand (Amersham Corp., Arlington Heights, IL) scintillation cocktail, and the ^{14}C-radioactivity is measured for 10 min in a Beckman Instruments (Palo Alto, CA) LS-330 liquid scintillation counter. Recovery of radioactivity was 85%. Each enzyme assay was conducted in duplicate or triplicate.

3β-Reductase activity (reduction of 5α-dihydrotestosterone to 5α-androstan-3β,17β-diol) is assayed under similar conditions to the 20α-activity assays (above) except that 0.1 μmol of 5α-androstan-17β-ol-3-one containing 100,000 cpm of the corresponding ^{14}C-steroid is substituted for progesterone. Also, the TLC sheet is developed with toluene/ethyl acetate (9:1.5). Then the dried, developed sheet is vizualized with iodine vapor (5α-androstanes do not absorb ultraviolet light), and the appropriate spots are transferred to scintillation fluid for measuring ^{14}C-radioactivity. Prior to measuring radioactivity, the silica gel-scintillation cocktail mixture is allowed to stand at room temperature for *at least* 5 h to allow for complete extraction by the cocktail of 5α-androstan-3β,17β-diol. Each enzyme assay was conducted in duplicate or triplicate. Confirmation of identity of the C_{19} and C_{21} steroid products of 3β- and 20α-reductase activity was accomplished by crystallization of the radioactive products with authentic carrier steroids (described below).

2. High performance liquid chromatography (HPLC) method. Measuring the conversion of progesterone to 4-pregnen-20α-ol-3-one by HPLC has advantages over the ^{14}C-isotope method in terms of precision, accuracy, rapidity, reproducibility, and avoidance of handling radioactivity. The 20α-enzyme activity assays are conducted precisely as described above *except* for exclusion from the assay mixture of [4-^{14}C]progesterone. The dried steroid residue from the diethyl ether/ethyl acetate extract is dissolved in 200 μL of HPLC

grade acetonitrile (Fisher Scientific Co., St. Louis, MO) and then injected in an HPLC system containing a Lichrosorp RP-18 column (4 mm x 250 mm). The column is eluted with acetonitrile at a flow rate of 2 mL/min (under 1,000-1,500 psi of pressure). The steroids are measured by a light absorption detector (set at 240 nm) located after the RP-18 column. Computer automated recording of the detected light absorption profile and integration of the areas under each emergent peak (which is proportional to the concentration of each steroid) provides the primary data. The molar extinction coefficients (at 240 nm) of progesterone and 4-pregnen-20α-ol-3-one are practically equal. This permits taking the ratios of the integrated peak areas as the molar ratios of the two steroids. Results from the radioisotope and HPLC enzyme assay methods for the same enzyme preparation were found to coincide exactly. Accordingly, all of the kinetic experiments that involved measuring 20α-enzyme activity were conducted by the HPLC method. Protein concentrations were determined by measuring optical density of solutions at 280 nm in a Beckman model 25 spectrophotometer, or by the methods of Lowry *et al* (6).

Identification by crystallization of products from 3β- or 20α-reductase activity with 3β,20α-hydroxysteroid oxidoreductase

1. Single isotope method with [4-^{14}C]progesterone. Radioactive [4–^{14}C]progesterone was incubated with the isolated 3β,20α-hydroxysteroid oxidoreductase and NADPH as described under *Methods of enzyme assays* (above). The steroidal product was isolated by TLC, extracted from the silica gel with ethanol, and the extract was filtered through a fine sintered glass filter. Then 20 mg of non-radioactive 4-pregnen-20α-ol-3-one was added as carrier, the solvent was evaporated, and the residue was recrystallized three times from a mixture of ethanol-water. At each step of crystallization, the steroid in the mother liquor and crystals was quantitated by measuring its light absorption at 240 nm and radioactivity by liquid scintillation counting. The results are shown in Table 2.

