PREGNENOLONE AND PREGNENOLONE SULFATE METABOLISM BY HUMAN FETAL TESTES IN VITRO [1]

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ABSTRACT

Homogenates of testes from human fetuses of 12-16 weeks gestation were incubated in vitro with pregnenolone-4-^{14}C and pregnenolone-7-^{3}H-sulfate. Dehydroepiandrosterone sulfate and pregnenediol sulfate, each bearing the tritium label only, were identified in the conjugated fraction. Progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, dehydroepiandrosterone, androstenedione and testosterone, each bearing both a tritium and ^{14}C-label, were identified in the organic fraction. In a similar incubation, with pregnenolone-7-^{3}H-sulfate-^{35}S as substrate, the direct conversion to 17α-hydroxypregnenolone sulfate was demonstrated. The presence of slight sulfatase activity was also noted. The direct metabolism of steroid sulfates and the presence of sulfatase activity in this fetal tissue and the possible significance of these findings in the developing male fetus are discussed.

INTRODUCTION

Steroid sulfates are present in relatively large quantities in the human fetal circulation (1). Steroid sulfokinase activity has been demonstrated both in vivo and in vitro in the human fetal adrenal (2-5). Normal adult human testes possess this capacity as well (6,7). Neutral steroid sulfate secretion by normal human adult testes has been demonstrated recently (8). Although the metabolism of certain unconjugated steroids by human fetal testes has been studied (9-11), the metabolism of steroid sulfates by this tissue has not.

Since steroid sulfates can be transformed to other steroid sulfates as well as to biologically active hormones in both the fetus and the adult (12-23), it was felt of interest to study in vitro the metabolic

fate of pregnenolone[5] and pregnenolone sulfate (PS) in human fetal testes.

MATERIALS AND METHODS

Human fetal testes were obtained from 3 fetuses of 12-16 weeks gestation at the time of hysterotomy for therapeutic termination of pregnancy. A segment of testis was removed from each fetus for histologic examination. Testicular tissue, weighing 17.6 mg from one fetus (crown-rump and crown-heel lengths of 75 mm and 102 mm respectively), used in incubation 1 was homogenized and immediately incubated, while testicular tissue weighing 43 mg from the two remaining fetuses (crown-rump and crown-heel lengths of one 86 mm and 125 mm respectively, and of the other 93 mm and 138 mm respectively), used in incubation 2, was frozen at -70°C in liquid nitrogen and thawed immediately prior to homogenization and incubation. Tissues were homogenized in an all glass Potter-Elvehjem apparatus.

Materials: Radioactive steroids were obtained from New England Nuclear Co. Pregnenolone-4-^{14}C, specific activity (SA) 56 mCi/mmole (lot 236-75A-14) was purified by thin layer chromatography (TLC) in systems 1 and 2 (Table 1). Pregnenolone-7-^{3}H-sulfate, SA 20 Ci/mmole (lot 321-190) was purified by Celite column partition chromatography (CPC) in system 3 and paper chromatography (PC) in system 4. Pregnenolone-7-^{3}H-sulfate-^{35}S was prepared by combining pregnenolone-7-^{3}H-sulfate and pregnenolone sulfate-^{35}S, (SA 12 mCi/mmole, lot 523004). The double labeled PS was purified in systems 3 and 4 and partitioned prior to use between organic solvent and water to remove unconjugated steroid.

All organic solvents were analytical grade and redistilled prior to use. Crystalline steroids used as standards and carriers were obtained commercially and assessed for purity by recrystallization, chromatographic behavior and melting point determination before use. Co-factors were purchased from Sigma Chemical Co.

The interconversion between ammonium and pyridinium salts of steroid sulfates was accomplished by the method of McKenna and Norymberski (24).

Incubation 1: The homogenized tissue was incubated in a medium containing 1.74 ml of 0.15 M phosphate buffer pH 7.4, 1.8 µCi pregnenolone-4-^{14}C, 10 µCi pregnenolone-7-^{3}H-sulfate, 1.49 µmoles ATP, 1.13 µmoles DPN, 1.01 µmoles TPN, 3.96 µmoles DL-isocitric acid and 200 µg isocitric dehydrogenase. Two drops of propylene glycol were added to dissolve the pregnenolone-4-^{14}C. The incubation was carried out in a Dubnoff metabolic incubator at 37°C for 3 hours using 95% O_2 and 5% CO_2 as the gas phase. The reaction was stopped by the addition

of dichloromethane, and the material was then partitioned between dichloromethane and water.

