PROGESTERONE METABOLISM IN OVINE BLOOD : THE FORMATION OF 3α-HYDROXY-PREGN-4-EN-20-ONE AND OTHER SUBSTANCES

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ABSTRACT

The metabolism of 4^{14}C-progesterone in sheep blood has been investigated. Sheep foetal blood cells contain in addition to the 20α-hydroxysteroid oxidoreductase (20αHSD) previously reported at least three other enzymes reacting with progesterone. Most active of these enzymes is a 3α-reductase (3αHSD) which catalyses the reaction:- progesterone ——> 3α-hydroxy-pregn-4-en-20-one. In addition evidence is presented to suggest that 20β-reductase and 6β-hydroxylase activity are present in fetal blood.

Adult ovine blood cells contain both 20αHSD and 3α-reductase activity although 20αHSD activity was much reduced.

The possible significance of blood gestagen metabolism in the ovine is discussed.

INTRODUCTION

Differences observed in the metabolism of gestagens in the sheep fetal-placental unit compared with the human, may in part, be attributed to active gestagen metabolism in sheep fetal blood (2). We have previously described the occurrence in ovine fetal blood cells of an active 20α-hydroxysteroid oxidoreductase (20αHSD) (3). In this communication we wish to report that ovine fetal blood cells also contain three other enzymes reacting with progesterone, namely a 3α-reductase, a 20β-reductase and a 6β-hydroxylase. The occurrence of a 3α-reductase in adult ovine blood is also reported and the presence of 20αHSD confirmed (4).

MATERIALS AND METHODS

Blood Samples were obtained from sheep fetuses salvaged from freshly slaughtered animals at the city abbatoirs or directly from live fetuses of known gestational age via cannulae placed in the umbilical artery by surgical procedures (3).

Other samples were taken by venepuncture under sterile conditions from conscious unanaethesitised animals. Samples were collected in sterile containers and stored on ice until used (maximum period 2 hours).

Chemicals 4-^{14}C progesterone (36.1 mCi/mM) and 7α-^{3}H-progesterone (2.26 Ci/mM) were obtained from the Radio-Chemical Centre, Amersham and purified by chromatographic procedures immediately before use. 3β-hydroxy-pregn-4-en-20-one (Δ^4-3βHP) was prepared by sodium borohydride reduction of progesterone in isopropanol as described by Kupfer (1961) (5). 6β-hydroxyprogesterone was obtained from Steraloids. The progesterone and other nonlabelled steroids used in this study were purchased from Ikapharm (Israel). All other reagents were of analytical grade. Organic solvents were redistilled before use.

7α-^{3}H-3α-hydroxy-pregn-4-en-20-one (Δ^4-3αHP) was isolated as a minor product during preparation of Δ^4-3βHP from progesterone (200 mg) to which had been added 0.05 mCi 7α-^{3}H-progesterone.

The progesterone was reacted at room temperature (36 hours) with 25 mg sodium borohydride in isopropanol as described by Kupfer (5) and the extract treated with digitonin. Following collection of the digitonides the supernatant containing Δ^4-3αHP, was concentrated in vacuo and subjected to preparative thin layer chromatography (system 1) on 20 x 20 cm plates.

A band of material was identified (Rf 0.37) which reacted with concentrated H_2SO_4 to give a characteristic pink color. The material in this band was eluted and rechromatographed several times (system 6 RF 0.61; system 7, Rf 0.65) to assure resolution from other reaction products present. Final purification was achieved by column (30 x 1 cm) chromatography over 10 gm benzene washed alumina (4.7% moisture content). The following fractions were collected : 20 ml benzene and twelve 10 ml fractions of 0.2% ethanol in benzene. The fractions were monitored by TLC; the fractions showing only one compound reacting with I_2 vapor and phosphomolybdate spray were combined and concentrated in vacuo. Recrystallization (3 x) from hexane-benzene mixtures gave 2-5 mgm material mp: 144-146°C; treatment with trichloroacetic acid (TCA) in chloroform gave the expected red color (positive Rosenheim). The infra red spectrum of this material was determined in chloroform solution and the following main identification bands detected:- saturated acyclic oxone (C20) 1702 cm^{-1}; free hydroxyl, 2620 cm^{-1}; isolated double bond 1640 cm^{-1}. The absence of a band at 1668 cm^{-1} which would have indicated an unsaturated oxo group at C-3 was also noted. λ Max. concentrated H_2SO_4, 1 hour at room temperature) at 325 mμ with weaker peaks at 412 and 490 mμ; other properties of this material are described in results section.