2. Single isotope method with 5α-[1,2-^{3}H]androstan-17β-ol-3-one. After reducing 5α-[1,2-^{3}H]androstan-17β-ol-3-one with 3β,20α-

hydroxysteroid oxidoreductase and NADPH the product was isolated by TLC. The crystallization procedure was similar to that described above except for quantitation during each step of crystallization of the radioactive product that had been isolated by TLC. After 20 mg of carrier 5α-androstan-3β,17β-diol was added to the radioactive product, the resulting mixture was crystallized three times from the solvents (shown in Table 2). After each crystallization, the mother liquor and separated crystals were dried at 38°C. The dry residue (mother liquor) and crystals were weighed, and then the radioactivity of each was measured by liquid scintillation counting. The results are shown in Table 2.

3. Double isotope ratio method with 5α-[4-^{14}C]androstan-17β-ol-3-one and 5α-[1,2-^{3}H]androstan-3β,17β-diol. First, 5α-[4-^{14}C]androstan-17β-ol-3-one was converted to product with 3β,20α-hydroxysteroid oxidoreductase, and the product was isolated by TLC as described above. Then authentic 5α-[1,2-^{3}H]androstan-3β,17β-diol was added to the product, and the mixture was crystallized three times with the solvents shown in Table 2. After each crystallization, the mother liquor and crystals were separated, dried at 38°C, and the dry solids were weighed. The ^{14}C- and ^{3}H-radioactivity were measured in each sample. Then the quotients from (^{3}H/mg)÷(^{14}C/mg) corresponding to the residue from the mother liquor and the crystals were tabulated. The results of the calculated ^{3}H/^{14}C ratios are shown in Table 2.

Isolation of ovine 3β,20α-hydroxysteroid oxidoreductase

All of the following operations were carried out at 4°C.

1. Reprecipitation of 3β,20α-HSD with ammonium sulfate. The lyophilized (ammonium sulfate-fractionated) enzyme preparation (50 g) was mixed with 2 L 10 mM phosphate buffer, pH 6.0 (1 g/40 mL), and then stirred for 3 h. Then sufficient ammonium sulfate was added to produce 30% saturation, the mixture was stirred for 40 min, then centrifuged at 25,000 x g for 30 min, and the supernatant (containing 3β,20α-HSD) was collected. Sufficient ammonium sulfate was added to the supernatant to provide 80% saturation, the mixture was stirred for 40 min, and then centrifuged at 25,000 x g for 30 min. The supernatant was discarded, the pellet was dissolved in 10 mM phosphate buffer, pH 6.0, and the resulting

solution was dialyzed against several changes of the same buffer. The retentate was then subjected to the calcium phosphate step.

2. Calcium phosphate (Ca–P) gel adsorption of 3β,20α-HSD. The retentate was mixed with freshly made Ca–P gel (1:1 v/v), stirred for 1 h, and then centrifuged at 1,500 rpm for 5 min (3). The supernatant was similarly treated twice with additional Ca–P gel, the pellets were pooled, and then washed: a) twice with 10 mM phosphate buffer, pH 6.0 (3 vol buffer/vol Ca–P gel); b) twice with 20 mM phosphate buffer, pH 6.0 (3:1, v/v). After each wash, the Ca–P gel was collected by centrifugation at 1,500 rpm for 5 min. After the final wash, 3β,20α-HSD was recovered by stirring 1 vol of the Ca-P in 2 vol of 100 mM phosphate buffer, pH6.0 (containing 50 mM NaCl) for 1 h, and then centrifuging the mixture at 1,500 rpm for 5 min. The supernatant (3β,20α-HSD) was adjusted to 80% saturation with ammonium sulfate, stirred for 40 min, and then centrifuged at 25,000 x g for 30 min. The pellet was dissolved in a minimum volume of 10 mM phosphate buffer, pH 6.0, and then dialyzed against the same buffer. The retentate was subjected to a complete, *second* Ca–P adsorption treatment, and then the final recovered 3β,20α-HSD was stored at –20°C.