Incubation 2: The homogenized tissue was added to a flask containing pregnenolone-7-^{3}H-sulfate-^{35}S (2936 x 10^{4}cpm ^{3}H and 2343 x 10^{3} cpm ^{35}S).

The incubation medium contained Krebs-Ringer bicarbonate buffer, 2.98 μmoles ATP, 2.26 μmoles DPN, 2.02 μmoles TPN, 7.92 μmoles DL-isocitric acid and 400 μg isocitric dehydrogenase. The ratio of tissue (mg) to buffer (ml) was 20/1. Three tissueless control flasks and 2 inactivated tissue control flasks were prepared in a similar manner, but with 0.002 x the amount of PS. All flasks were incubated simultaneously in a Dubnoff metabolic incubator at 37°C for 3 hours using air as the gas phase. The reaction was stopped by the addition of 10 ml of dichloromethane to each flask. Following extraction, the material from each flask was submitted to purification by CPC in system 3 without prior partition into conjugated and unconjugated fractions.

Table 1. CHROMATOGRAPHIC SYSTEMS EMPLOYED

No.	Solvents	Ratios	Type of Chromatography
1	Cyclohexane, ethyl acetate, ethanol	9:9:2	TLC *
2	Benzene, ethanol	9:1	TLC
3	Isooctane, t-butanol, IM NH_4OH	3:5:5	CPC **
4	Isopropyl ether, t-butanol, NH_4OH, H_2O	6:4:1:9	PC ***
5	Heptane, methanol, H_2O	5:4:1	PC
6	Toluene, isooctane, methanol, H_2O	6.7:3.3:6:4	PC
7	Ligroine, propylene glycol		PC
8	Cyclohexane, ethanol, H_2O	5:3:2	CCD ****
9	Heptane, benzene, methanol, H_2O	3.3:1.7:4:1	PC
10	Isooctane, chloroform, n-butanol, methanol, pyridinium sulfate, pyridine	40:20:4:20:19:1	CPC

* TLC = Thin layer chromatography
** CPC = Column partition chromatography
*** PC = Paper chromatography
**** CCD = Countercurrent distribution

Isolation and identification of metabolites were performed using reverse isotope dilution techniques which included identical chromatographic behavior of the radioactive material and the steroid carrier under study in various chromatographic systems and successive recrystallization until crystals and mother liquor agreed within 5% and were within 5% of mean value.

Chromatography: CPC using Johns-Manville Celite No. 545, previously washed as described by Kelly et al (25), as the support and PC using Whatman No. 1 paper were performed as previously described (22). Thin layer chromatography (TLC) was carried out on silica gel G (Merck & Co., Darmstadt) as described by Lisboa and Diczfalusy (26). The systems used are listed in Table 1 and will be referred to in the text by number.

Estimation of Steroids: The identification of non-radioactive samples has been described previously (23). Polar material was dissolved in 1 ml methanol and 0.1 ml of solubilizer (Nuclear Chicago Corp.) prior to the addition of the scintillation medium. Quench corrections were performed using an internal standard technique. ^{3}H- and ^{14}C-activity were expressed as dpm. ^{35}S-activity is expressed as cpm after correcting for decay. Thin layer and paper chromatograms were scanned on a Packard radiochromatogram scanner Model 7200.

Derivative formation was performed when sufficient material was available. Solvolysis was carried out using the method of Segal et al (27). Acetylation using acetic anhydride and anhydrous pyridine (1:2) was carried out at room temperature overnight. Saponification was accomplished by the method of Neher et al (28).

RESULTS

Incubation 1

Aqueous Fraction. Following dichloromethane/water partition, 94.4% of the recovered ^{3}H-labeled material and 1.2% of the recovered ^{14}C-labeled material were found in the aqueous fraction. However, after PC in system 4, 5 zones of radioactivity were observed, all of which contained only the tritium label. All of the ^{14}C-labeled material migrated to the solvent front and was found to be pregnenolone.