Incubation and extraction procedure Incubations were carried out in Ehrlenmeyer flasks. The radioactive substrate (0.01 μCi-100 μCi) plus any unlabelled steroid was added to the flask in ethanol and dried under a stream of N_2. The blood or blood fraction (usually 2-5 ml) was added to the flask which, after mixing, was incubated with gentle shaking at 37° in an air atmosphere. Water or 0.9% saline was used to replace the blood in incubation mixtures in control flasks.

The reaction was stopped and extracts prepared as described previously (3). In some experiments, the radioactivity was extracted from the incubation mixture directly into ethyl acetate (3 vols), following dilution with 2 vol of water.

Chromatography Thin layer chromatrography (TLC) was carried out on silica gel G (Merck) plates in the following systems (6,7,8). (Table 1).

1. Benzene:ethyl acetate (3:1, unsaturated atmosphere)
2. Ethylacetate:n-hexane:ethanol:acetic acid (72.0:13.5:4.5:10.6)
3. Cyclohexane:ethylacetate (1:1)
4. N-hexane:ethylacetate (3:1)
5. Benzene:absolute ethanol (9:1)
6. Chloroform:absolute ethanol (9:1)
7. Ethylacetate:cyclohexane:absolute ethanol (45:45:10)
8. Benzene:absolute ethanol (19:1)
9. Chloroform:absolute ethanol (19:1)

Paper chromatography (PC) was undertaken with the Bush A systems of light petroleum:methanol:water (5:4:1) and a slightly modified Bush system of toluene:light petroleum (b.p. 60:80°C):methanol water (1:5:7:3) after overnight equilibration at 29°C (9). Methods used in localization of radioactive areas and reference steroids and the procedure for elution, assay of radioactivity and derivative formation have been described previously (3, 10). Melting point determinations were made using a micro heating stage (Kofler). Vapor phase chromatography (VPC) was carried out using a Perkin Elmer Model 811 gas chromatograph. Fractions were collected for radioactivity determination using a fraction collector (Packard Model 850). In addition the following detection reagents were used for TLC. A) Phosphomolydic acid (10% in EtOH) at room temperature, B) Trichloroacetic acid (25%) in $CHCl_3$ and C) concentrated H_2SO_4.

RESULTS

Metabolism of progesterone by sheep blood

In experiments previously reported (3) a significant

TABLE 1

Rf values of a 3α-hydroxy-pregn-4-en-20-one and six other closely related compounds in chromatographic systems* used in the present studies

	SYSTEM						DETECTION REAGENT		
	1	3	5	7	8	9	A	B	C
3α-hydroxy-pregn-4-en-20-one	0.37	0.39	0.47	0.65	0.29	0.53	purple	red	yellow —> pink
3β-hydroxy-pregn-4-en-20-one	0.43	0.43	0.52	0.62	0.30	0.55	purple	red	yellow —> pink
Progesterone	0.48	0.42	0.55	0.69	0.40	0.71	-	-	-
20α-hydroxy-pregn-4-en-3-one	0.26	0.35	0.45	0.61	0.23	0.45	-	-	-
3β-hydroxy-pregn-5-en-20-one	0.37	0.37	0.45	0.66	0.24	0.46	green	-	yellow —> pink
3β-hydroxy-5α-pregnan-20-one	0.37	0.39	0.43	0.62	0.25	0.44	pale green	-	yellow
3β-hydroxy-5β-pregnan-20-one	0.45	0.46	0.49	0.65	0.30	0.52	-	-	pale yellow
5β-pregnan-3α,20α-diol	0.11	0.23	0.34	0.51	0.02	0.34	-	-	-
5β-pregnan-3α,20β-diol	0.15	0.30	0.35	0.56	0.15	0.38	-	-	-

* See Methods for details of system and detection reagents

TABLE 2

VAPOR PHASE CHROMATOGRAPHY (V.P.C.) DATA FOR

3α-HYDROXY-PREGN-4-EN-20-ONE AND OTHER

CHEMICALLY RELATED COMPOUNDS

Chromatography was carried out on a 6 ft glass column (i.d. 1.5 mm) using 0.8% Neopentylglycol Adipate (Applied Science Labs) on a support phase of 100/120 mesh Gas Chrom Q. Operating temperatures:- injector 279°C, Column 235°C, Detector 270°C. Carrier gas N_2 flow rate 30 ml/min. Flame ionisation detector.