3. Cibacron Blue Agarose affinity chromatography of 3β,20α-HSD. Prior to use, the Blue Agarose was washed with 6 M urea and then equilibrated at room temperature with 10 mM phosphate buffer, pH 6.0. The 3β,20α-HSD from the Ca–P purification was applied to a column of blue agarose (1.2 cm x 30 cm). The column was then eluted with 10 mM phosphate buffer, pH 6.0 containing stepwise increasing concentrations of NaCl (50 to 200 mM). At each stage, elution was continued until the light absorption (280 nm) of the effluent was negligible; then the NaCl concentration in the buffer was increased. Following the 200 mM NaCl elution, the blue agarose was transferred to a beaker, and 3β,20α-HSD was recovered from the adsorbent by stirring it at room temperature for 30 min in 10 mM phosphate buffer, pH 6.0 *containing* progesterone (0.10 mM) and NADPH (1.0 mM). The solution containing recovered 3β,20α-HSD was dialyzed against 10 mM phosphate buffer, pH 7.0 (containing 0.5 mM of 2-mercaptoethanol) at 4°C to prepare material for fast

performance liquid chromatography (FPLC). The retentate was concentrated to about 1/10 vol by packing the dialysis bag in polyethylene glycol, and then the purified enzyme was stored at –20°C to await final purification.

4. FPLC® chromatography and isolation of 3β,20α-HSD. The 3β,20α-HSD from Blue Agarose was dialyzed against 10 m**M** phosphate buffer, pH 7.0 (containing 10% glycerol) and then injected into a Pharmacia FPLC® system containing a Mono-Q column (previously washed with 2 **N** NaOH, 2 **N** NaCl, and then equilibrated with the above glycerol-phosphate buffer). During chromatography, the column was eluted with a 0.0 to 600 m**M** NaCl gradient in the glycerol-phosphate buffer, at a flow rate of 0.5 mL/min (under a pressure of 20 mPa). The 3β,20α-HSD was eluted at about 150 m**M** of NaCl. Fractions containing 3β,20α-HSD activity were pooled, concentrated with polyethylene glycol, and then stored at –20°C.

Molecular weight determination of 3β,20α-hydroxysteroid oxidoreductase

1. By gel filtration. The molecular weight of ovine 3β,20α-HSD was estimated by gel filtration of the enzyme together with protein standards through a Sephadex G-100 column (1.6 cm x 100 cm), eluted with 0.1 **M** phosphate buffer, pH 6.0 (containing 500 m**M** NaCl) at 25°C. The eluate was fractionated, and each fraction was assayed for 20α-reductase activity and protein content. The protein standards were carbonic anhydrase (M_r 29,000), ovalbumin (M_r 45,000), bovine albumin (M_r 67,000), and alcohol dehydrogenase (M_r 141,000). 3β,20α-HSD emerged in a single, narrow region (*i.e.*, two tubes) of the 20α-reductase activity profile. This profile was superimposed on the protein profile which, together with a standard curve derived from the protein standards, provided the molecular weight of ovine 3β,20α-HSD as M_r 35,000. This value was consistently obtained for 3β,20α-HSD, isolated in three different enzyme purifications, each measured by gel filtration.

2. By slab gel electrophoresis. The 3β,20α-HSD, isolated by FPLC, was heated at 100°C for 1 min in 3% sodium dodecyl sulfate and 500 m**M** of 2-mercaptoethanol. The resulting material was subjected

to gel electrophoresis on 10% polyacrylamide. A mixture containing 1 mg each of the following standard proteins was included in each analysis: bovine serum albumin (M_r 66,200); carbonic anhydrase (M_r 29,000); lysozyme (M_r 13,900); and cytochrome (M_r 12,800). A *single* band due to 3β,20α-HSD (stained with Coomassie Blue or $AgNO_3$) migrated to a position estimated as M_r 36,000. Each experiment was repeated at least three times with 3β,20α-HSD isolated from at least three different enzyme preparations.