Zone 1 (Rf=0.80): The radioactive material in this zone behaved in a similar fashion to carrier PS. This material was solvolyzed and submitted to PC in system 5. Two peaks were identified (Rf=0.45 and 0.02). The less polar area, which behaved like pregnenolone,

was rerun in system 5 after authentic pregnenolone carrier was added. Both radioactive and crystalline material exhibited identical mobility. It was concluded that this radioactive material represented unconverted substrate.

The more polar area was submitted to PC in system 6. The major peak of radioactivity (Rf=0.90) was acetylated and run in system 7. An area similar to 5-pregnene-3β,20α-diol diacetate (pregnenediol diacetate) (Rf=0.85) was identified. A portion of this material was submitted to countercurrent distribution (CCD) in system 8. A single peak (K=6.69) was observed associated with authentic carrier pregnenediol diacetate. After additional carrier was added, the radioactive material was recrystallized to constant SA (Table 2).

Zone 2 (Rf=0.64): This area was similar to 17α-hydroxypregnenolone sulfate (17α-OH-PS). However, following solvolysis and chromatography in several systems, the identity of this metabolite could not be established definitely.

Zone 3 (Rf=0.46): A portion of the radioactive material from this zone, which behaved like dehydroepiandrosterone sulfate (DHAS), was solvolyzed. After the addition of authentic dehydroepiandrosterone (DHA) carrier, this material was submitted to PC in system 7 for 50 hours, and both radioactive and added crystalline material had identical mobilities (Rt=0.32). The material was then submitted to PC in system 9 and the mobilities of carrier and radioactive material were again identical (Rf=0.57). The material was mixed with additional authentic DHA and recrystallized to constant SA as the unconjugated compound (Table 2).

Table 2. Radiochemical purity of steroid sulfates derived from incubation of fetal testicular homogenate with pregnenolone-7-^{3}H sulfate and pregnenolone-4-^{14}C.

5-pregnene-3β,20α-diol diacetate (following solvolysis and acetylation)			Dehydroepiandrosterone (following solvolysis)		
Solvent	Successive Recrystallization	dpm ^{3}H*/mg	Solvent	Successive Recrystallization	dpm ^{3}H*/mg
methanol	C_1	411	methanol, petroleum ether	C_1	87
methanol	C_2	397	methanol, petroleum ether	C_2	71
methanol	C_3	404	methanol	C_3	84
	ML_3	411		ML_3	91

*^{14}C-label was not found in these two metabolites
C = Crystal ML = Mother Liquor

Zone 4 and 5 (Rf=0.19 and 0.03 respectively). Despite further chromatography, the identity of this highly polar material could not be established before or after solvolysis.

Organic Fraction. Initial chromatography was performed in system 9. Several zones were identified. After further chromatography in appropriate systems and the addition of suitable cold carriers, the identity of the following unconjugated steroids was established by recrystallization to constant SA: pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, DHA, androstenedione and testosterone. Although losses were not calculated, DHA, 17α-hydroxypregnenolone and androstenedione appeared quantitatively to be the most important metabolites isolated (Table 3).

Table 3. Radiochemical purity of unconjugated steroids identified from incubation of fetal testicular homogenate with pregnenolone-7-^{3}H sulfate and pregnenolone-4-^{14}C ($^{3}H/^{14}C$ = 5.56).

Steroid	Solvent	Successive Recrystallization	dpm ^{3}H/mg	dpm ^{14}C/mg	$^{3}H/^{14}C$
	Methanol	C_1	2450	15336	
Pregnenolone	Ethanol	C_2	2492	15509	
	Methanol	C_3	2454	15622	0.16
		ML_3	2429	15703	0.15
17α-hydroxy-	Methanol, acetone	C_1	253	3322	
pregnenolone*	Methanol, acetone	C_2	197	3522	
	Methanol, acetone	C_3	102	3524	0.03
		ML_3	105	3507	0.03
	Ethanol, Petroleum ether	C_1	96	913	
Progesterone	N-hexane, acetone	C_2	93	901	
	N-hexane, acetone	C_3	96	912	0.11
		ML_3	89	915	0.10
	Ethanol	C_1	1069	707	
17α-hydroxy-	Methanol	C_2	1079	712	
progesterone	Ethanol	C_3	1128	724	1.56
		ML_3	1097	719	1.53
	Ethyl acetate, Methanol,				
Dehydroepi-	petroleum ether	C_1	1701	8146	
androsterone	Methanol, petroleum ether	C_2	1747	8392	
	Methanol	C_3	1693	8300	0.20
		ML_3	1803	8476	0.21
	Methanol	C_1	1943	2008	
Androst-	Ethanol	C_2	1898	1997	
enedione	Methanol	C_3	1922	1981	0.97
		ML_3	1940	1981	0.98
	N-hexane, acetone	C_1	278	462	
Testosterone*	N-hexane, acetone	C_2	271	468	
	N-hexane, acetone	C_3	278	459	0.61
		ML_3	299	479	0.62