Compound	Retention time (min) relative to progesterone*
3α-hydroxy-pregn-4-en-20-one	5.5
3β-hydroxy-pregn-4-en-20-one	6.4
3β-hydroxy-pregn-5-en-20-one	6.5
3α-hydroxy-5β-pregnan-20-one	5.4
3β-hydroxy-5β-pregnan-20-one	5.1
3β-hydroxy-5α-pregnan-20-one	6.2
5β-pregnan-3,20-dione	6.0
5β-pregnan-3α,20α-diol	6.7
5α-pregnan-3β,20α-diol	7.6

* Mean retention time progesterone 10.7 min (± 0.01 S.E.M.)

proportion (16%) of radioactivity recovered from incubation mixtures of both adult and fetal blood with ^{14}C-progesterone was present in metabolites other than 20αHP. On radiochromatogram scans (system 1) this material was detected as two and occasionally three peaks of activity in addition to the peaks corresponding to 20αHP and progesterone substrate.

One peak (I) was present between 20αHP and progesterone where the pregnanolones and pregnenolones are found and the second (II) was more polar than 20αHP and was present in the pregnanediol region. The other peak (III) occasionally seen did not move from the origin in system 1.

Identification of metabolites

3α-hydroxy-pregn-4-en-20-one (Δ^4-3αHP)

Peak I. Material for several experiments present in this area of the chromatograms was pooled and rechromatographed in system 1 and 4. A single peak of radioactivity was detected which corresponded to pregnenolone (3β-hydroxy-pregn-5-en-20-one). On acetylation the material ran with pregnenolone-3-acetate (system 1), the change in RF on acetylation (ΔRf = 0.37) indicating a single OH function. However, the material was resolved from pregnenolone by chromatography (system 9) (Table 1) and after treatment with CrO_3 in acetic acid, it underwent a change to form compound(s) which did not move off the origin of chromatograms (system 1 and Bush A). This apparent decomposition probably reflects the acid lability of the metabolite as it was sub-

sequently found that when treated at room temperature with acetic acid containing about 6% of concentrated HCl (11) rapid decomposition occured to afford more polar products (system 1).

The acid lability of the compound suggested the possibility of the Δ^4-3-hydroxy configuration as both the Δ^4-3α and Δ^4-3β compounds are known to be labile under the oxidising condition employed (11).

Treatment of the material with a slight excess (1.5 equivalents) of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in tert-butanol at room temperature (12) led to the formation of progesterone in about 25% yield. The product (0.079 μCi) was refractive to acetylation and was identified by chromatography in several systems (systems 1, 6 and Bush A), and finally by recrystallization with 50 mg authentic progesterone containing ^{3}H-progesterone (10 μCi) (Table 3) to constant ^{3}H/^{14}C ratio.

Table 3

CRYSTALLIZATION DATA OF ^{14}C-LABELLED DDQ OXIDATION PRODUCT OF Δ^4-3αHP WITH AUTHENTIC 7α-^{3}H-PROGESTERONE (See text for details)

		^{3}H/^{14}C Ratio
Hexane-Benzene	ML	192.8
	C	99.6
Hexane-Benzene	ML	136.9
	C	116.3
Light Petroleum-Benzene	ML	130.6
	C	123.5
Methanol-water	ML	120.0
	C	125.4

Values are expressed as d.p.m. ^{3}H/d.p.m. ^{14}C determined on samples of mother liquor (ML) or crystals (C).

Both Δ^4-3βHP and Δ^4-3αHP but not pregnenolone are oxidised under the above conditions with DDQ. However, the material was shown to be the Δ^4-3αHP compound by chromatography (systems 1,3).

Additional evidence that the metabolite was Δ^4-3αHP was obtained by i) establishing that the chromatographic mobilites of the ^{14}C-labelled metabolite were identical to authentic Δ^4-3αHP in several systems (Table 1) both before and after acetylation procedures (ΔRf on acetylation = 0.37, system 1) and ii) that retention time on VPC was identical (Table 2) and finally by iii) recrystallizing the material (0.29 μCi) to constant $^3H/^{14}C$ with the authentic material (4.53 mg; 1.14 μCi) prepared from 7α-^{3}H-progesterone (see methods and Table 4).