RESULTS AND DISCUSSION

Measurement of 3β- and 20α-reductase activity

Initially, the radioisotopic TLC-based enzyme assay was used to monitor 20α-reductase activity during isolation of 3β,20α-HSD, similar to that which we reported earlier (3,4). However, a new HPLC system for assaying 20α-reductase activity was developed with many advantages over the radioisotope method. HPLC is much more rapid than TLC for separating the steroids, the results are exactly reproducible, and the problems of radioactive background and radioisotope handling are eliminated. Analyses of the steroids for assaying 20α-reductase activity by the radioisotope method take about 45 to 50 min for TLC separation and ^{14}C-counting. The HPLC method gives more precise results within 5 min, representing a 10-fold savings in time and effort.

HPLC could not be used for assaying 3β-reductase activity because steroids emerging from the chromatographic column are analyzed with a *photometric* detector. Unlike progesterone and its

20α-reductase product 4-pregnen-20α-ol-3-one, both the 3β-reductase substrate 5α-dihydrotestosterone and also the product 5α-androstan-3β,17β-diol lack chromophores that absorb UV light, essential for detecting the steroids by HPLC. However, HPLC is superior to the radioisotope method for 20α-reductase kinetic studies in which 5α-dihydrotestosterone serves as an inhibitor of 20α-reductase activity (Table 3). 4-Pregnen-20α-ol-3-one and 5α-dihydrotestosterone have similar R_f values, but only 4-pregnen-20α-ol-3-one is photometrically detected as it emerges from the HPLC column.

Purification of 3β,20α-hydroxysteroid oxidoreductase

3β,20α-HSD was isolated from ovine fetal blood according to the purification scheme described under Materials and Methods and summarized in Table 1. The method for purifying ovine 3β,20α-HSD was similar to that used by us earlier for the corresponding bovine enzyme (3,4).

The calcium phosphate (Ca–P) step is effective in separating the large amounts of hemoglobin (about 85% of the protein in the ammonium sulfate fractionated material) from crude 3β,20α-HSD. It is important to freshly prepare Ca–P gel (from $CaCl_2$ and Na_3PO_4) in the laboratory for this purification step. Suspending the commercially available Ca–P in water was found ineffective. The newly prepared Ca–P must be exhaustively washed with distilled (or deionized) water for removing the soluble ions. This is confirmed by

testing aliquots from the wash water with a solution of 0.1 **M** silver nitrate solution. The tested aliquots progress from an initial, heavy precipitate to a slightly cloudy solution. When further washings do not decrease the cloudiness in the test solutions, the Ca–P gel is usable for separating the enzyme from hemoglobin.

Losses of at least 80% of the enzyme during the Ca–P gel purification process were disappointing. Many attempts were made to improve the yield of enzyme in the Ca–P step by systematically varying the following: the ratio of Ca–P gel to the crude enzyme mixture, pH, ionic strength, the time for each manipulation, and temperature. In each attempt, enzyme activity was monitored during all phases of the procedure. Generally, about 20-30% of the enzyme is lost with the hemoglobin during the initial adsorption of an enzyme to the Ca–P. Another 20-30% of the enzyme is released from the Ca–P gel while residual hemoglobin is washed from the gel. Finally, 20-30% of the enzyme is lost during its recovery from the Ca–P gel. Most likely, we are dealing with differences in the equilibrium of binding of the enzyme and hemoglobin to Ca–P. Separation of the two proteins with Ca–P evidently relies on the enzyme more strongly binding to the Ca–P gel than hemoglobin. Thus the initial large amounts of hemoglobin relative to the enzyme in the crude preparation, the subsequent wash steps, and the position of equilibrium associated with distribution of the enzyme between adsorption to the Ca–P gel and the 100 m**M** phosphate buffer used for

its recovery from Ca–P each contribute to reducing the yield of the enzyme.