* following acetylation

Incubation 2

Following initial chromatography in system 3, the material in hold back volume (HBV) I from the experimental and control flasks was found to contain only a ^{3}H-label. The amount of radioactivity recovered expressed as a percent of the ^{3}H-labeled radioactivity incubated, represents the degree of solvolysis occurring under these conditions. The amount of solvolysis in the control flasks was 0.31% and 0.32%.(tissueless and inactivated tissue control flasks respectively) and in the experimental flask 0.59%. The amount of sulfatase activity present in fetal testes under these incubation conditions is slight (approximately 0.27%).

In addition to this small amount of radioactivity in HBV I of each control incubation, an area of activity corresponding to the starting material (PS) was identified. No other areas of radioactivity were noted in the control incubations. The material in HBV I from the experimental flask was submitted to PC in a variety of systems. Pregnenolone and androstenedione were tentatively identified.

From the experimental flask, areas of radioactivity were noted in HBV IV to VI and HBV X to XII. The largest (HBV IV to VI) corresponded in mobility to carrier PS. The mobility of the radioactive material in the other area (HBV X to XIII), corresponded to carrier 17α-OH-PS and DHAS. After conversion to the pyridinium salt, the PS-like material was submitted to CPC in system 10. The area (HBV II to III) corresponding to carrier PS was submitted to PC in system 4 and behaved in an identical manner to PS. This material was recrystallized to constant SA after further addition of crystalline PS (Table 4).

Table 4. Radiochemical purity of 17α-hydroxypregnenolone sulfate identified after incubation of fetal testicular homogenate with pregnenolone-7α-^{3}H-sulfate-^{35}S.

Steroid	Solvent	Successive Recrystallization	cpm ^{3}H/mg	cpm ^{35}S/mg*	^{3}H/^{35}S
	Methanol	C_1	13683	1875	
Pregnenolone sulfate**	Ethanol	C_2	13021	1994	
	Methanol	C_3	12820	1919	6.68
		ML_3	13638	2000	6.82
	Methanol	C_1	999	157	
17α-hydroxy-pregnenolone sulfate	Methanol, petroleum ether	C_2	986	152	
	Methanol, petroleum ether	C_3	994	147	6.76
		ML_3	972	147	6.61

* cpm of ^{35}S are corrected for decay
** starting material

The area corresponding to 17α-OH-PS and DHAS was submitted to CPC in system 10. Four zones of radioactivity were isolated. Zone I (HBV VIII to X) was resubmitted to CPC in system 3. Two areas of radioactivity were found. The less polar (HBV IX and X) was identical to carrier 17α-OH-PS and, after further addition of cold carrier, was recrystallized to constant SA (Table 4).

Zone 2 (HBV IV to VI) was solvolyzed and submitted to PC in system 4. An area similar to DHAS carrier was rechromatographed in system 4 after authentic DHAS carrier had been added. This material was solvolyzed and submitted to PC in system 9. A small peak of radioactivity with a mobility identical to carrier DHA was noted, but the small amount of radioactivity precluded definitive identification.

The remaining two zones of polar radioactive material (HBV I and HBV II to III) were investigated further but the identity of this material could not be established.