Table 4

CRYSTALLIZATION DATA OF ^{14}C-LABELLED Δ^4-3αHP FORMED ON INCUBATION OF 4^{14}C PROGESTERONE WITH OVINE BLOOD AND 7α-^{3}H-Δ^4-3αHP PREPARED SYNTHETICALLY

(See text for details)

		$^3H/^{14}C$ ratio
Hexane-Benzene	ML	18.3
	C	16.0
Light petroleum-benzene	ML	16.9
	C	12.0
*Methanol-water	ML	14.0
	C	13.6

* As acetate

Values expressed as d.p.m. ^3H/d.p.m. ^{14}C determined on samples of mother liquor (ML) or crystals (C).

Pregn-4-en-3 ,20α-diol (Δ^4-pregnenediol)

Peak II. Material present in this area of the chromatogram corresponded in chromatographic mobility (system 3) to 5α-pregnane-3β,20α-diol. On acetylation the ΔRf values 0.67 (system 1) were comparable indicating two hydroxy functions. The metabolite was decomposed on treatment with CrO_3 in acetic acid and the possibility of the Δ^4-3ξ,20α-diol structure was considered.

Consistent with this possibility was the finding that mild oxidation with DDQ lead to the formation (15-20% yield) of 20α-hydroxy pregn-4-en-3-one (20αHP). Identification of the oxidation product was achieved by its identical behaviour to authentic 20αHP on:- 1) chromatography in several systems (Bush A, and TLC systems 1,3,9), both before and after acetylation procedures, 2) refraction to oxidation with 20βHSD, 3) and finally oxidation with CrO_3 in acetic acid to form progesterone.

Other Metabolites

In one experiment where 100 μCi (1.43 mg) 4^{14}C-progesterone was incubated with 5 ml whole fetal blood for one hour, several of the minor products were found in sufficient quantities for preliminary identification to be achieved. 0.26% of the recovered radioactivity in this experiment was chromatographically identified as 20β-hydroxy-pregn-4-en-3-one (20βHP). The chromatographic mobility of the U.V. light absorbing metabolite (λmax 241 mμ) corresponded exactly with authentic 20βHP in both the Bush A and TLC system 1. The identity of this

compound was confirmed by reacting the metabolite with the specific 20βHSD enzyme and characterizing the product as progesterone (3).

Several other metabolites formed remained on or near the origin in system 1. On rechromatography in the Bush A system the location of the major metabolites (approximately 0.3% recovered radioactivity) corresponded to a U.V. absorbing area (λmax 241 mμ). This metabolite behaved identically to 6βHP when chromatographed in the modified Bush A system and in system 2 and further evidence was obtained when it was shown that after acetylation, the material behaved identically to authentic 6β-acetoxy progesterone in system 1.

Factors influencing progesterone metabolising activity in sheep blood

a. Fetal vs adult blood Blood samples obtained from fetal sheep (70-140 days gestational age), lambs and adult sheep were examined for progesterone metabolising activity. In these assays a 2 ml sample of whole blood was incubated with 25 μg of 4^{14}C-progesterone for 30 minutes at 37°C in air. As previously described (3) the major metabolite formed in all fetal samples was 20αHP, the percentage formation being constant (70.9 ± 2.3 S.E.M.). After parturition the amount of 20αHP found decreased reaching a low adult level (<2%) within 60 days. In contrast there was no significant difference ($P > 0.50$) in the total amounts of other metabolites formed between fetal lamb and adult blood (13.2 ± 1.5 S.E.M. and 10.5 ± 0.5 S.E.M. respectively). There were differences, however, in the distribution of radioactivity as determined on the initial chromatogram (system 1)

between Δ^4-3αHP and the presumptive 3β,20α-diol. The mean values for lamb and adult blood expressed as a percentage of recovered radioactivity being 10.4 ± 0.5 S.E.M. and 1.5 ± 0.1 S.E.M. respectively for peak I (i.e. Δ^4-3αHP) and <1 and 11.7 ± 1.3 S.E.M. for peak II (i.e. the presumptive diol).

b. Localization, cell lysis and addition of cofactors The progesterone metabolising activity was shown to be present in the red blood cell fraction of whole blood. When plasma (fetal or adult) was incubated with progesterone either with or without cofactor no detectable metabolism occured. No 3α-reductase activity was detected in incubation mixtures containing progesterone and lysed cell preparations from either adult or fetal blood unless the coenzyme NADPH (0.5 μmol) was added then a 2-3 fold increase in the formation of both 20αHP and the other metabolites was obtained : NAD was ineffective in restoring the activity.