Attempts to improve the recovery of 3β,20α-HSD during its purification led us to bypass the Ca–P step and apply the crude mixture to the Cibacron Blue Agarose affinity column. This approach was unsuccessful. We speculate that the copious amounts of hemoglobin overloaded the affinity adsorbent, preventing the enzyme from binding to the column. However, after most of the hemoglobin has been removed by the Ca–P gel treatment, affinity chromatography with Cibacron Blue Agarose provides a 15-fold purification of 3β,20α-HSD (Table 1).

Table 1. Purification of 3β,20α-hydroxysteroid oxidoreductase

	Total Protein (g)	Total 3β,20α-HSD (mU)[a]	Spec. Act. (μU/mg)	Purif. Factor
$(NH_4)_2SO_4$ [b]	50.0	900	22	1
Ca–P [c]	0.53	129	240	11
Aff. Chrom. [d]	0.02	71	3,550	161
FPLC	0.008	57	7,100	323

[a] 1 U = μmol of substrate reduced per min (3). [b] Material from 30%-60% saturation of ammonium sulfate fractionated enzyme. [c] Material from calcium phosphate (Ca–P) gel purification. [d] From Cibacron Blue Agarose, affinity chromatography step.

Following the final FPLC step, the specific activity of isolated 3β,20α-HSD is 7.1 mU/mg. The specific activity of 3β,20α-HSD in whole fetal blood is 3 or 22 μU/mg in the $(NH_4)_2SO_4$ fraction. Thus we have achieved an overall 2,370-fold purification of ovine 3β,20α-HSD from whole blood, or 323-fold purification from the crude $(NH_4)_2SO_4$ fraction.

Characterization of 3β,20α-hydroxysteroid oxidoreductase

The identities of the products from the enzyme's NADPH-dependent reductase activity were established by crystallization methods. The results of the crystallization experiments are summarized in Table 2. 3β-Reductase and 20α-reductase activity were continuously measured throughout purification of ovine fetal 3β,20α-HSD. The ratio of the two reductase activities remained essentially the same during the four purification steps. The kinetics of 20α-reductase and 3β-reductase activity with isolated 3β,20α-HSD are summarized in Table 3 (discussed below).

The molecular weight of native, ovine 3β,20α-HSD was determined by its filtration together with standard proteins through a Sephadex G-100 column. The experiment, repeated three times, consistently provided a molecular weight for ovine 3β,20α-HSD of M_r 35,000. Polyacrylamide slab gel electrophoresis of 3β,20α-HSD

Table 2. Reduction by 3β,20α-hydroxysteroid oxidoreductase of progesterone or 5α-dihydrotestosterone

Step	Solvent	Mother liquor (cpm/mg)	Crystals (cpm/mg)
	4-[^{14}C]pregnen-20α-ol-3-one [a]		
1	ethanol-water	16,500	4,115
2	ethanol-water	19,800	4,105
3	ethanol-water	16,800	4,095
	5α-[^{3}H]androstan-3β,17β-diol [a]		
1	ethyl acetate-ethanol	2,468	809
2	ethyl acetate-ethanol	1,746	805
3	ethanol-water	1,779	788

Step	Solvent	Mother liquor ([^{3}H] cpm/[^{14}C] cpm)	Crystals ([^{3}H] cpm/[^{14}C] cpm)
	5α-[^{3}H/^{14}C]androstan-3β,17β-diol [b]		
1	ethyl acetate-ethanol	126	304
2	ethyl acetate-ethanol	329	332
3	ethanol	274	314

[a] Single isotope method. [b] Double isotope ratio method.

(treated with sodium dodecylsulfate) produced a single band corresponding to M_r 36,000 with respect to four standard proteins. The electrophoresis experiment was repeated three times. These data suggest that ovine 3β,20α-HSD is a single-stranded protein of M_r 35,000.