DISCUSSION

The demonstration in the first incubation that approximately 6% of the recovered tritium was in the organic fraction suggests the presence of sulfatase activity. The $^{3}H/^{14}C$ ratios of the unconjugated C-21 and C-19 steroids identified (Table 3) are noted to vary, but both labels are present in the final recrystallization of each metabolite indicating that both conjugated and unconjugated pregnenolone served as precursors in the formation of these unconjugated metabolites. The inconsistency in the $^{3}H/^{14}C$ ratios derived from the recrystallization data for 17α-hydroxypregnenolone and 17α-hydroxyprogesterone cannot be explained readily. Sulfatase activity was demonstrated in the second incubation as well, albeit to a lesser degree.

Sulfatase activity has been found in placenta (29,30), human fetal adrenals near term (29), and adult testes (23,31). That fetal perfusion studies have failed to demonstrate sulfatase activity may be explained either by the inability of the steroid sulfate to gain entrance to the site of enzyme action or by the fact that whole body perfusions were performed and the activity of any one organ, particularly one which may contribute quantitatively small amounts to the total steroid pool, may be difficult to assess.

^{14}C could not be demonstrated in either of the two steroid sulfate metabolites identified (DHAS and pregnenediol sulfate). In in vivo perfusion studies with labeled pregnenolone Solomon et al (32) isolated several sulfurylated steroids from a variety of fetal tissues; however,

the fetal gonad was not specifically studied. In a fetal perfusion with labeled 17α-hydroxypregnenolone, Pion et al recovered 17α-OH-PS and DHAS from adrenals, perfusate and residual tissue (5). Recently, the de novo formation of testosterone and cholesterol from acetate was demonstrated in the human fetal testis, but no sulfo-conjugated steroids were found (33). These findings are in contrast to our earlier studies in the human fetal adrenal (34) in which the de novo synthesis of both free and sulfurylated steroids was demonstrated. A high degree of sulfurylating activity in endocrine and non-endocrine fetal tissues has been reported (1-4). Our findings would suggest that under the experimental conditions employed the fetal testis, unlike other fetal tissues, possess little sulfokinase activity.

In the second incubation, the direct conversion of pregnenolone -^{3}H-sulfate-^{35}S to 17α-hydroxypregnenolone-^{3}H-sulfate-^{35}S has been demonstrated. Conversion of PS, which is present in large amounts in the umbilical artery (1), to 17α-OH-PS, DHAS, and 16α-hydroxydehydroepiandrosterone sulfate has been demonstrated in a similar fetal adrenal incubation in this laboratory (22). The importance of steroid sulfates in other tissues as precursors of other steroid sulfates (12-16,21,35) as well as biologically active hormones (17-20) has been noted. Steroid sulfates may play similar important roles in the fetal testis.

The work of Jost (36) has offered convincing evidence that androgens are necessary for the development and differentiation of the internal and external genitalia in the male fetus. Since in the fetal circulation steroids are present largely as sulfates, sulfatase activity in the fetal testis as demonstrated in this study may play a significant role in the biogenesis of free, biologically active androgens for this purpose.

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FOOTNOTES

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(5) The following abbreviations and trivial names are used: pregnenolone (3β-hydroxypregn-5-en-20-one), pregnenolone sulfate (PS) (20-oxo-pregn-5-en-3β-yl-sulfate), 17α-hydroxypregnenolone (3β,17α-dihydroxypregn-5-en-20-one), 17α-hydroxypregnenolone acetate (17α-hydroxy-20-oxo-pregn-5-en-3β-yl-acetate), 17α-hydroxypregnenolone sulfate (17α-OH-PS) (17α-hydroxy-20-oxo-pregn-5-en-3β-yl-sulfate), pregnenediol diacetate (5-pregnene-3β,20α-diol diacetate), pregnenediol sulfate (20α-hydroxy-pregn-5-en-3β-yl-sulfate), dehydroepiandrosterone (DHA) (3β-hydroxyandrost-5-en-17-one), dehydroepiandrosterone sulfate (DHAS) (17-oxo-androst-5-en-3β-yl-sulfate), 16α-hydroxydehydroepiandrosterone sulfate (16α-hydroxy-17-oxo-androst-5-en-3β-yl-sulfate), progesterone (pregn-4-en-3,20-dione), 17α-hydroxyprogesterone (17α-hydroxypregn-4-en-3,20-dione), androstenedione (androst-4-en-3,17-dione), testosterone (17β-hydroxyandrost-4-en-3-one).

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