DISCUSSION

3αHSD in ovine blood

Blood of the foetal sheep possess a remarkable capacity to metabolise progesterone. The main enzyme involved in this function is a 20αHSD present in the blood cells but significant levels of activity of the other enzymes, particularly the 3α-reductase, are also present. Whilst 3α-reductase activity has not been previously reported as occuring in blood cells it is known to be widely distributed elsewhere in animal tissues particularly the liver and kidney (13). However, the

activity towards Δ^4-3 oxosteroids is generally thought to follow Δ^{4-5} reductase activity. Neeman and his colleagues (14) were first to obtain evidence that biological conversion of Δ^4-3 oxosteroids into Δ^4-3-hydroxysteroids may take place, in animal tissue. These authors isolated 11β-hydroxy-androst-3,5-diene-17-one from the urine of a normal male to whom 11β-hydroxy-androst-4-en-3,17-dione has been administered. Later unequivocable evidence identifying 3α,11β-dihydroxy-androst-4-en-17-one as a metabolite of 11β-hydroxy-androst-4-en-3,17-dione in the urine of man was obtained (16). The Δ^4-3β-hydroxy product was also isolated from the urine extracts but was shown to have formed as an artifact resulting from the mild acid conditions of hydrolysis employed. Ringold and coworkers (15) found evidence for Δ^4,3-hydroxy (3α- and 3β-) formation in rat liver with the synthetic compound 6β-fluorotestosterone as substrate and Farnsworth (17) reported about the same time chromatographic evidence to indicate that similar activity was present in human prostate tissue. More recently Levy and coworkers (11) described the metabolism of 17α-hydroxy progesterone (17αHP) to 3α,17α-dihydroxy-pregn-4-en-20-one and the corresponding 20α-hydroxy-triol, by bovine adrenals and ovaries. The experimental method involved the incubation of 37 mg 17αHP with 650-750 ml whole cow blood for 2.5 to 3.8 hr. It is now possible, from the data presented in this paper, that the transformations reported may have occurred in the blood itself (3), although tentative evidence has been presented for the formation of 3α or 3β-hydroxy-pregn-4-en-20-one in incubation experiments of progesterone with human ovarian tissue (18).

To our knowledge no further reports of the formation in vivo of Δ^4-3α-hydroxy steroids exist. Levy and coworkers comment that these compounds may be widely formed in animal tissues but that the reaction may not be recognised due to the acid lability of the product.

We are able to confirm this acid lability, and data from our chromatographic studies indicates the ease with which these substances may be confused with closely related steroids. In these studies, the strong reaction of Δ^4-3-hydroxy compounds with TCA in chloroform (Rosenheim reaction) and with phosphomolybdic acid spray, to give a characteristic purple colour at room temperature, or with concentrated H_2SO_4 to give a pink color proved particularly helpful in chromatographic studies.

The significance of 3α-reductase in ovine blood remains to be determined. It is of interest that whilst the high fetal blood levels of 20αHSD declines over the first 60 days of adult life the 3α-reductase activities persist and appears to be equally active in the mature erythrocytes. 20αHSD activity in adult ovine blood cells has previously been described by van der Molen and Groen (4).

The 3α-reductase has not been tested for reversal of reaction either with Δ^4-3α-HP or with 3α-hydroxy-5ξ-pregnane steroids. It may be that the reaction progesterone $\rightleftharpoons$ Δ^4-3αHP occurs, and the significance of the activity may be to conserve gestagens.

Recent application of sensitive progesterone assay methods to adult ovine blood (19) reveal progesterone concentrations which would

only account for 1/1000 of the gestagen levels determined using bio-assay techniques (20). It remains to be determined if Δ^4-3αHP occurs in ovine blood and contributes towards gestagen activity.

Other activities

The significance of the presumptive 20β-reductase and 6β-hydroxylase activity in fetal blood is unknown. ^{14}C-labelled 20β-hydroxysteroids are found in fetal blood and tissues following infusion of 4-^{14}C progesterone into the ovine fetal placental unit (2). However, 20β-reductase occurs as a major activity in fetal liver (2) and the observed transformations probably take place in tissues. No 20β-reductase activity was detected in the adult ovine blood although this is the major activity of adult bovine blood cells (21).

Chromatographic evidence only is presented for the formation of 6βHP in fetal blood. 6β-hydroxylase activity has been described for human blood cells (22) and is known to be present in human placental tissues (23). The activity in ovine blood in comparison to 20αHSD and 3α-reductase activities are slight but may be significant in any long term infusion or incubation experiments in vitro.

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