Kinetics of 20α-reductase and 3β-reductase activity of ovine 3β,20α-HSD

A series of kinetic experiments was carried out to characterize the 20α-reductase and 3β-reductase activity of ovine 3β,20α-HSD. The results are summarized in Table 3 and reveal that progesterone and 5α-dihydrotestosterone are somewhat similar substrates (*viz.* K_m and V_{max} values). Based on the calculated Π values, progesterone has a utilization efficiency about 1.3 times that of 5α-dihydrotestosterone.

5α-Dihydrotestosterone was tested as an inhibitor of 20α-reductase activity. Dixon plots of the kinetic data revealed that the C_{19} steroid is a competitive inhibitor against progesterone with a K_i value of 102 μM. These data suggest that 20α-reductase and 3β-reductase activity may share the same catalytically active site of ovine 3β,20α-HSD. To prove the dual catalytic nature of a single active site will require further evidence from affinity labeling experiments.

Table 3. Comparison of the kinetic parameters of ovine and bovine 3β,20α-hydroxysteroid oxidoreductase

Substrate (activity)	K_m (μM)	V_{max} (μmol/min/μmol)	Π[a] (min-μmol/L)	K_i [b,c] (μM)
		Ovine		
progesterone (20α) [c]	30.8	0.7	44.0	
5α-DHT [d] (3β) [e]	74.0	1.3	56.9	102
		Bovine [f]		
progesterone (20α)	2.5	2.4	1.04	
5α-DHT (3β)	9.4	2.4	3.92	35

[a] Parameter of substrate utilization efficiency ($\Pi = K_m/V_{max}$), reflects increasing utilization with decreasing value of Π (7). [b] The K_i values were derived from measurements of 20α-reductase activity (progesterone as substrate) with 5α-dihydrotestosterone as inhibitor. [c] From the HPLC method. [d] 5α-Dihydrotestosterone (5α-DHT). [e] From the radioisotope method. [f] Data from Sharaf and Sweet (4).

Comparison of ovine and bovine 3β,20α-hydroxysteroid oxidoreductase

From the physico-chemical standpoint of isolation (Table 1), ovine and bovine 3β,20α-HSD exhibit similar properties with respect

to their precipitation with 30 to 60% saturation of $(NH_4)_2SO_4$ at 0 to 4°C, adsorption with calcium phosphate gel (which removes most of the hemoglobin), and affinity chromatography with Cibacron Blue Agarose (recovery with NADPH + progesterone mixtures). However, the two enzymes differ by about 20,000 in their molecular weights: ovine 3β,20α-HSD was found to have M_r 35,000 while bovine 3β,20α-HSD has M_r 55,000 (4). Both enzymes appear to be single stranded by SDS disc gel electrophoresis.

Comparing the kinetics of the corresponding 3β-reductase and 20α-reductase activities of ovine and bovine 3β,20α-HSDs is interesting (Table 3). Both ovine and also bovine 3β,20α-HSD require NADPH for reductase activity, and each enzyme utilizes progesterone or 5α-dihydrotestosterone as a substrate. Under similar assay conditions, the corresponding K_m and V_{max} values for progesterone and 5α-dihydrotestosterone differ considerably for ovine and bovine 3β,20α-HSD. The corresponding parameters of substrate utilization efficiency (Π) reveal that bovine 3β,20α-HSD utilizes progesterone some 3.8 times more efficiently than 5α-dihydrotestosterone. Ovine 3β,20α-HSD utilizes progesterone about 1.3 times more efficiently than 5α-dihydrotestosterone. These data show a parallelism between the two 3β,20α-HSD's in their relative utilization efficiencies of C_{21} and C_{19} substrates.

The 20α-reductase activity of ovine and bovine 3β,20α-HSD is competitively inhibited by 5α-dihydrotestosterone with K_i values of 102

and 35 μM, respectively. These data suggest that the C_{19} and C_{21} substrates compete for functionally similar catalytic sites in ovine or bovine 3β,20α-HSD. Earlier, bovine 3β,20α-HSD was affinity labeled with 19-nortestosterone 17-bromoacetate, and it was demonstrated that 3β-reductase and 20α-reductase activities share the same catalytic site (4).

A pattern of dual substrate activity has emerged among enzymes involved in steroid biosynthesis. Earlier, evidence from affinity labeling of bacterial 3α,20β-hydroxysteroid oxidoreductase demonstrated that the same catalytically active site contains *both* 3α- and 20β-reductase activity (7). Subsequently, a single active site of human placental estradiol 17β-dehydrogenase was reported to promote both 17β- and 20α-reductase activity (8). More recently, a cytochrome P-450 enzyme from porcine fetal testes that converts progesterone to 4-androstene-3,17-dione was shown by affinity labeling to carry out *both* a 17-hydroxylase and also a $C_{17,20}$-lyase step at the *same* catalytically active site (9). The present finding that 3β- and 20α-reductase activities are produced by the same ovine fetal 3β,20α-HSD enzyme and that the two activities may share a common site is consistent with this pattern.

Affinity labeling experiments are in progress to locate the site of 3β-reductase and 20α-reductase activity in ovine 3β,20α-HSD and to determine its amino acid topography (10). We have also begun to produce antibodies against the isolated ovine fetal 3β,20α-HSD for use

in cross reaction experiments to determine whether ovine and bovine 3β,20α-HSDs have common features. Furthermore, the anti-ovine 3β,20α-HSD will be used in an attempt to learn the role of this blood enzyme during fetal development by measuring evolution of the fetal enzyme in growing ovine embryos and fetuses.

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REFERENCES

1. Nancarrow CD and Seamark RF (1968). Progesterone metabolism in fetal blood. STEROIDS **12**:367-379.
2. Nancarrow CD, Connell PJ, and Stevens D (1981). Observations on the activity of 20α-hydroxysteroid oxidoreductase in ovine foetal erythrocytes. PROC AUST SOC REPROD BIOL **13**:101.
3. Nancarrow CD, Sharaf MA, and Sweet F (1981). Purification of 20α-hydroxysteroid oxidoreductase from bovine fetal erythrocytes. STEROIDS **37**:539-553.
4. Sharaf MA and Sweet F (1982). Dual activity at an enzyme site: 3β,20α-hydroxysteroid oxidoreductase from fetal blood. BIOCHEMISTRY **21**:4615-4620.
5. Nancarrow CD (1983). Decline with age in the rate of reduction of progesterone to 20α-hydroxypregn-4-en-3-one in the blood of perinatal ruminants. AUST J BIOL SCI **36**:183-190.
6. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951). Protein measurement with Folin phenol reagent. J BIOL CHEM **193**:265-275.
7. Sweet F and Samant BR (1980). Bifunctional enzyme activity at the same active site. Study of 3α- and 20β-activity by affinity alkylation of 3α,20β-hydroxysteroid dehydrogenase with 17-bromoacetoxy steroids. BIOCHEMISTRY **19**:978-986.
8. Thomas JL, LaRochelle MC, Asibey-Berko E, and Strickler RC (1985). Reactivation of human placental 17β,20α-hydroxysteroid dehydrogenase affinity alkylated by estrone 3-bromoacetate: topographical studies with 16α-bromoacetoxy-estradiol-17β 3-methyl ether. BIOCHEMISTRY **24**:5361-5367.
9. Onoda M, Haniu M, Yanagibashi K, Sweet F, Shively JE, and Hall PF (1987). Affinity alkylation of the active site of C_{21} steroid side-chain cleavage cytochrome P-450 from neonatal porcine testis: a unique cysteine residue alkylated by 17-(bromoacetoxy)-progesterone. BIOCHEMISTRY **26**:657-665.
10. Sweet F and Murdock GM (1987). Affinity labeling of hormone-specific proteins. ENDOCR REV **8**:154-184.